Pyroglutamate-Amyloid-β and Glutaminyl Cyclase Are Colocalized with Amyloid-β in Secretory Vesicles and Undergo Activity-Dependent, Regulated Secretion

Holger Cynis\textsuperscript{a} Lydiane Funkelstein\textsuperscript{b,c} Thomas Toneff\textsuperscript{b,c} Charles Mosier\textsuperscript{b,c} Michael Ziegler\textsuperscript{b,c} Birgit Koch\textsuperscript{a} Hans-Ulrich Demuth\textsuperscript{a,d} Vivian Hook\textsuperscript{b,c}

\textsuperscript{a}Fraunhofer IZJ-MWT, Halle, Germany; \textsuperscript{b}Skaggs School of Pharmacy and Pharmaceutical Sciences and \textsuperscript{c}School of Medicine, University of California, San Diego, La Jolla, Calif., USA; \textsuperscript{d}Probiodrug AG, Halle, Germany

\section*{Key Words}
Pyroglutamate · Glutaminyl cyclase · Amyloid-β · Secretory vesicles

\section*{Abstract}
\textbf{Background and Aims:} N-truncated pyroglutamate (pGlu)-amyloid-β [Aβ(3–40/42)] peptides are key components that promote Aβ peptide accumulation, leading to neurodegeneration and memory loss in Alzheimer’s disease. Because Aβ deposition in the brain occurs in an activity-dependent manner, it is important to define the subcellular organelle for pGlu-Aβ(3–40/42) production by glutaminyl cyclase (QC) and their colocalization with full-length Aβ(1–40/42) peptides for activity-dependent, regulated secretion. Therefore, the objective of this study was to investigate the hypothesis that pGlu-Aβ and QC are colocalized with Aβ in dense-core secretory vesicles (DCSV) for activity-dependent secretion with neurotransmitters. \textbf{Methods:} Purified DCSV were assessed for pGlu-Aβ(3–40/42), Aβ(1–40/42), QC, and neurotransmitter secretion. Neuron-like chromaffin cells were analyzed for cosecretion of pGlu-Aβ, QC, Aβ, and neuropeptides. The cells were treated with a QC inhibitor, and pGlu-Aβ production was measured. Human neuroblastoma cells were also examined for pGlu-Aβ and QC secretion. \textbf{Results:} Isolated DCSV contain pGlu-Aβ(3–40/42), QC, and Aβ(1–40/42) with neuropeptide and catecholamine neurotransmitters. Cellular pGlu-Aβ and QC undergo activity-dependent cosecretion with Aβ and enkephalin and galanin neurotransmitters. The QC inhibitor decreased the level of secreted pGlu-Aβ. The human neuroblastoma cells displayed regulated secretion of pGlu-Aβ that was colocalized with QC. \textbf{Conclusions:} pGlu-Aβ and QC are present with Aβ in DCSV and undergo activity-dependent, regulated cosecretion with neurotransmitters.

\section*{Introduction}
Accumulation of neurotoxic β-amyloid (Aβ) peptides in the brain represents a key factor in the development of memory deficits in Alzheimer’s disease (AD) \cite{1–4}. Aβ peptides of multiple forms are present in AD brains. Notably, affected AD brains contain the N-terminally trun-
cated pyroglutamate (pGlu) forms of Aβ(3–40/42) (pGlu-Aβ) as a major portion of the total Aβ peptides, which includes the known full-length Aβ(1–40/42) peptides [5–7]. The accumulation of pGlu-Aβ(3–40/42) in the brain occurs before that of the Aβ(1–40/42) peptides [5]. Significantly, pGlu-Aβ facilitates the seeding of Aβ peptides into neurotoxic oligomers [8] that are thought to participate in the development of memory deficits in AD [9]. Increasing the brain levels of pGlu-Aβ(3–42) resulted in aggravated memory deficits and amyloid plaque accumulation in the 5XFAD mouse model of AD [10], illustrating the key role of N-truncated pGlu-modified forms of Aβ in AD.

