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Identification of thyrotropin-releasing hormone as hippocampal glutaminyl cyclase substrate in neurons and reactive astrocytes

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ABSTRACT

Recently, Aβ peptide variants with an N-terminal truncation and pyroglutamate modification were identified and shown to be highly neurotoxic and prone to aggregation. This modification of Aβ is catalyzed by glutaminyl cyclase (QC) and pharmacological inhibition of QC diminishes Aβ deposition and accompanying gliosis and ameliorates memory impairment in transgenic mouse models of Alzheimer's disease (AD). QC expression was initially described in the hypothalamus, where thyrotropin-releasing hormone (TRH) is one of its physiological substrates. In addition to its hormonal role, a novel neuroprotective function of TRH following excitotoxicity and Aβ-mediated neurotoxicity has been reported in the hippocampus. Functionally matching this finding, we recently demonstrated QC expression by hippocampal interneurons in mouse brain.
Here, we detected neuronal co-expression of QC and TRH in the hippocampus of young adult wild type mice using double immunofluorescence labeling. This provides evidence for TRH being a physiological QC substrate in hippocampus. Additionally, in neocortex of aged but not of young mice transgenic for amyloid precursor protein an increase of QC mRNA levels was found compared to wild type littermates. This phenomenon was not observed in hippocampus, which is later affected by Aβ pathology. However, in hippocampus of transgenic — but not of wild type mice — a correlation between QC and TRH mRNA levels was revealed. This co-regulation of the enzyme QC and its substrate TRH was reflected by a co-induction of both proteins in reactive astrocytes in proximity of Aβ deposits. Also, in primary mouse astrocytes a co-induction of QC and TRH was demonstrated upon Aβ stimulation.

1. Introduction

Alzheimer's disease (AD) is characterized by the formation of Aβ plaques and neurofibrillary tangles [1,2]. In particular neocortex and hippocampus are affected by AD pathology leading to clinical symptoms like cognitive decline and loss of memory function [2–4]. The knowledge about the structural diversity of Aβ peptides was extended in the 1990s by the identification of N-terminally truncated and pyroglutamate (pGlu)-modified Aβ peptides (pGlu-Aβ) in *post mortem* brains of AD patients [5,6]. These pGluAβ-peptides exhibit altered biochemical properties causing increased neurotoxicity, resistance to proteolysis and accelerated tendency to aggregate [7–10].

Subsequently, the enzyme glutaminyl cyclase (QC) was demonstrated to catalyze this post-translational modification of Aβ *in vitro* [11] and *in vivo* [12]. Furthermore, pharmacological inhibition of QC leads to a significant reduction of overall Aβ aggregation in transgenic animal models of AD and to better performance in experimental tasks of learning and memory [12].

Recently, we reported QC expression in a subset of hippocampal interneurons of wild type mice [13] as well as deposition of pGlu-Aβ in the core of Aβ plaques in human amyloid precursor protein (APP) transgenic Tg2576 mice [14]. An understanding of the physiological function and substrate specificity of QC in brain regions such as the hippocampus appears important in order to (i) gain insight into the involvement of QC in AD-related pathological changes and (ii) estimate the effects of pharmacological inhibition of QC in the course of a possible therapeutic intervention.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-site APP-cleaving enzyme 1; HPT, hypothalamus–pituitary–thyroid; IFN-γ, interferon-γ; LPS, lipopolysaccharide; pGlu, pyroglutamate; QC, glutaminyl cyclase; TRH, thyrotropin-releasing hormone

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There is substantial knowledge about QC function in the hypothalamus, where the enzyme catalyzes the pGlu modification of neuropeptides like neurotensin and thyrotropin-releasing hormone (TRH) [15–17]. Notably, QC-mediated pGlu modification of TRH is known to occur as the latest step of a processing cascade which is essential for the biological activity of TRH enabling its interaction with specific TRH receptors in pituitary gland and decreasing its degradation rate [18–21]. In this hypothalamus–pituitary–thyroid (HPT) axis, activation of TRH receptors increases the release of TSH, which in turn stimulates the secretion of thyroid hormones [22]. Accordingly, QC knock-out mice show reduced plasma thyroxine concentration [23].

However, there is evidence for a function of TRH independent of the HPT axis. In this context, TRH was shown to exhibit neuromodulatory as well as neuroprotective effects. The expression of TRH in hippocampus, raphe nuclei and the hypothalamic nuclei [24–27] as well as the co-localization with neurotransmitters like choleocystokinin, galanin, neuropeptide Y and serotonin point towards a function of TRH as neuromodulator [25,28–30]. More recently, the potential of TRH to act as a neuroprotective compound has been discussed. TRH was reported to prevent neurodegenerative effects of excitotoxic substances like kainate, glutamate and NMDA in cell culture studies of organotypic brain slices and primary neurons [31–33]. In this context, it is also notable that TRH decreases the release of excitatory glutamate [34] but increases secretion of inhibitory GABA [35] as shown in electrophysiological studies using organotypic brain slice cultures and primary neurons.

