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## Functional Amyloids as Natural Storage of Peptide Hormones in Pituitary Secretory Granules

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### Abstract

Amyloids are highly organized cross  $\beta$ -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. Here, we show that peptide and protein hormones in secretory granules of the endocrine system are stored in an amyloid-like cross  $\beta$ -sheet-rich conformation. Thus, in contrast to the original association of amyloids with diseases, 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- $\beta$ -sheet structures that are primarily associated with several neurodegenerative diseases including Alzheimer's disease. However, amyloid fibril formation

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**One-sentence summary:** Peptide and protein hormones are stored in secretory granules in an amyloid conformation

also provides biologically functional entities termed functional amyloids (9) and are present in *Escherichia coli* (10), silkworm (11), fungi (12), and mammalian skin (13). The cross- $\beta$ -sheet motif is composed of intermolecular  $\beta$ -sheets along the fibril axis with the  $\beta$ -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 whether peptide and protein hormones are stored in secretory granules in an amyloid-like aggregation state, we first asked if a diverse set of peptide and protein hormones could form amyloids in vitro at granule-relevant pH 5.5. 42 peptide and protein hormones from multiple species and organs were selected randomly, 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), congo red (CR), luminescent conjugated polyelectrolyte probes (LCP), by the conformational transition into  $\beta$ -sheet-rich structure measured by circular dichroism (CD), 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 hormones out of the 42 showed significant formation of amyloids (figs. S1 and S2, Table S1).

Given that only one quarter of peptides spontaneously formed amyloid fibrils in vitro (Table S1) and the possible involvement of glycosaminoglycans (GAG)s 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. Indeed, after two weeks of incubation in the presence of heparin, most hormones formed amyloid fibrils (Table S1) based on EM (Fig. 1A and fig. S3), Thio T binding (fig. S1), CD (Table S1, fig. S4), CR binding (Table S1, 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, out of the 42 hormones, 31 formed amyloid fibrils in the presence of heparin by all methods studied. Nonconclusive data was obtained for four hormones (see SOM) and the alpha-helical CRF served as a negative control (see SOM). 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 fibrilization conditions were not optimal, or/and 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) (Table S1, Fig. 1A and fig. S1).

None of the adrenocorticotrophic hormone's (ACTHs) studied formed amyloid-like aggregates (Table S1). Because ACTH and  $\beta$ -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  $\beta$ -endorphin as an aggregation partner for storage in secretory granules. A 1:1 ACTH -  $\beta$ -endorphin mixture in the presence of heparin formed amyloid fibrils (fig. 1B) that comprise ACTH as measured by dot plot (Fig. 1C) and Trp fluorescence (Fig. 1D). The Trp/Tyr measurements also indicate that the ACTH -  $\beta$ -endorphin amyloid fibrils are composed of a 1:2 ratio of ACTH and  $\beta$ -endorphin present either in mixed or in individual fibrils (Fig. 1D). Since seeding experiments of ACTH with  $\beta$ -endorphin fibrils failed, we suggest that ACTH and  $\beta$ -endorphin form mixed fibrils. Since fibril-forming hypothalamic human CRF was not able to induce aggregation of the pituitary ACTH, the  $\beta$ -endorphin - ACTH aggregation appeared to be specific (figs. S8D and S8E, see also SOM and fig. S7). Coaggregation between  $\beta$ -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  $\beta$ -endorphin, where as some  $\beta$ -endorphin did not colocalize with ACTH supporting the in vitro observation that ACTH aggregates only in the presence of  $\beta$ -

endorphin. To show that the ACTH co-aggregation with  $\beta$ -endorphin was not an isolated case, coaggregation of Ghrelin with obestatin is documented in SOM (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, an amyloid release assay was performed (21) for a selection of hormone fibrils both at granule-relevant pH 6 and pH 7.4 at which the hormone aggregates are exposed upon secretion (Fig. 2 and fig. S9). The monomer release was monitored outside the membrane either by CD (Fig. 2B), UV or Trp fluorescence (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, the monomer release of human CRF from its amyloid entity was investigated in more detail. The CD spectrum (Fig. 2B) indicated that the released human CRF comprises its functional helical structure. Furthermore, monomeric and aggregated human CRF activate cyclic AMP 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 (23).

