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Functional Amyloids As Natural Storage of Peptide Hormones in Pituitary Secretory Granules

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Amyloids are highly organized cross- β -sheet-rich protein or peptide aggregates that are associated with pathological conditions including Alzheimer's disease and type II diabetes. However, amyloids may also have a normal biological function, as demonstrated by fungal prions, which are involved in prion replication, and the amyloid protein Pmel17, which is involved in mammalian skin pigmentation. We found that peptide and protein hormones in secretory granules of the endocrine system are stored in an amyloid-like cross- β -sheet-rich conformation. Thus, functional amyloids in the pituitary and other organs can contribute to normal cell and tissue physiology.

Cells transport newly synthesized secretory proteins and peptides in vesicles via the endoplasmic reticulum (ER) and Golgi for release into the extracellular space (1, 2). Some secretory cells, such as neuroendocrine cells and exocrine cells, store secretory proteins and peptides for extended time periods in a highly concentrated form in membrane-enclosed electron-dense cores termed "secretory granules" (1, 3, 4), which are derived from the Golgi complex. The dense cores of these granules are made up of large, insoluble secretory protein and peptide aggregates that are formed by self-association (4–6). The granules are not amorphous but possess a distinct molecular organization, possibly of crystalline structures (7) or large intermolecular aggregates (5, 8).

Amyloid fibrils are cross- β -sheet structures that are primarily associated with several neurodegenerative diseases including Alzheimer's disease. However, amyloid fibril formation also provides biologically functional entities termed functional amyloids (9) that are present in *Escherichia coli* (10), silkworms (11), fungi (12), and mammalian skin (13). The cross- β -sheet motif is composed of intermolecular β sheets along

the fibril axis with the β strands aligned perpendicularly to the fibril axis. An amyloid-like structure of peptide and protein hormones in secretory granules could explain most of their properties.

To address the question of whether peptide and protein hormones are stored in secretory granules in an amyloid-like aggregation state, we first asked whether a diverse set of peptide and protein hormones could form amyloids in vitro at granule-relevant pH 5.5. We randomly selected 42 peptide and protein hormones from multiple species and organs, some linear and some cyclic, with a variety of different three-dimensional structures (table S2). This set of hormones was assayed for a capacity to form amyloids by the amyloid-specific dyes thioflavin T (Thio T) and Congo Red (CR), by luminescent conjugated polyelectrolyte probes (LCPs), by the conformational transition into β -sheet-rich structure measured by circular dichroism (CD) spectroscopy, and by the presence of fibrils in electron microscopy (EM) images. Furthermore, x-ray fiber diffraction was measured for a subset of hormones (table S1). Only 10 of the 42 hormones showed considerable formation of amyloids (table S1 and figs. S1 and S2).

Given that only one-quarter of peptides spontaneously formed amyloid fibrils in vitro (table S1) and the possible involvement of glycosaminoglycans (GAGs) in the formation of both secretory granules and amyloid fibrils (14–22), the amyloid formation of all 42 peptides and proteins was monitored in the presence of low molecular weight (LMW) heparin as a representative of GAGs. After 2 weeks of incubation in the presence of heparin, most hormones formed amyloid fibrils (table S1) as assessed by EM (Fig. 1A and fig. S3), Thio T binding (fig. S1), CD (table S1 and fig. S4), CR binding (table S1 and figs. S5A and S5B), LCP binding (fig. S5C), and x-ray fiber diffraction (fig. S5D). In addition, the algorithm TANGO predicted 18 hormones to be aggregation-prone

(fig. S6). Thus, of the 42 hormones, 31 formed amyloid fibrils in the presence of heparin by all methods studied. Nonconclusive data were obtained for four hormones (23) and the α -helical corticotropin-releasing factor (α -CRF) served as a negative control (23). Possible explanations for the lack of amyloid aggregation of the remaining six hormones may be that these hormones do not form amyloid-like aggregates when stored in secretory granules, that fibrillization conditions were not optimal, and/or that another substance might be required for aggregation. Indeed, human prolactin did not form amyloids in the presence of heparin, but did in the presence of chondroitin sulfate A, which is a GAG found in prolactin-specific granules (16) (Fig. 1A, table S1, and fig. S1).

