Fibrillar aggregations of pathogenic pro-vasopressin mutants

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Summary

Diabetes insipidus is a disregulation of water homeostasis characterized by large fluid turnover in the kidney. Water homeostasis is regulated by the hormone vasopressin by increasing reapsorption of water in the renal collecting duct. Autosomal dominant diabetes insipidus is caused by mutations in the precursor protein of vasopressin. More than 50 different such mutations are known. Mutant prohormones are retained within the cell and cause degeneration of vasopressinergic neurons in the hypothalamus by an unknown mechanism.

In this work we identified three novel dominant mutations of the pro-vasopressin gene. We further show that different mutations in the precursor lead to the formation of disulfide-linked homo-oligomers and large aggregates with fibrillar morphology in the endoplasmic reticulum of transfected cultured cells, both fibroblasts and neuronal. In addition, bacterially expressed and purified pro-vasopressin spontaneously formed fibrils *in vitro* under oxidizing conditions. Further mutagenesis experiments showed that the presence of cysteines, but no specific single cysteine, is essential for disulfide oligomerization and aggregation *in vivo*. Our findings place autosomal dominant neurohypophyseal diabetes insipidus in the class of neurodegenerative diseases associated with amyloid-like protein aggregations.

To identify the sequence responsible for aggregation, segments comprising ten amino acids throughout the precursor were replaced by a sequence composed almost entirely of prolines and glycines. Prolines and glycines have been shown to disrupt the formation of amyloids. Formation of large aggregates required the intact N-terminal ten amino acids. Disulfide-linked oligomers, however, were not abolished. These results indicate that the vasopressin moiety is essential for precursor aggregation and that disulfide-linked oligomers not necessarily lead to the formation of large aggregates.

1 General Introduction

1.1 Water homeostasis

For a living organism, stability of the composition and the volume of body fluids is critical to survival and function. This stability of the internal environment is known as homeostasis. Relatively small changes in physiologically important variables activate a series of feedback mechanisms which sets off events that will promptly restore the altered variable to the normal level. In water balance, homeostasis can be maintained despite large variations in water intake and loss. The largest part of this loss of water is due to urination, which is dependent on the kidney activity. 90% of the primary kidney filtrate is held back by the proximal compartments of the nephron, the remaining 10% is absorbed in the distal collecting tubules where water reabsorption is controlled by the antidiuretic hormone (ADH).

The antidiuretic hormone of humans and most other mammals is vasopressin. Its synthesis occurs by the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus from where it is transported to the neurohypophysis. Vasopressin secretion is regulated primarily by plasma osmolality, which is monitored by osmolality receptors located in the hypothalamus. These receptors are not very well characterized and react on hypovolemia and/or hypernatremia by releasing vasopressin to the blood stream.

In the kidney, vasopressin from the bloodstream binds primarily to the vasopressin receptor V2 (V2R) at the basolateral side of the epithelial cells in the collecting duct (Fig 1). As a G-protein coupled receptor, it induces nucleotide exchange in G-proteins at Gα. Gα-GTP activates adenylate cyclase thus generating elevated cAMP levels. This leads to subsequent phosphorylation of the water channel aquaporin 2 (AQP2) at its cytoplasmic C-terminus by protein kinase A. As a consequence, transport vesicles containing AQP2 fuse with the apical plasma membrane. The resulting channels permit increased water reabsorption from the renal collecting ducts. Through AQP3 and AQP4 channels at the basolateral side,

water molecules can leave the cell again into the bloodstream. If vasopressin levels decrease, AQP2 is reinternalized (Fig 1) (Levin 2001; Rutishauser & Kopp 1999).

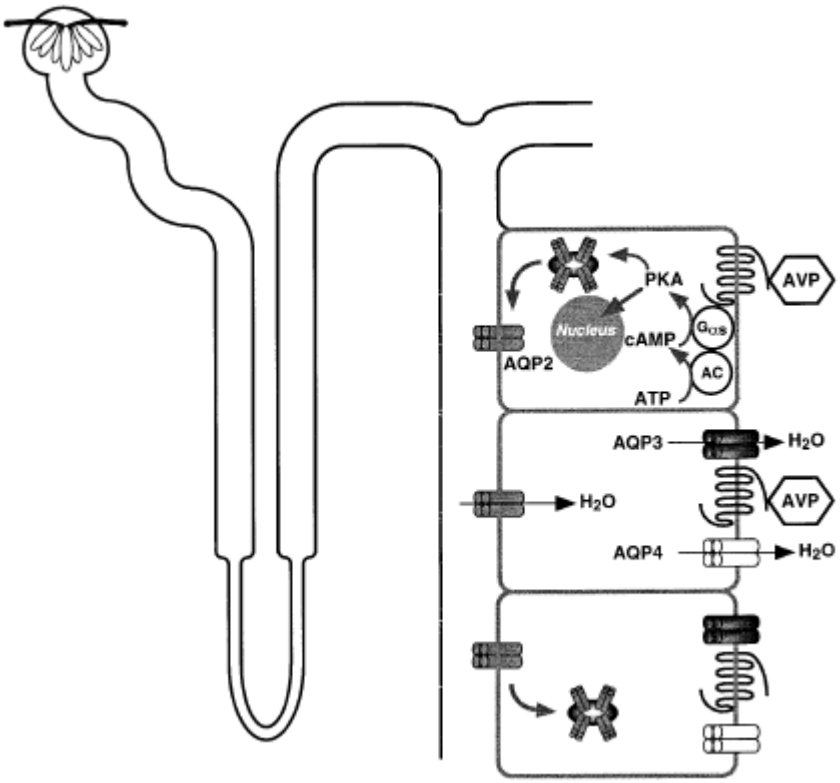

Fig 1 Vasopressin binds to its receptor and activates protein kinase A (PKA) which induces fusion of aquaporin 2 containing vesicles with the plasma membrane. Water reapsorption persists as long as vasopressin remains bound to its receptor. (Rutishauser & Kopp 1999).

1.2 Diabetes insipidus

If the concentration of the urine in the kidneys fails, polyuria and polydipsia are the consequence. This clinical condition is known as diabetes insipidus (DI). DI is characterized by excretion of abnormally large volumes (>30 ml/kg body weight/d for adults) of dilute urine. Three basic mechanisms are known to be the cause for DI. Firstly, absence of vasopressin from the blood due to a deficiency in the vasopressin production is referred to as neurohypophyseal diabetes insipidus. Secondly, renal resistance due to injury of the nephron or mutations in the vasopressin receptor is the cause for nephrogenic diabetes insipidus. Nephrogenic DI can be either an acquired disorder due to hypokalemia, hypercalcemia, or drugs (with lithium being the most notable example), or of hereditary origin. Almost 90% of hereditary nephrogenic DI disorders are due to mutations in the vasopressin receptor-2 gene (V2R), which causes a disease with X-linked recessive inheritance (XNDI or DIN type 1) (Morello & Bichet 2001). Furthermore mutations in AQP2 can also lead to DI. These mutations can be inherited in a dominant or recessive manner. Dominant AQP2 mutants are retained in the Golgi compartment. Since AQPs are thought to tetramerize, the retention of mutant AQP2 together with wild-type AQP2 in mixed tetramers in the Golgi compartment is a likely explanation for the dominant inheritance of DI in this patients (Mulders et al. 1998). In a third mechanism, vasopressin can be suppressed by excessive water uptake without need. This condition is referred to as dipsogenic diabetes insipidus and is most often caused by an abnormality in the part of the brain that regulates thirst.

Diagnosis of all kinds of DI takes place via a water deprivation test. Patients go through a defined period of water deprivation during which osmolality of the plasma and urine are measured and compared to each other. Assessment of the response to exogenous vasopressin can provide information on the DI subtype.

1.2.1 Neurohypophyseal diabetes insipidus

Limited capacity to secrete vasopressin during dehydration can be due to mutations in the pro-vasopressin gene. During the last decade a great diversity of peptide

producing neurons has been described in the brain and in the peripheral nervous system representing the so called peptidergic neurons. These are a group of interneurons which contain and secrete peptide hormones such as enkephalins, somatostatins, oxytocin and vasopressin. A major site of secretion for this type of hormones is the pituitary gland which consists of three different parts, the anterior, intermediate and the posterior lobe (Table 1). The posterior lobe is characterized by an enrichment of neuronal processes, essentially axons, and their terminals, comprising about 42% of the total volume. These processes are derived from magnocellular neurons located in the supraoptic and paraventricular nuclei of the hypothalamus. The secretory products of this type of neurons are stored in neurosecretory granules at the nerve terminals in the posterior pituitary and are released from there into the blood stream.

Table 1: Pituitary hormones and their major targets and physiologic effect

	Hormone	Major target organ(s)	Major physiological effects
Anterior Pituitary	Growth hormone	Liver, adipose tissue	Promotes growth (indirectly), control of protein, lipid and carbohydrate metabolism
	Thyroid stimulating hormone	Thyroid gland	Stimulates secretion of thyroid hormones
	Adrenocorticotropic hormone	Adrenal gland (cortex)	Stimulates secretion of glucocorticoids
	Prolactin	Mammary gland	Milk production
	Luteinizing hormone	Ovary and testis	Control of reproductive function
	Follicle stimulating hormone	Ovary and testis	Control of reproductive function
Intermediate Pituitary	Melanotropin	Melanocytes	Melanin synthesis
Posterior Pituitary	Vasopressin	Kidney	Conservation of body water
	Oxytocin	Uterus	Uterus contraction
		Mammary gland	Milk secretion

1.2.2 Vasopressin precursor

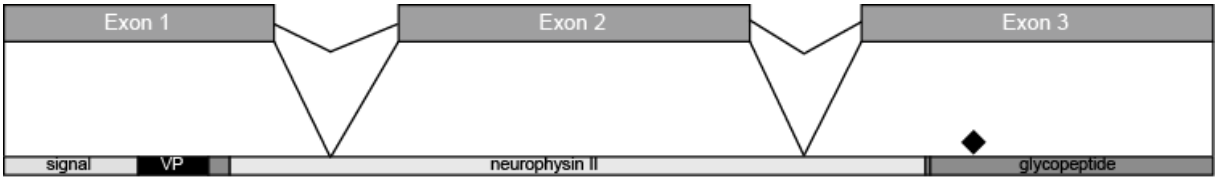

Fig 2: Gene structure and spliced precursor of vasopressin

Vasopressin is a nonapeptide synthesized as a preprohormone. It possesses a 19 amino acid signal sequence and a 93 amino acid carrier protein, the neurophysine II (NPII), followed by a glycopeptide of 39 amino acids, also referred to as copeptide, comprising a single N-linked glycosylation site. The gene encoding the precursor is located on chromosome 20p13 and consists of three exons (Fig 2)

Vasopressin and oxytocin are quite similar in their structure. The genes encoding vasopressin and oxytocin are in a tandem array on chromosome 20, separated by 8 Kb of DNA. The structure of oxytocin differs from that of vasopressin by only 2 amino acids: Ile for Phe at position 3, and Leu for Arg at position 8. This close structural relationship suggests that both derive from a common ancestral gene.

Mutation in the vasopressin gene can cause autosomal dominant neurohypophyseal diabetes insipidus. There are no hotspots for mutations responsible for the dominance. More than 50 mutations spread all over the precursor with exception of the glycopeptide are known and only one recessive mutation has been discovered so far in humans (Willcutts, Felner & White 1999). This exception results from reduced binding affinity to the vasopressin receptor in the collecting duct.

Interestingly, most mutations in the vasopressin gene have a dominant phenotype. This type of DI is referred to as autosomal dominant neurohypophyseal diabetes insipidus (ADNDI). ADNDI usually has a delayed onset. The first symptoms appear during the first months and years of life. The mechanism by which mutations in the vasopressin gene can cause dominant DI despite the presence of a functional wild-type allele, may be due to magnocellular cell death caused by an accumulation of

mutant hormone precursor in the endoplasmic reticulum. Death of magnocellular neurons could also explain the delayed onset of the disease since in early life enough vasopressin producing cells are present.

1.2.3 ER retention of mutant vasopressin precursors

The vasopressin precursor is cotranslationally targeted to the endoplasmic reticulum (ER). After entering the ER, the signal is cleaved off by signal peptidase. Upon translocation of the precursor, folding occurs, where chaperones play an important role of assisting in the folding process.

The folded precursor forms a dimer. Dimerization is stabilized by binding of vasopressin into its binding pocket of the folded NPII (de Bree & Burbach 1998). The copeptide is modified at the Asp at position 5, where a glycan is attached. In the Golgi the glycan is further modified. Reaching the trans Golgi network (TGN) the prohormone is sorted into the regulated secretory pathway. Immature secretory granules bud off from the TGN. During maturation, prohormone convertase I cleaves the prohormone between the vasopressin peptide and NPII. Mature secretory granules containing processed vasopressin move along the axon from the cell periphery and are stored near the synapse prepared for regulated release.

The effects of the DI mutations in the precursor have been studied by several groups using heterologous expression systems. Olias et al. (1996) showed that expression of the wilde-type and a mutant (G17V) in the pituitary cell line AtT-20 exhibit distinct differences in processing and secretion of the molecules. The precursor of the wild-type was correctly glycosylated and processed. However, the mutant precursor, allthough core glycosylated remained endoglycosidase H sensitive. This indicated that the protein never reached the TGN. This was supported by the fact that the G17V mutant protein was restricted to the ER, in contrast to cells expressing the wild-type, where NPII was concentrated in the tips, as shown by immunofluorescence.

The ΔG227 mutation leading to a truncated signal peptide was shown to be glycosylated (Beuret et al. 1999), suggesting the truncated signal to be sufficient for

ER import. The fact that most of the precursor showed a higher molecular weight suggested failure in signal peptide cleavage. Secretion was drastically reduced and the protein was completely retained inside the ER. This was supported by co-staining for the ER resident protein p63.

Another signal peptide mutant A(-1)T examined by Siggaard et al. (1999) in Neuro2a cells showed an eight-fold reduction in secretion when compared to the wild-type. This precursor was also shown to be retained in the ER by the co-localization with glucose-regulated protein 78 (GRP78).

To elucidate the mechanism of ADNDI, Nijenhuis et al. (1999) studied five mutants, G14R, ΔE47, E47G, G57S and G65V by stably expressing them in the neuroendocrine cell lines Neuro2a and PC12/PC2. All of the five mutants examined showed impairment of secretion, but to different extents. Retention of the precursors in the ER was indicated by sensitivity to endoglycosidase H and co-staining with the ER marker protein disulfide isomerase (PDI).

The same results were obtained for the E87X mutant in PC12/PC2 and AtT-20 cells (Nijenhuis, Zalm & Burbach 2000), and the C85G mutant in PC12/PC2 (Nijenhuis et al. 2001), where reduced processing and secretion as well as co-localization with PDI were observed. The high level of expression of the mutant pro-hormone caused strong accumulation in the ER subsequently leading to enlarged ER sub-compartments.
These morphological changes of the ER could be evidence for severe disturbance and dysfunction of the ER which in turn could be responsible for dominance (Aridor & Hannan 2000; Aridor & Hannan 2002; Rutishauser & Spiess 2002).

Since the phenotype is not linked to a specific site, any change in the amino acid sequence seems to be sufficient to induce misfolding and thus leads to retention. This retention, although variable between different mutations (Nijenhuis, Zalm & Burbach 1999), seems to be a hallmark for ADNDI and thus critical for pathogenesis. ADNDI is hence a trafficking defect. In order to understand this defect, comprehension of trafficking and thus the secretory pathway is crucial.

1.3 The secretory pathway

1.3.1 ER entry

If a protein is destined for secretion it is targeted to the ER, where it is, in the case of a mammalian cell, inserted into the lumen via a co-translational process. The endoplasmic reticulum consists of a netlike labyrinth of branching tubules and flattened sacs and is extended throughout the cytosol. The highly convoluted space within the tubules and sacs is called the ER lumen. The ER captures two types of proteins from the cytosol as they are being synthesized. Transmembrane proteins are only partly translocated across the ER membrane and are finally embedded in it. Water soluble proteins are fully translocated across the ER membrane, and released into the ER lumen. To ensure the direction of these proteins to the ER membrane, both kinds of protein possess the same kind of signal sequence which exhibits a stretch of hydrophobic amino acids. As these amino acids emerge from the ribosome, they are recognized and bound by the signal recognition particle (SRP). Translation is attenuated by binding of the signal sequence to SRP, and tight binding of SRP to its receptor targets the nascent peptide chain to the ER. The polypeptide chain enters the ER lumen via a gated channel. This channel is formed by the heterotrimeric Sec61 αβγ complex. On the luminal side of the ER membrane, the signal is cleaved by a signal peptidase. Entry is dependent on additional components. Requirement of translocating chain-associated membrane protein (TRAM) has been shown in reconstitution experiments in lipid vesicles (Görlich & Rapoport 1993).

During translocation, proteins are also glycosylated on specific aspargine residues (N-linked glycosylation). Oligosaccharide units consisting of 14 sugar residues are added to acceptor asparagine residues of growing polypeptide chains as they are translocated into the ER.

1.3.2 Quality control

There are distinct biophysical features, distinguishing native from non-native conformation. These differences like exposed hydrophobic regions, unpaired

cysteines and the tendency to aggregate are the basis for primary quality control. Before newly synthesized polypeptides reach their final destinations, they may undergo five principal modifications: Formation of disulfide bonds, proper folding, addition and processing of carbohydrates, specific proteolytic cleavages, and assembly into multimeric proteins. Essential for normal structure and activity in proteins that contain more than one disulfide bond is the proper pairing of cysteine residues. The reorganization of disulfide bonds is accelerated by the enzyme protein disulfide isomerase (PDI).

Important molecular chaperones and folding sensors used in the primary quality control are abundant in the ER. BiP, calnexin, calreticulin, glucose-regulated protein 94 (GRP94) and the thiol-disulfide oxidoreductases protein disulphide isomerase (PDI) and ERp57 are part of this group. Important for the folding of glycoproteins is the calnexin/calreticulin cycle. During synthesis of glycoproteins with N-glycans, the oligosaccharide Glc3Man9GlcNAc2 is attached to the asparagine residue in the consensus sequence NXS/T (X≠P) of newly synthesized proteins in the ER. Two glucoses are removed by glucosidase I and II. By this step a monoglucosylated (Glc1Man9GlcNAc2) glycoprotein is generated, which can interact with calnexin and calreticulin. Both chaperones are associated with the thiol-disulfide oxidoreductase ERp57 through an extended arm-like domain. During disulfide-bond formation, ERp57 forms interchain disulfide bonds (S-S) with bound glycoproteins. The decision about retention or release from the control cycle is made by two independent enzymes. The terminal glucose is removed by glucosidase II. This permits dissociation of the glycoprotein from the respective lectin. If folding fails, the glycoprotein becomes substrate for UDP-glucose:glycoprotein glucosyltransferase (UGGT), which reattaches a terminal glucose, and thus causes the proteins to reassociate with the lectin. Patches of hydrophobic amino acids are suggested to be the recognition motif for reglucosylation (Caramelo et al. 2003).

If the protein permanently fails to fold, ERα1,2-mannosidase I removes a mannose from the middle branch of the oligosaccharide. This is the recognition pattern for the ER degradation-enhancing 1,2-mannosidase-like protein (EDEM), which is probably responsible for the targeting of glycoproteins for ER-associated degradation (ERAD). Only If the folding deficiency is final after multiple cycles of deglucosylation

and reglucosylation the proteins are removed from the calnexin/calreticulin cycle and eliminated (Molinari et al. 2003).

1.3.3 ER associated degradation (ERAD)

It is not surprising that protein misfolding can lead to a multitude of diseases, such as cystic fibrosis, antitrypsin deficiency and protein aggregation diseases, such as Huntington's, Parkinson's, Alzheimer's and prion-associated diseases. To prevent misfolding-induced toxicity, aberrant proteins are often destroyed. If folding is delayed, or an illegitimate conformation arises, the substrate is either subjected to additional folding cycles (see 1.3.2) or is selected for a process termed ER-associated degradation (ERAD). ERAD involves a large number of components, which have been identified through both genetic and biochemical approaches (Sayeed & Ng 2005; Hebert & Molinari 2007; Nakatsukasa & Brodsky 2008). These efforts revealed that ERAD substrates are recognized, targeted, retrotranslocated, polyubiquitylated, and then degraded by the 26S proteasome (Fig 3). Individual components have been found to catalyze unique steps during ERAD. Most of these components are conserved from yeast to humans.

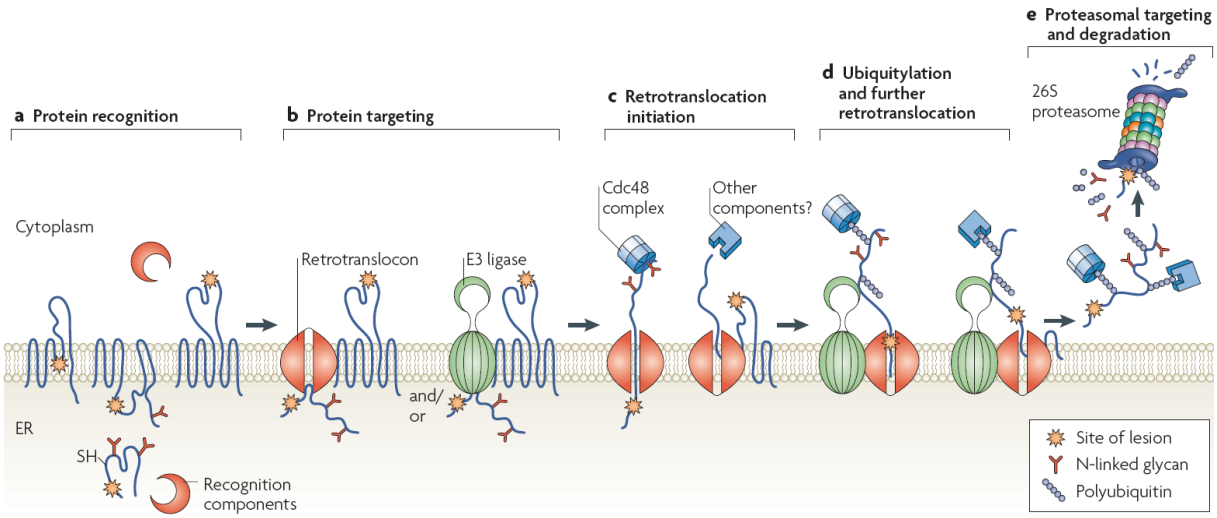

Fig 3: The ERAD pathway (Vembar & Brodsky 2008)

The first step of the ERAD pathway is the recognition of the substrate. Proteins, including soluble and integral proteins, polypeptides which have failed to become post-translationally modified or are otherwise damaged or misfolded, and unassembled members of multiprotein complexes are potential ERAD substrates. It is not quite clear how ERAD substrates are distinguished from proteins that are properly folded or that are on the correct folding pathway, probably many proteins are mistakenly targeted for ERAD. Misfolded proteins are recognized by cytoplasmic and luminal chaperones and associated factors, such as 70 kDa heat-shock protein (Hsp70)-family members, calnexin and calreticulin, and PDI. In a next step ERAD substrates are targeted to the retrotranslocation machinery (the retrotranslocon) and/or to E3 ligases. Cell-division cycle-48 (Cdc48) complex and other components such as molecular chaperones or the proteasome initiate the substrate retrotranslocation into the cytoplasm. In a subsequent step, ubiquitylation by E3 ubiquitin ligases takes place, as proteins exit the retrotranslocon. This promotes further retrotranslocation and is assisted by cytoplasmic ubiquitin-binding protein complexes. The receptor of 19S cap of the 26S proteasome recognized polyubiquitylated substrate, once displaced into the cytoplasm, and the polyubiquitin tag is removed by de-ubiquitylating enzymes. The substrate is then inserted into the 20S catalytic core of the proteasome, where it is broken up into peptide fragments. The ubiquitin generated by this process can be recycled for subsequent rounds of modification (Vembar & Brodsky 2008).