Interestingly, TRH-mediated neuroprotection has not only been reported with respect to excitotoxicity [31–33], Parkinson's disease [36,37] and following brain injury [38,39], but also in response to Aβ neurotoxicity [39,40]. Furthermore, in *post mortem* hippocampal tissue of AD patients diminished levels of TRH were demonstrated and depletion of TRH in cell culture studies was shown to result in hyperphosphorylation of tau [41], another hallmark of AD.

In summary, due to its presumed neuroprotective effects, hippocampal TRH expression and function deserves a thorough investigation, especially since the TRH-modifying enzyme QC also contributes to Aβ pathology. In order to study a possible spatial and temporal relationship between hippocampal QC and TRH expression, quantitative mRNA analysis and immunohistochemical labeling were performed in young and aged wild type and Tg2576 mice with distinct hippocampal Aβ pathology. Additionally, activated astrocytes were used as an *in vitro* model to study a possible co-regulation of QC and TRH under pathogenic conditions. The relevance of TRH in the hippocampus, a brain area of particular importance in learning and memory which is strongly affected in AD, is discussed.

2. Materials and methods

2.1. Experimental animals

In order to analyze hippocampal QC and TRH expression, transgenic Tg2576 mice and wild type littermates at postnatal ages of 4, 8, 10, 13, 17 and 21 months were used for immunohistochemical and for qPCR experiments (N = 3–6 per age group). Due to overexpression of human APP comprising Swedish double mutation (Lys670→Asn, Met671→Leu) Tg2576 mice develop Aβ pathology first detectable by 10 months of age in entorhinal cortex and by about 13 months in hippocampus.

2.2. Preparation of mouse brains for immunohistochemistry and qPCR

The mice were deeply anesthetized with pentobarbital and transcardially perfused with 50 ml 0.9% saline containing 0.1% heparin followed by perfusion with 80 ml 4% paraformaldehyde in PBS (0.1 M; pH 7.4). The brains were removed from the skull and post-fixed by immersion in the same fixative overnight at 4 °C. After cryoprotection in 30% sucrose in 0.1 M PBS for 3 days, the brains were snap-frozen in n-hexane at −68 °C and stored at −20 °C. Coronal sections (30 μm) were cut on a sliding microtome and collected in 0.1 M PBS.

In order to perform qPCR mice were sacrificed, brains were removed from the skull and subsequently neocortex, hippocampus and ventral brain were dissected. Dissection of the ventral brain was done by cutting off the rostral part of the brain at the level of the anterior commissure, followed by removal of the overlaying neocortex, hippocampus and cerebellum. Thus, it contains subcortical nuclei and hypothalamus with well-described QC and TRH expression.

2.3. Immunohistochemical labeling

In order to perform immunohistochemistry, mouse brain slices were pre-treated with 60% methanol (30 min), followed by washes in 0.1 M TBS and blocked in TBS containing 0.3% TritonX-100 and 5% normal donkey serum for 30 min to reduce unspecific binding of antibodies. Incubation with primary antibodies was performed in TBS containing 0.1% TritonX-100 and 5% normal donkey serum over 1–3 days at 4 °C. For detection of QC a rabbit anti-mouse QC antibody (AB1301, Probiodrug AG, Halle/Saale, Germany) or goat anti-mouse QC antibody (10269, Probiodrug AG) was used as described earlier [13,14]. Immunohistochemical labeling of TRH was obtained with different commercially available rabbit anti-TRH antibodies raised either against proTRH (BP5066, Acris) or against synthetic pGlu-His-Pro conjugated to KLH (PAB13482, Abnova; TRH, BioLogo). Staining with these antibodies displayed a very similar pattern of fibers and single neurons in mouse hypothalamus indicating specific labeling since this brain region is described to show robust TRH immunoreactivity [22]. pGlu-Aβ peptides in mouse hippocampus were detected using the mouse monoclonal antibody mab2-48 (Synaptic Systems, Göttingen, Germany), which has been well characterized [42]. A goat anti-GFAP antibody (Santa Cruz) was used for labeling of astroglia. According to the host species of the primary antibody appropriate secondary antibodies (Dianova) were used to visualize respective antigens either with chromogen (3,3′DAB) or fluorescent dyes (Cy2, Cy3, Cy5).

2.4. Confocal laser scanning microscopy

Laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) was performed to analyze co-localization of QC and TRH in mouse brain tissue. For Cy2-labeled antigens (green fluorescence), an argon laser with 488 nm excitation was used and emission from Cy2 was recorded at 510 nm applying a low-range band pass (505–550 nm). For Cy3-labeled antigens (red fluorescence), a helium–neon laser with 543 nm excitation was used and emission from Cy3 at 570 nm was detected applying high-range band pass (560–615 nm). The Cy5-labeled antigens were visualized using excitation at 650 nm and emission at 670 nm.