None of the adrenocorticotrophic hormones (ACTHs) studied formed amyloid-like aggregates (table S1). Because ACTH and β -endorphin are processed from the same prohormone (pro-opiomelanocortin) and secreted together in a regulated secretory pathway, we hypothesized that ACTH might need the amyloid-forming β -endorphin as an aggregation partner for storage in secretory granules. An ACTH- β -endorphin 1:1 mixture in the presence of heparin formed amyloid fibrils (Fig. 1B) that comprised ACTH, as measured by dot plot (Fig. 1C) and Trp fluorescence (Fig. 1D). The Trp/Tyr measurements also indicate that the ACTH- β -endorphin amyloid fibrils are composed of a 1:2 ratio of ACTH and β -endorphin present either in mixed or individual fibrils (Fig. 1D). Because seeding experiments of ACTH with β -endorphin fibrils failed, we suggest that ACTH and β -endorphin form mixed fibrils. Because fibril-forming hypothalamic human CRF was not able to induce aggregation of the pituitary ACTH, the ACTH- β -endorphin aggregation appeared to be specific [fig. S8, D and E; see also (23) and fig. S7]. Coaggregation between β -endorphin and ACTH was further supported by a colocalization study of the two hormones in the tumor cell line AtT20 using double immunohistochemistry (Fig. 1E and fig. S7). A detailed analysis revealed that all the stained ACTH colocalized with β -endorphin, whereas some β -endorphin did not colocalize with ACTH, which supports the in vitro observation that ACTH aggregates only in the presence of β -endorphin. To show that the ACTH coaggregation with β -endorphin was not an isolated case, we document coaggregation of ghrelin with obestatin in (23) and fig. S8.

The concept that peptide and protein hormones are stored in secretory granules in an amyloid-like aggregation state is challenged by the notion that amyloid fibrils are very stable and do not release monomers [but see (21)], a prerequisite upon granule secretion. To demonstrate that amyloid fibrils formed by hormones are able to release monomers, we performed an amyloid release assay (21) for a selection of hormone fibrils both at granule-relevant pH 6 and at pH 7.4, at which the hormone aggregates

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are exposed upon secretion (Fig. 2 and fig. S9). The monomer release was monitored outside the membrane by CD (Fig. 2B) and by ultraviolet absorption spectroscopy or Trp fluorescence spectroscopy (fig. S9), and the remaining aggregates (inside the dialysis membrane) were monitored by Thio T binding (Fig. 2A and fig. S9). All hormone fibrils studied appeared to release monomeric hormone upon dilution, because with time a drastic decrease of the Thio T binding was apparent and monomers were present outside the membrane. To show that the released monomers were functional, we investigated the monomer release of human CRF from its amyloid entity in more detail. The CD spectrum (Fig. 2B) indicated that the released human CRF comprises its functional helical structure. Furthermore, mono-

meric and aggregated human CRF activate cyclic adenosine monophosphate (cAMP) with similar efficacy in CHO cells expressing stably human type 1 CRF receptor (Fig. 2C), indicating that CRF amyloid fibrils release active monomer. The *in vitro* dialysis experiments with amyloid fibrils therefore resemble qualitatively the release abilities of isolated secretory granules (24).

Amyloid fibrils are thought to be toxic to neuronal cells (25). To test whether amyloids of hormones are toxic, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan assay (MTT assay) with the neuronal cell line B12 (25). Upon addition of 20 μ M A β (1-40) fibrils, MTT reduction was decreased by 40% (fig. S10A). In contrast, many of the hormone amyloids formed in the presence

of LMW heparin and all hormones that did not aggregate into amyloids induced no considerable decrease in MTT reduction at 20 μ M. However, in contrast to monomers, amyloids of ovine CRF, mUcnIII, hUcnIII, human growth hormone-releasing factor, and GLP-2 showed a response similar to that of A β (1-40), whereas glucagon amyloids interfered with the reduction of MTT almost twice as much (26). To further support the observation that some amyloids from hormones are moderately toxic, we measured the neuronal cell survival of E18 rat hippocampal neurons after the addition of amyloid fibrils of A β (1-42) and human CRF, which showed a moderate effect in the MTT assay (fig. S10, B and C). Although A β (1-42) fibrils appeared to be one order of magnitude more