The pGlu-Aβ(3–40/42) peptides start with N-terminal glutamate, the third amino-terminal residue of Aβ(1–40/42). Notably, this residue is converted to pGlu by the enzyme glutaminyl cyclase (QC) [8, 11, 12]. QC is elevated in AD brains [5–7]. Moreover, inhibition of QC [13, 14] results in decreased brain pGlu-Aβ levels, and QC gene knockout results in decreased brain pGlu-Aβ levels with improved behavioral deficits in 5XFAD mice [12]. These findings indicate that QC is involved in the development of AD via formation of pGlu-Aβ.

Aβ peptides have been demonstrated to be released from brain neurons in an electrical activity-dependent manner [15–19]. Neural activity modulates the formation and secretion of Aβ [20, 21]. Recent evidence indicates that endogenous neuronal activity regulates regional brain vulnerability to Aβ deposition [22]. Significantly, neuronal activity is fundamental for the regulated secretion of neurotransmitters [23, 24]. These features of Aβ peptides raise the question of whether pGlu-Aβ and its biosynthetic enzyme QC are cosecreted with Aβ and neurotransmitters in a regulated, activity-dependent manner. Therefore, this study investigated the hypothesis that cosecretion of truncated pGlu-Aβ and full-length Aβ occurs with neurotransmitters stored and released from secretory vesicles, resulting in extracellular Aβ peptide forms. These studies utilized neuronal chromaffin cells that display activity-dependent secretion of neurotransmitters and also produce Aβ [17, 19], as well as human IMR32 neuroblastoma cells.

Our results show that secretory vesicles of the dense-core secretory vesicle (DCSV) type contain pGlu-Aβ with QC and Aβ combined with catecholamine and peptide neurotransmitters. Cellular pGlu-Aβ and QC undergo cosecretion from the regulated secretory pathway of neuron-like chromaffin cells stimulated by nicotine or KCl depolarization. pGlu-Aβ also undergoes regulated cosecretion with Aβ and the peptide neurotransmitters methionine Met-enkephalin and galanin peptide. Treatment of cells with a QC inhibitor resulted in decreased levels of pGlu-Aβ released from the regulated secretory pathway. Furthermore, human neuroblastoma cells displayed regulated cosecretion of pGlu-Aβ(3–40) with Aβ(1–40/42) combined with colocalization of pGlu-Aβ and QC. These findings support the hypothesis that pGlu-Aβ and QC undergo cosecretion with Aβ and peptide neurotransmitters from the activity-dependent, regulated secretory pathway.

**Experimental Procedures**

**Content of pGlu-Aβ, QC, Aβ Peptides, and Neurotransmitters in DCSV**

Secretory vesicles were purified from fresh bovine adrenal medulla to evaluate the content of QC, pGlu-Aβ, Aβ peptides, Met-enkephalin and galanin peptide neurotransmitters, and the catecholamine neurotransmitters dopamine, noradrenaline, and epinephrine. These DCSV, also known as chromaffin granules, were isolated by differential sucrose density gradient centrifugation, as previously described [25–29]. The density gradient isolation procedure has been established to yield secretory vesicles of high purity based on the assessment of organelle markers and on electron microscopy [25–29]. The procedure results in purified secretory vesicles that lack biochemical markers for other subcellular organelles of lysosomes (acid phosphatase marker), cytoplasm (lactate dehydrogenase marker), mitochondria (fumarase and glutamate dehydrogenase markers), and the endoplasmic reticulum (glucose 6-phosphatase marker). Enzyme markers in the purified secretory vesicle preparation represent less than 1% of the total amount of homogenate markers. Furthermore, electron microscopy and enzyme markers have confirmed the integrity and purity of isolated DCSV [25–29].

The presence of QC in isolated DCSV was assessed by monitoring QC enzymatic activity as previously described [30–32]. QC in chromaffin granules was subjected to immunoprecipitation with anti-QC – conducted as previously described [33] – followed by Western blotting with anti-QC sera (rabbit CPC1301, from Probiobug), using Western blot procedures as described [25, 33–35].