1.3.4 ER Stress

The ER stress response is an important adaptive cellular signaling response. It aims to maintain the balance between secretory protein synthesis in the ER against protein folding capacity. If an imbalance occurs between these 2 factors, the ER stress response, also known as the unfolded protein response (UPR), is activated. The UPR consists of different pathways which in the end, aim to restore ER homeostasis by down-regulating global protein translation, increasing the ER folding capacity by up-regulating ER chaperones, and the degradation of misfolded proteins. It has been estimated that approximately 30% of all newly synthesized proteins are rapidly degraded, possibly as a result of improper folding (Schubert et

al. 2000). In mammalian cells, the UPR may also activate genes and pathways that are not directly linked to the expression of resident ER proteins. For example, treatment of cells with the glycosylation inhibitor tunicamycin, a potent inducer of the UPR, leads to the induction of growth arrest and programmed cell death in many cell types (Larsson, Carlberg & Zetterberg 1993; Pérez-Sala & Mollinedo 1995; Carlberg et al. 1996).

A build up of misfolded protein would impose a major problem for the cell, such accumulations can lead to perturbations in the ER environment, such as alterations in redox state or calcium levels. In response to ER stress, 3 ER localized transmembrane signal transducers are activated (Tirasophon, Welihinda & Kaufman 1998) (Fig 4). These transducers are inositol requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK) and the activating transcription factor 6 (ATF6). All three inducers of ER stress response are inactive as long as they are bound to the ER chaperone BiP.

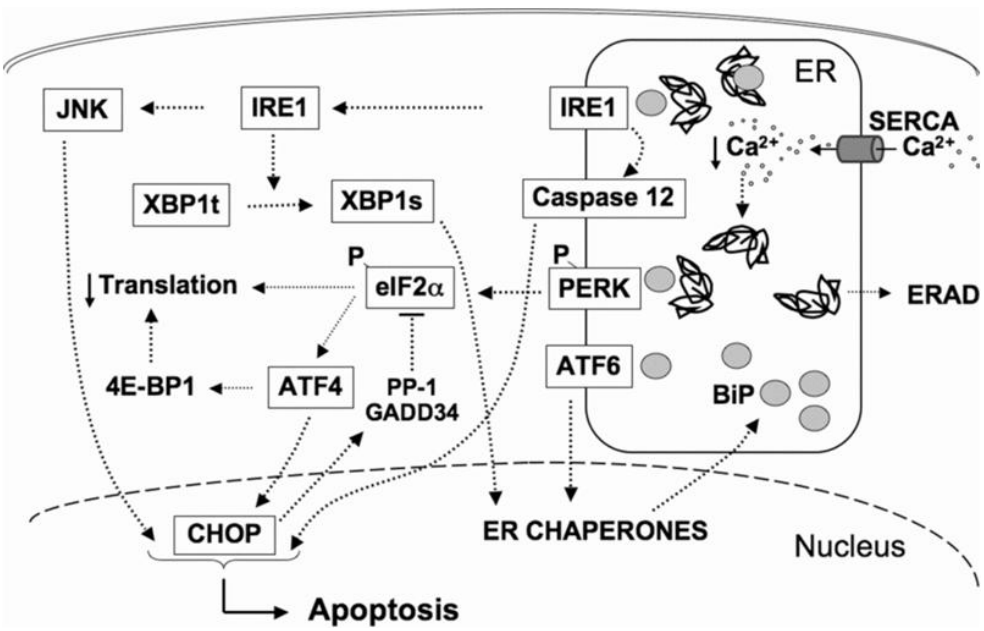

Fig 4: ER stress pathway (Cnop et al. 2008)

Activated IRE1 leads to alternative splicing of XBP1, which is an inducer of genes involved in ER expansion, protein folding and the degradation of misfolded protein.

The IRE1 gene was duplicated in mammals, in contrast to yeast. This duplication gave rise to IRE1α and IRE1β. Whereas IRE1α is expressed in all mammalian cells, IREβ is primarily expressed in epithelial cells (Tirasophon, Welihinda & Kaufman 1998; Wang et al. 1998; Bertolotti et al. 2001). The two isoforms seem to have the same *in vitro* activities, subcellular localization and downstream target (XBP1 mRNA). However, whereas IREα is essential for mammalian development (Zhang et al. 2005), IRE1β deletion does not lead to significant defects (Bertolotti et al. 2001).

Activated ATF6 is exported to the Golgi apparatus. There it is cleaved from the membrane. This free form translocates to the nucleus, where it induces the transcription of ER chaperones such as BiP.

PERK in its activated form phosphorylates eIF2α and thereby prevents ternary complex formation and suppresses global translation initiation. Activation of eIF2α facilitates translation of proteins like ATF4 which in turn activates the CCAAT/enhancer binding protein (C/EBP) homologous transcription factor (CHOP), the Bcl-2 family members (Bak/Bax), caspase-12, and c-Jun NH_2-terminal kinase (JNK) are induced. JNK and caspase-12 induce growth arrest and DNA damage-inducible protein (GADD34), which associates with protein phosphatase 1 (PP-1) and dephosphorylates eIF2α, acting as a negative feedback loop. Consequences of the UPR are either reconstitution of normal function or apoptosis.

1.3.5 ER export and ERGIC

A properly folded, and thus functional protein is packed into vesicles covered by cytosolic coat protein II (COPII) and transported out of the ER to the Golgi. The coat proteins form a shell around the forming vesicle, shaping it into a transport vesicle (Bonifacino & Lippincott-Schwartz 2003) destined for the cis Golgi. The COPII coat consists of five subunits: Sar1, Sec23, Sec24 and Sec13, Sec31. Sar1 is a small GTPase that inserts its N-terminal amphipathic helix into the cytoplasmic leaflet of the ER membrane bilayer when bound to GTP. Sec12 serves as the guanine-nucleotide exchange-factor (GEF) for Sar. Sar1 recruits Sec23 and Sec24, which form a tightly associated heterodimeric complex, and bind directly to the ER

membrane, forming an inner coat. The Sar1-Sec23-Sec24 complex recruits the heterotetrameric Sec13-Sec31 complex which builds the outer coat. This outer coat is not in direct contact with the ER membrane surface. Cargo proteins are primarily selected by Sec24 to be incorporated into COPII vesicles via binding to the cytoplasmic domains of ER transmembrane proteins destined for anterograde compartments. Sar1 and Sec23 are also known to bind cargo molecules, but their role in discrimination of COPII cargo versus ER-resident proteins is less clear. Sec23 is a GTP-activating protein (GAP) for Sar1, its activity is stimulated by binding of the outer coat. If the coat is completely polymerized, hydrolysis of Sar1-bound GTP to GDP occurs, which leads to the depolymerization of the coat, and the vesicle is uncoated, so that the COPII subunits can be recycled for another round of vesicle formation.

The ER-derived COPII vesicles fuse with each other or with a target compartment, to transport cargo to the next station (Beckers et al. 1989; Kaiser & Schekman 1990; Rexach & Schekman 1991). In yeast and higher eukaryotes the ER to Golgi transport is restricted to specialized, long-lived subdomains of the ER, the ER-exit sites (ERES) (Bannykh, Rowe & Balch 1996; Hammond & Glick 2000).In yeast, fusion occurs directly with the cis-Golgi whereas in higher eukaryotes ER to Golgi transport is directed via the tubulovesicular membrane clusters of the ER-Golgi intermediate compartment (ERGIC), the marker of which is the lectin ERGIC-53 (Hauri et al. 2000; Schweizer et al. 1988). ERGIC constitutes an independent structure, that is not continuous with the ER or the cis-Golgi (Sesso et al. 1994; Bannykh, Rowe & Balch 1996; Fan, Roth & Zuber 2003; Klumperman et al. 1998). The ERGIC serves as the first post ER sorting station for anterograde and retrograde protein traffic. (Appenzeller et al. 1999; Aridor et al. 1995; Ben-Tekaya et al. 2005). This sorting functions via transport signals such as KDEL in soluble proteins in retrograde transport. KDEL binds to the KDEL receptor, which in turn, is recognized by COPI. About the anterograde transport little is known. Like in ER export, anterograde exit from the ERGIC might be signal mediated or occur by default. Successful anterograde delivery from the ERGIC to the Golgi requires dissociation of cargo proteins from their receptors in the ERGIC. This suggests, that the ERGIC exhibit different features, compared with the ER, where cargo-receptor interaction occurs. pH has been shown to be one of these features. Acidification of

cells in culture inhibits the association of ERGIC-53 with its cargo procathepsin Z, and neutralization of organelle pH by chloroquine specifically impairs the dissociation of this glycoprotein in the ERGIC (Appenzeller-Herzog et al. 2004). The lumen of the ER has been shown to be neutral (pH 7.4) (Wu et al. 2001), the ERGIC however, is the earliest low-pH site in the secretory pathway. Its exact pH is unknown, but it is unlikely to be lower than that of the TGN (pH 6.4) (Machen et al. 2003). Only slight reduction in pH compared to the pH of the ER would be sufficient to act as a general trigger for the dissociation of cargo-receptor complexes in combination with other factors, such as calcium concentrations.

1.3.6 Intra Golgi transport

The central sorting and processing station of the secretory pathway is the Golgi apparatus. It is composed of 4 to 6 flat cisternae, which are arranged into several stacks, linked by tubular connections. Within these stacks, it is ensured, that cargo proteins, synthesized in the ER are modified and packaged into carriers for transport to their final destination. Post-translational modifications of secretory cargo proteins are assured by processing enzymes, such as glycosidases and glycosyltransferases. Thus, forward cargo movement must be coordinated with an assembly line of processing enzymes that sequentially modify the cargo proteins (Dean 1999; Helenius & Aebi 2001; Lowe & Marth 2003; Opat, van Vliet & Gleeson 2001).

Two models for the movement of cargo through the Golgi have been proposed. Important for both models is coatomer protein complex I (COPI). COPI-coated vesicles are positioned laterally from the Golgi cisternae. According to the *vesicular transport model*, the Golgi is a relatively static structure. The molecules are transported through the cisternae in sequence, carried by specific COPI transport vesicle (Rothman & Wieland 1996). COPI vesicles carry cargo and move in an anterograde fashion from one Golgi cisterna to the next (Ostermann et al. 1993; Rothman 1994). In the *cisternal maturation model*, however, the Golgi is viewed as a dynamic structure. The cisternae themselves move through the Golgi stack (Glick & Malhotra 1998; Matsuura-Tokita et al. 2006). At the cis-Golgi new cisternae would

assemble and old cisternae would disassemble at the trans-Golgi. Transport of resident enzymes from the more trans side to the more cis side would be achieved via COPI coated vesicles. The characteristic distribution of Golgi enzymes is explained by retrograde flow. the COPI vesicles move in a retrograde fashion and function as a retrieving device that is used by Golgi enzymes to maintain their specific and differential localization over the Golgi stack (Glick, Elston & Oster 1997; Pelham 1998). The two models are not mutually exclusive. Transport may occur by a combination of both models.

COPI coat assembly begins with the membrane recruitment and activation of the small soluble GTPase Arf1. This is mediated by guanine nucleotide exchange factors (GEFs) that convert Arf1 to its active, GTP-bound form. Upon activation at the membrane, Arf1 recruits a coatomer complex from the cytoplasm. Coatomer bound to Arf1 then binds membrane-associated ArfGAP, the GTPase activating protein for Arf1. The complex of coatomer–Arf–ArfGAP polymerizes into a coat on the membrane, concentrating cargo proteins directly beneath it. The coat deforms the membrane into a bud. This bud pinches off the membrane as a coated vesicle. Disassembly of the coat occurs when ArfGAP hydrolyzes the GTP on Arf1. This releases Arf1 into the cytoplasm, which in turn triggers the release of coatomer and ArfGAP from the membrane. The components are then free to be reused for coat reassembly.

1.3.7 Granule formation

Endocrine and neuro-endocrine cells possess a regulated secretory pathway, in addition to the constitutive pathway present in all cells (Burgess & Kelly 1987). A hallmark of these cells are dense-core secretory granules which are responsible for regulated secretion of cargo molecules. Peptide hormones are synthesized as large precursors at the ER and transported to the Golgi apparatus. At the TGN, the precursors are packaged into secretory granules where they are processed to active peptides and secreted in a regulated manner in response to stimuli. The dense core of this granules consist of large insoluble secretory proteins and peptide

aggregates, that are formed by self-association (Dannies 2001; Arvan et al. 2002; Tooze 1998).

There are two non-exclusive models proposed on how secretory granules are formed and how specific cargo selection is achieved. The first one, “sorting for entry”, is analogous to receptor-mediated endocytosis and mannose 6-phosphate receptor-dependent lysosomal transport (Schmid 1997). In this model cargo binds to receptors, which in turn recruit a cytosolic coat. The alternative model is termed "sorting-by-retention". Here it is proposed that selective aggregation of regulated cargo in the TGN results in an immature granule. Captured non-granule molecules are subsequently removed in vesicles budding from maturing secretory granules by clathrin-coated vesicles and by so-called constitutive-like secretion, whereas specific granule cargo is retained (Kuliawat & Arvan 1992). It has been proposed that a single protein of endocrine and neuronal cells, chromogranin A (CgA), controls secretory granule biogenesis (Kim et al. 2001). Expression of CgA was even found to induce granular structures in transfected CV-1 fibroblast cells. It has been shown that several other cargo proteins of the regulated secretory pathway of endocrine cells, peptide hormone precursors as well as granins, induce the formation of granule-like structures when expressed in cell lines normally lacking regulated secretion. It has also been shown that expression of cargo is sufficient to drive segregation of regulated and constitutive secretory proteins and the formation of dense membrane-bound accumulations. These accumulations showed a similar ultrastructural appearance as secretory granules, suggesting that initial granule formation requires no additional machinery specific to regulated secretory cells. The regulated cargo itself is sufficient (Beuret et al. 2004).

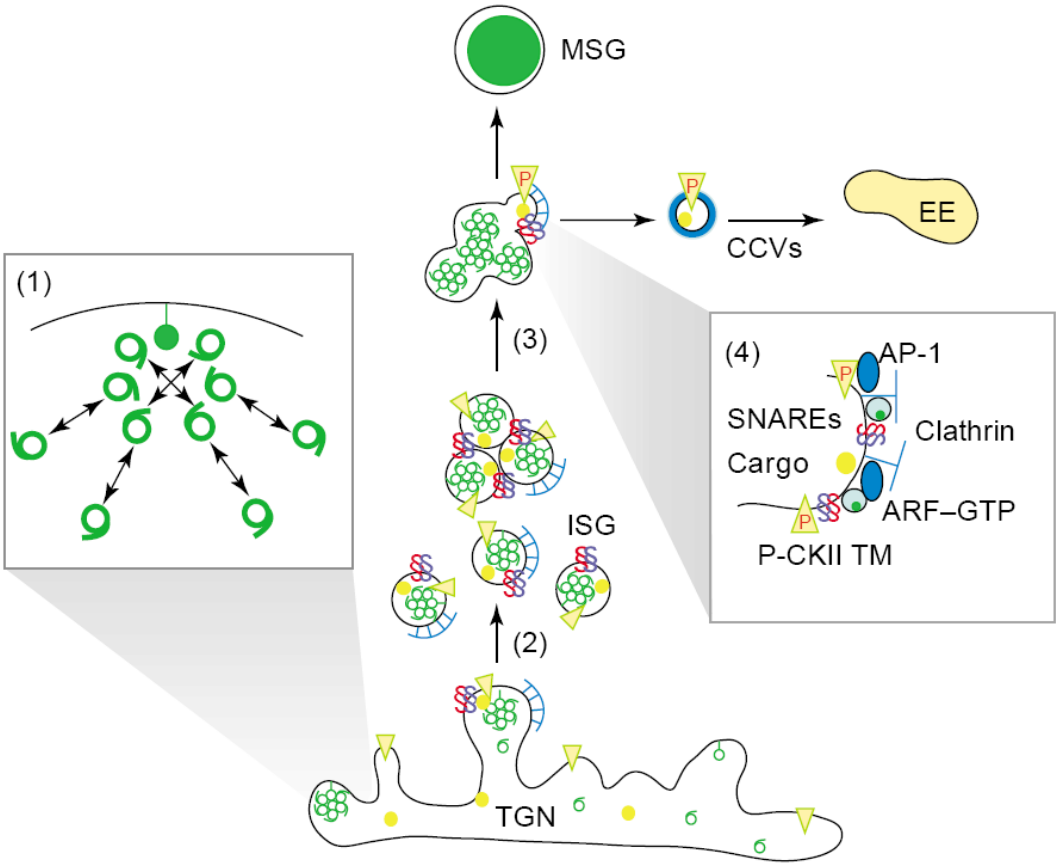

Fig 5: Biogenesis of secretory granules in neuroendocrine cells. Step (1) Regulated secretory proteins (RSPs; green spheres with curly tail) become associated with a specialized region of the TGN and (filled green circle with stalk). (2) Additional components, including SNARE molecules (blue and red '§'), other soluble non-secretory granule proteins, or cargo (yellow spheres) and non-secretory granule membrane proteins (yellow triangles), are included into the nascent bud. (3) After formation, ISGs undergo homotypic fusion mediated by SNAREs, followed by membrane remodeling (4). Remodeling to the mature secretory granule (MSG). Abbreviation: EE, early endosome. (Tooze, Martens & Huttner 2001)

1.4 Neurodegeneration

A common cellular and molecular mechanism of neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's disease, is the formation of inclusion bodies and protein aggregations, which can be found in the cytoplasm, the nucleus or extracellularly.

The ability of proteins to fold into a structure, which is biologically active, is encoded in their amino acid sequence. Protein folding involves populations of transiently denatured, or partially folded intermediate structures that rapidly convert into the final native state. However, this optimal situation is often perturbed when the native fold is destabilized by mutation, or when changes occur in the cellular or physicochemical environment in which the polypeptide is produced or kept.

Huntington's disease is a neurodegenerative disorder, caused by an abnormal polyglutamine (polyQ) expansion within the protein huntingtin (Htt). Individuals with more than 40 polyQ repeats will always develop the disease within a normal life span. A characteristic feature of Huntington's is the death of striatal and cortical neurons, paralleled by aggregation of Htt into microscopic intracellular deposits. These aggregations are located in the nucleus and the cytoplasm. Formation of these inclusions has been associated with neurodegeneration (Davies et al. 1997; Becher et al. 1998; DiFiglia et al. 1997; Ordway et al. 1997). However, other reported no correlation or even a negative one (Saudou et al. 1998; Taylor et al. 2003; Shimohata et al. 2002). The correlation between inclusions and degeneration is thus not clear.

In Alzheimer's disease (AD), massive degeneration of the brain mass occurs. Over twenty years ago, the amyloid hypothesis, explaining the neuropathology was proposed, but the molecular pathogenesis still needs to be clarified. The loss of cerebral substance is often accompanied by the formation of extracellular plaques, composed of amyloid beta (Aβ) peptide and intracellular fibrillary tangels composed of hyperphosphorylated tau protein. Genetic animal modeling and biochemical data have brought up the suggestion that Aβ plays an important role in Alzheimer's disease. Cleavage of the amyloid precursor protein (APP) by the action of two aspartyl proteases (β- and γ-secretase) leads to the formation of Aβ fragments (Haass et al. 1992; Seubert et al. 1992; Shoji et al. 1992). After cleavage of APP by β-secretase, a 99 amino acid long stub is left in the membrane, which is subsequently cleaved by the γ-secretase, which leads to the release of Aβ. Three principal forms (Aβ38, 40 or 42) of Aβ are produced, depending on the exact point of cleavage. The longer form Aβ 42 is far more prone to oligomerize and form

amyloid fibrils than the Aβ40 peptide (Burdick et al. 1992; Jarrett, Berger & Lansbury 1993).

The familial form Parkinson's disease (PD) is caused by degeneration of dopaminergic neurons in the substancia nigra of the midbrain (Forno 1996). The pathological hallmark of adult-onset PD are Lewy bodies, inclusion bodies found in the cytoplasm of neurons, often near the nucleus. Lewy bodies contain large amounts of the protein α-synuclein.

Amytrophic lateral scleorsis (ALS) is a disease caused by degeneration of lower motor neurons in the lateral horn of the spinal cord and upper motor neurons of the cerebral cortex (Cleveland & Rothstein 2001). One familial form, which is of early onset but relatively rare can be caused by mutations in the superoxide dismutase (SOD1) gene. A murin model, overexpressing mutant SOD1, showed cytoplasmic inclusions, containing aggregated SOD1 protein (Bruijn et al. 1998; Rakhit et al. 2002).

Prions are exceptional infectious pathogens that cause a group of invariably fatal neurodegenerative diseases, mediated by an entirely novel mechanism. Prion diseases occur as genetic, infectious, or sporadic disorders, all of which involve misfolding of the prion protein (PrP), a constituent of normal mammalian cells (Prusiner 1991). Bovine spongiform encephalopathy (BSE), scrapie found in sheep, and Creutzfeldt–Jakob disease (CJD) of humans are among the most notable prion diseases. Prion disease is a prototypical protein conformation disease, where cellular PrP (PrP^{C}) is converted into PrP^{Sc} through a posttranslational process, during which it acquires a high β-sheet content (Pan et al. 1993). This leads to the formation of amyloid plaques appearing similar to those of AD.

It is likely that the formation of these various aggregates constitute an end-stage manifestation of a multistep process. The role of these aggregation in toxicity has been a matter of great controversy, although many lines of indirect evidence link aggregation to toxicity. However, it has been shown that inclusion bodies and other visible aggregates correlate poorly with the severity of neurodegenerative diseases such as Alzheimer's (Terry et al. 1991) or Parkinson's (Tompkins & Hill 1997). It has also been shown that cells expressing comparable levels of the huntingtin fragment, were less likely to die if they formed inclusion bodies (Arrasate et al. 2004). These

observations raise the question whether the formation of inclusion bodies and visible protein aggregations are protective, and even represent a cellular and molecular mechanism to deal with aggregated and misfolded proteins.

Proteins always have a certain tendency to aggregate. This forced the cell to evolve mechanisms to deal with misfolded or abnormal proteins. Molecular chaperones are able to provide the first line of defense against protein aggregation. The function of these molecules is to assist in folding of newly synthesized proteins and refold them if necessary which renders them non-toxic (section 1.3.2). A further defense mechanism lies in the function of the proteasome which degrades non functional proteins in the cytoplasm and the nucleus. Autophagy constitutes another way of dealing with wrongly synthesized proteins and aggregates.