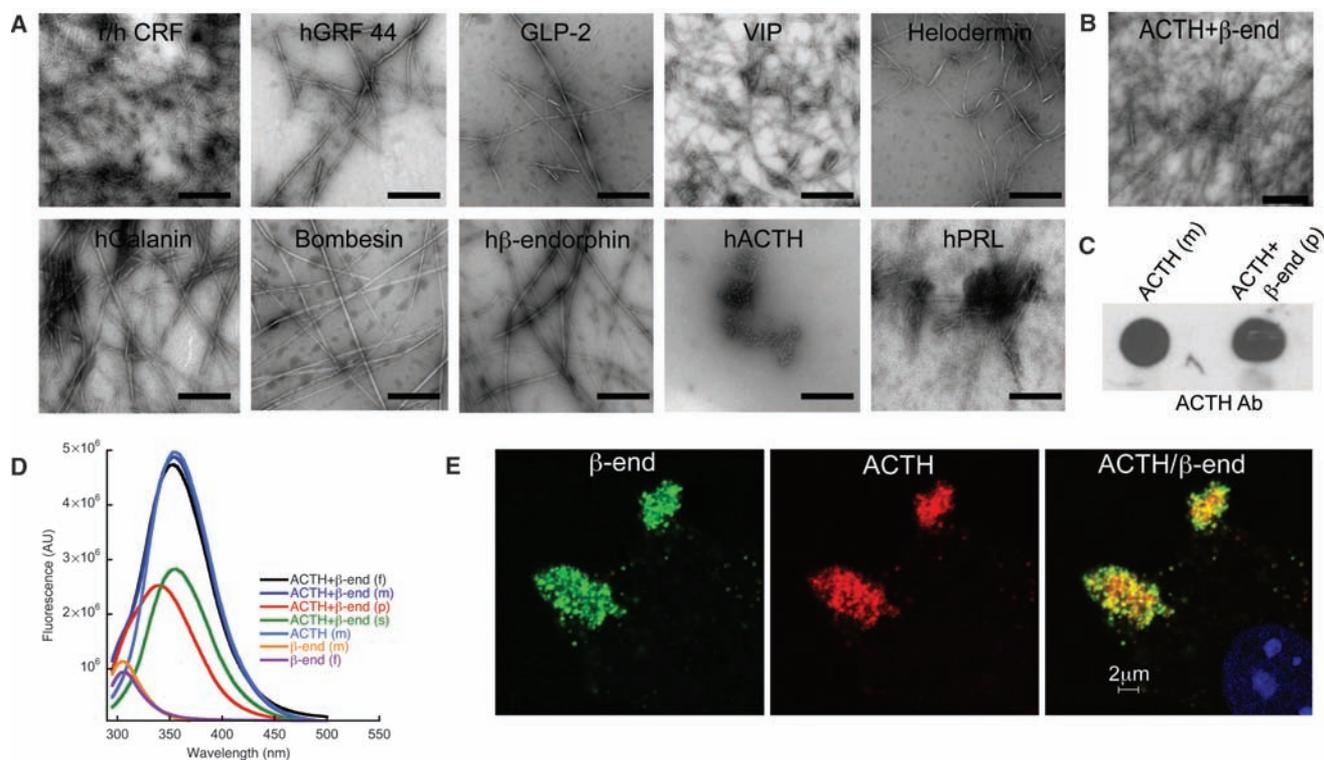


Fig. 1. Amyloid-like aggregation and coaggregation of hormones. **(A)** EM images of 10 hormones incubated for 14 days, indicating the formation of amyloid fibrils. The entire set of 42 hormones studied is shown in fig. S3. The aggregations of the hormones were followed at 37°C at a concentration of 2 mg/ml in the presence of 0.4 mM LMW heparin in 5% D-mannitol (pH 5.5) under slight agitation. Human prolactin (hPRL) was fibrillized in the presence of 400 μ M chondroitin sulfate A. Transmission electron microscopy (TEM) of negative stained samples was performed. Scale bars, 500 nm. **(B to E)** Coaggregation of ACTH with β -endorphin measured by **(B)** EM, **(C)** dot blot, **(D)** Trp and Tyr fluorescence, and **(E)** colocalization in AtT20 cells. **(B)** EM of ACTH- β -endorphin mixture at 37°C at hormone concentrations of 1 mg/ml each in the presence of 0.4 mM LMW heparin in 5% D-mannitol (pH 5.5) incubated under slight agitation for 14 days. **(C)** The presence of ACTH in these amyloid fibrils of the ACTH- β -endorphin mixture was identified by dot blot staining with ACTH antibody of the aggregates harvested by centrifugation (p). For a positive control of the antibody staining, ACTH monomer (m) was used. **(D)** Trp and Tyr fluorescence was measured for an aggregated sample of ACTH- β -endorphin mixture, labeled with (f) and shown in black. High-speed centrifugation at 120,000g for 1 hour was performed with this sample, and the Trp and Tyr fluorescence was measured for