For the measurement of Aβ peptides and neurotransmitters, purified secretory vesicles were lysed by freeze-thawing in buffer (50 mM Na acetate, 50 mM NaCl, 1 mM EDTA) containing a cocktail of protease inhibitors (peptatin A, leupeptin, chymostatin, and E64c at 10 μM each, and PMSF at 500 μM). An acid extract (0.1 M acetic acid) was prepared from the lysed secretory vesicles (as described previously [25, 26, 34, 35]) for the measurement of pGlu-Aβ(3–40), pGlu-Aβ(3–42), Aβ(1–40), Aβ(1–42), Met-enkephalin, and galanin, and of the catecholamines dopamine, noradrenaline, and epinephrine. DCSV samples were subjected to ELISA measurements of pGlu-Aβ(3–40) (No. 27418; IBL International, Toronto, Ont., Canada), pGlu-Aβ(3–42) (No. 27716; IBL), Aβ(1–40) (No. 27718; IBL), and Aβ(1–42) (No. 27712; IBL), and RIA for Met-enkephalin and galanin (the RIAs were conducted as previously reported [28, 33, 35]). The well-established ELISA kits have been characterized to demonstrate their specificities for the
Particular peptides (by the manufacturer IBL). The ELISAs for Aβ(1–40) and Aβ(1–42) do not cross-react with each other, nor with the pGlu-Aβ peptides; the ELISA for pGlu-Aβ(3–40) does not detect pGlu-Aβ(3–42) or Aβ(1–40/42), and the ELISA for pGlu-Aβ(3–42) specifically detects this N-truncated peptide rather than pGlu-Aβ(3–40) and does not detect Aβ(1–40/42). QC activity was measured in the media, as previously described [30–32], using H-Gln-βNA as the substrate in a fluorometric assay. Dopamine, norepinephrine, and epinephrine catecholamines were measured by radioenzymatic assays, as previously described.

Neuron-Like Chromaffin Cells in Primary Culture
Neuron-like chromaffin cells in primary culture were obtained from the adrenal medulla of the sympathetic nervous system. In our study, they were prepared from fresh adrenal medulla tissue (bovine), as previously described [25, 35, 38]. Briefly, chromaffin cells were dissected from fresh adrenal glands, dissociated in a collagenase/DNase solution at 37 °C, filtered, and centrifuged. The cells were plated onto fibronectin-coated dishes (EMD Chemicals, Gibbstown, N.J., USA) in medium containing DMEM (Cellgro, Manassas, Va., USA), 10% FBS (Invitrogen, Carlsbad, Calif., USA), and penicillin/streptomycin. The cells were maintained at 37 °C and 6% CO2.

Regulated Secretion of pGlu-Aβ, QC, Aβ, and Peptide Neurotransmitters from Chromaffin Cells
After 6 days in culture, the chromaffin cells were subjected to KCl depolarization or treatment with nicotine to stimulate regulated, activity-dependent secretion. They were incubated in standard release medium (as described by Bark et al. [28]) with 0.25 μg/ml BSA for 90 min at 37 °C, followed by collection of the medium, which represents basal secretion. Then they were stimulated for 90 min (at 37 °C) in standard release medium containing KCl (50 mM) or nicotine (10 μM), followed by collection of the medium, which represents regulated secretion. The secretion medium was collected with addition of a cocktail of protease inhibitors (1 mM EDTA, 500 μM AEBSF, and 10 μM each of E64c, leupeptin, pepstatin A, and chymostatin). Then it was concentrated by ultrafiltration through a 2-kDa cut-off membrane (Vivaspin 2 2K MWCO HydroSart; Sartorius, Göttingen, Germany), which retains Aβ. The concentrated sample was subjected to measurements of pGlu-Aβ(3–40), pGlu-Aβ(3–42), Aβ(1–40), Aβ(1–42), Met-enkephalin, and galanin, as described above for DCSV. QC activity was measured in the medium, as previously described [30, 31], using H-Gln-βNA as the substrate in a fluorometric assay.

Inhibition of Endogenous QC in Chromaffin Cells and Levels of Secreted pGlu-Aβ(3–40)
The chromaffin cells were incubated with the QC inhibitor PQ529 (Probiodrug AG, Halle, Germany) at 50 μM for 18 h. They were then subjected to regulated secretion induced by treating cells with KCl (50 mM) for 90 min. Control incubation without KCl was also included. The cell culture medium was then collected for the measurement of pGlu-Aβ(3–40) by ELISA (as described above).