As a last resort aggregates can be sequestered by microtubule-mediated transport and concentrated at a special cytoplasmic site next to the centriole. These accumulations of aggregated material, usually rather big in size and thus visible by light microscopy, are called aggresomes. They are likely to constitute an end product of an active process to cope with misfolded proteins.

There is evidence that aggregates might be a protective response of the cell against misfolded proteins. However, there is also evidence that the aggregation process itself is likely to be related to toxicity in neurodegeneration, and that there may be common mechanisms of toxicity among the described neurodegenerative diseases.

1.5 Neurotoxicity in ADNDI

As already mentioned in section 1.3.2, misfolded proteins are usually degraded via the ubiquitin-proteasome pathway. Having this in mind, one would expect all the listed mutations in the vasopressin gene to be recessive, since they are all likely to be misfolded. Friberg et al. (2004) showed, that the mutants are retrotranslocated to the cytosol and degraded by the proteasome. Most of the cases are heterozygous for these mutations thus the correct allele of the gene is still present and one would expect to find fully functional vasopressin in circulation. However, all listed neurohypophyseal diabetes insipidus mutations are dominant. There is only one

recessive mutation known in humans. The resulting hormone has a low affinity for its receptor in the kidney and is thus to be seen as a null mutation. The mechanism of this dominance has currently not been determined. Ito et al. (1999) proposed a model in which vasopressin precursors form heterodimers with wild-type products to alter their function, and contribute to the pathogenesis of ADNDI. They examined the physical and functional interactions between wild-type and mutant vasopressin precursors, by expressing epitope-tagged precursors in cultured cells. Crosslinking experiments showed, that wild-type and mutant precursors were able to build homo- and hetero-dimers, and that mutant precursors inhibited the trafficking of the wild-type protein from the ER to the Golgi apparatus. According to this hypothesis, the secretion of vasopressin would be decreased. However, the cell would still be able to adapt to increasing demands of hormone by increasing the expression of vasopressin and the phenotype would thus still be a recessive one.

Another hypothesis to explain the phenomenon comes from clinical data from individuals affected by diabetes insipidus. Studies in patients revealed low levels of circulating vasopressin accompanied by gliosis and hypocellularity of vasopressinergic neurons in the hypothalamus (Hanhart 1940; Braverman, Mancini & McGoldrick 1965; Gaupp 1941; Bergeron et al. 1991). Blotner (1958) suggested that hereditary diabetes insipidus represents a selective neural system degeneration. Taken all together, this supports the hypothesis that vasopressin mutants exert a toxic effect on their host cells which in turn would be an explanation for the dominant inheritance. The nature of this cytotoxic effect of mutant pro-vasopressin is still not explained and needs further investigation.

Heterologous expression studies delivered more support for the cytotoxicity hypothesis. Neuro2a cells, stably expressing the mutants A(-1)T, ΔE47, G57S and C67X were not much affected in growth and viability. However, when valproic acid was used to initiate differentiation of the cell to a neuronal cell type, viability was significantly reduced. The reduction of viability differed between the different mutations (C67X>A(-1)T>G57S>ΔE47) (Ito & Jameson 1997).

Several animal models have been established to study ADNDI. The best-known mutation in rat is the Brattleboro mutation, where a deletion of a guanine at the position 65 of neurophysin causes a frameshift. This frameshift leads to a precursor

without a stop codon, and causes a read-through into the poly A tail. However, since the resulting protein is poorly expressed, the resulting phenotype is recessive and thus not directly comparable with the situation in ADNDI patients (Schmale et al. 1984). Si-Hoe et al. (2000) created a transgenic rat model expressing the C67X mutation. This mutation did not show a disease phenotype in rats. The mutant was retained in the ER. However, cell death or atrophy of magnocellular neurons could not be observed. In a subsequent study using the same transgenic rat strain, the authors showed autophagy to accompany accumulation of mutant precursor in the ER (Davies & Murphy 2002). Autophagy was claimed to act as a prosurvival mechanism in ADNDI since inhibition of autophagy decreased cell viability (Castino et al. 2005).

Russel et al. (2003) showed in their work that knock-in mice expressing the C67X mutation revealed a clear ADNDI phenotype with polyuria and polydipsia worsening with age, in parallel with extensive loss of neurons in the supraoptic and paraventricular nuclei. Interestingly, the A(-1)T mutation did not cause any defects. In a recent publication a novel murine knock-in model expressing the mutant C67X was established. These heterozygous mice manifested progressive polyuria. Large inclusion bodies with gradually increasing size were present in the supraoptic nucleus (SON). The size of these inclusions developed in parallel with the increases in urine volume. Localization of these aggregates to the ER was shown by electron microscopy. However, counting cells expressing vasopressin mRNA in the SON indicated that polyuria had progressed substantially in the absence of neuronal loss (Hayashi et al. 2009). Taking these different studies together, the use of animal models seems to provide only contradictory information about whether cytotoxicity is the cause for ADNDI or not. Experiments in cell culture seemed to look more promising so up until now.

1.6 Aim of this thesis

Different experiments have shown that ADNDI is linked to a trafficking defect probably caused by misfolding of the mutant precursor. The mutant precursor was shown to be retained and formed disulfide-linked oligomers (Beuret et al. 1999;

Christensen et al. 2004; Friberg, Spiess & Rutishauser 2004; Ito & Jameson 1997; Nijenhuis, Zalm & Burbach 1999; Olias, Richter & Schmale 1996; Siggaard et al. 1999). The role of these oligomers in terms of disease progression is still not clear.

The aim of this thesis was to gain further insights into the mechanism causing ADNDI mutants to be dominant. Using a cell culture approach, we wanted to characterize the structural properties of the precursor leading to retention and inter protein disulfide formation.

3994 Research Article

Dominant pro-vasopressin mutants that cause diabetes insipidus form disulfide-linked fibrillar aggregates in the endoplasmic reticulum

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Summary

Autosomal dominant neurohypophyseal diabetes insipidus results from mutations in the precursor protein of the antidiuretic hormone arginine vasopressin. Mutant prohormone is retained in the endoplasmic reticulum of vasopressinergic neurons and causes their progressive degeneration by an unknown mechanism. Here, we show that several dominant pro-vasopressin mutants form disulfide-linked homo-oligomers and develop large aggregations visible by immunofluorescence and immunogold electron microscopy, both in a fibroblast and a neuronal cell line. Double-labeling showed the pro-vasopressin aggregates to colocalize with the chaperone calreticulin, indicating that they originated from the endoplasmic reticulum. The aggregates revealed a remarkable fibrillar substructure. Bacterially expressed and purified mutant pro-vasopressin spontaneously formed fibrils under oxidizing conditions. Mutagenesis experiments showed that the presence of cysteines, but no specific single cysteine, is essential for disulfide oligomerization and aggregation in vivo. Our findings assign autosomal dominant diabetes insipidus to the group of neurodegenerative diseases associated with the formation of fibrillar protein aggregates.

Key words: Aggregates, Diabetes insipidus, Fibrils, Neurophysin, Vasopressin

Introduction

Familial neurohypophyseal diabetes insipidus, a disorder of water homeostasis, results from lack of the circulating antidiuretic hormone vasopressin, normally produced in vasopressinergic neurons in the hypothalamic magno- and parvocellular nuclei. As a consequence, vasopressin-mediated reabsorption of water from the renal collecting ducts is deficient, causing patients to lose large amounts of unconcentrated urine and to suffer from increased thirst. The disease is caused by mutations in the gene encoding the vasopressin pre-prohormone. The vasopressin precursor consists of a signal peptide of 19 amino acids, the nine-amino acid hormone, a three-amino acid linker, the 93-amino acid carrier protein neurophysin II (NPII), a single linker amino acid and a C-terminal glycopeptide of 39 amino acids (Fig. 1). The precursor is stabilized by the formation of eight disulfide bridges, one in the hormone and seven in the NPII moiety.

Over 50 pathogenic mutations of the human vasopressin gene have been reported that alter the signal peptide, the hormone, or the NPII moieties (Christensen and Rittig, 2006). So far, no mutations have been found in the glycopeptide. Almost all mutations are dominant. Only the P7L mutant (Pro7 of vasopressin mutated to Leu; Willcutts et al., 1999) causes a recessive form of the disease. Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) shows a high penetrance, with symptoms beginning weeks to months after birth. Postmortem histological studies of affected individuals have shown degeneration of the vasopressinergic magnocellular neurons (Bergeron et al., 1991; Braverman et al., 1965; Green et al., 1967; Nagai et al., 1984). A knock-in mouse model expressing the human pathogenic mutant C67X (Cys67 of NPII mutated to a stop codon) confirmed the neurotoxic effect of the mutant protein on vasopressinergic cells (Russell et al., 2003), but the mechanism causing cell death remains unknown.

Expression studies have shown that dominant pro-vasopressin mutants are not secreted, but are retained in the endoplasmic reticulum (ER) (Beuret et al., 1999; Christensen et al., 2004; Friberg et al., 2004; Ito and Jameson, 1997; Nijenhuis et al., 1999; Olias et al., 1996; Siggaard et al., 1999). Most of the retained mutant protein is degraded by cytosolic proteasomes after retrotranslocation (Friberg et al., 2004). Here, we show that newly synthesized mutant precursors form disulfide-linked homo-oligomers and large ER-derived accumulations with a fibrillar ultrastructural appearance. Fibril formation was reproduced by the purified precursor in vitro. The results suggest that disulfide-linked oligomers in part escape degradation and gradually aggregate. Autosomal dominant diabetes insipidus thus belongs to the neurodegenerative diseases associated with fibrillar protein aggregation.

Results

Secretion-deficient dominant mutants form disulfide-linked homo-oligomers

To assay for secretion, wild-type pro-vasopressin, the recessive mutant P7L, and the dominant mutants ΔE47, Y2H, C28Y and ΔG227 were expressed in COS-1 cells, pulse-labeled with [^{35}S]methionine/cysteine, chased for 2 hours, isolated from the cells and from the media by immunoprecipitation, and analyzed by SDS-gel electrophoresis and autoradiography (Fig. 2A). As expected, a considerable fraction of the wild type and of P7L was recovered from the media, reflecting their ability to fold and pass ER quality control. By contrast, the dominant mutants ΔE47, Y2H and C28Y

Journal of Cell Science

2 Part I: Dominant pro-vasopressin mutants that cause diabetes insipidus form disulfide-linked fibrillar aggregates in the endoplasmic reticulum

2.1 Summary

Autosomal dominant neurohypophyseal diabetes insipidus results from mutations in the precursor protein of the antidiuretic hormone arginine vasopressin. Mutant prohormone is retained in the endoplasmic reticulum of vasopressinergic neurons and causes their progressive degeneration by an unknown mechanism. Here, we show that several dominant pro-vasopressin mutants form disulfide-linked homo-oligomers and develop large aggregations visible by immunofluorescence and immunogold electron microscopy, both in a fibroblast and a neuronal cell line. Double-labeling showed the pro-vasopressin aggregates to colocalize with the chaperone calreticulin, indicating that they originated from the endoplasmic reticulum. The aggregates revealed a remarkable fibrillar substructure. Bacterially expressed and purified mutant pro-vasopressin spontaneously formed fibrils under oxidizing conditions. Mutagenesis experiments showed that the presence of cysteines, but no specific single cysteine, is essential for disulfide oligomerization and aggregation *in vivo*. Our findings assign autosomal dominant diabetes insipidus to the group of neurodegenerative diseases associated with the formation of fibrillar protein aggregates.

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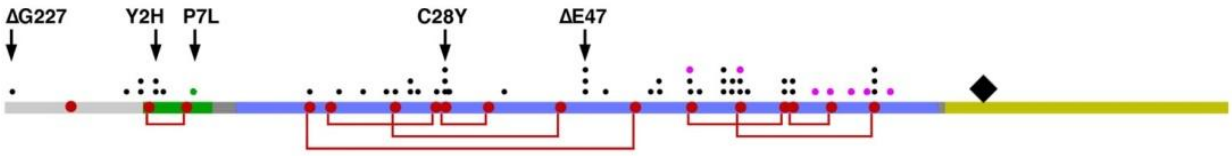

Fig 6: Wild-type and mutant vasopressin precursors. The domain organization of the vasopressin precursor (signal sequence in gray, vasopressin in green, NPII in blue, glycopeptide in yellow) is shown with cysteines as red dots, disulfide bridges as red lines, and the glycosylation site as a diamond. The positions of known ADNDI mutations are indicated as dots above the sequence (black for missense, pink for stop codon mutations). The only recessive mutation is indicated by a green dot. The mutations analyzed in this study are indicated by arrows.

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2.3 Results

2.3.1 Secretion-deficient dominant mutants form disulfide-linked homo-oligomers

To assay for secretion, wild-type pro-vasopressin, the recessive mutant P7L, and the dominant mutants ΔE47, Y2H, C28Y, and ΔG227 were expressed in COS-1 cells, pulse-labeled with [^{35}S]methionine/cysteine, chased for 2 h, isolated from the cells and from the media by immunoprecipitation, and analyzed by SDS-gel electrophoresis and autoradiography (Fig 7A). As expected, a considerable fraction of the wild-type and of P7L was recovered from the media, reflecting their ability to fold and pass ER quality control. In contrast, the dominant mutants ΔE47, Y2H, and C28Y were completely retained in the cells. Only the dominant mutant ΔG227, in which the signal sequence is inefficiently cleaved, was also partially secreted (lanes 13 and 14). The reduced molecular weight of the secreted protein indicates that the uncleaved pre-pro-vasopressin was retained and only cleaved pro-vasopressin, which is identical to wild-type, was allowed to exit.

We have previously observed the formation of disulfide-linked products of retained ΔG227 in COS cells (Beuret et al. 1999). Here, we investigated the formation of disulfide complexes for the wild-type and all five pro-vasopressin mutants in COS-1 and Neuro2a mouse neuroblastoma cells. Transiently transfected cells were radiolabeled with [^{35}S]methionine/cysteine for 1 h and then incubated with iodoacetamide to alkylate free SH-groups and prevent post-lysis oxidation. Vasopressin precursors were immunoprecipitated and separated by gel electrophoresis either under non-reducing conditions or after reduction of disulfide bonds with β-mercaptoethanol. Under reducing conditions, the precursors expressed in Neuro2a cells were found as a ~21 kDa species representing glycosylated pro-vasopressin (Fig 7B). In contrast, non-reducing conditions (Fig 7C) revealed a ladder of bands corresponding in size to prohormone oligomers. Whereas wild-type pro-vasopressin and the recessive mutant P7L produced less than 20% of high-molecular weight forms, the most of the dominant mutant proteins (50–90%) was found in covalent oligomers (Fig 7D). Qualitatively the same result was obtained with expression in COS-1 cells (not shown), indicating that disulfide-linked oligomerization of mutant pro-vasopressin is not a phenomenon specific to neuronal cells.

The strikingly regular spacing of the disulfide-linked products suggested homo-oligomerization of the precursor protein. To test this hypothesis, a lane containing the non-reduced ΔE47 products was cut out, placed horizontally onto a second gel, and overlayed with reducing sample buffer before electrophoresis. All disulfide-linked oligomers collapsed to bands of the same mobility as the monomeric protein (Fig 7E). The same result was observed for the ΔG227 mutant (not shown). The disulfide-linked products generated predominantly by the dominant mutants thus represent homo-oligomers of glycosylated vasopressin precursor.

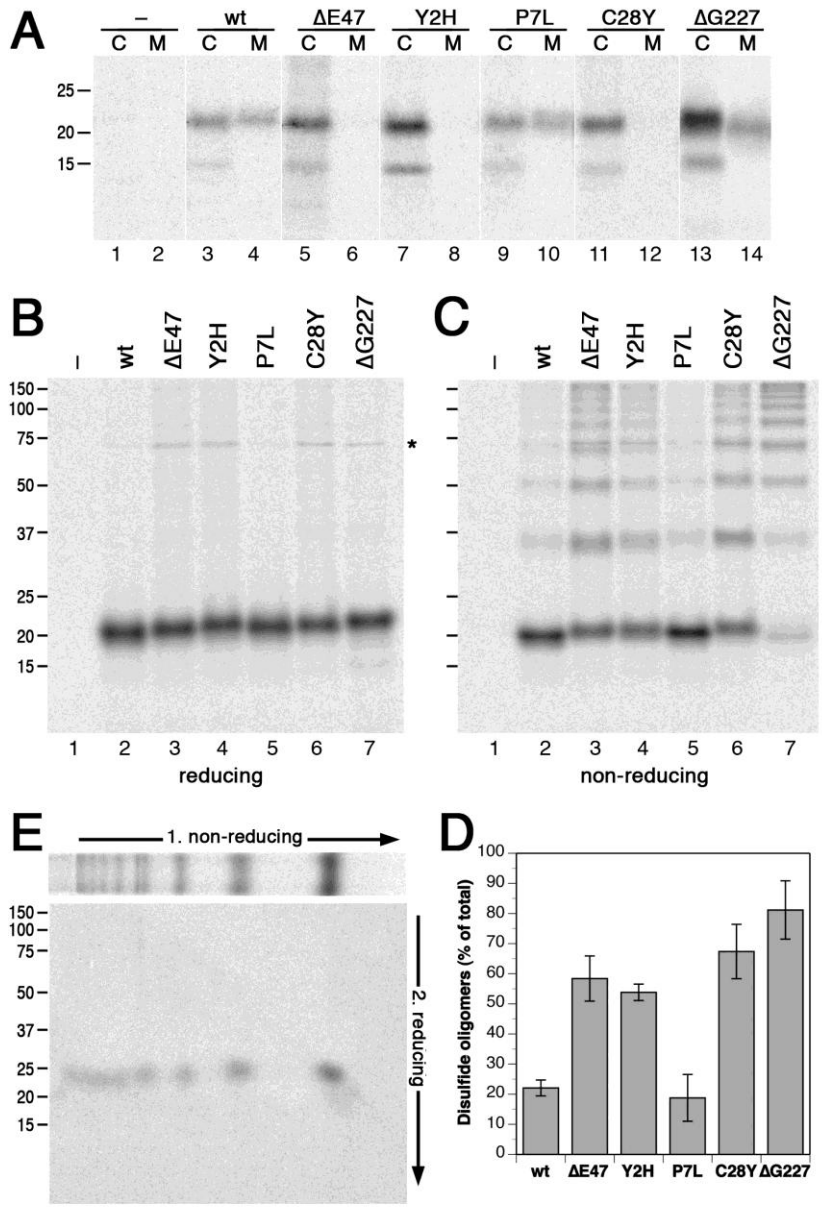

Fig 7: Disulfide-linked pro-vasopressin homo-oligomers. A: Wild-type (wt) vasopressin precursor and the mutants ΔE47, Y2H, P7L, C28Y, and ΔG227were expressed in COS-1 cells, pulse-labeled with [^{35}S]methionine/cysteine for 1 h, and chased for 2 h. Pro-vasopressin was immunoprecipitated from cell lysates (C) and media (M), and analyzed by SDS-gel electrophoresis and autoradiography. The positions of marker proteins are indicated with their molecular masses in kDa. B–E: Untransfected Neuro2a cells (–) and cells expressing wild-type (wt) or mutant vasopressin precursor were pulse-labeled and immunoprecipitated. Precursors were analyzed after reduction of disulfide bonds (panel B) or under non-reducing conditions (panel C) by SDS-gel electrophoresis and autoradiography. The asterisk indicates the position where the spacer gel ends and the separating gel begins. Panel D shows the fraction of disulfide-linked oligomers in percent of the total protein (average and standard deviation of three independent experiments). In panel E, ΔE47 products separated in a first dimension by non-reducing gel electrophoresis were subjected to a second gel electrophoresis after reduction of disulfide bonds.

2.3.2 Dominant pro-vasopressin mutants progressively accumulate as ER-associated aggregates

To characterize the intracellular localization of the vasopressin precursors, transfected COS-1 cells were analyzed by immunofluorescence staining 48 h after transfection (Fig 8). COS cells are particularly suitable to visualize intracellular organelles and aggregations due to their flat cell body and large cytoplasm. As previously shown (Beuret et al. 2004), expression of regulated secretory proteins such as granins and prohormones, including pro-vasopressin, is sufficient to generate granule-like structures in a variety of nonendocrine cell lines due to aggregation in the trans-Golgi. Accordingly, wild-type precursor was concentrated in granular accumulations in the cell periphery of about half the expressing cells (Fig 8A). The recessive mutant P7L showed the same immunofluorescence pattern as the wild-type (Fig 8B), which is in agreement with the observation that P7L was not retained in the ER, but was secreted into the culture medium as was the wild-type prohormone (Fig 7A).

In many cells, the dominant mutants showed strong reticular staining typical for proteins retained in the ER. However, in up to 60% of the transfected cells, they also produced condensed accumulations dispersed throughout the cell (Fig 8C, D, E and F), with morphologies distinct from that of granule-like structures formed by the wild-type protein or the recessive P7L mutant. These accumulations had a coarse, short tubular (Fig 8C, D, and E) or irregular, laminar appearance (Fig 8F). The different morphologies of the aggregates were not specific to particular mutants, but were similarly found for all of them. The accumulations were distinct from mitochondria as there was no costaining with the mitochondrial marker cytochrome c (not shown).

To unambiguously distinguish between granule-like structures and accumulations formed in the ER, co-staining experiments were performed. When wild-type pro-vasopressin and other regulated secretory proteins, for example secretogranin II, were co-expressed in COS-1 cells, they colocalized in granule-like structures (Beuret et al. 2004). This was also the case for the P7L mutant (Fig 9A). By contrast, the secretogranin structures and the accumulations of the dominant mutants were clearly distinct (shown in Fig 9C for C28Y). When COS-1 cells expressing pro-vasopressin

were co-stained with antibodies against NPII and against BAP31 (B cell receptor – associated protein 31), an endogenous ER membrane protein, the granule-like structures of P7L (like those of the wild-type (Beuret et al. 2004)) were not positive for BAP31 (Fig 9B). In contrast, there was extensive co-staining of the C28Y accumulations with BAP31 (Fig 9D), indicating that the aggregations produced by the dominant mutant are located within the ER. The same was observed with all dominant mutants.

COS-1 cells transfected with the four dominant mutants were analyzed by immunofluorescence at different times after transfection (Fig 9E). All mutants produced similar results. At 24 h after transfection, anti-NPII immunostaining showed the reticular ER pattern, identical to that produced by anti-BAP31 antibody staining, in a majority of the cells, whereas less than 30% of transfected cells showed distinct aggregates. After 48 and 72 h, the percentage of aggregate-forming cells increased to ~55% and ~75%, respectively, and the aggregations generally increased in size. As a control, we also analyzed the immunofluorescence staining of COS-1 cells expressing an ER-retained mutant of cathepsin Z, a secretory protein with 5 disulfide bonds. In these experiments, the frequency of apparent aggregations did not increase with time above the control levels of ~ 20%. Large aggregates and disulfide-oligomers were also produced by dominant vasopressin mutants expressed in CV-1 cells, the parental cell line of COS-1 cells, lacking the large T-antigen needed for high level expression (not shown). Thus, the formation of aggregates was not an artefact of excessive expression levels due to plasmid amplification.