2.5. Stimulation of cultured primary astrocytes

Astrocyte primary cell cultures were started with brains of newborn mice according to Löffner et al. [43] and were maintained in DMEM-based medium at 37 °C in a humidified atmosphere with 95% air/5% CO_2. The medium was renewed once a week.

Activation of astrocytes was induced when cells had reached 80–90% confluency by incubation with (i) Aβ1–42 (5 μM) or (ii) lipopolysaccharide derived from *Escherichia coli* (LPS; 1 μg/ml; O55:B5, Sigma) and interferon-γ (IFN-γ; 20 ng/ml; Preprotech) for 48 h. Cell viability was analyzed with a commercially available lactate dehydrogenase assay kit from Promega.

2.6. mRNA quantification by qPCR

RNA was isolated using the NucleoSpin RNA II kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. RNA

was obtained from different brain regions as well as from cultured primary astrocytes. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Peqlab, Erlangen, Germany). RNA (0.1–1 mg) was reverse transcribed into cDNA using random primers (Roche, Penzberg, Germany) and Superscript III (Life Technologies). Quantitative PCR was performed in a Rotorgene 3000 (Corbett Research, Sydney, Australia) using the Rotor-Gene SYBR Green PCR kit and specific primers for QC (NM_027455.2, 5′-GGGAGGCAGACACAAT CAAT and 3′-TCAGATTCCCAGCTGTCAGA), TRH (NM_009426.2, 5′-GTGCCAACCAAGACAAGGAT and 3′-TTCTTCCCAGCTTCTTTGGA) and GFAP (NM_010277.3, 5′-ACATCGAGATCGCCACCTAC and 3′-TCACATCA CCACGTCCTTGT) synthesized by Metabion (Martinsried, Germany). Relative gene expression was determined using the Rotorgene Software version 6.1 in comparative quantitation mode. TTl (NM_027192.2), Umps (NM_009471.2) and Sys1 (NM_025575.3) were used as reference genes for expression analysis. The PCR was verified by product melting curves and single amplicons were confirmed by agarose gel electrophoresis.

2.7. Quantification of immunocytochemistry

After treatment of primary astrocytes, cells were fixed in 4% paraformaldehyde, labeled with antibodies against TRH and QC as described in 2.3 and cell nuclei were counterstained with Hoechst dye (1:10,000; Invitrogen). From each sample 5 images were taken by means of fluorescence microscope (Keyence Biorevo BZ-9000) and approx. 10 cells/sample were densitometrically analyzed by Keyence BZ-9000 Analyzer software. Values obtained from control astrocytes were set to 100%.

2.8. Statistical analyses

Data that were obtained from qPCR experiments were analyzed by GraphPad Prism4 Software with respect to statistical significance in Student's t-test and correlation analysis of data was performed with Pearson's correlation coefficient.

3. Results

3.1. Co-localization of QC and TRH in hippocampus of wild type mice

In order to obtain insight into the biological significance of the QC expression in the hippocampus as reported by Hartlage-Rübsamen et al. [13], we focused on the hypothalamic QC substrate TRH, since this well known releasing hormone of the HPT axis has also been shown to play a role in the hippocampus [33–35]. Therefore, immunohistochemistry was used to reveal the cellular expression pattern of TRH in the hippocampus of young adult postnatal day 42 wild type mice.

Immunohistochemical labelings displayed a considerable number of TRH positive interneurons dispersed over different layers of the hippocampus (Fig. 1B). The regional expression pattern strongly resembled the distribution of QC positive neurons, particularly in *stratum lacunosum moleculare* and in *stratum radiatum* of hippocampus proper (Fig. 1A). In the polymorphic cell layer of dentate gyrus TRH immunoreactivity was low in labeling intensity compared to the relatively strong QC labeling. Double immunofluorescent staining demonstrated co-localization of QC and its substrate TRH in a subpopulation of hippocampal interneurons (Fig. 1C).

3.2. Temporal expression pattern of QC and TRH mRNA in different brain regions during aging of Tg2576 mice

QC was shown to be involved in the development of Aβ pathology by modifying N-terminally truncated Aβ peptides. The resulting pGlu-Aβ peptides exhibit altered biochemical properties which lead to increased neurotoxicity. TRH, a known hypothalamic substrate of QC, was demonstrated to have neuromodulatory properties in the hippocampus and to provide protection towards Aβ-mediated neurotoxicity as well as glutamatergic excitotoxicity. In order to study the interrelationship between TRH and QC we investigated the temporal gene expression pattern of these two proteins in Tg2576 mice before and after the onset of Aβ pathology and compared to non-transgenic littermates.