Cellular Immunofluorescence Localization of pGlu-Aβ and QC with Met-Enkephalin Neurotransmitter Present in Secretory Vesicles
Immunofluorescence microscopy of the chromaffin cells was conducted to assess the subcellular localization of pGlu-Aβ and QC with Met-enkephalin in the DCSV that undergo regulated secretion. The chromaffin cells were fixed for immunofluorescence deconvolution microscopy, conducted as previously described [33–35]. The primary antibodies used for immunocytochemistry were rabbit anti-QC 1301 (1:250; Probiodrug AG), mouse anti-Met-enkephalin (1:100, Abcam No. 23503; Abcam, Cambridge, Mass., USA), mouse anti-pGlu-Aβ (1:50; Probiodrug AG) detecting the N-terminus of both pGlu-Aβ(3–40) and pGlu-Aβ(3–42), rabbit anti-Met-enkephalin (1:50; Millipore, Billerica, Mass., USA), and mouse anti-amyloid precursor protein (APP) clone 6E10 (1:100; Covance, Princeton, N.J., USA). The primary antibodies were detected with the secondary antibodies anti-rabbit IgG-Alexa Fluor 594 (goat, 1:200 dilution, red fluorescence; Molecular Probes, Eugene, Ore., USA) and anti-mouse IgG-Alexa Fluor 488 (goat, 1:200 dilution, green fluorescence; Molecular Probes). The immunofluorescent images were analyzed by DeltaVision Spectris Image Deconvolution Systems on an Olympus IX70 epifluorescence microscope using the software softWoRx Explorer by Applied Precision (GE Healthcare Company, Issaquah, Wash., USA), as previously described [33, 39]. As a control procedure, immunostaining with only secondary antibody was performed, and the controls obtained demonstrated a lack of immunofluorescence, indicating the specificity of the primary immunoreactivity observed.

Human IMR32 Neuroblastoma Cells and Regulated Secretion of pGlu-Aβ with Aβ Peptides
Human IMR32 neuroblastoma cells were obtained from ATCC (American Type Culture Collection, Crystal City, Va., USA) and cultured according to the manufacturer’s instructions. Activity-dependent secretion was achieved by stimulating secretion in the medium by incubation with KCl (50 mM) for 90 min. Control secretion for 90 min without KCl was included. The secretion media were collected, concentrated, and subjected to measurements of pGlu-Aβ(3–40), Aβ(1–40), and Aβ(1–42) (ELISA kits from IBL). Cellular localization of pGlu-Aβ and QC was conducted by immunofluorescence confocal microscopy, as described above for chromaffin cells.

Statistical Analysis of Data
The secretion of Aβ peptides and neurotransmitters was conducted in triplicate in each experiment, and the experiments were repeated 3 times. Data were considered statistically significant at p < 0.05 by Student’s t test.

Results
Secretory Vesicles Contain pGlu-Aβ and QC with Aβ and Neurotransmitters
Several studies in the field have demonstrated that Aβ undergoes activity-dependent, regulated secretion from neurons [15–18], implicating release from secretory vesicles (by the manufacturer IBL). The ELISAs for Aβ(1–40) and Aβ(1–42) do not cross-react with each other, nor with the pGlu-Aβ peptides; the ELISA for pGlu-Aβ(3–40) does not detect pGlu-Aβ(3–42) or Aβ(1–40/42), and the ELISA for pGlu-Aβ(3–42) specifically detects this N-truncated peptide rather than pGlu-Aβ(3–40) and does not detect Aβ(1–40/42). QC activity was measured in the media, as previously described [30–32], using H-Gln-βNA as the substrate in a fluorometric assay. Dopamine, norepinephrine, and epinephrine catecholamines were measured by radioenzymatic assays, as previously described. Dopamine, norepinephrine, and epinephrine catecholamines were measured by radioenzymatic assays, as previously described. Dopamine, norepinephrine, and epinephrine catecholamines were measured by radioenzymatic assays, as previously described.
icles that store and release neurotransmitters (fig. 1a). Therefore, our study investigated the hypothesis that pGlu-Aβ and its biosynthetic enzyme QC may be present with Aβ and neurotransmitters in secretory vesicles for extracellular release.