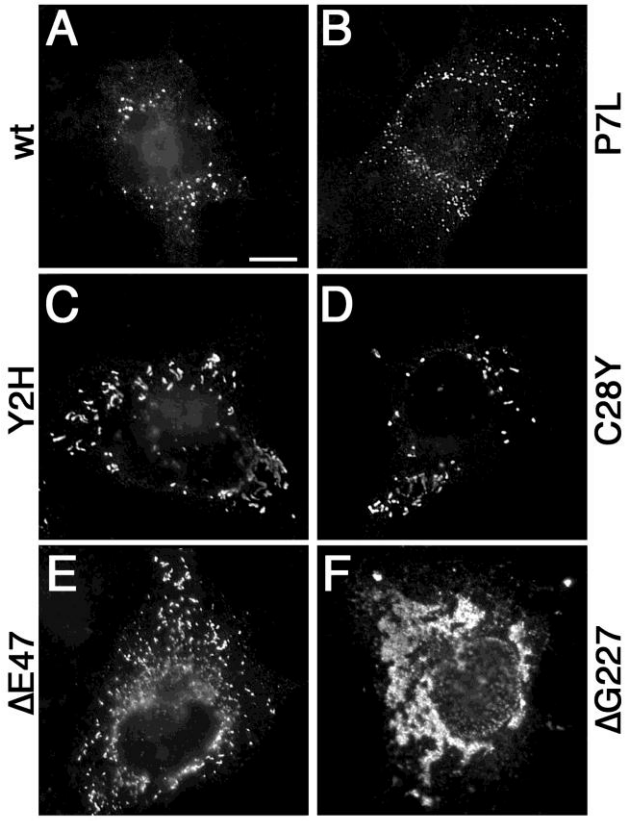

Fig 8: Intracellular accumulation of wild-type and mutant pro-vasopressin in COS-1 cells. COS-1 cells expressing wild-type pro-vasopressin or the indicated mutants were subjected to immunofluorescence staining against NPII 48 h post transfection. As expected, approximately half the cells expressing wild-type pro-vasopressin or P7L produced mostly round granule-like structures (A and B). Conversely, the dominant mutants showed accumulations that were mostly elongated or laminar (C–F). The different shapes did not correlate with specific mutations. Scale bar, 20 µm.CatZ mut), another ER-retained secretory protein.

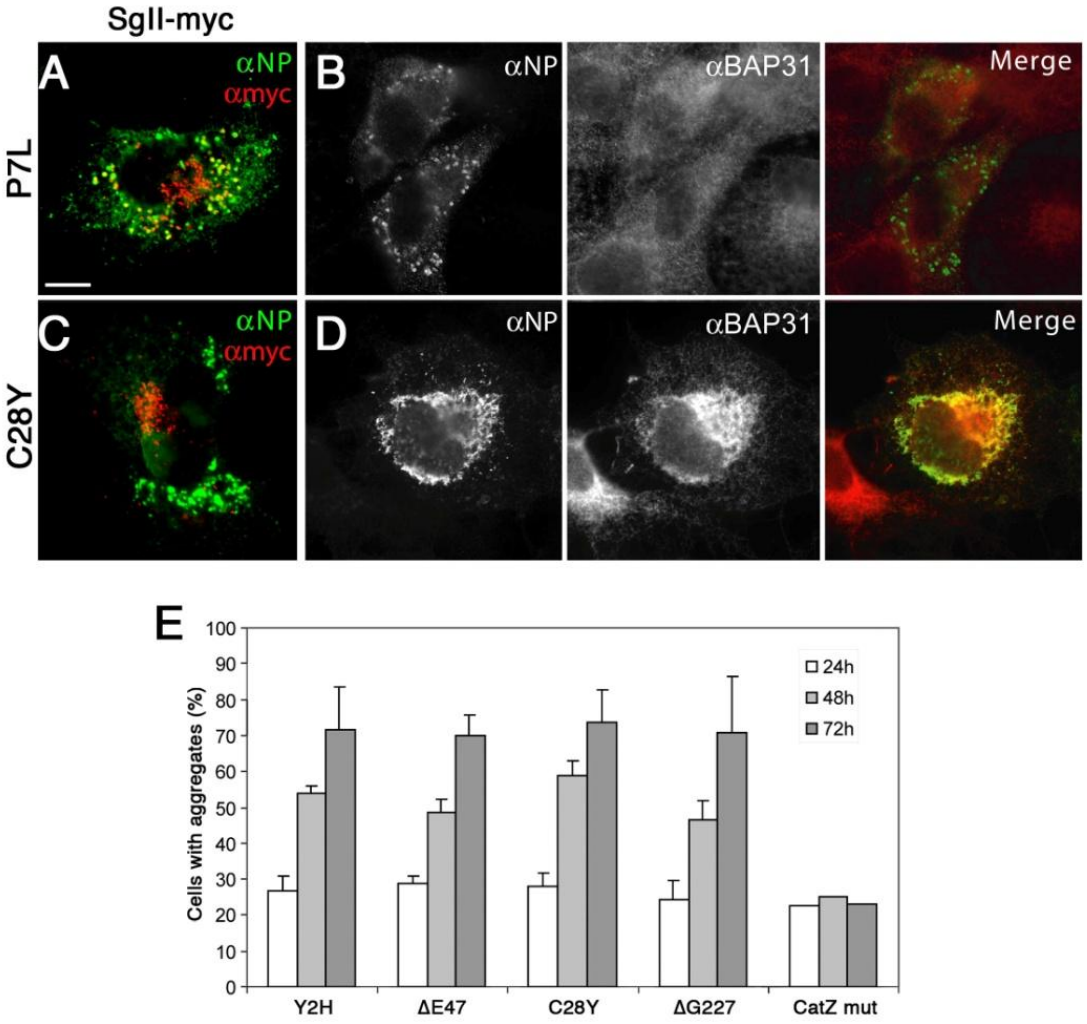

Fig 9: Gradual development of aggregations of dominant pro-vasopressin mutants in the ER. COS-1 cells expressing the recessive pro-vasopressin mutant P7L (A and B) or the dominant mutant C28Y (C and D) were stained 48 h post transfection for NPII in green, and either for cotransfected secretogranin II-myc (SgIImyc; A and C) as a marker for granule-like structures, or against endogenous BAP31 (B and D) in red. Scale bar, 20 µm. In panel E, COS-1 cells transfected with each of the four dominant pro-vasopressin mutants were analyzed by immunofluorescence staining after 24, 48, or 72 h and the cells with visible aggregates of any kind were quantified. As a control, the experiment was performed in parallel for a mutant cathepsin Z (CatZ mut), another ER-retained secretory protein.

2.3.3 Mutant pro-vasopressin aggregates show filamentous ultrastructure

COS-1 cells expressing dominant mutants were analyzed by immunogold electron microscopy 48 h after transfection (Fig 10). As expected, gold staining was found in many cells in ER tubules cut longitudinally or across, some of which had a dilated appearance (panels A and A'). Other cells contained multiple large structures of ~1 µm in diameter decorated with 15-nm gold for pro-vasopressin (panels B, B', and C), corresponding to the bright accumulations seen by immunofluorescence (Fig 8C, D, and E). In some cells, large regions were densely filled with labeled structures (Fig 10D and D') that might appear almost continuous in immunofluorescence (as in Fig 8F), adjacent to regions with normal-sized ER tubules of ~60-90 nm diameter. With double-labeling, the pro-vasopressin aggregations stained for NPII with 15-nm gold were also found to be decorated with 10-nm gold for the ER chaperone calreticulin (Fig 10C), confirming that these are ER-derived structures. In some micrographs, the core of the aggregates appeared to be structured and gold particles were aligned in continuous rows (Fig 10B'), suggesting a filamentous arrangement of the antigen.

To test their behavior also in a neuronal cell type mimicking the physiological situation more closely, dominant pro-vasopressin mutants were expressed in Neuro2a cells and analyzed by immunofluorescence and electron microscopy. By confocal microscopy, again dispersed accumulations of pro-vasopressin could be observed that were strongly costained for the ER chaperone Hsp47 (heat shock protein 47; Fig 11). At the ultrastructural level, pro-vasopressin was found by immunogold staining in compact accumulations of similar size to those in COS-1 cells (Fig 11A and enlarged areas in B and C). The aggregations are structured and appear to be composed of tangled filaments of approximately 10–15 nm diameter (Fig 12B–F). Using double-labeling, again both mutant pro-vasopressin and calreticulin were found to decorate these structures (Fig 12E and F), demonstrating that they are derived from the ER. Clearly, aggregations containing mutant pro-vasopressin are formed not only in fibroblasts, but also in neuronally derived cells, which more closely mimic the situation *in vivo*.

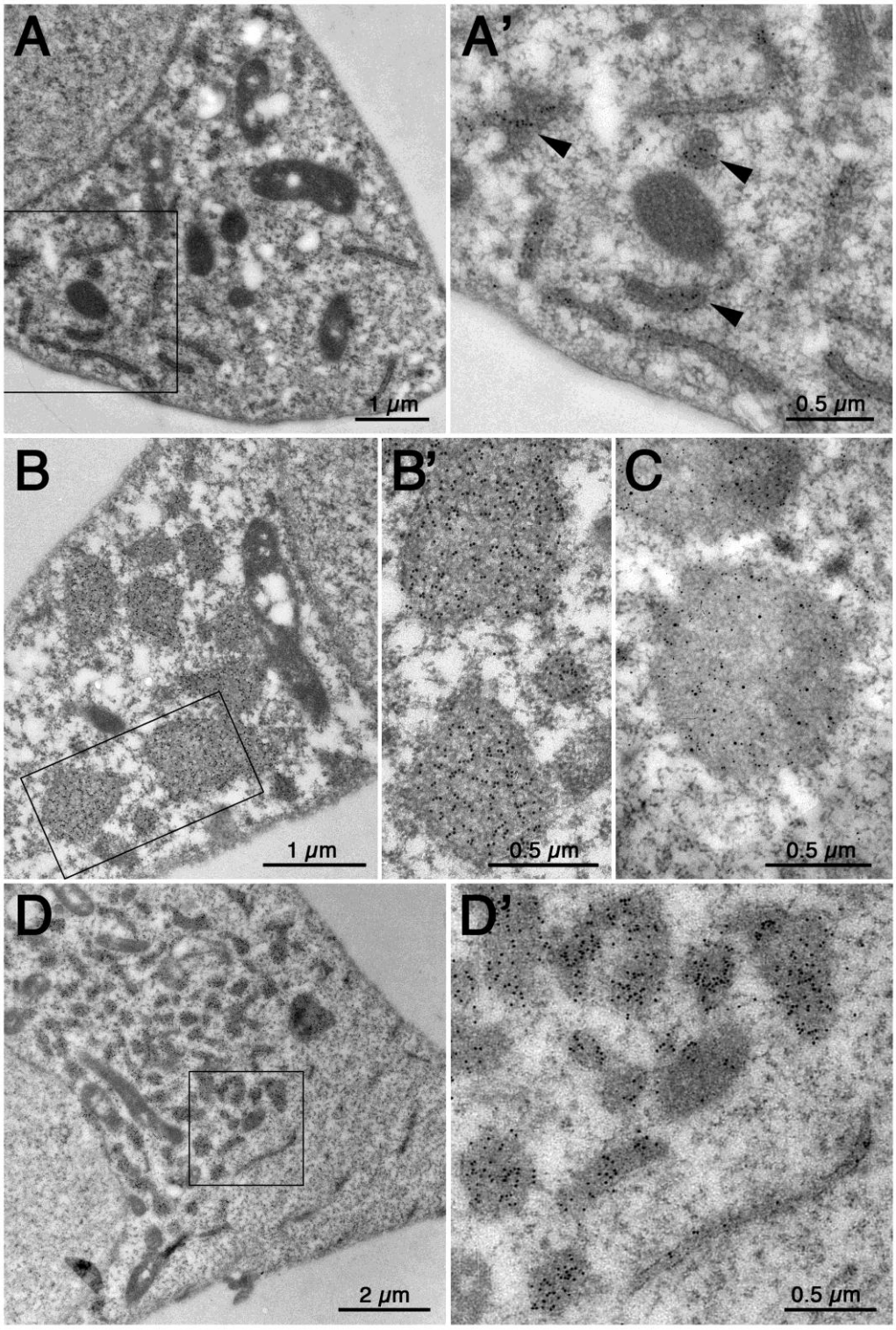

Fig 10: Mutant pro-vasopressin aggregations in COS-1 cells visualized by electron microscopy. COS-1 cells transfected with ΔE47 (A–C) or ΔG227 (D) were analyzed by immunogold electron microscopy using 15-nm gold to decorate pro-vasopressin. In panel C, the section was double labeled in addition with 10-nm gold for the ER chaperone, calreticulin. Boxed areas in A, B, and D are shown as enlargements in A', B', and D'. Arrowheads in panel A' point out dilated ER elements.

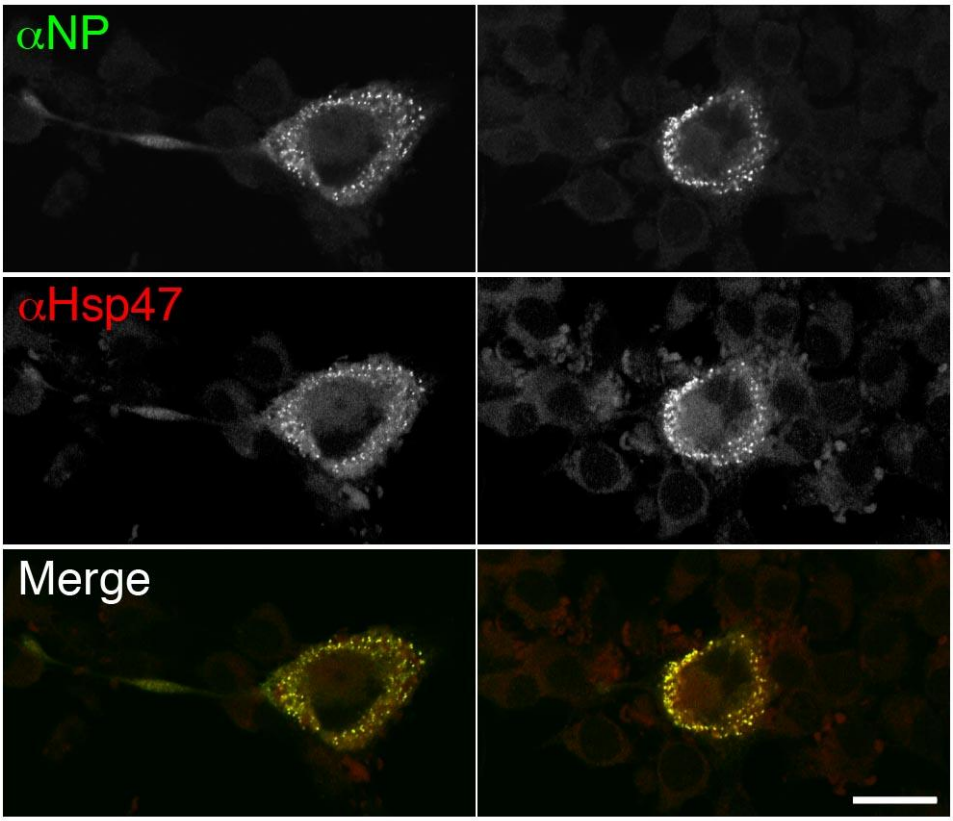

Fig 11: Accumulation of mutant pro-vasopressin in Neuro2a cells. Differentiated Neuro2a cells expressing C28Y pro-vasopressin was stained 48 h post transfection for NPII in green, and for the ER chaperone Hsp47 in red and analyzed by confocal microscopy. Scale bar, 20 µm.

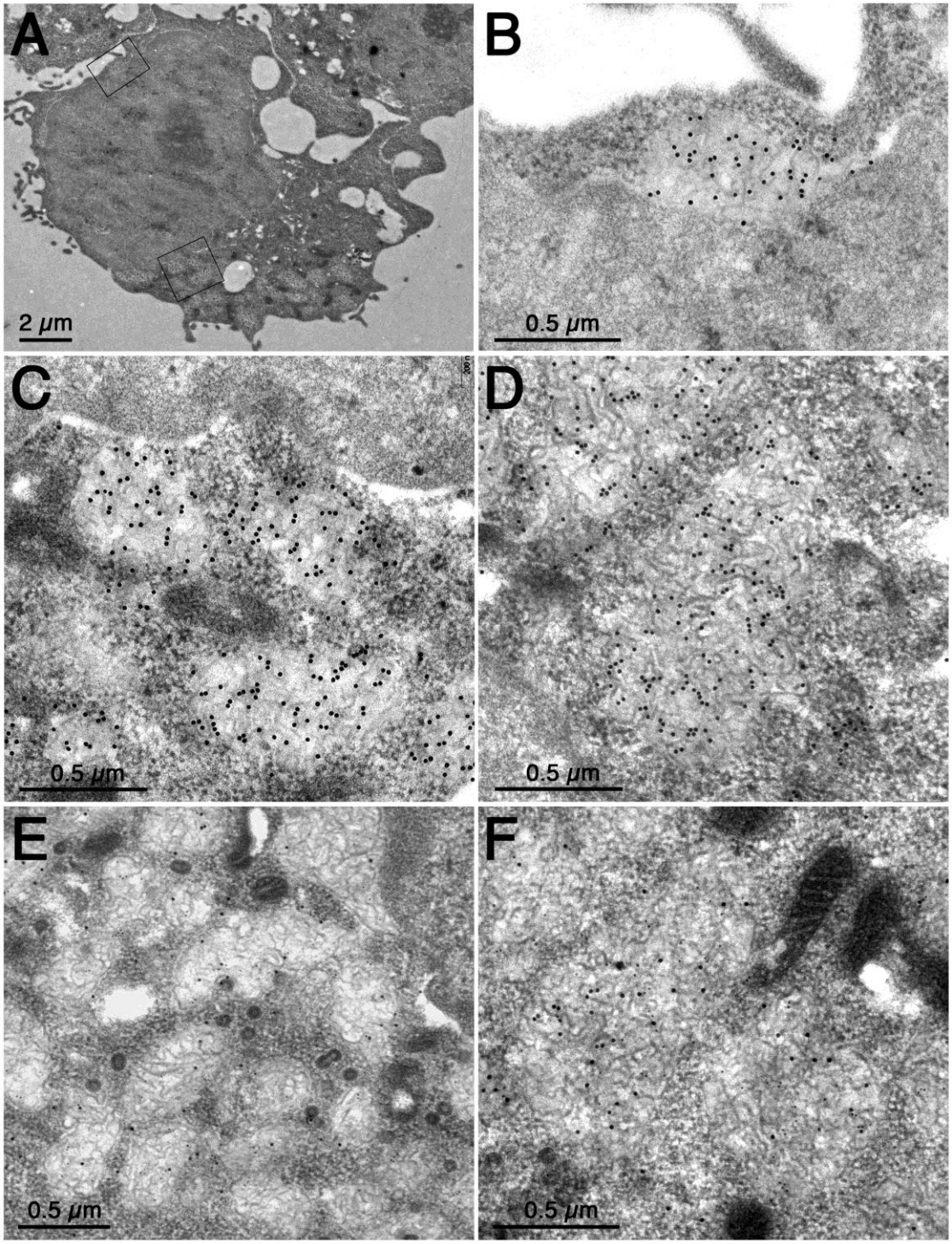

Fig 12: Fibrillar ultrastructure of mutant pro-vasopressin aggregations in Neuro2a cells. Immunogold electron microscopy of Neuro2a cells expressing ΔE47. Pro-vasopressin was stained with 15-nm gold. In E and F, in addition to the pro-vasopressin staining the ER marker calreticulin was stained with 10-nm gold. Boxed areas in A are shown as enlargements in B and C.

2.3.4 Purified pro-vasopressin spontaneously forms fibrils after removal of denaturant

To test the ability of pro-vasopressin to form fibrils in isolation, the ΔE47 mutant was expressed with a C-terminal His_6 tag replacing the signal sequence in *Escherichia coli*, recovered in inclusion bodies, solubilized with 8 M urea, and purified under reducing conditions by metal chelate affinity chromatography. After removal of urea and reducing agent by dialysis and after pelleting amorphous precipitates by centrifugation, fibrillar structures of defined diameter were detected in the supernatant by electron microscopy (Fig 13). No fibrils were observed when reducing conditions were maintained throughout. These results show that pro-vasopressin can form fibrils *in vitro* in the absence of any other cellular proteins and that oxidation is necessary to stabilize them.

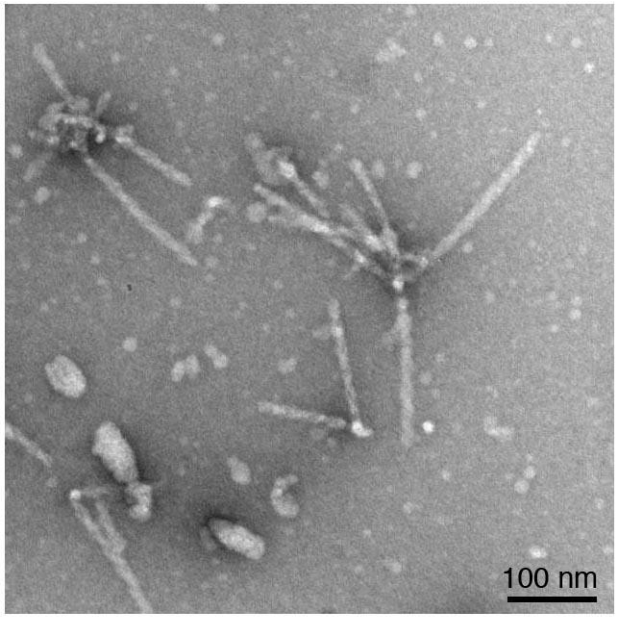

Fig 13: *In vitro* formation of fibrils from purified pro-vasopressin ΔE47. Bacterially expressed pro-vasopressin ΔE47 with an C-terminal His_6-tag was purified by metal chelate affinity chromatography in 8 M urea and 1 mM DTT. Upon removal of urea and DTT (as described in Materials and Methods), fibrils of defined diameter were produced visible by electron microscopy.

2.3.5 Cysteine residues are required for aggregate formation

To investigate whether the formation of disulfide-linked oligomers and of large aggregations is dependent on specific cysteines, we generated artificial mutants in which the cysteines in any of four segments of the precursor protein were replaced by serines or in which all 16 cysteines were mutated (Fig 14A). Mutation of cysteines is almost certainly preventing native folding of the precursor. (Indeed, 10 of the 16 have been found mutated in diabetes insipidus alleles.) These mutants were tested for the formation of disulfide-linked oligomers in Neuro2a cells (Fig 14B and C) and of aggregates in COS-1 cells (Fig 14D and E). Mutation of any one group of cysteines still allowed efficient formation of disulfide oligomers and the appearance of large aggregations. None of the cysteines is thus essential for disulfide oligomerization or aggregation. As expected, the mutant without any cysteines did not produce covalent homo-oligomers (Fig 14B, abcd). This cysteine-less mutant also did not induce the formation of aggregates detectable by immunofluorescence above background (Fig 14D and E). When these mutants were tested for ER retention, two constructs, aBCD and abcd, surprisingly were found to be partially secreted into the medium (Fig 14G, lanes 10 and 14).