Amyloid fibrils are thought to be toxic to neuronal cells (24). To test whether amyloids of hormones are toxic the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan assay (MTT assay) with the neuronal cell line B12 was employed (24). Upon addition of 20  $\mu$ M of A $\beta$ (1–40) fibrils, the 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 do not aggregate into amyloids induced no significant decrease in MTT reduction at 20  $\mu$ M concentration. However, in contrast to monomers amyloids of ovine CRF, mUcnIII, hUcnIII, human GRF, and GLP-2 showed a similar response as A $\beta$ (1–40), whereas glucagon amyloids interfered with the reduction of MTT almost twice as much (25). To further support the observation that some amyloids from hormones are moderately toxic, the neuronal cell survival of E18 rat hippocampal neurons was measured following the addition of amyloid fibrils of A $\beta$ (1–42) and human CRF, which showed a moderate effect in the MTT assay (figs. S10B and S10C). Although A $\beta$ (1–42) fibrils appeared to be one order of magnitude more toxic than human CRF fibrils, human CRF amyloids also influenced the survival of neurons with an IC<sub>50</sub> of approximately  $\sim$ 20  $\mu$ 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. In this paragraph, direct evidence is provided 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  $\beta$ -endorphin), and correctly glycosylates, cleaves, and stores them in secretory granules (26). 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 200–600 nm surrounded by membrane (figs. S11B, S11C, and S11D). 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 membrane-less secretory granules (fig. S11B). A near membrane-free preparation of granules was used because membrane lipids attribute a very strong reflection at 4.1 Å close to the 4.7 Å reflection expected for a cross- $\beta$ -sheet structure.

The major reflections observed were at 4.7 Å interpreted as the spacing between strands in a  $\beta$ -sheet and a diffuse reflection at  $\sim 10$  Å interpreted as the spacing between  $\beta$ -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, 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 (figs. S12C and S12D) and birefringence (Figs. 3G, see also SOM). Furthermore, the x-ray diffraction pattern with membrane-less granules of type light show the typical cross  $\beta$ -sheet reflections at 4.7 Å and at  $\sim 10$  Å in addition to a 4.1 Å reflection attributed to remaining membrane (Figs. 3H and 3J). 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 pituitary of mouse (Fig. 4). To examine if the Thio-S detected amyloids were composed of endocrine hormones colocalization of Thio-S with hormone-specific antibodies was measured. There was almost complete colocalization between Thio-S and the hormones ACTH,  $\beta$ -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 as well as the inert hormone storage, and subsequently the release of hormones from the granules (fig. S14): It is proposed that in the Golgi, amyloid aggregation of the (pro) hormone is initiated spontaneously above a critical (pro)hormone concentration or/and in presence of helper molecules such as GAGs in parallel to a possible prohormone processing. Alternatively, since the prohormone may aggregate less into an amyloid entity than its hormone counterpart (27), the prohormone processing at critical hormone concentrations may initiate the aggregation. Since the formation of amyloid fibrils is amino acid sequence-specific, the initiated amyloid aggregation of the (pro)hormone is selective yielding granule cores composed of specific hormones only. Specific coaggregation of some hormones may be possible since some amyloid proteins are able to cross-seed (28, Fig. 1). The amyloid aggregation sorts thereby 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 get surrounded by membrane, separate from the golgi and form mature granules. The membrane attraction may be spontaneous since membrane binding seems to be an inherent property of amyloid aggregates (29). Alternatively, since the cross- $\beta$  sheet represents a single structural epitope it may serve as a possible recognition motive of an unknown granule-recruiting membrane protein. Once the secretory granules are formed they can be stored for long durations since the amyloid entity provides a very stable depot. Upon signaling, secretory granules are secreted and the cross- $\beta$  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 pituitary (fig. 4) and possibly hypothalamus (i.e. CRF) and pancreas (for example somatostatin)