DCSV of neuron-like chromaffin cells store neurotransmitter contents for activity-dependent, regulated secretion; thus, they were isolated for this study. The DCSV were purified by sucrose density gradient centrifugation and the integrity of the isolated DCSV was illustrated by electron microscopy (fig. 1b). Previous reports have documented the high purity of these isolated DCSV by assessing enzyme markers for subcellular organelles [25, 28, 29].

The isolated DCSV contained pGlu-Aβ(3–40), pGlu-Aβ(3–42), Aβ(1–40), and Aβ(1–42), quantitated by ELISA assays (table 1). The concentrations of pGlu-Aβ(3–40) and pGlu-Aβ(3–42) (at approx. 0.022 and 0.070 pg/mg protein) represented 10 and 82% of the concentrations of Aβ(1–40) and Aβ(1–42), respectively. Moreover, the DCSV contained enkephalin and galanin peptide neurotransmitters, as well as the catecholamines dopamine, norepinephrine, and epinephrine (table 1). These data show that the DCSV contained pGlu-Aβ and Aβ peptides with peptide and catecholamine neurotransmitters.

The presence of QC activity in the isolated DCSV was demonstrated by an activity assay using H-Gln-βNA as substrate. The time-dependent formation of the QC product is illustrated in figure 2a. Heat inactivation as well as omitting the auxiliary enzyme of the assay underlined the specificity of the test. Furthermore, QC protein is present in DCSV, as observed by anti-QC Western blots (fig. 2b).

**Table 1.** Aβ peptides and neurotransmitters in purified DCSV

<table>
<thead>
<tr>
<th>Aβ peptides</th>
<th>Content, pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGlu-Aβ(3–40)</td>
<td>0.022±0.004</td>
</tr>
<tr>
<td>pGlu-Aβ(3–42)</td>
<td>0.070±0.009</td>
</tr>
<tr>
<td>Aβ(1–40)</td>
<td>0.220±0.010</td>
</tr>
<tr>
<td>Aβ(1–42)</td>
<td>0.085±0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide neurotransmitters</th>
<th>Content, pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-enkephalin</td>
<td>215,000±14.4</td>
</tr>
<tr>
<td>Galanin</td>
<td>408±0.015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Catecholamine neurotransmitters</th>
<th>Content, pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>1,150±0.13</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>4,560±0.34</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>6,490±1.02</td>
</tr>
</tbody>
</table>

The concentrations of Aβ peptides and neurotransmitters in purified chromaffin DCSV were measured by assays as described in the Experimental Procedures section.
stimulated the predominate portion of secreted Aβ(3–40) compared with the condition of basal constitutive secretion. Notably, QC, the biosynthetic enzyme for pGlu-Aβ, undergoes activity-dependent cosecretion with pGlu-Aβ(3–40) (fig. 3b).

Furthermore, KCl and nicotine stimulated the secretion of Aβ(1–40) and Aβ(1–42) (fig. 4). Because regulated, activity-dependent secretion is fundamental for the release of neurotransmitters, the peptide neurotransmitters Met-enkephalin and galanin were evaluated in these

Fig. 2. a QC activity in DCSV. QC activity (black symbols) in purified DCSV of chromaffin cells (also known as chromaffin granules) was measured in time-course assays. The secretory vesicles showed no activity after heat inactivation (red symbols; colors in online version only) and after omission of the auxiliary enzyme of the test (green symbols). b QC protein in DCSV. Purified DCSV were subjected to anti-QC Western blots. Endogenous QC immunoreactive bands of approximately 60 and 48 kDa were observed. The selectivity of anti-QC for detecting these immunoreactive bands was demonstrated by conducting a Western blot with only the secondary anti-rabbit serum (omitting the primary anti-QC serum), which resulted in the absence of immunoreactivity (unpublished data). It is noted that, on SDS-PAGE, endogenous bovine pituitary QC has been observed with an apparent molecular weight of approximately 40–45 kDa [101], and recombinant non-glycosylated murine QC at a molecular weight of 37–40 kDa [31]. Glycosylation of endogenous QC is known to modify its apparent molecular weight [102].
experiments. Met-enkephalin and galanin were secreted in an activity-dependent manner, stimulated by KCl depolarization and by nicotine, as conducted in figure 3. Aβ(1–40) and Aβ(1–42) were measured in the secretion media. c, d Regulated secretion of Met-enkephalin (c) and galanin peptide (d) neurotransmitters from chromaffin cells was stimulated by KCl depolarization and by nicotine, as conducted in figure 3, and the neuropeptides Met-enkephalin and galanin were measured in the secretion media.