the corresponding pellet (p) in red, as well as the supernatant (s) in green. Furthermore, a fresh solution of a mixture of ACTH- β -endorphin (m) in blue, a fresh solution of ACTH (m) in cyan, a fresh solution of β -endorphin (m) in yellow, and an aggregated β -endorphin fibrillar sample (f) in violet were measured accordingly. The Trp fluorescence signal in pellet suggests that ACTH is present in fibrils because only ACTH has a Trp. The λ_{max} of the Trp signal is blue-shifted by 15 nm when compared to monomeric/nonfibrillated ACTH, which suggests that the Trp is less solvent-exposed in the fibril structure than in its monomeric state. The fluorescence study suggests the aggregation of about half of the ACTH, because the Trp fluorescence intensities of the pellet containing fibrils and the corresponding supernatant containing ACTH monomer were similar. Because the Tyr fluorescence of monomeric β -endorphin, the β -endorphin fibrils harvested by centrifugation, and the harvested ACTH- β -endorphin fibrils were similar in intensity and no Tyr fluorescence signal was observed in the corresponding supernatant, the entire β -endorphin population appeared to have aggregated into amyloid fibrils. **(E)** Colocalization of the two hormones in the tumor cell line AtT20 by double immunohistochemistry with mouse ACTH (red) and rabbit endorphin antibodies (green). The nuclear marker 4',6'-diamidino-2-phenylindole is shown in blue.

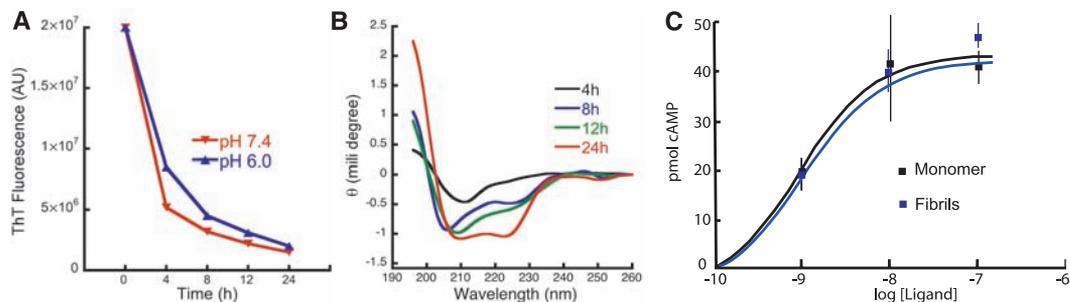
toxic than human CRF fibrils, human CRF amyloids also influenced the survival of neurons with a median inhibitory concentration of $\sim 20 \mu\text{M}$. Although some hormone amyloids appear to be moderately toxic, their actual toxicity might be substantially diminished *in vivo* by their membrane-encapsulated state in the granules.

The above biophysical analysis of hormones supports the hypothesis that hormones are stored as amyloids in secretory granules. Below we offer direct evidence that secretory granules of the mouse pituitary tumor neuroendocrine cell line AtT20 are composed of amyloids. The AtT20

cell line synthesizes precursors to corticotropin (i.e., ACTH and β -endorphin) and correctly glycosylates, cleaves, and stores them in secretory granules (27). Secretory granules of AtT20 cells with and without membrane were purified (fig. S11) and showed typical ACTH-containing electron-dense granules with a diameter of 200 to 600 nm surrounded by membrane (fig. S11, B to D). The purified granules from AtT20 cells were amyloid-like in nature, as determined by amyloid-specific antibody (Fig. 3A), Thio T binding (Fig. 3B), CR binding (fig. S12), and CR birefringence (Fig. 3C). Finally, x-ray fiber diffraction was

measured of purified membraneless secretory granules (fig. S11B). A near-membrane-free preparation of granules was used because membrane lipids contribute a very strong reflection at 4.1 Å, close to the 4.7 Å reflection expected for a cross- β -sheet structure. The major reflections observed were at 4.7 Å, interpreted as the spacing between strands in a β sheet, and a diffuse reflection at ~ 10 Å, interpreted as the spacing between β sheets (Fig. 3D). These reflections are typically observed for amyloid-like fibrils. The circular profiles of these reflections, rather than the normal orthogonal positions for the two reflections,