contrasts directly the historical disease-association of amyloids both in the brain (such as tau,  $\alpha$ -synuclein, A $\beta$ , and prion protein) and in the pancreas (such as insulin and amylin). On the one hand, this challenges once more the “amyloid hypothesis” that amyloids are the most toxic culprit in amyloid diseases (30). On the other hand, hormone amyloids may not be (very) toxic since 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 slower than the hormone counterpart (27) or the required presence of helper molecules to induce aggregation as demonstrated for prolactin (note: prolactin lacks a prohormone). Furthermore, the hormone amyloids are stored in an “inert” membrane container and the amyloid fibrils dissociate upon secretion. Such regulations require a functional protein homeostasis. If the protein homeostasis is altered under certain conditions such as diet, stress and 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 (31). Similarly, A $\beta$  may be present in granules (32) and A $\beta$  aggregation may be initiated intracellularly and ends up as a pathological hallmark in the extracellular space (30). (ii) The malaria drug chloroquine both interferes with the formation of secretory granules as well as prion infectivity (33,34). (iii) The natural compound curcumin both interferes with the progression of Alzheimer’s disease (35) and the release of endocrine hormones (36). (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 (37). 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 require a rethinking of the relationship between aggregation and function of polypeptides, and the correlation between amyloid aggregation and toxicity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

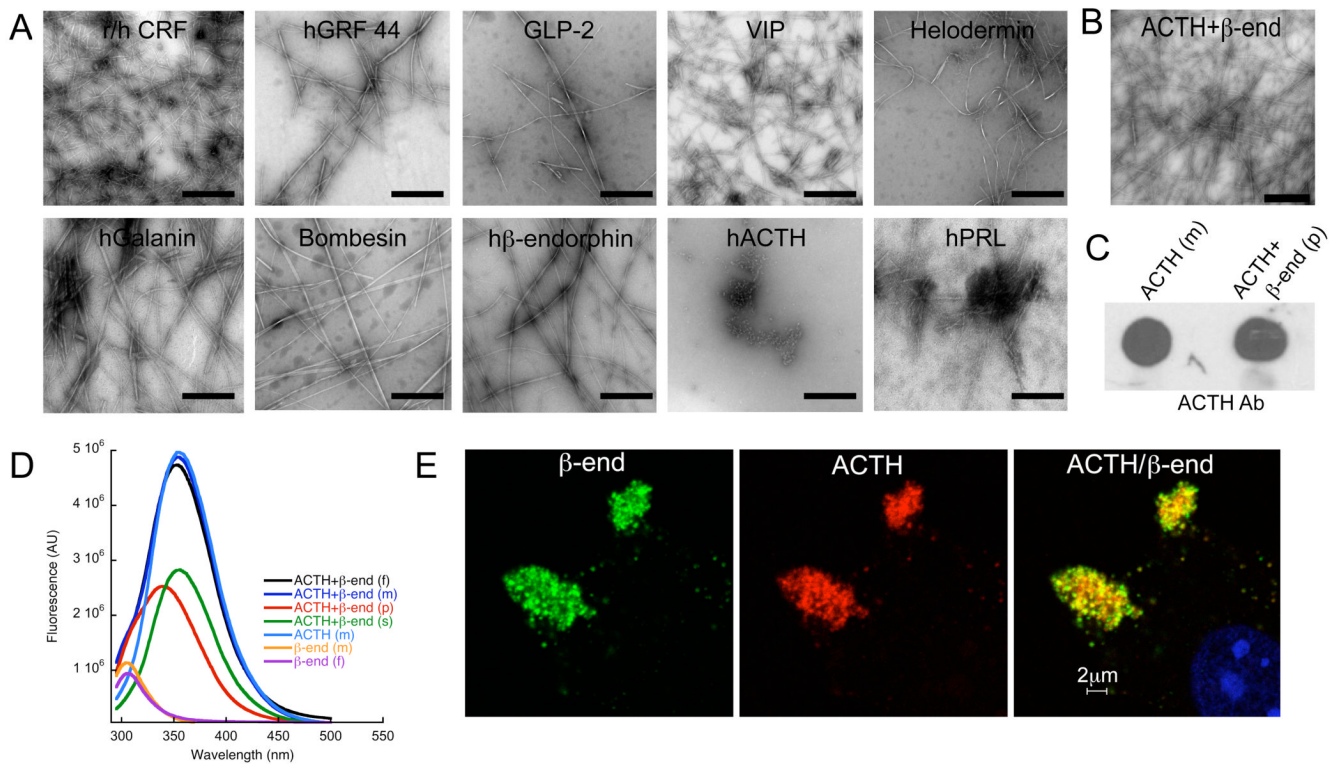
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## References and Notes