Inhibition of Endogenous QC Reduces pGlu-Aβ in the Regulated Secretory Pathway

The role of QC in the production of pGlu-Aβ was evaluated with the QC inhibitor PQ529 [51]. Chromaffin cells were treated with PQ529 (50 μM) for 18 h, and then subjected to regulated secretion stimulated by KCl depolarization. The results demonstrated that PQ529 significantly reduced the amount of pGlu-Aβ(3–40) released via the regulated secretory pathway (fig. 5). Basal, constitutive levels of secreted pGlu-Aβ(3–40) were not reduced by PQ529, although a modest, insignificant decrease was observed with PQ529. These findings support the role of QC in producing pGlu-Aβ in DCSV via the regulated secretory pathway that provides activity-dependent secretion.
Cellular Localization of pGlu-Aβ and QC with Enkephalin in Secretory Vesicles

Neurotransmitter and neurosecretory components that undergo regulated secretion are stored in secretory vesicles. Therefore, the colocalization of pGlu-Aβ and QC with the Met-enkephalin neurotransmitter was demonstrated by immunofluorescence confocal microscopy (fig. 6). The data show the cellular colocalization of pGlu-Aβ with Met-enkephalin in chromaffin cells (fig. 6a). The punctate pattern of subcellular pGlu-Aβ localization coincides with that of Met-enkephalin which is present in secretory vesicles, shown by their merged yellow immunofluorescence (fig. 6a). Furthermore, QC is also colocalized with enkephalin (fig. 6b), indicating the presence of both QC and pGlu-Aβ in enkephalin neurotransmitter-containing secretory vesicles. These data are consistent with the presence of pGlu-Aβ and QC in secretory vesicles that contain neurotransmitters (table 1; fig. 2).

The colocalization of QC with Aβ- and APP-related forms was assessed by the 6E10 antibody, which detects Aβ- and APP-related immunoreactivity. The data show a punctate localization of QC and 6E10 immunoreactivity, showing colocalization (fig. 6c). These results support the hypothesis on the combined presence of pGlu-Aβ and QC with APP- and Aβ-related peptide forms in secretory vesicles.

Human Neuroblastoma Cells Display Activity-Dependent, Regulated Secretion of pGlu-Aβ with Aβ Peptides

An evaluation in a human neuroblastoma cell line (IMR32) was conducted to assess the regulated secretion of pGlu-Aβ in a human neuronal model. The secretion of pGlu-Aβ(3–40) was stimulated by KCl and compared with basal control cells (no KCl; fig. 7a). In addition, regulated cosecretion of Aβ(1–40) and Aβ(1–42) was stimu-
lated by KCl (fig. 7b, c). Immunofluorescence microscopy illustrated the colocalization of pGlu-Aβ with QC (fig. 7d). pGlu-Aβ and QC were each observed in discrete, punctate patterns of subcellular distribution. Merging of their images displayed their colocalization (shown by the yellow immunofluorescence). These data demonstrate the regulated cosecretion of pGlu-Aβ with QC, Aβ, and peptide neurotransmitters from the regulated secretory pathway of neuron-like chromaffin cells. Cellular immunofluorescence microscopy also illustrated the colocalization of pGlu-Aβ with QC and enkephalin, and of QC with APP- and Aβ-related immunoreactivity. Treatment of cells with a QC inhibitor resulted in reduced levels of pGlu-Aβ released from the regulated secretory pathway, indicating the role of QC in the production of pGlu-Aβ. Furthermore, human neuroblastoma cells displayed regulated secretion of pGlu-Aβ and Aβ, and pGlu-Aβ was colocalized with QC. These data demonstrate that pGlu-Aβ and QC undergo regulated cosecretion with Aβ from neurotransmitter secretory vesicles to provide extracellular pGlu-Aβ with Aβ that accumulates in AD brains.