Fig. 2. Release of monomeric, α -helical, and functional CRF from its amyloid fibrils. CRF amyloid fibrils were dialyzed against buffer with a 10-kD cutoff membrane. (A) Time-dependent decrease of Thio T fluorescence inside the membrane at two pHs as labeled. The decrease of Thio T indicates loss of amyloid fibrils due to dialysis. (B) Time-resolved CD spectroscopy outside the membrane measuring released CRF. The time-dependent increase of the signal indicates release of CRF from the amyloid. The released CRF is likely to be monomeric because of the 10-kD cut-off of the dialysis membrane. The CD spectrum of the released CRF is of helical structure, which corresponds to the active conformation of



CRF. (C) Functional studies of monomeric and amyloid fibrillar sample of CRF by measuring in a hormone concentration-dependent manner the activation of intracellular cAMP in CHO cells stably expressing CRF-R1. Both samples show similar potencies.

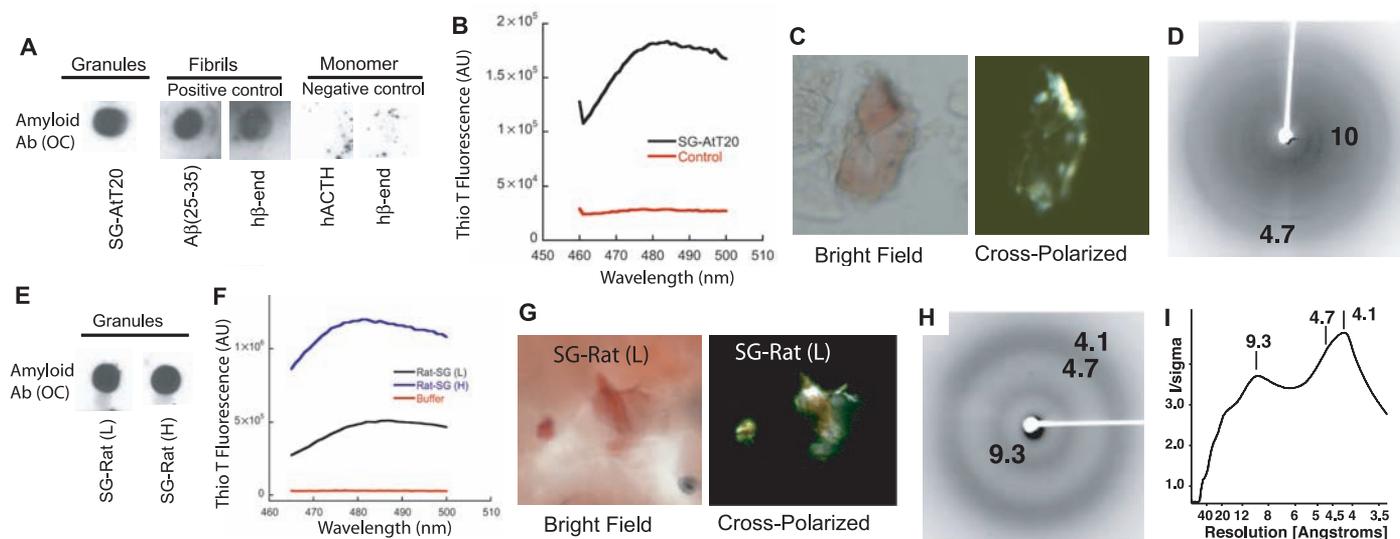


Fig. 3. Purified secretory granules from the AtT20 cell line and from rat pituitary are composed of an amyloid-like structure as determined by an amyloid-specific antibody OC (A and E), the amyloid-specific dye Thio T (B and F), CR birefringence (C and G), and x-ray fiber diffraction (D, H, and I). (A) Dot blot staining of purified secretory granules from AtT20 cells against the amyloid-specific antibody OC. As positive controls, stains of amyloid fibrils of A β (25–35) fibrils and β -endorphin fibrils (in presence of LMW heparin) are shown. As negative controls, stains of monomeric human ACTH and β -endorphin are shown. (B) Thio T fluorescence between 460 and 500 nm is shown for purified secretory granules (black) and buffer only (red). Thio T was excited at 450 nm. The strong binding of Thio T to secretory granules suggests that they are composed of amyloid-like structures. (C) Congo Red birefringence of purified secretory granules. CR birefringence is shown that suggests the presence of ordered (amyloid) aggregates. The picture