1. Kelly RB. *Science* 1985;230:25. [PubMed: 2994224]
2. Kelly RB. *Nature* 1987;326:14. [PubMed: 3821873]
3. Arvan P, Castle D. *Biochem. J* 1998;332(Pt 3):593. [PubMed: 9620860]
4. Dannies PS. *Mol. Cell. Endocrinol* 2001;177:87. [PubMed: 11377824]
5. Arvan P, Zhang BY, Feng L, Liu M, Kuliawat R. *Curr. Opin. Cell. Biol* 2002;14:448. [PubMed: 12383795]

6. Tooze SA. *Biochim. Biophys. Acta* 1998;1404:231. [PubMed: 9714820]
7. Miller F, Harven deE, Palade GE. *J. Cell Biol* 1966;31:349. [PubMed: 19866705]
8. Keeler C, Hodsdon ME, Dannies PS. *J. Mol. Neurosci* 2004;22:43. [PubMed: 14742909]
9. Fowler DM, Koulov AV, Balch WE, Kelly JW. *Trends Biochem. Sci* 2007;32:217. [PubMed: 17412596]
10. Barnhart MM, Chapman MR. *Annu. Rev. Microbiol* 2006;60:131. [PubMed: 16704339]
11. Iconomidou VA, Vriend G, Hamodrakas SJ. *FEBS Lett* 2000;479:141. [PubMed: 10981723]
12. Maddelein ML, Dos Reis S, Duvezin-Caubet S, Coulary-Salin B, Saupe SJ. *Proc. Natl. Acad. Sci. U.S.A* 2002;99:7402. [PubMed: 12032295]
13. Fowler DM, et al. *PLoS Biol* 2006;4:e6. [PubMed: 16300414]
14. Kolset SO, Prydz K, Pejler G. *Biochem. J* 2004;379:217. [PubMed: 14759226]
15. Reggio HA, Palade GE. *J. Cell. Biol* 1978;77:288. [PubMed: 649653]
16. Zanini A, et al. *J. Cell. Biol* 1980;86:260. [PubMed: 7419576]
17. Rosa P, Zanini A. *Eur. J. Cell. Biol* 1983;31:94. [PubMed: 6413211]
18. Carlson SS, Kelly RB. *J. Biol. Chem* 1983;258:11082. [PubMed: 6193120]
19. Kisilevsky R. *J. Struct. Biol* 2000;130:99. [PubMed: 10940218]
20. Cohlberg JA, Li J, Uversky VN, Fink AL. *Biochemistry* 2002;41:1502. [PubMed: 11814343]
21. Maji SK, et al. *Plos Biology* 2008;6:e17. [PubMed: 18254658]
22. Suk JY, Zhang F, Balch WE, Linhardt RJ, Kelly JW. *Biochemistry* 2006;45:2234. [PubMed: 16475811]
23. Giannattasio G, Zanini A, Meldolesi J. *J. Cell. Biol* 1975;64:246. [PubMed: 45846]
24. Liu Y, Schubert D. *J. Neurochem* 1998;71:2322. [PubMed: 9832130]
25. Onoue S, et al. *Pharm. Res* 2004;21:1274. [PubMed: 15290870]
26. Gumbiner B, Kelly RB. *Proc. Natl. Acad. Sci. U. S. A* 1981;78:318. [PubMed: 6264438]
27. Yonemoto IT, Kroon GJ, Dyson HJ, Balch WE, Kelly JW. *Biochemistry* 2008;47:9900. [PubMed: 18710262]
28. Giasson BI, et al. *Science* 2003;300:636. [PubMed: 12714745]
29. Gellermann GP, et al. *Proc. Natl. Acad. Sci. U. S. A* 2005;102:6297. [PubMed: 15851687]
30. Hardy JA, Higgins GA. *Science* 1992;256:184. [PubMed: 1566067]
31. Farquhar, MG.; Heller, HaL; K, editors. Vol. vol. 79. Cambridge: University Press; 1971.
32. Hook V, Schechter I, Demuth HU, Hook G. *Biol. Chem* 2008;389:993. [PubMed: 18979625]
33. Moore HP, Gumbiner B, Kelly RB. *Nature* 1983;302:434. [PubMed: 6300682]
34. Korth C, May BC, Cohen FE, Prusiner SB. *Proc. Natl. Acad. Sci. U. S. A* 2001;98:9836. [PubMed: 11504948]
35. Yang F, et al. *J. Biol. Chem* 2005;280:5892. [PubMed: 15590663]
36. Miller M, Chen S, Woodliff J, Kansra S. *Endocrinology* 2008;149:4158. [PubMed: 18450960]
37. Chiti F, Dobson CM. *Annu. Rev. Biochem* 2006;75:333. [PubMed: 16756495]