The combined presence of pGlu-Aβ and QC with Aβ in neurotransmitter secretory vesicles raises the question of how Aβ and pGlu-Aβ are produced in this organelle.
The presenilin 1 γ-secretase component is present in secretory vesicles containing neuropeptides and catecholamines. The components of the γ-secretase complex are also present in DCSV together function as γ-secretase containing neuropeptides and catecholamines. pGlu-Aβ and Aβ peptides in neurotransmitter secretory vesicles are produced by QC (fig. 8). Prior studies have shown that these secretory vesicles contain pGlu-Aβ by QC (fig. 8). Our previous studies have indicated the presence of β-, γ-, and α-secretases in DCSV [58, 59, 62]. DCSV contain cathepsin B, which has been identified as a new alternative β-secretase [59–61], and the well-known β-secretase BACE1 [58, 62], an aspartyl protease [54–57]. The components of the γ-secretase complex are also present in DCSV [58], i.e. presenilins 1 and 2, nicastrin, Aph-1, and Pen-2, which together function as γ-secretase [63, 64]. The α-secretase ADAM10 protease is also present in DCSV [58]. These findings illustrate the presence of the APP-processing machinery in the production of pGlu-Aβ and Aβ peptides in neurotransmitter secretory vesicles containing neuropeptides and catecholamines.

The reason for this question is that it may be hypothesized that APP and its processing secretases may be present in these secretory vesicles for the production of Aβ, and for the production of pGlu-Aβ by QC (fig. 8). Our previous studies have shown that these secretory vesicles contain APP [17, 52, 53] and Aβ peptides, indicating the presence of secretases that convert APP to Aβ. Indeed, β- and γ-secretases are present in the secretory vesicles with APP. The β-secretase BACE1 [54–57] is present in DCSV [19, 58]. A wild-type β-secretase was recently identified as cathepsin B [59–61] and is present in DCSV [58, 59]. The presenilin 1 γ-secretase component is present in secretory vesicles [58, 62], as well as the nicastrin, Aph-1, and Pen-2 components [58] of the γ-secretase complex [63, 64]. Subsequent to the secretases, N-terminal truncation of full-length Aβ is predicted to occur in order to generate Aβ(3–40/42), which serves as a substrate for QC to produce pGlu-Aβ(3–40/42) [65–68]. In future studies, it will be of interest to determine the relative levels of the N-truncated Aβ peptides Aβ(2–40/42) and Aβ(3–40/42) compared with pGlu-Aβ(3–40/42), utilizing mass spectrometry to distinguish these peptide species. Thus, secretory vesicles contain the APP-processing proteases that produce Aβ peptides combined with QC for the production of pGlu-Aβ(3–40/42) (fig. 8). The range of pH conditions necessary for the activities of these secretases [69–74] coincides with the intracellular pH conditions of these secretory vesicles [75–77]. The secretory vesicle organelle, thus, contains the APP-processing machinery for producing Aβ and pGlu-Aβ peptide forms.

These data support the hypothesis that pGlu-Aβ is produced by QC in secretory vesicles that release neurotransmitter components in a regulated, activity-dependent manner. QC converts the N-terminal glutamate of Aβ(3–40/42) peptides to the pyroglutamate of pGlu-Aβ(3–40/42). The QC substrates Aβ(3–40/42) are presumably produced by N-terminal truncation of Aβ(1–40/42). The presence of full-length Aβ(1–40/42) in secretory vesicles [19, 58, 59] is compatible with biosynthesis of pGlu-Aβ from Aβ in these secretory vesicles.

The DCSV is a key organelle for the regulated, activity-dependent release of peptide and catecholamine neurotransmitters. Indeed, regulated secretion of pGlu-Aβ and Aβ occurs with the peptide neurotransmitters Met-enkephalin and galanin. Evidence for cosecretion of Aβ peptides with neurotransmitters supports involvement of activity-dependent deposition of Aβ peptides in AD brains, as demonstrated in vivo [21, 22]. It will be of interest in future studies to examine the variety of secretagogues known to induce neurotransmitter secretion [78–83] for effects on the stimulation of the regulated secretion of pGlu-Aβ peptides in order to gain further understanding of factors controlling pGlu-Aβ release. It is noted that the DCSV isolated from the peripheral sympathetic nervous system (from adrenal medulla) contain pGlu-Aβ, whereas this is not detectable in normal aged human brains [84]. Thus, the DCSV utilized in this study provide a model for understanding the neurobiology of pGlu-Aβ.