represents the bright-field microscope image with 10 \times resolution. The same section is shown under cross-polarized light with 10 \times magnification, respectively. (D) X-ray fiber diffraction of purified membrane-depleted secretory granules that were treated with 1% Lubrol PX. The two reflections at 4.7 Å and ~ 10 Å consistent with cross- β -sheet structure are labeled. [(E) to (I)] Purified light (L) and heavy (H) secretory granules from rat pituitary are composed of amyloid-like structure as assessed by an amyloid-specific antibody OC (E), the amyloid-specific dye Thio T (F), CR birefringence (G), and x-ray fiber diffraction [(H) and (I)]. The same measurement parameters as in [(A) to (D)] were used. In (I) the x-ray fiber diffraction measurements of purified membraneless secretory granules are shown as a full image radial profile. The two reflections at ~ 4.7 Å and ~ 9.3 Å consistent with cross- β -sheet structure are labeled. In addition, a strong reflection at 4.1 Å is present, attributed to the remaining lipid content of the granules under study.

show that the amyloid-like entities in granules are not strongly aligned.

Similarly, secretory granules of type light (L) and heavy (H) purified from rat pituitary (fig. S13) also contained amyloid-like material, as evidenced by amyloid-specific antibody binding (Fig. 3E), Thio T binding (Fig. 3F), CR binding (fig. S12, C and D), and birefringence (Fig. 3G) (23). Furthermore, the x-ray diffraction pattern with membraneless granules of type L show the typical cross- β -sheet reflections at 4.7 Å and at \sim 10 Å, in addition to a 4.1 Å reflection attributed to remaining membrane (Fig. 3, H and I). The circular profiles for these reflections indicate that the amyloid-like entities in granules are not strongly aligned.

Immunohistochemical studies were performed with mouse pituitary tissue to probe whether secretory granules in neuroendocrine tissues are amyloid-like in nature. The positive binding of the amyloid-specific dye Thio S both in the anterior and posterior lobes of the pituitary was indicative of the abundant presence of amyloids in the mouse pituitary (Fig. 4). To examine whether the Thio S-detected amyloids were composed of endocrine hormones, we measured colocalization of Thio S with hormone-specific antibodies. There was almost complete colocalization between