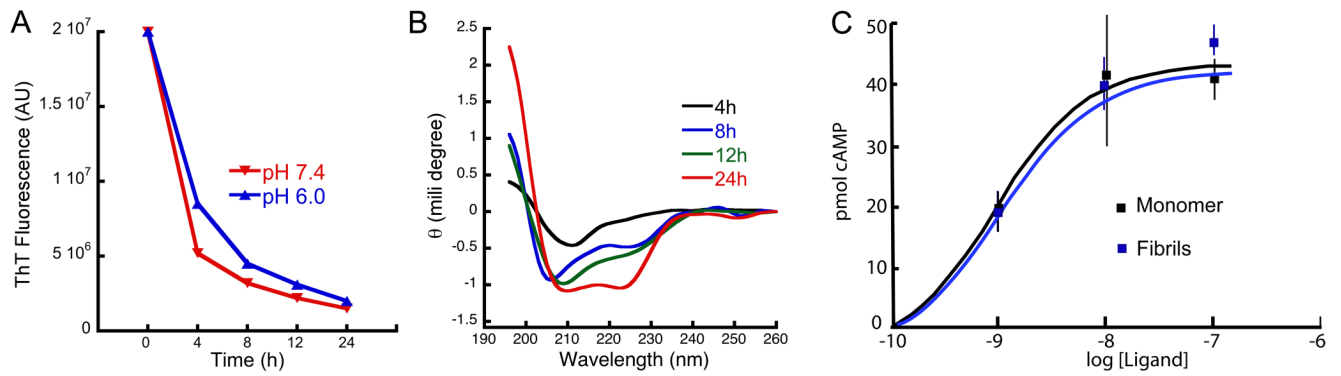


**Figure 1.**

Amyloid-like aggregation and coaggregation of hormones. (A) EM of ten hormones incubated for 30 days indicate the formation of amyloid fibrils. In fig. S3 the entire set of 42 hormones studied are shown. 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. The human prolactin (hPRL) was fibrillized in presence of 400 μM chondroitin sulfate A. Transmission electron microscopy of negative stained samples was performed. Scale bars are 500 nm. (B-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 displayed in black. High speed centrifugation at 120,000 g for 1 h was performed with this sample and the Trp and Tyr fluorescence was measured both for the corresponding pellet (p) displayed in red, as well as the supernatant (s) displayed in green. Furthermore, a fresh solution of a mixture of ACTH – β-endorphin (m) displayed in blue, a fresh solution of ACTH (m) displayed in blue, a fresh solution of β-endorphin (m) displayed in yellow, and an aggregated β-endorphin fibrillar sample (f) displayed in violet were measured accordingly. The Trp fluorescence signal in pellet suggests that ACTH is present in fibrils since only ACTH has a Trp. The λ<sub>max</sub> of the Trp signal is blue shifted by 15 nm when compared to monomeric/non-fibrillated ACTH suggested that the Trp is less solvent exposed in the fibril structure than in its monomeric state. Furthermore, the fluorescence study suggested 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. Since the Tyr fluorescence of monomeric β-endorphin, the β-endorphin fibrils