To exclude the possibility that the absence of aggregates with construct abcd is due to reduced ER concentrations, the ER retention signal KDEL was fused to the C-terminus. This effectively prevented secretion of both aBCD^{KDEL} and abcd^{KDEL} (lanes 12 and 16). The cysteine-less construct, even when retained in the ER as abcd^{KDEL}, did not increase aggregate formation above backgound levels (Fig 14D and E). Even at the ultrastructural level, no aggregations could be observed for abcd, but there was dispersed staining in the ER (mostly tubular elements in the cytosol and the nuclear envelope; Fig 14F).

To test the ability of the cysteine-free construct to form fibrils *in vitro*, abcd without the signal sequence was expressed in bacteria. Unlike ΔE47 pro-vasopressin, which accumulated in inclusion bodies, abcd remained soluble in the bacterial cytoplasm. After purification in 8 M urea, dialysis to remove the denaturant and dithiolreitol (DTT), and analysis by electron microscopy, hardly any structures resembling the fibrillar aggregates as found for ΔE47 in a parallel experiment could be detected (not shown). Together with the *in vivo* results this suggests that, whereas no individual

cysteine in the precursor is necessary for oligomerization or aggregation, the formation of fibrils and aggregates is dependent on the presence of cysteine residues, irrespective of their positions within the precursor protein.

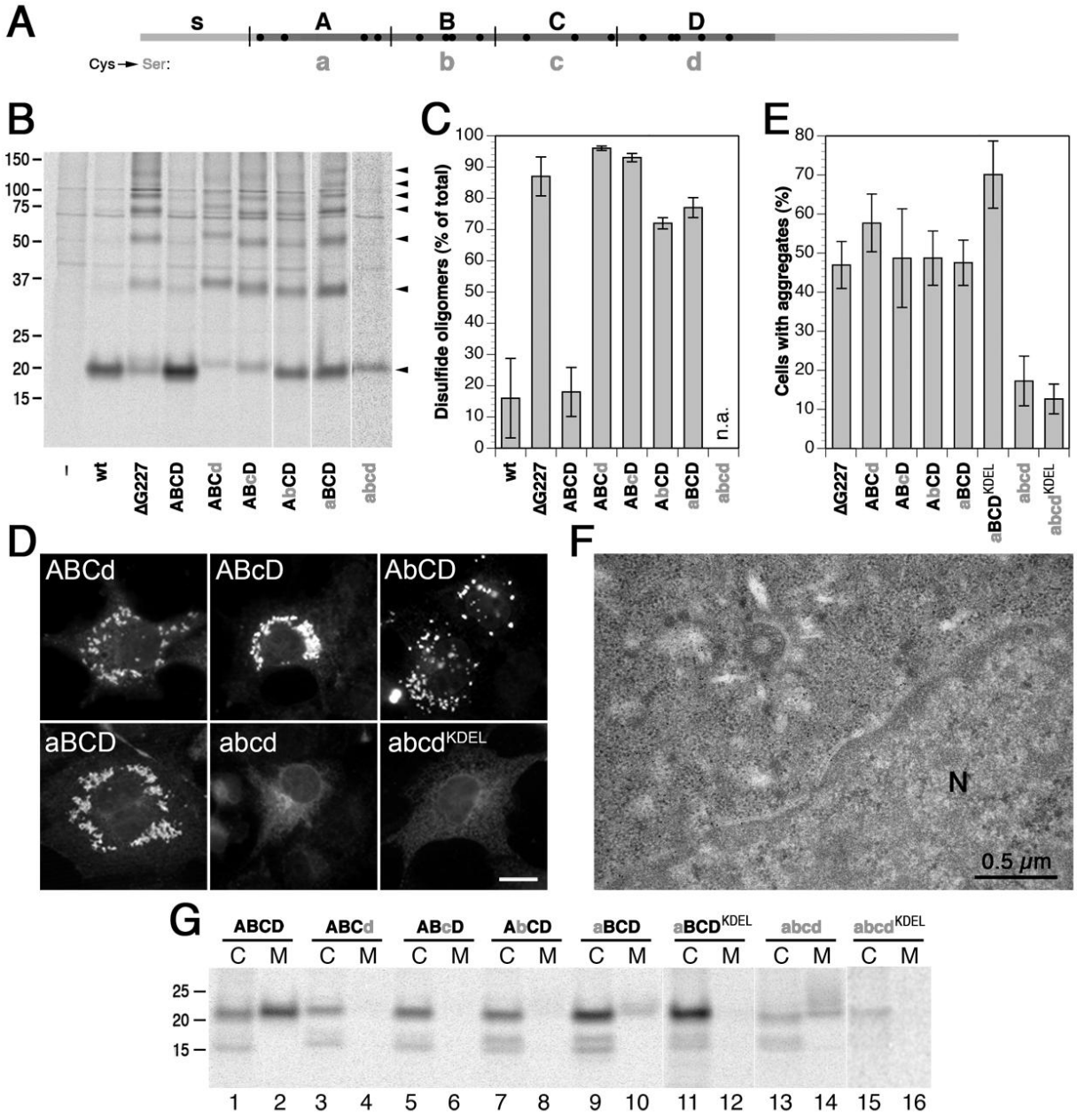

Fig 14: Contribution of cysteines to the formation of aggregates. A: The 16 cysteines in pro-vasopressin (indicated as black dots) were mutated in groups corresponding to four segments (A, B, C, D) to serines (a, b, c, d). s indicates the signal sequence (in these constructs, the pre-pro-enkephalin signal replaced the natural signal peptide). B: To analyze the formation of disulfide-linked oligomers, Neuro2a cells were transfected with the indicated constructs and labeled for 1 h with [^{35}S]methionine/cysteine. After alkylation of free SH groups, pro-vasopressin was immunoprecipitated

and analyzed by SDS-gel electrophoresis under non-reducing conditions. The experiment with untransfected cells (–) reveals the non-specific bands. The positions of molecular weight standards (in kDa) are indicated. The absence of cysteines in construct abcd (with a single methionine in NPII) accounts for its low labeling signal. C: The signal of homo-oligomers (arrowheads in panel B; not applicable [n.a.] for abcd) were quantified and shown as percent of the total (mean and standard deviation of three independent experiments). No specific cysteine cluster was necessary for the formation of oligomers. D: COS-1 cells expressing the indicated constructs were amalyzed by immunofluorescence microscopy after staining for pro-vasopressin 2 d after transfection. Typical examples are shown. Scale bar, 20 µm. E: The frequency of aggregate formation was quantified from immunofluorescence micrographs as in panel D (mean and standard deviation of three independent experiments). Wild-type pro-vasopressin (from the original cDNA [wt] and from the cDNA containing silent mutations introducing additional restriction sites [ABCD]) produced post-Golgi granule-like structures and were not included in this analysis. Background as determined for mutant CatZ in Fig 9E is ~15–20%. F: Electron micrograph of a Neuro2a cell expressing the cysteine-free construct abcd stained for pro-vasopressin with 15-nm gold . Distributed staining on the nuclear envelope and cytoplasmic ER is observed, but no aggreagations. N: nucleus. G: Secretion of the cysteine mutants expressed in COS-1 cells was analyzed in a pulse-chase experiment as in Fig 7A. Constructs $aBCD^{KDEL}$ and $abcd^{KDEL}$ correspond to aBCD and abcd with a C-terminal ER retention sequence.

2.4 Discussion

2.4.1 Dominant pro-vasopressin mutants cause ER retention due to misfolding

Many different mutations in the vasopressin precursor have been shown to cause diabetes insipidus. Only two of these are recessive. The human P7L mutation in the vasopressin nonapeptide (Willcutts, Felner & White 1999) greatly reduces hormone binding to its renal receptor, but does not affect precursor folding and secretion. In addition, the Brattleboro mutation in rat, a frameshift mutation in the gene segment encoding NPII, shows a recessive phenotype (Schmale & Richter 1984). Here, the complete absence of a stop codon in the new reading frame results in highly inefficient translation of the mutant protein, resulting essentially in a null allele. All other known mutations in the vasopressin precursor are dominant, eventually causing death of the vasopressin-producing neurons.

The mutations are diverse and not localized to a specific region in the precursor protein, except that no pathogenic mutations have been identified within the C-terminal glycopeptide. Cytotoxicity appears therefore not to be caused by a specific effect of each individual mutation within the prohormone, but to result from consequences common to all the mutations. This notion is supported by two mutations in the signal sequence, ΔG227 (resulting in a signal truncation; (Beuret et al. 1999)) and A(-1)T (mutation of the signal cleavage site (Ito et al. 1993)) In both cases, removal of the signal peptide is impaired, but the pro-vasopressin sequence itself remains unchanged. A common effect of all dominant mutants tested is their retention in the ER, most likely due to misfolding. For frameshift and point mutations affecting cysteine residues normally involved in disulfide bonds, misfolding is an obvious consequence. Failure to cleave the signal sequence has a more subtle effect, as it prevents the N-terminus of the vasopressin sequence from folding into NPII, a requirement for the stabilization of the disulfide bond within the hormone segment (Beuret et al. 1999; Chen et al. 1992). How can misfolding and ER retention cause cytotoxicity?

2.4.2 Mutant pro-vasopressins form disulfide-linked homo-oligomers, fibrils and large aggregations

At least half of newly synthesized precursor proteins of all four dominant mutants were found to be part of disulfide-linked homo-oligomers. This indicates a specific interaction between unfolded pro-vasopressin polypeptides, bringing together cysteines to form intermolecular crosslinks. By *in vitro* mutagenesis, we tested which cysteines might be essential for this process. Whereas the presence of some cysteines was required for the formation of disulfide-linked oligomers as well as aggregations visible by immunofluorescence, no single cysteine was specifically necessary.

Disulfide-linked homo-oligomers were also observed for wild-type pro-vasopressin and the recessive mutant P7L, but to a much lower extent. Not yet folded protein appears to be transiently crosslinked into disulfide-linked homo-oligomers, but subsequently to be rescued when the free molecules successfully fold into the native structure. Indeed, even the mutant proteins are not irreversibly trapped in disulfide aggregates because we have previously found them to be quite efficiently degraded by the cytosolic proteasome (Friberg, Spiess & Rutishauser 2004). Only a small fraction thus escapes ER-associated degradation to accumulate in large aggregates visible by fluorescence and electron microscopy. Consistent with this, it takes 2–3 days for a majority of expressing cultured cells to develop visible aggregates.

Electron microscopic analysis showed that the pro-vasopressin accumulations have a fibrillar ultrastructure. The ability of pro-vasopressin to form ordered, linear polymers by self-assembly was confirmed *in vitro* with bacterially expressed and purified protein. The vasopressin precursor thus resembles a whole family of proteins that generate amyloid fibers and are associated with neurodegenerative disorders.

2.4.3 A model for the formation of fibrillar aggregates in the ER

Taken together, our results suggest a model for aggregate formation in ADNDI as illustrated in Fig 15. Upon translocation into the ER lumen, vasopressin precursor

might acquire a conformation that leads to homo-oligomers which are stabilized by intermolecular disulfide-links between exposed cysteine residues. This process is reversible and thus allows wild-type proteins to fold into their native structure and exit the ER, and allows folding-deficient mutants to be retrotranslocated into the cytosol for proteasomal degradation. A significant fraction of mutant precursors accumulate as growing oligo- and polymers, forming fibrils and eventually large visible aggregates aqssociated with ER chaperones.

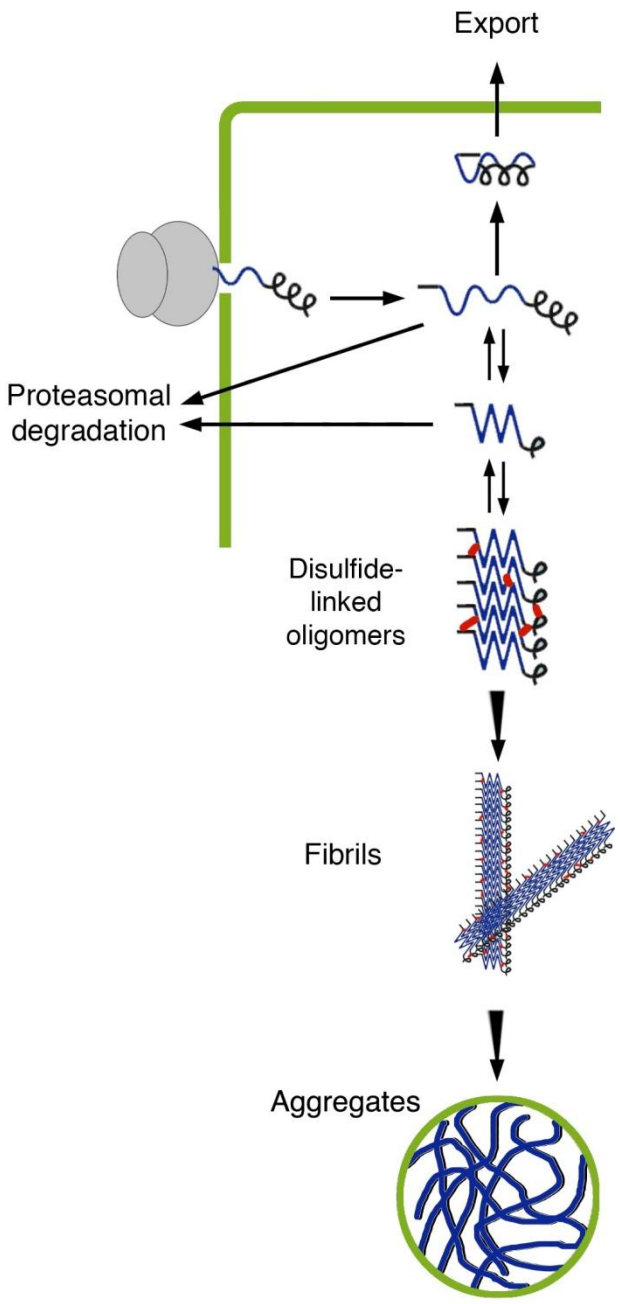

Fig 15: Model for the development of fibrillar aggregates in the ER. Pre-pro-vasopressin is synthesized into the ER to undergo folding and quality control. Natively folded precursor leaves the ER and exits the cell via the secretory pathway and secretory granules. Misfolded precursor may either be retrotranslocated for subsequent proteasomal degradation, or form specific oligomers and polymers, stabilized by disulfide-links (in red), growing to fibrils and generating inclusions within the ER, visible by light microscopy.

2.4.4 Role of aggregates for cytotoxicity

The formation of fibrillar aggregations and inclusion bodies are frequent pathological features of neurodegenerative disorders (Ross & Poirier 2005). In contrast to the extensively studied aggregations associated with Alzheimer's, Parkinson's, and Huntington's disease that are extracellular, cytoplasmic, or intranuclear, the aggregations formed by dominant pro-vasopressin mutants are produced within the lumen of the ER.

The role of aggregation in degenerative disease processes is controversial because there is often no direct correlation between the amount of inclusions and cell degeneration (Chun et al. 2002; Ross & Poirier 2005). The formation of large aggregates and inclusion bodies might represent a cellular protective response and early protofibrils might be the neuropathogenic species triggering disease (Lansbury & Lashuel 2006; Ross & Poirier 2005). In a diabetes insipidus knock-in mouse expressing the cytotoxic pro-vasopressin mutant C67X, induction of the ER chaperone BiP was detected, but no obvious aggregations were apparent (Russell et al. 2003). No upregulation of the ER stress-induced proapoptotic transcription factor C/EBP homologous protein (CHOP) or other evidence for apoptosis was observed. However, aggregate-producing cells might have been rapidly eliminated. In contrast, vasopressinergic neurons expressing the same C67X mutant in transgenic rats were resistant to a cytotoxic effect, but produced distended ER structures containing the mutant protein and markers of autophagy (Davies & Murphy 2002; Si-Hoe et al. 2000). This lysosomal degradation mechanism is thought to protect against toxic effects of altered or aggregated intracellular proteins (Martinez-Vicente & Cuervo 2007). Inhibition of autophagy was found to compromise viability of C67X-expressing rat neurons and Neuro2a cells (Castino et al. 2005). Interestingly, we could not detect significant colocalization of the dominant mutant C28Y with Lamp1 or LC3 (markers for lysosomes and autophagosomes respectively) neither in COS-1 nor Neuro2a cells (unpublished results). It remains to be analyzed in detail to which extent the disulfide-linked homo-oligomers of pro-vasopressin mutants detected biochemically and the large aggregations observed by light and electron microscopy contribute to cytotoxicity.

2.5 Materials and methods

2.5.1 Plasmids and constructs

The cDNAs of the human wild-type vasopressin precursor and the mutants ΔG227, ΔE47, and C67X, as well as of myc-tagged secretogranin II have been described before (Beuret et al. 1999; Friberg, Spiess & Rutishauser 2004). The cDNAs encoding the vasopressin mutants Y2H and P7L were a gift from Jane Christensen (Århus University Hospital, Denmark). The C28Y mutant was generated by polymerase chain reaction. The expression plasmid of human pre-pro-cathepsin Z mutant (N184Q, N224Q) (Appenzeller-Herzog et al. 2005) was a gift from Hans-Peter Hauri and Beat Nyfeler (Biozentrum, University of Basel). All human cDNAs were subcloned into the pRc/RSV expression plasmid (Invitrogen).

The 16 cysteines in pro-vasopressin were mutated to serines by PCR mutagenesis. For this purpose, the signal sequence was replaced by that of pre-pro-enkephalin as described (Cescato et al. 2000; Friberg, Spiess & Rutishauser 2004), providing a *Bsr*GI restriction site near the start of pro-vasopressin. Silent mutations were introduced to insert a *Kpn*I site at codons 49/50 of the pro-vasopressin coding sequence and a *Bam*HI site at codons 64/65. Together with a natural *Sma*I site at codons 27/28 and external *Sal*I site, these restriction sites served to clone and recombine PCR amplified mutant segments. All constructs were verified by DNA sequencing.

2.5.2 Cell culture and transient transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37°C in 7.5% CO_2. COS-1 cells were transiently transfected in 6-well plates using Lipofectine (Life Technologies) or FuGENE HD (Roche) and analyzed 2–3 days after transfection or as indicated. Neuro2a cells were grown in DMEM containing 4500 mg/l glucose, supplemented as above. They were transfected using Metafectene (Biontex Laboratories). To induce differentiation, cells

were cultured from 1 d before transfection in 1 mM valproic acid in DMEM with 3% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine.

2.5.3 Metabolic labeling and immunoprecipitation

For labeling experiments, transfected cells were starved for 30 min. in DMEM without methionine and cysteine (Sigma) supplemented with 2 mM L-glutamine. Cells were labeled with 100 μCi/ml [^{35}S]protein labeling mix (PerkinElmer) and chased with excess methionine and cysteine. Cells were washed in phosphate-buffered saline (PBS), lysed in 500 μl of lysis buffer (PBS, 1% TritonX-100, 0.5% deoxycholate, 2 mM phenylmethylsulfonylfluoride), and scraped. Cell lysates or media were incubated with rabbit polyclonal anti-NPII (Friberg, Spiess & Rutishauser 2004) in lysis buffer containing 1 mM phenylmethylsulfonylfluoride, 0.5% SDS and 1 mg/ml BSA. Bound proteins were immunoprecipitated with protein A-Sepharose (Zymed) and analyzed by electrophoresis on 10% polyacrylamide Tris/tricine SDS-gels and autoradiography.

2.5.4 Analysis of disulfide-linked oligomers

Transfected and labeled cells were washed with cold PBS containing 100 mM iodoacetamide, incubated at 4°C for 1 h in the dark with 200 μl 200 mM Tris, pH 8.0, 100 mM iodoacetamide, 2mM phenylmethylsulfonyl fluoride, lysed in lysis buffer containing 100 mM iodoacetamide, and immunoprecipitated. One half of each sample was boiled in SDS-sample buffer with and the other half without β-mercaptoethanol before gel electrophoresis and autoradiography. The amount of protein present as disulfide-linked aggregates (signal of high molecular weight oligomers as percent of total signal for each construct) was quantified by phosphorimager.

For analysis in the second-dimension, a lane containing a non-reduced sample was cut out and soaked in sample buffer containing 200 mM β-mercaptoethanol for 1 min. The gel strip was then loaded horizontally onto a second Tris/tricine SDS-gel, whose

stacking gel was supplemented with 50 mM β-mercaptoethanol, and overlayed with reducing sample buffer for electrophoresis.

2.5.5 Immunofluorescence

Transfected cells were grown for 24–72 h on glass coverslips, fixed with 3% paraformaldehyde for 30 min at room temperature, washed in PBS, permeabilized and blocked with 0.1% saponin, 20 mM glycine, 1% bovine serum albumin (BSA) in PBS for 20 min, incubated at room temperature with primary antibodies for 2 h in PBS containing 0.1% saponin, 20mM glycine and 1% BSA; washed, and stained with fluorescent secondary antibodies in PBS containing 0.1% saponin for 30 min After several washes with saponine/PBS, the coverslips were mounted in Mowiol 4-88 (Hoechst). As primary antibodies, we used the polyclonal rabbit anti-NPII antiserum, mouse monoclonal anti-myc antibody 9E10, and a mouse monoclonal antibody against the endogenous ER membrane protein BAP31 (a gift from Hans-Peter Hauri; (Klumperman et al. 1998)). As secondary antibodies, non-cross-reacting Cy3-labeled goat anti-mouse and Cy2-labeled goat anti-rabbit immunoglobulin antibodies (Jackson Immunoresearch and Amersham Biosciences, respectively) were used according to the manufacturers' recommendations. Staining patterns were analyzed with a Zeiss Axioplan 2 microscope with a Leica DFC420C imaging system or for Neuro2a cells with a Zeiss Confocal LSM510 Meta microscope.

2.5.6 Electron microscopy

Cultured cells were fixed in 3 % formaldehyde and 0.2% glutaraldehyde for 2 h at room temperature and after addition 1% picric acid overnight at 4°C, scraped, pelleted, resuspended and washed 3 times in PBS, treated with 50 mM NH_4Cl in PBS for 30 min to quench free aldehydes, and washed 3 times in PBS. The final pellet was resuspended in 2% warm agarose and left on ice to solidify. Agarose pieces were dehydrated and infiltrated with LR-gold resin (London Resin, London, UK) according to the manufacturer's instructions and allowed to polymerize for 1 day at 4°C. For immunogold labeling, sections of about 60 nm were collected on carbon-

coated Formvar-Ni-grids, incubated with rabbit anti-NPII in PBS, 2% BSA, 0.1% Tween-20 for 2 h, washed with PBS, and incubated with 15-nm colloidal gold-conjugated goat anti-rabbit immunoglobulin antibodies (BioCell, Cardiff) in PBS, 2% BSA, 0.1% Tween-20 for 90 min Grids were washed 5 times for 5 min in PBS and then 5 times in H_2O, before staining for 10 min in 2% uranylacetate followed by 1 min in Reynolds lead citrate solution. Sections were viewed in a Phillips CM100 electron microscope.