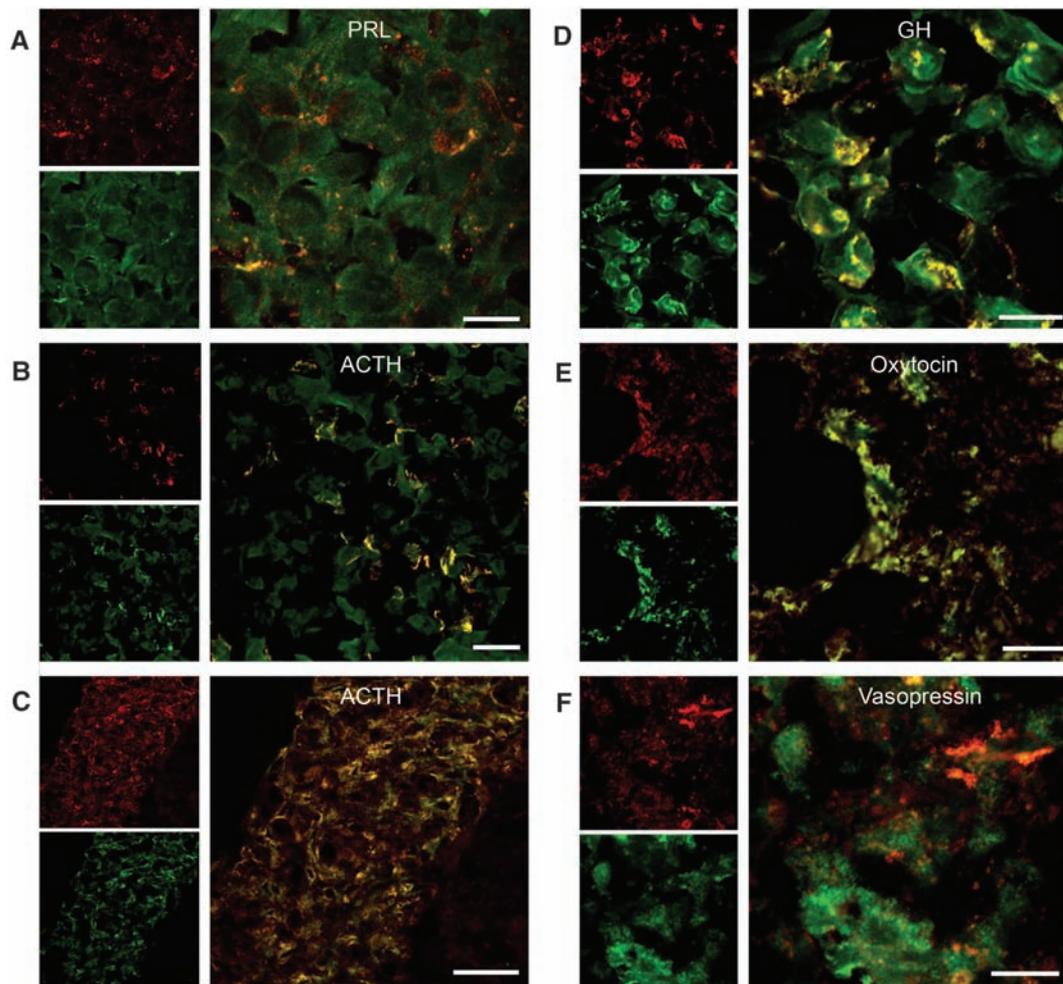
Thio S and the hormones ACTH, β -endorphin, prolactin, and growth hormone (GH) in the anterior lobe, ACTH in the intermediate lobe, and oxytocin and vasopressin in the posterior lobe of the pituitary, respectively (Fig. 4), strongly supporting their storage in the secretory granules to be extensively amyloid-like. Similar results were obtained for a colocalization study between the fibril-specific antibody OC and the corresponding hormone antibodies.

The proposed amyloid-like conformation of peptide/protein hormones in secretory granules may explain the processes of granule formation including hormone selection, membrane surrounding and inert hormone storage, and subsequently the release of hormones from the granules (fig. S14). We propose that in the Golgi, amyloid aggregation of the (pro)hormone is initiated spontaneously above a critical (pro)hormone concentration and/or in the presence of helper molecules such as GAGs in parallel to a possible prohormone processing. Alternatively, because the prohormone may aggregate less into an amyloid entity than its hormone counterpart (28), the prohormone processing at critical hormone concentrations may initiate the aggregation. Because the formation of amyloid fibrils is amino acid sequence-specific, the initiated amyloid aggre-

gation of the (pro)hormone is selective, yielding granule cores composed of specific hormones only. Specific coaggregation of some hormones may be possible because some amyloid proteins are able to cross-seed (29) (Fig. 1). The amyloid aggregation thereby sorts the protein/peptide hormones into secretory granule cores, concentrates them to the highest density possible, and excludes non-aggregation-prone, constitutively secreted proteins. During the aggregation process, the hormone amyloids become surrounded by membrane, separate from the Golgi, and form mature granules. The membrane attraction may be spontaneous because membrane binding seems to be an inherent property of amyloid aggregates (30). Alternatively, because the cross- β -sheet represents a single structural epitope, it may serve as a possible recognition motif of an unknown granule-recruiting membrane protein. Once the secretory granules are formed, they can be stored for long durations because the amyloid entity provides a very stable depot. Upon signaling, secretory granules are secreted and the cross- β -sheet structure of the amyloid enables a controlled release of monomeric, functional hormone (21), which might be supported by chaperones.