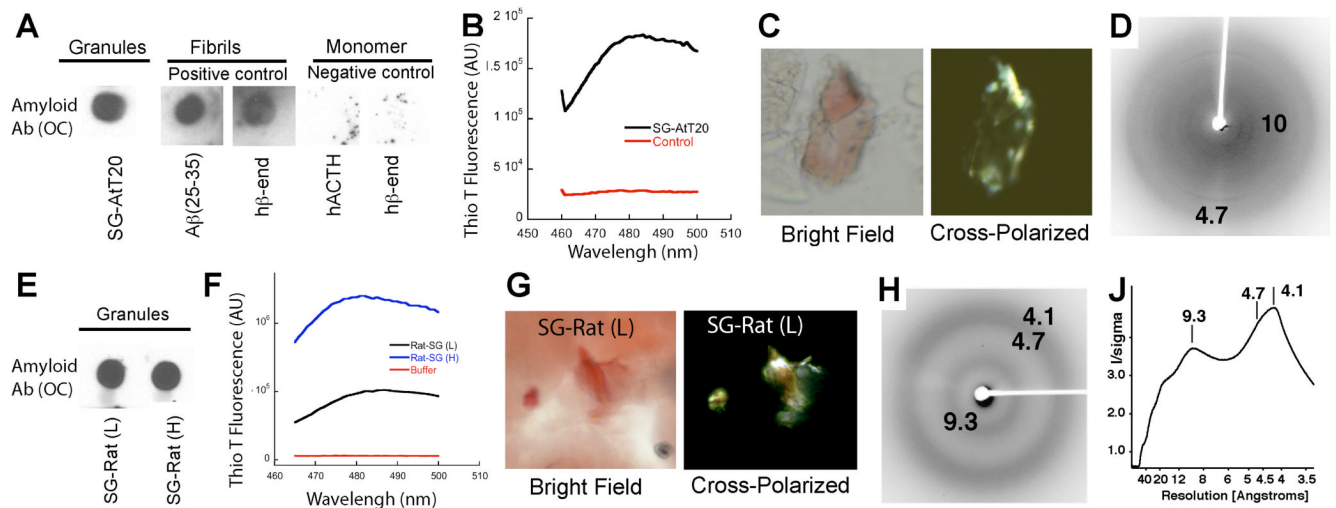
harvested by centrifugation, as well as the harvested ACTH –  $\beta$ -endorphin fibrils were similar in intensity and no Tyr fluorescence signal was observed in the corresponding supernatant, the entire  $\beta$ -endorphin population appeared to have aggregated into amyloid fibrils. (E) Colocalization 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 DAPI is shown in blue.