For double labeling, the first labeling was performed using rabbit anti-human calreticulin antibody (Stressgen) and 10-nm colloidal gold-conjugated goat anti-rabbit immunoglobulin antibodies as described above. After washing 6 times for 2 min with PBS, the sections were fixed again for 2 h with 1% formaldehyde and 1% glutaraldehyde in PBS, washed with PBS, and quenched for 10 min with 50 mM NH_4Cl. The sections were incubated with PBS, 2% BSA, 0.1% Tween-20 for 10 min followed by incubation for 90 min with rabbit anti-NPII immunoglobulins that had been purified from serum by a protein A–Sepharose column (Amersham Pharmacia Biotech), pre-coupled to 15-nm gold–protein A (BioCell) at a molar ratio of 2:1, and incubated with a twofold excess of unrelated mouse IgG. Grids were washed and stained as above. As negative controls to ensure specificity of labeling, cells were stained as above, except that 15-nm gold–protein A was used that had not been loaded with anti-NPII immunoglobulins. This produced very low background labeling (not shown).

2.5.7 Bacterial expression and *in vitro* fibril formation

For bacterial expression, the coding sequence of ΔE47 and cysteine-free (abcd) pro-vasopressin (without the signal sequence) were cloned into the plasmid pET21b (Novagen) and thus provided with a C-terminal His_6 tag. The proteins were expressed in E. coli BL21(DE3) after induction with 1 mM isopropyl-D-thiogalactoside for 2–3 h, solubilized from inclusion bodies (ΔE47) or the cytosol fraction (abcd) in 8 M urea, 10 mM Tris-HCl, pH 8, 1 mM DTT, and protease inhibitors (10 µg/ml each of leupeptin, pepstatin and aprotinin, 0.5 mM orthovanadate, 1 mM PMSF), and purified by Ni-NTA chromatography. Protein at 1 mg/ml was dialyzed stepwise for 8–16 h each to 6, 5, 4,

2, 1 and 0.5 M urea, and finally to 10 mM Tris-HCl, pH 7.4, 100 mM NaCl without DTT overnight. Large precipitates were removed by centrifugation in a tabletop centifuge for 5 min. The supernatant was analyzed by electron microscopy. 10 µl were adsorbed to Formvar carbon-coated Ni grids, washed, dried, stained with 0.5% uranyl acetate for 1 min dried, and imaged by a Phillips CM100 electron microscope.

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3 Part II: Aggregates of mutant vasopressin precursors: an amyloid-like structure?

3.1 Introduction

3.1.1 Amyloids

Many neurodegenerative diseases including Alzheimer's, Parkinson's, and mammalian prion disease are characterized by the formation of fibrillar protein aggregates (Kelly 1996). They are usually formed by normally soluble proteins, which assemble into insoluble fibers. These fibers are generally resistant to proteolytic digestion. In Alzheimer's disease, accumulation of amyloid fibers and their deposition in plaques has long been associated with cell death and disease progression. However, there is recent evidence suggesting the non fibrillar protein oligomers to be the cytotoxic species (Bucciantini et al. 2002; Kayed et al. 2003).

High-resolution solid state nuclear magnetic resonance (NMR) studies and X-ray crystallography revealed the structure of amyloid fibrils formed by several proteins (Jaroniec et al. 2004; Luca et al. 2007; Petkova, Yau & Tycko 2006; Sawaya et al. 2007; Walsh et al. 2009). All these structures share a common cross-β architecture, in which β-strands run perpendicular to the fibril axis. The β-sheets self-assemble into protofilaments, which may comprise a bundle of twisted β-sheets, which further packs into amyloid fibers (Sunde & Blake 1998).

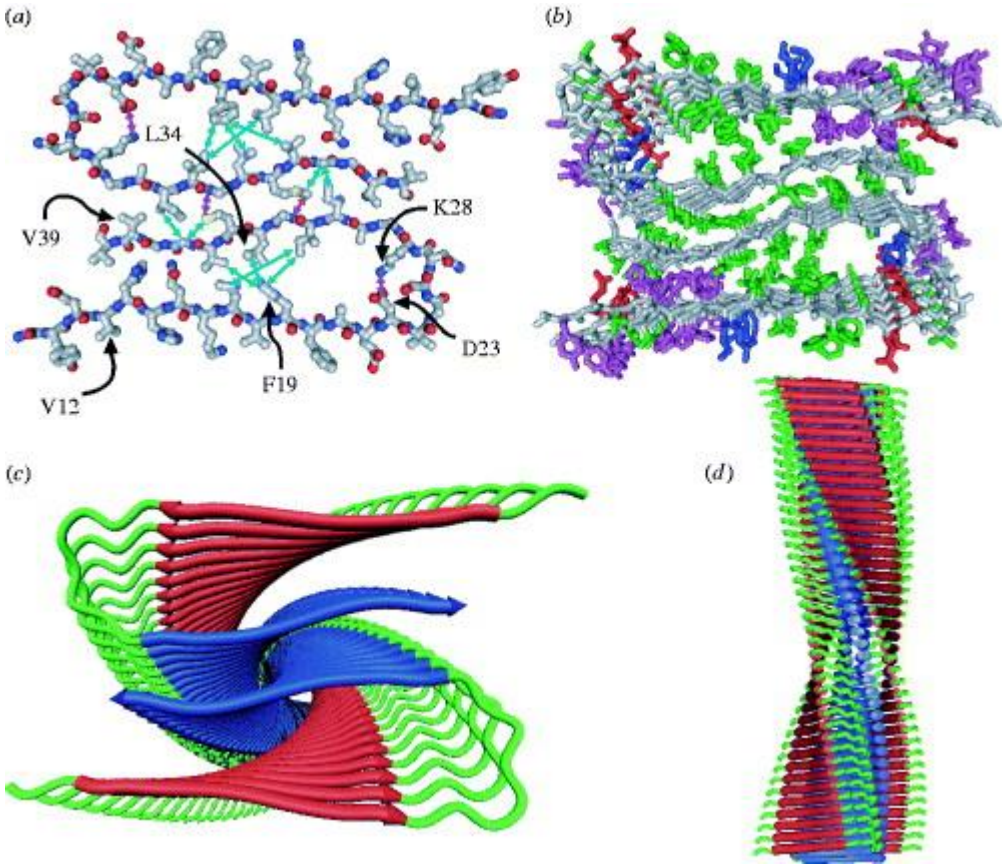

Fig 16: Structural model for the protofilament in $A\beta_{1-40}$ fibrils: a) All-atom representation of a pair of peptide molecules. Residues 10–22 and 30–40 have β-strand conformations, forming two separate in-register, parallel β-sheets. The protofilament is a four-layered β-sheet structure with C_2 symmetry about its long axis. b) Average structure resulting from 10 independent molecular dynamics/energy minimization runs on a cluster of 12 peptide molecules, with interatomic distance and backbone torsion angle restraints dictated by solid-state NMR data. Hydrophobic, polar, negatively charged, and positively charged side-chains are colored green, purple, red, and blue respectively. c) and d) Cartoon representations, with residues 12–21 in red and residues 30–40 in blue **(Tycko 2006)**

3.1.2 Disruption of β-sheets by proline insertions

Proline is a very potent candidate to disrupt β-sheet formation. Minor and Kim (1994) showed that the amino acids glycine and proline have a very low propensity to form β-sheets. This property has been used to determine the regions important for amyloid formation in different proteins. Williams et al. (2004) generated a series of single-

proline replacement mutants of Amyloid(A)β(1–40), and determined the thermodynamic stabilities of amyloid fibrils formed from these mutants to characterize the susceptibility of different residue positions of the Aβ sequence to proline substitution. Their results suggested that the Aβ peptide, when engaged in the amyloid fibril, folds into a conformation containing three highly structured segments, consisting of contiguous sequence elements 15–21, 24–28, and 31–36, that are sensitive to proline replacement and thus likely to include the β-sheet portions of the fibrils.

Abedini and Raleigh (2006) designed and compared a triple proline mutated peptide variant of the amyloidogenic 8–37 region of the human islet amyloid polypeptide (hIAPP) with proline substitutions at positions 17, 19 and 30. They could show, that proline residues outside the 20–29 region reduce ordered fibril formation significantly.

Rauscher et al. (2006) analyzed diverse sequences, including those of elastin amyloids, spider silks, wheat gluten, and insect resilin. This analysis revealed a threshold in proline and glycine composition, above which amyloid formation was impeded and elastomeric properties became apparent.

Parrini et al. (2005) found that glycine residues are highly conserved in the acylphosphatase-like structural family. They constructed six variants of human muscle acylphosphatase (AcP), where six glycines were exchanged to alanine. All six mutants were enzymatically active and had conformational stabilities similar to those of other mutants with non-conserved residues. However, all variants, with the exception of one were found to form amyloid aggregates more rapidly than the wild-type protein under both native and denaturing conditions. Glycine residues have a high level of conformational flexibility and a high entropic cost associated with their secondary structure formation. The authors thus concluded that the high level of conversion of these four glycine residues in this structural family arises from the need to possess inhibitors of aggregation at strategic positions, which act at both the unfolded and folded levels.

In Parkinson’s disease the protein α-synuclein forms fibrillar structures enriched in the core of so-called Lewy bodies, a histologically defining lesion of Parkinson’s disease. It has been shown that incorporation of prolines, especially in the region

spanning residues 37-89 of α-synuclein, reduces fibril formation (Koo, Lee & Im 2008).

3.1.3 Proline/glycine substitution in pro-vasopressin

Since we could show that none of the 4 groups of cysteines of the precursor was exclusively necessary for aggregation of pro-vasopressin (section 2.3.5), we suggested that disulfide bonds were only important to stabilize the fibrils. Thus, the question arose which part of the protein sequence itself could promote aggregation and fibril formation. Due to the apparently ordered structure of the fibrils formed *in vitro* (2.3.4, Fig 13), formation of an amyloid-like structure can be suggested.

In order to define the sequence necessary for aggregation of pro-vasopressin, 10 mutants were designed. In these mutants sequential stretches of 10 amino acids were substituted by the following sequence: PGPGPGTPGP (Fig 17) (a single threonin was included to facilitate cloning), thereby disrupting any potential aggregation promoted by the respective amino acid stretch.

3.2 Material and methods

3.2.1 Cloning strategy:

The Pro constructs were cloned from pECE vasopressin wild type as template using the following primers:

Table 2: Primers for Pro mutants

	N-terminal portion reverse	C-terminal portion forward
Pro1	CGCGGTACCAGGTCCAGGTCCAGGCGCGGAGGAGAAGGC	GCGTGTACACCAGGTCCAAAGAGGGCCATGTCC
Pro2	CGCGGTACCAGGTCCAGGTCCAGGGCCCCTCGGGC	GCGTGTACACCAGGTCCACAGTGCCTCCCCTGC
Pro3	CGCGGTACCAGGTCCAGGTCCAGGTCTCAGCTCCAGGTCG	GCGTGTACACCAGGTCCAGGCCGCTGCTTCGGG
Pro4	CGCGGTACCAGGTCCAGGTCCAGGTTTGCCCCCGGGGC	GCGTGTACACCAGGTCCAGCGGACGAGCTGGGC
Pro5	GCAGCAGATGCTGGGCCGCGGTACCAGGTCCAGGTCCAGG	GCGTGTACACCAGGTCCAGCTGAGGCGCTGCGC
Pro6	CGCGGTACCAGGCCCAGGGCCAGGCGTGCCCACGAAGCA	GCGTGTACACCAGGTCCATACCTGCCGTCGCCC
Pro7	CGCGGTACCAGGTCCAGGTCCAGGGTTCTCCTCCTGGCAG	GCGTGTACACCAGGTCCAAAGGCGTGCGGGAGC
Pro8	CGCGGTACCAGGTCCAGGTCCAGGCTGGCCGGACTGGCAG	GCGTGTACACCAGGTCCAGCCTTCGGCGTTTGCTG
Pro9	CGCGGTACCAGGTCCAGGTCCAGGGGCGCAGCGGCCCC	GCGTGTACACCAGGTCCATGCGTGACCGAGCCC
Pro10	CGCGGTACCAGGTCCAGGTCCAGGGCTCTCGTCGTTGCAG	GCGTGTACACCAGGTCCATTTCACCGCCGCGCC

Forward flanking primer for the N-terminal portion: GAAGTAGTGAGGAGGC (ECEleft)
Reverse flanking primer for the C-terminal portion: CTACAAATGTGGTATGGC (ECEright)

The PCR products were purified from a 1% agarose gel using the NucleoSpin gel extraction kit (Macherey-Nagel) according to the manufacturer's protocol. The purified constructs were cut with the restriction enzyme *Asp*718 (Roche) for the N-terminal product and *Bsr*GI (NEB) for the C-terminal product. After gel electrophoresis and purification, the products were mixed and ligated using T4 ligase (Roche). The ligase was inactivated at 60°C. PCR was performed on the ligation product using the flanking primers ECEleft and ECEright and the Expand PCR

system (Roche). The product was cut from an agarose gel, digested with *Hind*III and *Xba*I (Roche) and ligated into the pRc/RSV expression vector.

3.2.2 Cultivation and Transient Transfection of COS-1 cells

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in 7.5% CO_2.

The cells were transiently transfected in 6-well plates using polyethylenimin (PEI, Sigma). 240 µl complete DMEM were mixed with 8 µl PEI (Stock 1 mg/ml 25 kDa) and 1.5 µg DNA and incubated for 45 min at room temperature. 560 µl complete DMEM were added to the transfection mix and added to the cells. The medium was changed the next day, and the cells were analyzed two to three days after transfection.

3.2.3 Metabolic labeling and immunoprecipitation

48 h post transfection the cells were starved for 30 min in 500 µl DMEM without methionine and cysteine (Sigma) supplemented with 2 mM L-glutamine. Cells were labeled with 100 µCi/ml [^{35}S]protein labeling mix (PerkinElmer) im DMEM for another 30min. The labeling medium was removed and the cells washed with PBS. 500 µl chase medium containing an excess of methionine and cysteine were added and chased for 2 h. Cells were washed again with cold medium and lysed for 20 min with lysis buffer (1% Triton X-100, 0.5% Deoxycolate, 2 mM PMSF in PBS). Cells were scraped into a tube an incubated for 1 h on ice. After lysis, the mixture was centrifuged to remove the cellular debris and the supernatant and the medium were incubated over night with lysis buffer containing 1 mM PMSF, 0.5% SDS, 1 mg/ml BSA and rabbit polyclonal anti-NPII (Friberg, Spiess & Rutishauser 2004). The protein was precipitated using protein A-Sepharose (Zymed) for 30 min and washed several times with lysis buffer containing 1 mM PMSF, 1% SDS and 1 mg/ml BSA

followed by 2 washes with PBS. The precipitate was mixed with 100 µl 2x SDS sample buffer containing β-mercapto ethanol and cooked for 10 min. The samples were analyzed by electrophoresis on 10% polyacrylamide Tris/tricine SDS-gels and autoradiography.

3.2.4 Metabolic labeling and non-reducing immunoprecipitation

Metabolic labeling was performed as described above. After starvation and labeling the cells were washed with PBS containing 100 mM iodoacetamide (Fluka). The cells were incubated at 4°C for 1 h in the dark with 200 µl 200 mM Tris, pH 8.0, 100 mM iodoactamid and 2 mM PMSF. The cells were lysed by adding 500 ml lysis buffer (described above) containing 100 mM iodoacetamide for 1 h in the dark. The mixture was centrifuged for 15 min to remove cellular debris and the supernatant was mixed with 1 mg/ml BSA and rabbit polyclonal anti-NPII (Friberg, Spiess & Rutishauser 2004) overnight. The protein was precipitated using protein A-Sepharose (Zymed) for 30 min and washed several times with lysis buffer containing 1 mM PMSF, 1% SDS, 1 mg/ml BSA and 50 mM iodoactemide, followed by 2 washes with PBS containing 50 mM iodoactemide. The precipitate was mixed with 100 µl 2x SDS sample buffer without β-mercaptoethanol and cooked for 10 min. The samples were analyzed by electrophoresis on 10% polyacrylamide Tris/tricine SDS-gels and autoradiography.

3.2.5 Immunofluorescence

Cells were grown on coverslips and transfected as mentioned above. 48 h post transfection the cells were washed 3 times in PBS and fixed in 3% paraformaldehyde (Fluka) for 20 min. To prevent autofluorescence, the cells were quenched in 50 mM NH_4Cl in PBS for 10 min. Permeabilization was achieved using 0.1% Triton X-100 and 1% BSA in PBS for 15 min. The cells were stained using rabbit polyclonal anti-NPII (Friberg, Spiess & Rutishauser 2004) as primary antibody in 0.1% Triton X-100 and 1% BSA in PBS for 2 h in the dark. The cells were washed 4 times in PBS and stained with non-crossreacting Cy2 labeled goat anti rabbit (Jackson

Immunoresearche) according to the manufacturers recommendations. The coverslips were washed in PBS and mounted in Mowiol 4-88 (Hoechst). Analysis was performed using a Zeiss Axioplan 2 microscope with a Leica DFC420C imaging system.

3.3 Results

3.3.1 Proline/glycine replacements cause retention of the precursor in the ER but do not prevent the formation of disulfide-linked oligomers

To test the secretion ability of the Pro-mutants in COS-1 cells, transiently transfected cells were radiolabeled with [35S]methioninen/cysteine for 30 min and chased with excess unlabeled methionine/cysteine for 2 h. Cells and media were subjected to immunoprecipitation using anti-neurophysin II antiserum followed by SDS-gel electrophoresis an autoradiography (Fig 17B). The wild-type proteins and the mutants Pro1-10 (Fig 17A) were visualized as major species of 21 kDA, corresponding to N-glycosylated pro-vasopressin. The mutant Pro1, in which the first ten aminoacids after the signal peptide were mutated appeared as a larger product compared to glycosylated pre-pro-vasopressin (Fig 17B). This difference in size resulted from inhibited cleavage of the signal peptide due to the Pro at position +1 (Nilsson & von Heijne 1992; Barkocy-Gallagher & Bassford 1992). After 2 h of chase, wild-type pro-vasopressin was secreted into the medium but not the Pro mutants, indicating that Pro1-10 had been retained.

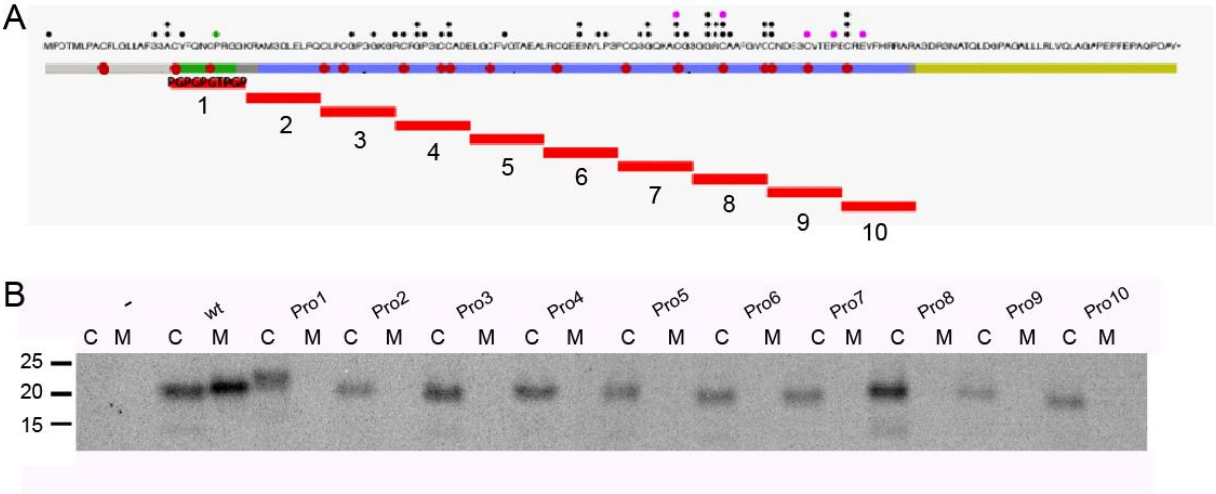

Fig 17: A: proline mutagenesis of the vasopressin precursor: stretches of ten amino acids were exchanged by a proline/glycine rich sequence (red), B: Wild-type (wt) vasopressin precursor and the Pro mutants 1-10 were expressed in COS-1 cells, pulse-labeled with [^{35}S]methionine/cysteine for 1 h, and chased for 2 h. Pro-vasopressin was immunoprecipitated from cell lysates (C) and media (M), and analyzed by SDS-gel electrophoresis and autoradiography. The positions of marker proteins are indicated with their molecular weights in kDa.

In order to characterize the Pro mutants in terms of their ability to form of disulfide-linked oligomers, transiently transfected COS-1 cells were radiolabeled with [35S]methionine/cysteine for 30 min and treated with iodoacetamide to prevent post-lysis oxidation. After immunoprecipitation and separation by SDS-gel electrophoresis under non-reducing conditions, all Pro mutants showed significant formation of disulfide-linked oligomers compared to the wild-type (Fig 18). This finding suggests that oligomer formation is not prevented by the inclusion of proline/glycine rich segments.

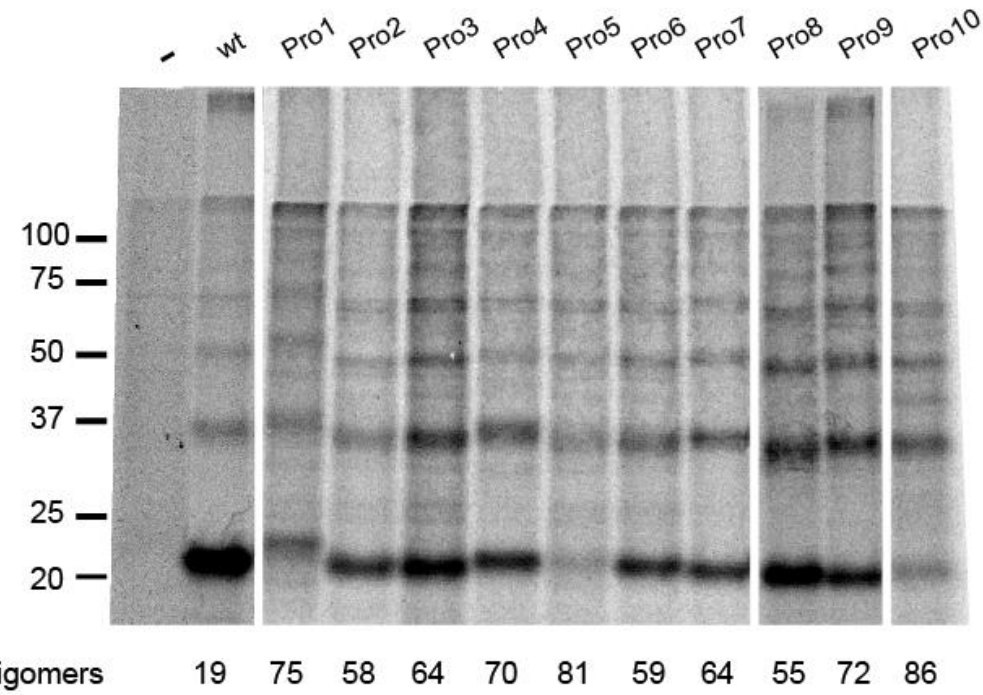

Fig 18: To analyze the formation of disulfide-linked oligomers, COS-1 cells were transfected with the indicated constructs and labeled for 1 h with [^{35}S]methionine/cysteine. After alkylation of free SH groups, pro-vasopressin was immunoprecipitated and analyzed by SDS-gel electrophoresis under non-reducing conditions. The positions of molecular weight standards (in kDa) are indicated.