The functional amyloid state of many endocrine hormones in secretory granules of the pi-

Fig. 4. Immunohistochemical staining of the mouse pituitary with Thio S (green) and polyclonal antibodies (red) to (A) prolactin, (B) and (C) ACTH, (D) growth hormone (GH), (E) oxytocin, and (F) vasopressin. Regions of the pituitary selected are anterior lobe [(A), (B), and (D)], intermediate lobe (C), and posterior lobe [(E) and (F)]. Scale bars, 20 μ m.



tuitary (Fig. 4), and possibly the hypothalamus (e.g., CRF) and pancreas (e.g., somatostatin), contrasts directly the historical disease association of amyloids both in the brain (e.g., tau, α -synuclein, A β , and prion protein) and in the pancreas (e.g., insulin and amylin). On the one hand, this challenges once more the “amyloid hypothesis” that amyloids are the most toxic culprit in amyloid diseases (31). On the other hand, hormone amyloids may not be (very) toxic because the hormone amyloids are stored inside the granules and the amyloid aggregation of hormones for secretory granule formation may be highly regulated. This regulation may include the processing of prohormones that aggregate more slowly than the hormone counterpart (28) or the required presence of helper molecules to induce aggregation, as demonstrated for prolactin (note that prolactin lacks a prohormone). Furthermore, the hormone amyloids are stored in an “inert” membrane container and the amyloid fibrils dissociate upon secretion. Such regulation requires a functional protein homeostasis. If the protein homeostasis is altered under certain conditions such as diet, stress, or age, hormone aggregation may be out of control and disease-associated amyloid aggregation of hormones may occur (see below). Whether such aggregations cause disease, or are an indirect effect of the protein homeostasis altered by disease, remains to be determined.

There are many associations between amyloid processing and function of secretory granules: (i) Aggregation of hormones into secretory granules is an intracellular process, and upon secretion not all of the aggregates dissolve completely (32). Similarly, A β may be present in granules (33), and A β aggregation may be initiated intracellularly and end up as a pathological hallmark in the extracellular space (31). (ii) The malaria drug chloroquine interferes with the formation of secretory granules as well as prion infectivity (34, 35). (iii) The natural compound curcumin interferes with both the progression of Alzheimer’s disease (36) and the release of endocrine hormones (37). (iv) Several endocrine hormones that may be stored in secretory granules in an amyloid-like state are present as amyloids in amyloid diseases. These include amylin associated with diabetes type II, calcitonin associated with medullary carcinoma of the thyroid, and atrial natriuretic factor with atrial amyloidosis (38). Hence, the presence of many functional amyloids in the body, together with the apparent tight link between functional and disease-associated amyloids in their processing, biophysical, and biochemical properties, requires a rethinking of the relationship between aggregation and function of polypeptides, and the correlation between amyloid aggregation and toxicity.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S14

Tables S1 and S2

References

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RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis

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Necrosis can be induced by stimulating death receptors with tumor necrosis factor (TNF) or other agonists; however, the underlying mechanism differentiating necrosis from apoptosis is largely unknown. We identified the protein kinase receptor-interacting protein 3 (RIP3) as a molecular switch between TNF-induced apoptosis and necrosis in NIH 3T3 cells and found that RIP3 was required for necrosis in other cells. RIP3 did not affect RIP1-mediated apoptosis but was required for RIP1-mediated necrosis and the enhancement of necrosis by the caspase inhibitor zVAD. By activating key enzymes of metabolic pathways, RIP3 regulates TNF-induced reactive oxygen species production, which partially accounts for RIP3’s ability to promote necrosis. Our data suggest that modulation of energy metabolism in response to death stimuli has an important role in the choice between apoptosis and necrosis.

Cell death occurs through morphologically distinct processes of apoptosis and necrosis (1). Some necrosis is regulated, via pathways differing from those controlling classical apoptosis, although necrosis/apoptosis interconnectivity has been observed (1–4). Caspase inhibition, which distinguishes apoptotic and non-apoptotic cell death, sometimes shifts apoptosis to necrosis or enhances necrosis (5–8). Receptors containing death domains can induce a form of

regulated necrosis through kinase activity of RIP1 (receptor-interacting protein 1) (3, 5, 6). Mitochondrial generation of reactive oxygen species (ROS) is essential for this type of necrosis (9). A genome-wide small interfering RNA (siRNA) screen has identified a number of genes involved in the signaling network of death domain receptor-mediated necrosis (10), but the precise mechanisms underlying programmed necrosis and the apoptosis/necrosis molecular switch remain unclear.