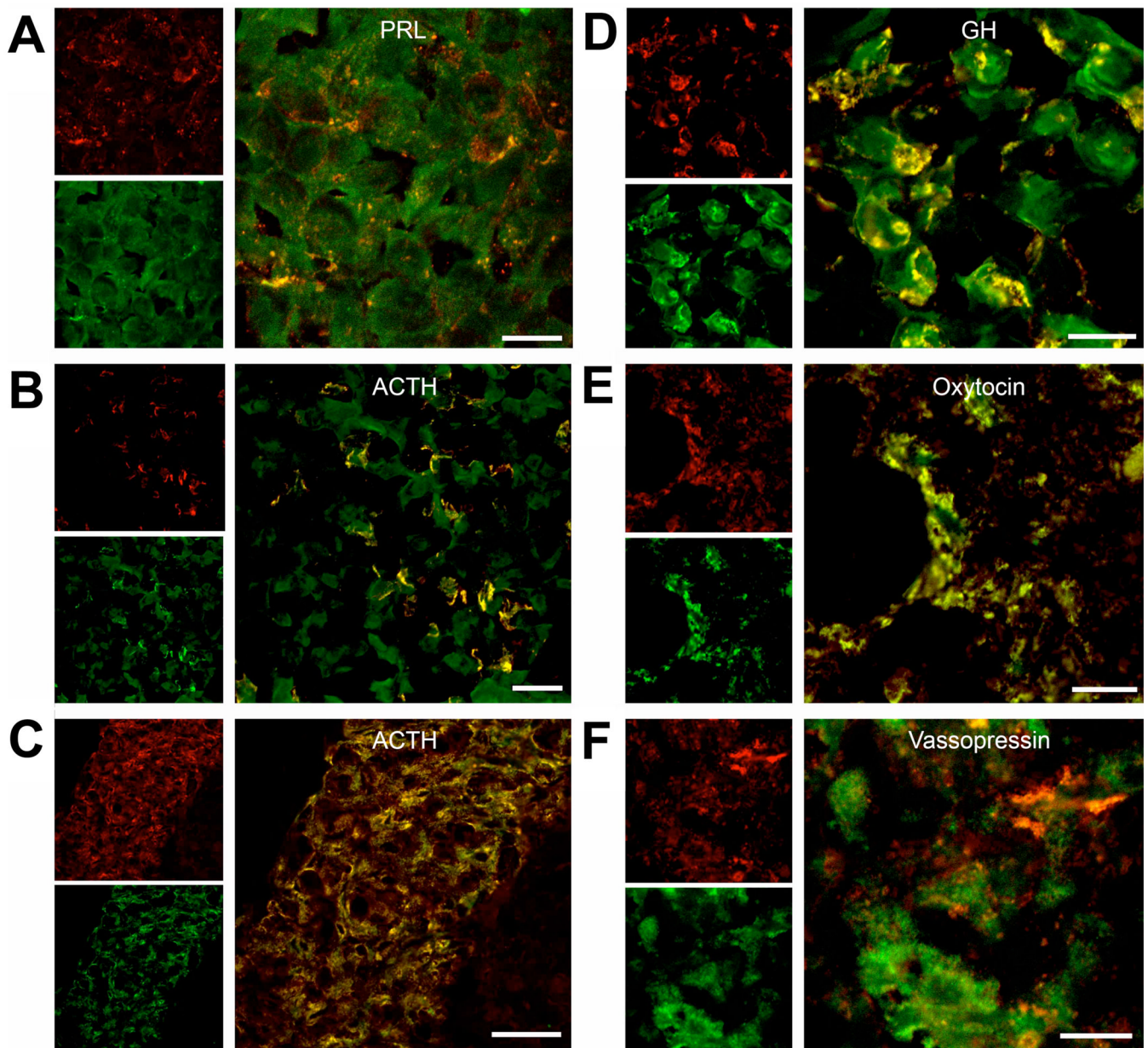
**Figure 2.**

Release of monomeric,  $\alpha$ -helical and functional CRF from its amyloid fibrils. CRF amyloid fibrils were dialyzed against buffer with a 10 kDa cut-off membrane. (A) Time-dependent decrease of Thio T fluorescence inside the membrane at two pH's 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 kDa 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 cyclic AMP in CHO cells stably expressing CRF-R1. Both samples show similar potencies.



**Figure 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, E), the amyloid-specific dyes Thio-T (B, F), CR (C-G), and x-ray fiber diffraction (D,H,J). (A) Dot blot staining of purified secretory granules from AtT20 cells against the amyloid-specific antibody OC. As positive controls, staining of amyloid fibrils of A $\beta$ (25–35) fibrils and  $\beta$ -endorphin fibrils (in presence of LMW heparin) are shown. As negative controls, staining of monomeric human ACTH and  $\beta$ -endorphin are presented. (B) Thio T fluorescence between 460–500 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 structure. (C) Congo Red birefringence of purified secretory granules. CR birefringence is shown that requests the presence of ordered (amyloid) aggregates. The picture represents the bright field microscope image with 10X resolutions. The same section is shown under cross-polarized light with 10X 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- $\beta$ -sheet structure are labeled. (E–J) Purified light (L) and heavy (H) secretory granules from rat pituitary are composed of amyloid-like structure using an amyloid-specific antibody OC (E), the amyloid-specific dyes Thio-T (F), CR birefringence (G), and x-ray fiber diffraction (H, J). The same measurement parameters as in (A–D) were used. In (J) the X-ray fiber diffraction measurements of purified membrane-less secretory granules are shown as a full image radial profile. The two reflections at ~4.7 Å and ~9.3 Å consistent with cross- $\beta$ -sheet structure are labeled. In addition, a strong 4.1 Å is present attributed to the remaining lipid content of the granules under study.



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