3.3.2 Proline insertion within the vasopressin domain inhibits formation of large aggregates

Proline/glycine insertion throughout the precursor sequence did not prevent the formation of disulfide-linked oligomers. To define the sequence involved in the formation of large aggregates, transiently transfected COS-1 cells were analyzed by immunofluorescence 72 h post transfection with the Pro mutants and the wild-type as

a control (Fig 19A). Only mutant Pro1, in which the first 10 amino acids were replaced by a proline/glycine rich sequence showed a reduction in the formation of visible aggregates, suggesting this region to be engaged in the formation of stable complexes (Fig 19B).

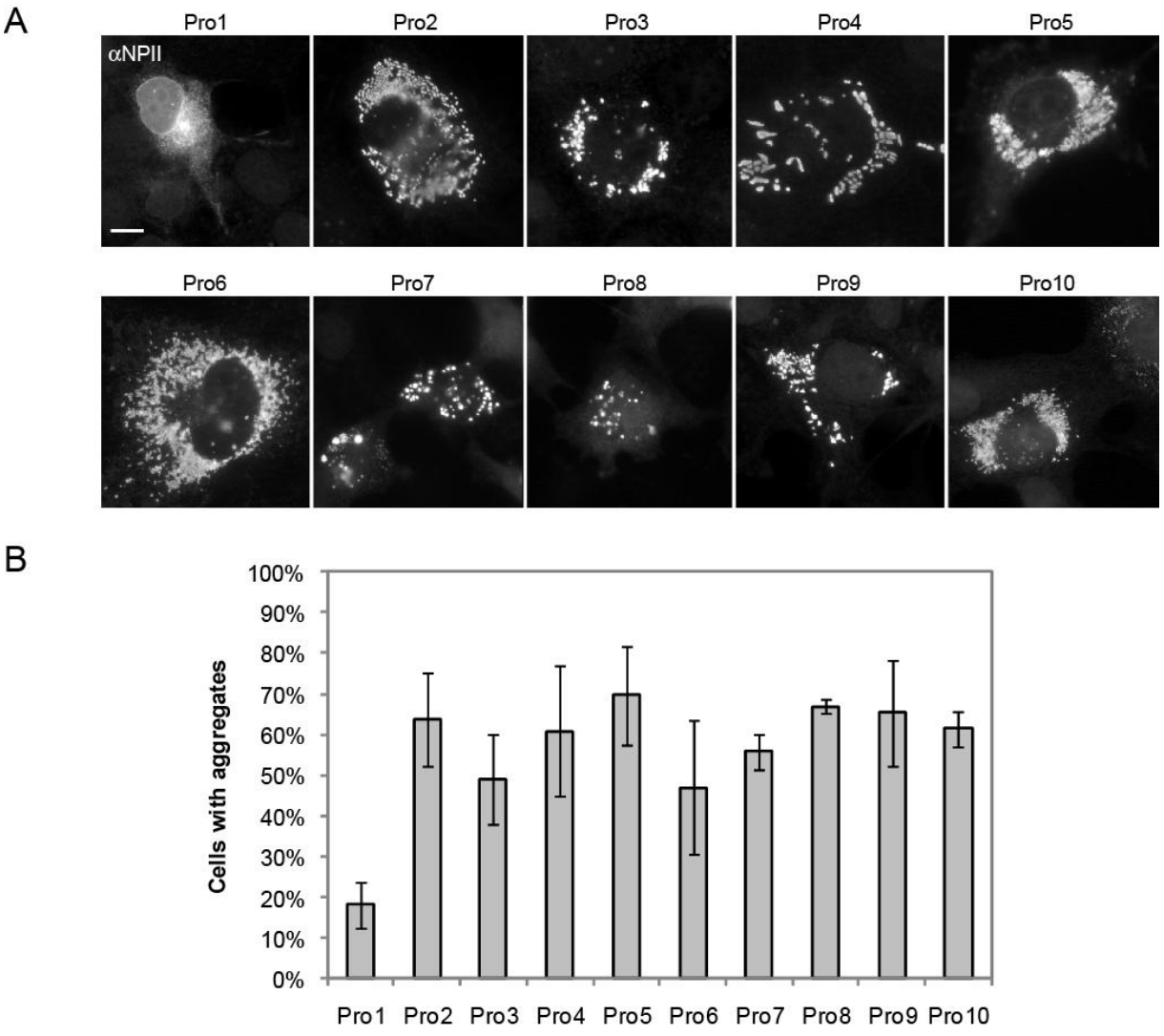

Fig 19: A: COS-1 cells expressing the indicated constructs were amalyzed by immunofluorescence microscopy after staining for pro-vasopressin 3 d after transfection. Representative cells are shown. Scale bar, 20 µm. B: The frequency of aggregate formation was quantified from immunofluorescence micrographs as in panel A (mean and standard deviation of three independent experiments. Background as determined for mutant CatZ in Fig 9E is ~15–20%.

3.4 Discussion

Fibrillar protein aggregates are a hallmark of many neurodegenerative diseases like Alzheimer's or prion disease. Usually soluble proteins assemble to insoluble fibres which are resistant to proteolytic cleavage. Depositions of these protein accumulations have long been thought to be associated with cell death and disease progression. However the pathogenic nature of these accumulations is still debated. Studies have also suggested that the toxic species may be the small oligomers rather than the large aggregates which may even represent a protection mechanism (Goldberg & Lansbury Jr 2000; Haass & Steiner 2001).

3.4.1 Insertion of 10 prolines and glycines inhibits the formation of large aggregates

A common feature of amyloids is their cross-β architecture, in which β-strands run perpendicular to the fibril axis (Sunde & Blake 1998) . These strands assemble into protofilaments which then comprise a structure resembling a bundle of twisted β-sheets. Many studies reported that insertion of proline and glycine caused disruption of β-sheets. (Minor & Kim 1994; Rauscher et al. 2006; Koo, Lee & Im 2008).

We constructed 10 artificial mutants of the vasopressin precursor where we inserted stretches of proline and glycine (P/G) to perform a scan for regions on the precursor necessary for aggregation and disulfide-linked oligomerisation. Only one of our proline constructs was able to disrupt the formation of large aggregates in COS-1 cells. In this construct, the vasopressin moiety was replaced by proline and glycine. The result suggests that the vasopressin domain is necessary for the formation of large aggregates. Beuret et al. (1999) found that the naturally occurring mutant with a truncation in the signal sequence (ΔG227) causes reduced cleavage of the signal peptide. In the resulting precursor, vasopressin is not able to fold into its binding pocket of NPII and disulfide linked oligomers as well as large aggregates (Fig 8) are formed, although NPII is folded and only vasopressin moiety is available. This

suggests that vasopressin is sufficient for the formation of disulfide linked oligomers and large aggregates which fits to our data.

The fact that the formation of disulfide-linked oligomers was not influenced by the insertion of a P/G rich sequence at any site suggests that disulfide-linked oligomerisation alone is not sufficient to initiate assembly of oligomers into fibres. Disulfide-linked oligomerisation might be also triggered by more than one site on the precursor. Since our approach would only be able to inactivate one of these sites at a time, we would not be able to see an effect on disulfide-linked oligomerisation.

To address this question, new constructs will be necessary. Since it is very likely that one of the sites sufficient for oligomerisation lies within the vasopressin moiety, new constructs would contain the replacement of the vasopressin domain for a P/G rich sequence as in mutant Pro1 in combination with additional sequential segments replaced by P/G. This would give rise to 9 additional constructs, screening for two putative sites necessary for oligomerisation. However, it is still possible that formation of disulfide-linked oligomers is not diagnostic for DI mutants. Still, this approach could provide further insights into the mechanism of aggregation in ADNDI.

4 Part III: Molecular analysis of novel mutations causing autosomal dominant neurohypophyseal diabetes insipidus

4.1 Introduction

The genetic linkage between ADNDI and the vasopressin gene was established by Repaske et al. (1990). Ito et al. (1991) described the first mutation in the pro-vasopressin gene in a Japanese family affected by ADNDI. Interestingly, most mutations fall within the coding sequence for NPII; a few signal mutations and mutations in vasopressin itself are known, but none fall within the coding sequence for the glycopeptide. The mutations that affect the precursor include both missense mutations changing a single amino acid, and nonsense mutations which truncate the precursor. No differences in clinical presentation have been described for individuals with different dominant mutations, and penetrance is high.

In cooperation with a group of Belgian pediatricians, we analyzed DNA samples from various patients with ADNDI and their relatives for mutations in the pro-vasopressin gene. One of these mutations was cloned and expressed in COS-1 cells.

4.2 Material and methods

4.2.1 Genomic DNA analysis in ADNDI patients

Each of the three exons of the vasopressin-NPII gene was amplified from genomic DNA by polymerase chain reaction (PCR) using a Biometra T3 thermocycler in 10% dimethyl sulfoxide (DMSO) using the Expand High Fidelity PCR System (Roche).

The following primers were used for amplification:

TGCCTGAATCACTGCTGACCGCTGGGGACC	forward PCR primer for genomic vasopressin ex.1
GCTATGGCTGCCCTGAGATGGCCCACAGTG	reverse PCR primer for genomic vasopressin ex.1
TCGCTGCGTTCCCCTCCAACCCCTCGACTC	forward PCR primer for genomic vasopressin ex.2+3
CCTCTCTCCCCTTCCCTCTTCCCGCCAGAG	reverse PCR primer for genomic vasopressin ex. 2+3

40 cycles of melting (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 3 min) were carried out, with a final 15 min at 72°C for extension. PCR products were run on 1% agarose gels, then the bands were excised and extracted from agarose using a commercial kit (NucleoSpin Gel extraction; Macherey-Nagel). Following purification of the PCR product, the nucleotide sequences of both coding and non-coding DNA strands were determined using the following primers:

TGCCTGAATCACTGCTGACCGCTGGGGACC	forward PCR primer for genomic vasopressin ex.1
GCTATGGCTGCCCTGAGATGGCCCACAGTG	reverse PCR primer for genomic vasopressin ex.1
TCGCTGCGTTCCCCTCCAACCCCTCGACTC	forward PCR primer for genomic vasopressin ex.2
CCTCTCTCCCCTTCCCTCTTCCCGCCAGAG	reverse PCR primer for genomic vasopressin ex. 3
CGCCCCCCCCCAGGCCCGCCCCCGCCGCGC	reverse sequencing primer for genomic vasopressin ex. 2
CCCAGGCGCCCGTGCTCACACGTCCTCCCG	forward sequencing primer for genomic vasopressin ex. 3

4.2.2 Plasmids and constructs

One of the mutants discovered by sequencing was cloned for further analysis. The cDNA of the human wild-type vasopressin precursor has been described before (Beuret et al., 1999; Friberg et al., 2004). The C28Y (as a control) and C61W mutants were generated by polymerase chain reaction. All human cDNAs were subcloned in the pECE expression plasmid. The constructs were confirmed by automated sequencing.

4.2.3 Cell culture and transient transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine at 37 °C in 7.5% CO_2. The cells were transiently transfected in 6-well plates using polyethylenimine (Sigma) and analyzed 2 days after transfection.

4.2.4 Metabolic labeling and immunoprecipitation

For labeling experiments, transfected cells were starved for 30 min in DMEM without cysteine and methionine (Sigma) supplemented with 2 mM L-glutamine. Cells were labeled for 30 min with 100 µCi/ml [^{35}S]-protein labeling mix (PerkinElmer Life Sciences) in starvation medium and chased in starvation medium supplemented with excess cysteine and methionine. Cells were transferred to 4 °C, washed with PBS, lysed in 500 µl of lysis buffer (PBS, 1% Triton X-100, 0.5% deoxycholate, 2 mM phenylmethylsulfonyl fluoride), and scraped. After 10 min of centrifugation in a microcentrifuge, the lysate was subjected to immunoprecipitation using rabbit polyclonal anti-neurophysin II (Friberg et al., 2004). The immune complexes were isolated with protein A-Sepharose (Zymed Laboratories Inc.) and analyzed by electrophoresis on 10% polyacrylamide Tris/Tricine SDS-gels and autoradiography.

4.2.5 Immunofluorescence

Transfected cells were grown for 48 h on glass coverslips, fixed with 3% paraformaldehyde for 20 min at room temperature, washed with PBS, permeabilized and blocked with 0.1% saponin, 1% bovine serum albumin (BSA) in PBS for 20 min, incubated at room temperature with primary antibodies for 2 h in saponin/BSA/PBS, washed, and stained with fluorescent secondary antibodies in saponin/PBS for 30 min. After several washes with saponin/PBS, the cover slips were mounted in Mowiol 4-88 (Hoechst). As primary antibodies we used the polyclonal rabbit anti-NPII antiserum and monoclonal antibody against the endogenous ER chaperone calnexin

(BD Bioscience). As secondary antibodies we used Cy2-labeled goat anti-rabbit and Cy3-labeled goat anti-mouse (Jackson Immunoresearch) according to the manufacturers' recommendation. Staining was analyzed using a Zeiss Axioplan 2 microscope with a Leica DFC420C imaging system.

4.3 Results

4.3.1 Direct sequence analysis of patient samples

Genomic DNA samples from 3 families with healthy and affected members were analyzed. For each person, exon 1, 2 and 3 were amplified, and both strands were sequenced. Amplification yielded fragments of 345 bp and 732 bp, respectively. In all patients a heterozygous single base substitution was detected, whereas all healthy family members showed wild-type sequence.

Patient 1 had developed DI as an infant. She carried a novel mutation of a G to a T at nucleotide 1919 (1919 G > T, genomic nucleotide numbering beginning with the A of initial ATG) in exon 2 (Fig 20). This mutation alters codon 77 from GAG to TAG, substituting glutamine for a stop codon, resulting in a truncated precursor.

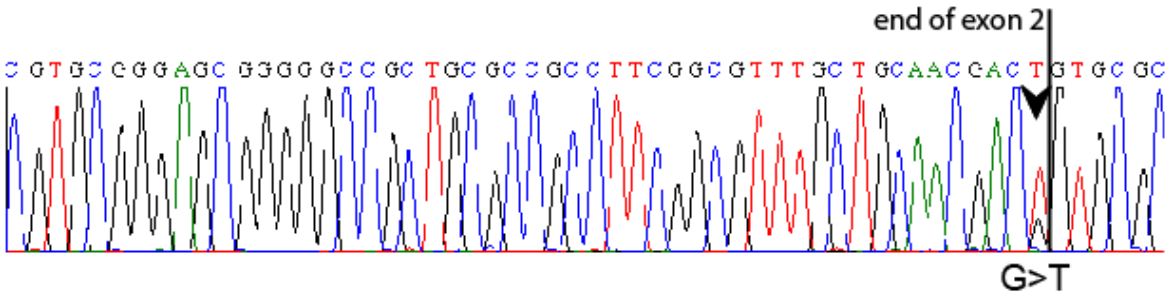

Fig 20: Genomic sequence of vasopressin-NPII in patient 1 showing a substitution of G to T at nucleotide 1919 (arrowhead)

Patient 2 is a member of a family with three affected and two asymptomatic family members. All affected members carried a novel mutation of a C to a G at nucleotide

1720 (1720 C > G, genomic nucleotide numbering beginning with the A of ATG) in exon 2. This mutation alters codon 10 from TGC to TGG (Fig 21B), resulting in a substitution of cysteine 10 for tryptophan.

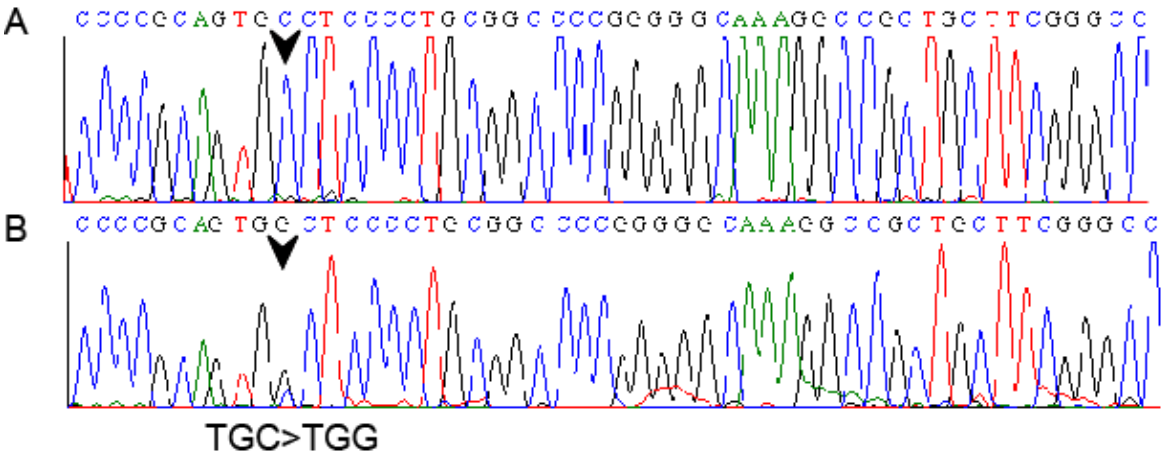

Fig 21: Genomic sequence of vasopressin-NPII in patient 2 (panel A) and an unaffected family member, showing a substitution of C to G at nucleotide 1720 (arrowhead)

Patient 3 is a member of a family with two asymptomatic and three affected family members (Fig 22). Each affected individual carried a novel a mutation of a C to a G at nucleotide 1873 (1873 C > G, genomic nucleotide numbering beginning with the A of initial ATG) in exon II (Fig 23 panel B). This mutation alters codon 61 of NPII from TGC to TGG, substituting cysteine 61 for tryptophan, C61W.

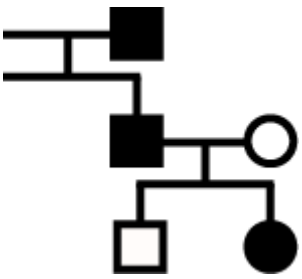

Fig 22: pedigree of a family with 3 affected members: squares: males, circles: females, closed symbols: affected

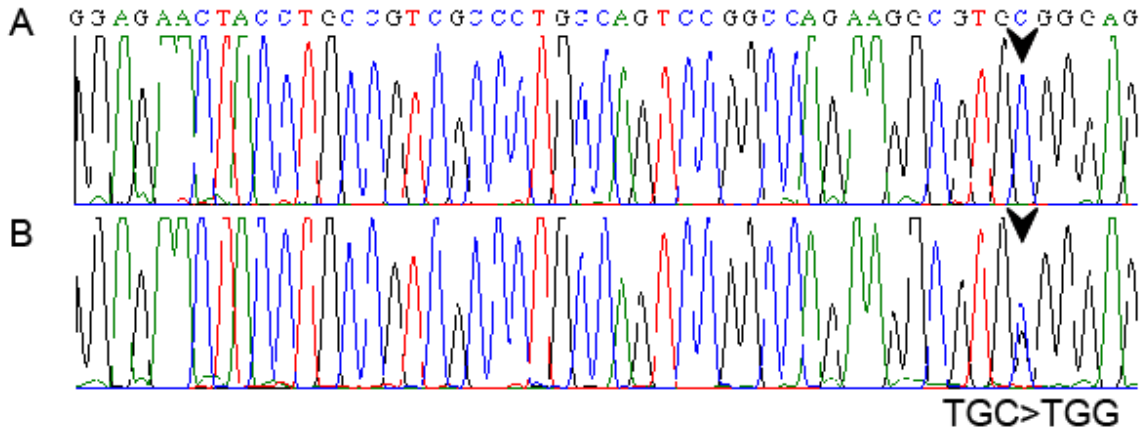

Fig 23: Genomic sequence of vasopressin-NPII in patient 3 (panel B) showing a substitution of C to G at nucleotide 1873 (arrowhead) and an unaffected family member (panel A)

4.3.2 Mutant pro-vasopressin is retained

To assay secretion, wild-type pro-vasopressin, the known dominant mutant C28Y and the novel mutant C61W were expressed in COS-1 cells, pulse labeled with [35S]methionine/cysteine, chased for 2 h, isolated from the cells and from the media by immunoprecipitation, and analyzed by SDS-gel electrophoresis and autoradiography. In contrast to the wild-type pro-vasopressin, where a considerable fraction could be recovered from the medium, the C28Y and C61W mutants were completely retained in the cell (Fig 24).

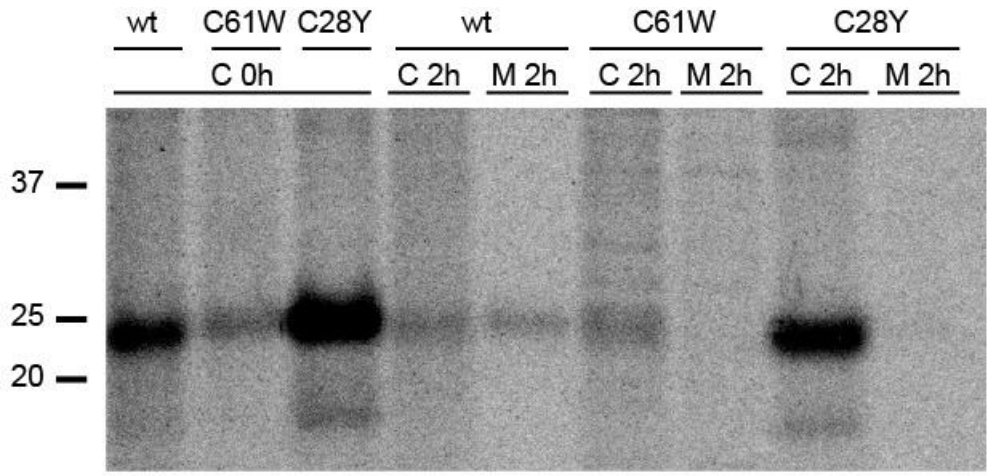

Fig 24: secretory capacity of mutant and wild-type pro-vasopressin: COS1 cells expressing the wild type and the two mutants C28Y and C61W were labeled with [35S]methionine/cysteine for 30 min and chased for 2 h. Pro-vasopressin was immunoprecipitated from cell lysates (C) and media (M), and analyzed by SDS-gel electrophoresis and autoradiography. The positions of marker proteins are indicated with their molecular weights in kDa.