Further, it is noted that it is not yet known whether pGlu-Aβ is located in other subcellular organelles that contain full-length Aβ(1–40/42). Full-length Aβ is present in endosomes [85–89], lysosomes [90–92], autophagosomes [91, 93–95], exosomes [96, 97], and other

---

**Fig. 8.** pGlu-Aβ and Aβ peptides with QC and the APP-processing machinery in secretory vesicles containing neurotransmitters. In this study, the secretory vesicles (DCSV type) isolated from model neuron-like chromaffin cells were demonstrated to contain pGlu-Aβ(3–40/42) and QC combined with Aβ(1–40/42). Prior studies have indicated the presence of β-, γ-, and α-secretases in DCSV [58, 59, 62]. DCSV contain cathepsin B, which has been identified as a new alternative β-secretase [59–61], and the well-known β-secretase BACE1 [58, 62], an aspartyl protease [54–57]. The components of the γ-secretase complex are also present in DCSV [58], i.e. presenilins 1 and 2, nicastrin, Aph-1, and Pen-2, which together function as γ-secretase [63, 64]. The α-secretase ADAM10 protease is also present in DCSV [58]. These findings illustrate the presence of the APP-processing machinery in the production of pGlu-Aβ and Aβ peptides in neurotransmitter secretory vesicles containing neuropeptides and catecholamines.
related subcellular organelles. It will be important in future studies to compare the organelle locations of pGlu-Aβ and Aβ peptides, which will provide an understanding of the neuronal trafficking of pGlu-Aβ compared with Aβ.

The neurobiology of the regulated, activity-dependent secretion of pGlu-Aβ and Aβ is significant with respect to the functional role of pGlu-Aβ in neurodegeneration and memory loss in AD. pGlu-Aβ comprises a major portion of the Aβ species in AD compared with Aβ(1–40) and Aβ(1–42) [5–7]. Quantification indicates the presence of pGlu-Aβ peptides at similar and greater levels than Aβ(1–40) and Aβ(1–42) in AD brains. Also, pGlu-Aβ is present as oligomeric complexes in AD brains [98]. In vitro studies have shown that pGlu-Aβ displays a higher aggregation propensity as well as a stronger tendency to seed the aggregation of other Aβ species [8, 9, 99]. Moreover, pGlu-Aβ is present in AD brains but not in normal aged brains [84].

In vivo studies have illustrated the neurotoxicity of pGlu-Aβ in memory deficits. Overexpression of pGlu-Aβ in mice correlates with Aβ and behavioral deficits [10, 12] as well as neuronal loss and impaired long-term potentiation [100]. These studies also showed that pGlu-Aβ formation is dependent on QC, since knockout of QC in transgenic mice resulted in reduced levels of brain pGlu-Aβ. These findings support a role of pGlu-Aβ in amyloid plaque formation and memory loss in AD.

Reduction of pGlu-Aβ formation may provide a therapeutic strategy for AD, since inhibition of QC results in decreased brain pGlu-Aβ [13, 14] and QC gene knockout decreases brain pGlu-Aβ with improved behavior [12]. These findings indicate that QC is involved in the development of AD via the formation of pGlu-Aβ.

Based on the results of this study, inhibition of QC reduces the amount of pGlu-Aβ released from the regulated secretory pathway. In future studies, it will be of interest to assess the effects of QC inhibition on the regulated, activity-dependent deposition of amyloid in animal AD model brains. The production of pGlu-Aβ by QC in secretory vesicles that produce and secrete Aβ peptides and neurotransmitters in an activity-dependent manner suggests a close association of pGlu-Aβ and Aβ neurotoxicity with neurotransmitter functions.

**Acknowledgements**

This study was supported by grants from the Alzheimer’s Association and the National Institutes of Health to Vivian Hook, and by National Institutes of Health grants to Michael Ziegler.

**References**

pGlu-Åβ and QC Colocalization with Åβ in Secretory Vesicles

Neurodegener Dis 2014;14:85–97
DOI: 10.1159/000358430


PGLu-Aβ and QC Colocalization with Aβ in Secretory Vesicles