4.3.3 Localization of mutant pro-vasopressin

To characterize the intracellular localization of the C61W precursor, transfected COS-1 cells were analyzed by immunofluorescence staining 48 h after transfection. In many cells expressing C61W, a strong reticular staining typical for proteins retained in the ER was visible. However, a considerable number of cells also showed larger aggregations, typical for mutant pro-vasopressin (see also Part I, Fig 8). These inclusions were of round, sometimes short tubular appearance and stained positive for the ER marker calnexin (Fig 25).

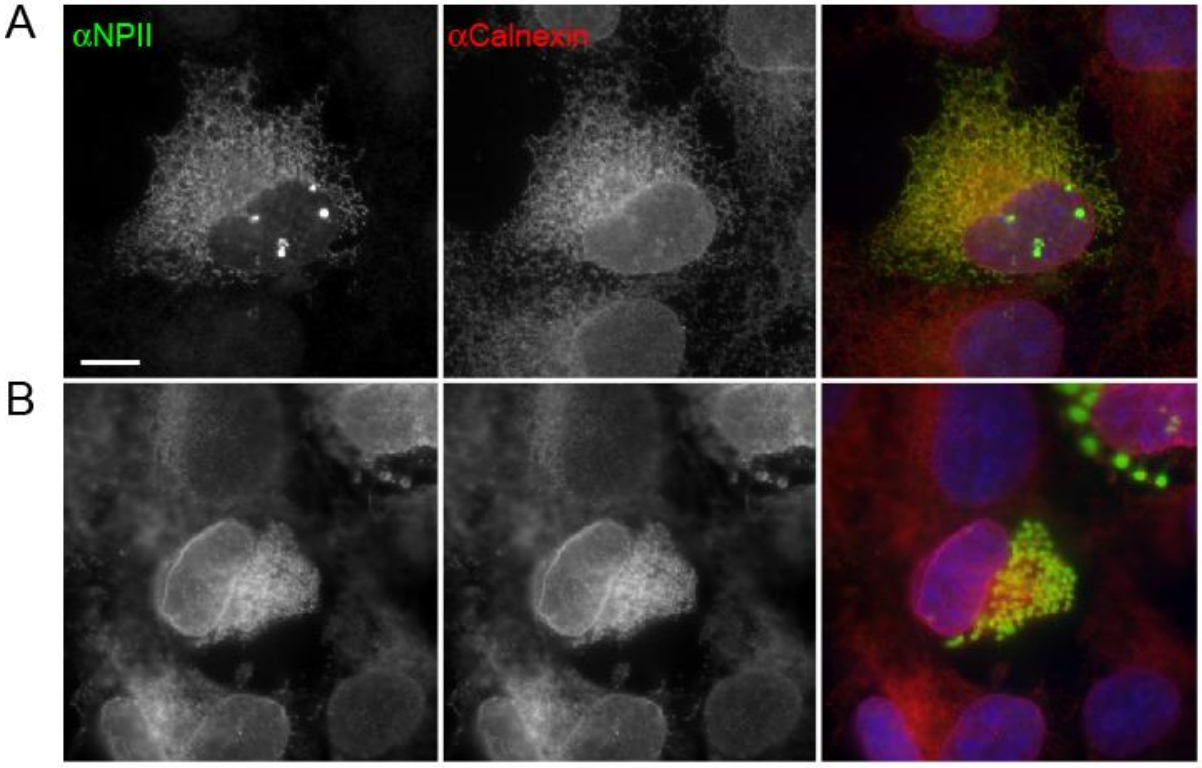

Fig 25: Intracellular accumulation of C61W pro-vasopressin in COS-1 cells. COS-1 cells vasopressin C61W were subjected to immunofluorescence staining against NPII 72 h post transfection. More than half of the expressing cells showed accumulations that were mostly round or elongated (panel B). The remaining cells showed a reticular staining typical for ER retained protein (panel A). Scale bar, 20 µm.

4.4 Discussion

Here, we present three additional ADNDI families with novel mutations in the vasopressin gene. In total, 58 pro-vasopressin mutations causing DI are now known (Table 3). The clinical presentation in patients with different mutations is similar and all affected individuals usually manifest with polyuria and polydipsia within their first months or years of life.

Table 3: ADNDI mutations; above described cases displayed in grey

Exon	Nucleotide number	Base Mutation	Amino Acid number	Amino Acid Substitution	Kindreds	Reference
1	225	A→G	SP -19 to -16	del MPDT	1	(Christensen et al. 2004)
	227	G→A	SP -19 to -16	del MPDT	1	(Christensen et al. 2004)
	227	del G	SP -19 to -16	del MPDT	1	(Rutishauser et al. 1996)
	274	C→T	SP -3	S→F	1	(Rittig et al. 1996)
	279	G→A	SP -1	A→T	8	(Ito et al. 1993) (McLeod et al. 1993) (Rittig et al. 1996) (Calvo et al. 1998) (Siggaard et al. 1999) (Boson et al. 2003) (Christensen et al. 2004)
	280	C→T	SP -1	A→V	3	(Rittig et al. 1996) (Heppner et al. 1998) (Christensen et al. 2004)
	285	T→C	VP 2	Y→H	2	(Rittig et al. 1996) (Rittig et al. 2002)
	286	A→C	VP 2	Y→S	1	(Kobayashi et al. 2006)
	287-289	del CTT	VP 3	del F	1	(Wahlstrom et al. 2004)
2	1720	C→G	NP 10	C→W	1	This work
	1730	G→C	NP 14	G→R	1	(Rittig et al. 1996)
	1740	G→T	NP 17	G→V	1	(Bahnsen et al. 1992)
	1748	C→T	NP 20	R→C	1	(Rittig et al. 1996)
	1751	T→C	NP 21	C→R	1	(Goking et al. 1997)
	1757	G→C	NP 23	G→R	3	(Heppner et al. 1998) (Rutishauser et al. 2002) (Christensen et al. 2004)
	1757	G→A	NP 23	G→R	1	(Calvo et al. 1999)
	1758	C→T	NP 23	G→V	1	(Gagliardi, Bernasconi & Repaske 1997)
	1761	C→T	NP 24	P→L	1	(Repaske & Browning 1994)
	1770	G→T	NP 27	C→F	1	(Wolf et al. 2003)
	1772	T→C	NP 28	C→R	1	(Hansen, Rittig & Robertson 1997)
	1773	G→A	NP 28	C→Y	1	(Skordis et al. 2000)
	1774-1776	del CGC	NP 28/29	del C/ A→W	1	(Flück et al. 2001)
	1797	T→C	NP 36	V→A	1	(Christensen et al. 2004)
	1824-1829	del GAG	NP 47	del E	7	(Yuasa et al. 1993) (Rittig et al. 1996) (Mahoney et al. 2002) (Christensen et al. 2004) (Ye et al. 2005)
	1829	G→A	NP 47	E→K	1	(Miyakoshi et al. 2004)

	1830	A→G	NP 47	E→G	2	(Rittig et al. 1996) (Christensen et al. 2004)
	1839	T→C	NP 50	L→P	1	(Rittig et al. 1996)
	1857	C→T	NP 56	S→F	1	(Grant et al. 1998)
	1859	G→A	NP 57	G→S	2	(Ito et al. 1991) (Rittig et al. 1996)
	1859	G→C	NP 57	G→R	1	(Rittig et al. 1996)
	1859	G→T	NP 57	G→V	9	(de Melo et al. 2008)
	1872	G→C	NP 61	C→S	2	(Rittig et al. 1996) (Bullmann et al. 2002)
	1872	G→A	NP 61	C→Y	2	(Grant et al. 1998) (Rutishauser et al. 2002)
	1873	C→A	NP 61	C→X	3	(Rittig et al. 1996) (Grant et al. 1998) (Christensen et al. 2004)
	1873	C→G	NP 61	C→W	3	This work
	1874	G→T	NP 62	G→W	1	(Nagasaki et al. 1995)
	1883	G→T	NP 65	G→C	2	(Rittig et al. 1996) (Christensen et al. 2004)
	1884	G→A	NP 65	G→D	1	(Christensen et al. 2004)
	1884	G→T	NP 65	G→V	2	(Ueta et al. 1996) (Rauch et al. 1996)
	1886	C→T	NP 66	R→C	1	(Rutishauser et al. 1999)
	1887	G→C	NP 66	R→P	1	(Mundschenk et al. 2001)
	1889	T→A	NP 67	C→S	1	(Baglioni et al. 2004)
	1889	T→G	NP 67	C→G	1	(DiMeglio et al. 2001)
	1890-1891	GC→CT	NP 67	C→S	2	(Davies et al. 2005)
	1891	C→A	NP 67	C→X	1	(Nagasaki et al. 1995)
	1892	G→C	NP 68	A→P	1	(Elias et al. 2003)
	1907	T→G	NP 73	C→G	1	(Christensen et al. 2004)
	1908	G→T	NP 73	C→F	1	(Santiprabhob, Browning & Repaske 2002)
	1910	T→C	NP 74	C→R	1	(Rutishauser et al. 2002)
	1911	G→A	NP 74	C→Y	1	(Fujii, Iida & Moriwaki 2000)
	1919	G→T	NP 77	E→X	1	This work
3	2094	C→A	NP 79	C→X	1	(Rittig et al. 1996)
	2101	G→T	NP 82	E→X	2	(Calvo et al. 1999)
	2106-2107	CG→GT	NP 83	E→X	2	(Rittig et al. 1996) (Bullmann et al. 2002)
	2110	T→G	NP 85	C→G	2	(Abbes et al. 2000) (Nijenhuis et al. 2001)
	2110	T→C	NP 85	C→R	1	(Abbes et al. 2000)
	2112	C→G	NP 85	C→W	1	(Christensen et al. 2004)
	2116	G→T	NP 87	E→X	1	(Rittig et al. 1996)

Out of the 58 different mutations in the coding sequence, 55 are causing different amino acid sequence alterations. 6 mutations are located in the signal sequence, 3 of them leading to deletion of the first methionine. The resulting signal peptide is truncated and products of these mutations show less efficient cleavage of the signal peptide. 3 mutations are located in the vasopressin sequence and 49 mutations are found in the NPII sequence. There are no sequence alterations known to be located within the sequence of the glycopeptide. There are 6 mutations causing deletions, 7 nonsense mutations and 45 missense mutations. In 22 cases a cysteine is affected, this represents a proportion of approximately 40%. This high percentage of cysteines points out the importance of cysteines in proper precursor folding and suggests cysteines to play a major role in ADNDI.

All these mutations have in common that they very likely interfere with protein folding and as a consequence with ER export. This is most obvious in truncations where entire regions necessary for folding are deleted, or in cases where a cysteine is mutated, preventing proper disulfide formation. A more particular case is represented by the deletion of the initial methionine. This has shown to cause truncation of the signal sequence. Translation thus starts at a methionine at position (-16), resulting in a cleavage defect (Beuret et al. 1999). This is similar to the mutation A(-1)T (Ito et al. 1993). The uncleaved signal prevents binding of the N-terminal vasopressin into its binding pocket of NPII. Several mutations involve the vasopressin binding sites of NPII including Glycine 14, Glycine 17, Glycine 23, Arginine 20, Proline 24, Glutamate 47, and Leucine 50 of NPII (Gagliardi, Bernasconi & Repaske 1997).

We showed that several mutants cause oligomerization (Beuret et al. 1999) and aggregation (see Part I Fig 8) of the mutant precursor which is hypothesized to cause cytotoxicity (Rittig et al. 1996; Ito et al. 1993; Ito & Jameson 1997). Accumulation of mutant vasopressin pro-hormone has been shown to cause an aberrant ER morphology (Nijenhuis, Zalm & Burbach 1999; Si-Hoe et al. 2000), thus it is likely to disturb the function of the ER and thus also the viability of the cell. Decreased viability of the magnocellular neurons producing vasopressin would explain the autosomal dominant inheritance of ADNDI. There are several observations supporting the possible mechanism for ADNDI. Strong reductions in the amount of magnocellular neurons in the paraventricular and suprapoptic nuclei of the

hypothalamus was observed autopsies of ADNDI patients (Braverman, Mancini & McGoldrick 1965; Nagai et al. 1984; Gaupp 1941; Hanhart 1940; Bergeron et al. 1991). It has also been shown that mutant vasopressin precursors caused cytotoxicity in stably transfected Neuro2a cells (Ito & Jameson 1997) and in a knock-in mouse model (Russell et al. 2003).

The 3 novel mutations described here fit into this pattern: mutation of cysteine 77 to stop in NPII, mutation of cysteine 10 to tryptophan, mutation of cysteine 61 to tryptophan. Metabolic labeling of transiently transfected COS-1 cells showed the C61W mutation to be not secreted after 2 hours, but retained inside the cell. The site of retention was identified by immunofluorescence studies as the ER, shown by colocalization with the ER marker calnexin. The staining revealed patterns ranging from reticular to bigger accumulations of NPII.

Our findings strengthens the hypothesis that there is no link of ADNDI to a specific set of mutations in the vasopressin gene, since all the mutations described above cause ADNDI, possibly due to misfolding and aggregation. How aberrant precursor processing leads to magnocellular neuron death remains to be determined.

5 General Discussion

5.1 Aggregation of mutant pro-vasopressin

Mutations in the pro-vasopressin gene causing DI are inherited in a dominant manner. This dominance is most likely due to cytotoxicity of the precursor leading to massive loss of vasopressinergic magnocellular neurons, which results in low or undetectable circulating levels of vasopressin. We were able to show that mutant vasopressin precursors are retained in the ER. This is a common effect of dominant DI mutations, independent from their nature or location. It is very likely that all of them are retained due to misfolding. We could also show that DI mutants form intracellular aggregates of mutant protein. Analysis under higher magnification by electron microscopy revealed a fibrillar substructure within the protein accumulations. *In vitro* expression of the vasopressin precursor confirmed the ability of pro-vasopressin to self assemble into ordered, linear polymers. This connects ADNDI to a whole group of neurodegenerative diseases characterized by the formation of protein aggregates and amyloid fibers.

Many proposals regarding the mechanism of toxicity induced by protein aggregations have been made (Ross & Poirier 2005). Aggregated proteins could inhibit the proteasome as shown for α-synuclein in Parkinson's disease (Tanaka et al. 2001). Another possible mechanism of toxicity could be impairment of autophagy, since mutant α-synuclein has been reported to impair chaperone-mediated autophagy (Cuervo et al. 2004). During the aggregation process aberrant proteins could interact with or even recruit other cellular constituents, thereby inactivating them. Finally, exposure of hydrophobic patches could cause interactions with other cellular components, for example cellular membranes, potentially leading to the formation of pores.

In contrast to Alzheimer's, Parkinson's or Huntington's disease where aggregates are found extracellularly, in the cytosol, or nucleus, aggregates of pro-vasopressin are located in the ER. Nevertheless the above mentioned mechanisms of action may similarly apply. Direct inhibition of the proteasome as discussed above is unlikely, since it has been shown that mutant pro-vasopressin is retrotranslocated and

degraded by the proteasome (Friberg, Spiess & Rutishauser 2004). Impairment would rather take place at the site of retrotranslocation. Accumulations of mutant pro-vasopressin could partly get stuck in the retrotranslocon and thus cause persistent blockage. Another possible mechanism could be the recruitment and sequestration of proteins important for proper ER function, mainly chaperones. During the aggregation process, mutant pro-vasopressin might recruit large amounts of chaperones like calnexin, calreticulin or PDI, making them unavailable for other proteins during their synthesis. Improperly folded pro-vasopressin could expose hydrophobic patches which could form pores in the ER membrane. Such pores would destroy the ER membrane, causing massive Ca^{2+} leakage into the cytoplasm which triggers apoptosis.

For the oligomerization process of mutant pro-vasopressin, cysteines turned out to be required by forming intermolecular disulfide crosslinks. However, no single cysteine is specifically necessary to form disulfide-linked oligomers and large protein aggregates. The formation of disulfide-linked oligomers was also observed in cells expressing either wild-type pro-vasopressin or the recessive mutant P7L; however, oligomerisation happened on a much lower level. This suggests that disulfide crosslinking of the precursor is a transient event and thus can be resolved again, e.g. by PDI, enabling the protein to assume its native structure. However, higher concentrations of mutant unfolded precursor promote the formation of homo-oligomers, since folding is not possible.

Importance of covalent crosslinks in neurodegenerative diseases has been reported for Cu/Zn-superoxide dismutase (SOD1) in a familial form of amyotrophic lateral sclerosis (ALS), where point mutations in SOD1 are the cause for neurodegeneration (Rosen et al. 1993; Deng et al. 1993). Aggregates of mutant SOD1 proteins are observed in histopathology and are invoked in several proposed mechanisms for motor neuronal death (Johnston et al. 2000). SOD1 contains 4 cysteine residues. Mutations in the SOD1 gene were reported to induce the formation of an incorrect disulfide bond upon mild oxidative stress. This intermolecular disulfide bond was shown to be important for high molecular weight aggregates in the cytosol. Recent biochemical studies suggest that it is the immature disulfide-reduced forms of the familial ALS mutant SOD1 proteins that play a critical role; these incorrect forms tend to misfold, oligomerize, and readily undergo incorrect disulfide formation. It is not

known how the disulfide bridge is formed in the reducing environment of the cytosol (Niwa et al. 2007).

Another example for the formation of covalent intermolecular crosslinks in neurodegeneration is the protein huntingtin. Expanded CAG repeats in the huntingtin gene are the cause for Huntington's disease. The disease manifests when 40 or more consecutive glutamines are present in the protein. Transglutaminases cause oligomerisation via transamination between huntingtin molecules, leading to protein aggregates in the nucleus and the cytoplasm (Kahlem, Green & Dijan 1998).

Andringa et al (2004) showed that in Parkinson's disease, α-synuclein is also covalently crosslinked by tissue transglutaminases. *In vitro* experiments showed that transglutaminase 1, 2 and 5 specifically crosslinked glutamine 99 and lysine 58. As shown by Thioflavine T fluorescence monitoring, the formation of this crosslink accelerated the aggregation of native α-synuclein in Lewy bodies (Nemes et al. 2009). Taken together these findings suggest that covalent crosslinks in aggregation diseases are more widespread than previously assumed.

5.2 The vasopressin domain of the precursor is necessary for aggregation but not oligomerisation

In order to further characterize the aggregation mechanism, we were looking for other factors influencing the aggregation competence of the vasopressin precursor. We already showed that cysteines play an important role in aggregation since replacement of all 16 cysteines by serines abolished aggregate formation. However, since no specific group of cysteines seemed to exquisitely promote aggregate formation, we were looking for a sequence necessary for aggregation. For this purpose we performed a proline/glycine scan where we replaced sequential stretches of 10 amino acids by a sequence riche of prolines and glycines (P/G). Surprisingly, the analysis of the resulting 10 mutant vasopressin precursors revealed that the formation of disulfide-linked homo-oligomers was not abolsihed by prolines. However, the formation of large aggregates was specifically prevented if the vasopressin domain was replaced by a P/G rich sequence, indicating that the

determinants for aggregation are located in the nonapeptide vasopressin and that oligomerisation not necessarily culminates in formation of large aggregates.

The detailed mechanism for the formation of fibrillar amyloid-like aggregates is not entirely clear. The first step is always represented by protein misfolding, followed in a second step by formation of protein oligomers. The process finally culminates in the formation of fibrils. Additionally, several other structures have been described to be part of the protein misfolding and aggregation process, including soluble oligomers, pores, annular structures, spherical micelles and protofibrils (Glabe & Kayed 2006; Caughey & Lansbury 2003; Haass & Selkoe 2007). There are studies supporting the notion that an intermediate-ordered oligomers rather than the fibrillar endproduct represents the pathogenic protein (Goldberg & Lansbury Jr 2000; Haass & Steiner 2001). The hypothesis that primarily early aggregates exhibit toxicity also provides an explanation for the poor correlation between inclusion bodies and other visible aggregates and the severity of neurodegenerative diseases such as Alzheimer's (Terry et al. 1991) or Parkinson's (Tompkins & Hill 1997). An assay to measure toxicity of dominant DI mutants would provide information about the toxic aggregate species in ADNDI. It remains to be further investigated to which extent disulfide-linked homo-oligomers and the larger aggregations in ADNDI contribute to cytotoxicity. Further analysis of the mechanisms and consequences of aggregation may provide deeper insight into the mechanisms leading to cytotoxicity in ADNDI.

5.3 Aggregation of mutant vasopressin precursors: a premature physiological event?

Although amyloids are associated with more than two-dozen human diseases, there are also amyloids with native biological activities. The Escherichia coli protein "curli" forms extra-cellular amyloid fibrils which are involved in surface and cell-cell contact (Barnhart & Chapman 2006). Pmel17 is a melanocyte protein necessary for eumelanin deposition in mammals and is found in melanosomes in a filamentous form. It forms a functional human amyloid that is important in the formation of skin pigmentation. An increasing number of proteins with no link to protein deposition diseases has been found to form, under some conditions *in vitro*, fibrillar aggregates

that have the morphological and structural properties classifying them as amyloid fibrils, e.g. endostatin or stefin B (Stefani & Dobson 2003). These results suggest that the ability to form amyloid fibrils is a generic property of many peptides and proteins.

Maji et al. (2009) proposed a model in which amyloid formation of peptide hormones is important and contributes to normal cell and tissue physiology. They analyzed 42 peptide hormones for their capacity to form amyloids and found 10 to be capable. Among them was the nonapeptide vasopressin. According to this model, transient aggregation of vasopressin into amyloid like structures is part of the maturation process of dense core secretory vesicles. The lower pH of the TGN and secretory granules causes vasopressin to exit its binding pocket of NPII and amyloid formation is initiated. Upon vasopressin leaving its binding pocket, prohormone convertase clipps the protein. The amyloid formed by vasopressin is desintegrated upon fusion of the secretory granule with the plasma membrane due to increase in pH in extracellular conditions. This model would propose a physiological role of amyloid formation. The finding of Maji et al. (2009) could explain previous and recent findings in ADNDI. Beuret et al. (1999) analyzed the molecular consequences of a mutation (ΔG227) that destroys the translation initiation codon. The precursor was completely retained within the ER but the uncleaved signal did not affect folding of the neurophysin portion. Preventing disulfide formation in the vasopressin nonapeptide by mutation of cysteine 6 to serine was shown to be sufficient to cause disulfide oligomerization and retention. The present data extend these earlier findings and confirms that pro-vasopressin is still able to aggregate even when NPII is folded and that vasopressin is the portion promoting aggregation. This would also fit to our findings that aggregate formation was disrupted by exchanging the vasopressin portion by a stretch of prolines and glycines (Fig 19). The role of the disulfide bond formation, however, would be to act as a stabilizing factor for aggregate formation, since conditions in the ER (pH7.4) are different from those in the TGN (pH 6.2) and granules (pH5.5). The higher pH of the ER resembles more extracellular conditions. Thus formation of pro-vasopressin amyloids in the ER without a stabilizing factor may be not possible.

Taken together, our finding could represent a premature aggregation event which takes place in the ER in contrast to the physiological aggregation event in the Golgi

as described by Maji et al. (2009). In the ER, aggregates cannot be resolved anymore. Important for aggregation initiation is a high concentration of the hormone. Mutant precursors can accumulate over time and thus form amyloid-like aggregates in the ER.

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