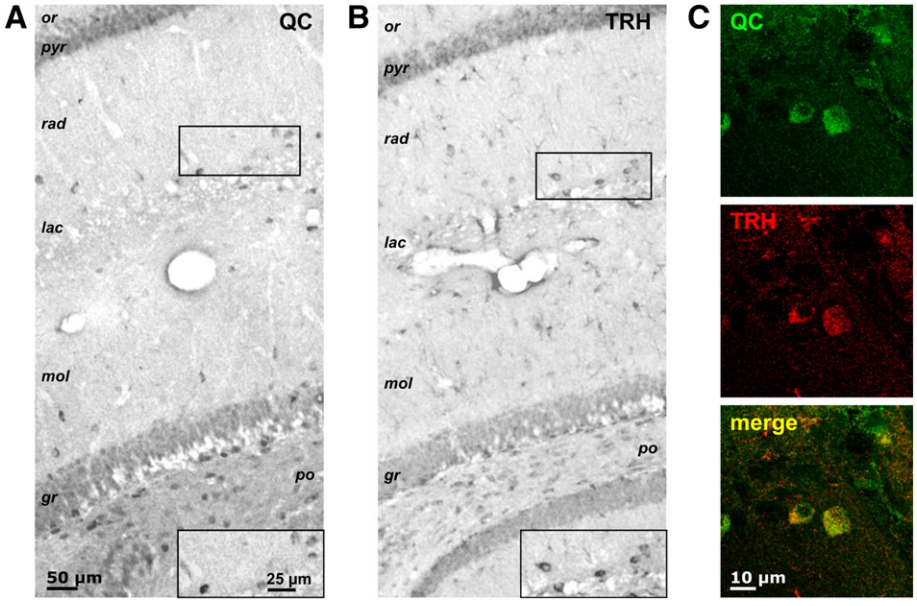

Fig. 1. QC and TRH co-localization in hippocampal neurons of adult wild type mice. Immunohistochemistry for QC (A) and TRH (B) in hippocampal slices of wild type mice revealed immunoreactive neurons in distinct layers of the hippocampus. The staining displayed a similar distribution of QC- and TRH-positive neurons in *stratum lacunosum moleculare* (lac) and in *stratum radiatum* (rad) of the hippocampus. In the polymorphic cell layer of dentate gyrus (po) strong labeling for QC occurred whereas TRH immunoreactivity was low. In the pyramidal cell layer of the hippocampus (pyr) as well as in the granular cell layer of dentate gyrus (gr) no specific labeling was detected (A, B). Double immunolabeling revealed co-localization of QC and TRH in a subpopulation of hippocampal interneurons (C). Abbreviations or, *stratum oriens;* lac, *stratum lacunosum moleculare;* rad, *stratum radiatum;* pyr, pyramidal cell layer; mol, molecular layer of *dentate gyrus;* po, polymorphic cell layer of *dentate gyrus;* gr, granular cell layer of *dentate gyrus.*

To this aim, QC and TRH mRNA levels of Tg2576 and wild type mice of different age groups (4, 8, 13 and 17 months) were quantified in distinct brain regions (ventral brain, cortex and hippocampus). Adult Tg2576 mice at the age of 4 months were used as control and 8-month-old Tg2576 mice were chosen since at this age excitotoxicity might be present due to the generation of high amounts of Aβ, whereas aggregated Aβ is first detectable in entorhinal cortex from 10 months on. In 13-month-old Tg2576 mice numerous Aβ deposits are already present in cortical areas, whereas Aβ plaque burden in hippocampus is still low. This allows studying in parallel a direct effect of ongoing Aβ deposition in two different brain areas affected by Aβ pathology. At the age of 17 months, in cortex as well as in hippocampus of Tg2576 mice high amounts of Aβ plaques are detectable, contrary to the ventral brain area where hardly any plaque pathology is detectable.

In the ventral brain, we observed an opposing trend comparing the age-related expression of QC and TRH mRNA of Tg2576 and wild type mice (Fig. 2). This was demonstrated by a constant decline of the amount of QC and TRH mRNA in Tg2576 mice from 4 months up to 17 months of age, whereas in wild type littermates both gene transcripts continuously increased from 8 months onwards. As a result, expression of QC (−67%) and TRH mRNA (−75%) in the ventral brain of 17 months old Tg2576 mice is significantly lower compared to wild type mice (Fig. 2).

In contrast, the neocortex, a region strongly affected by Aβ pathology displayed a significant increase of about 35% in the expression of QC mRNA in 17-month-old Tg2576 mice (Fig. 2). In the hippocampus expression of QC and TRH mRNA remained constant over all investigated ages revealing different expression patterns of QC and TRH mRNA in brain areas strongly affected by Aβ pathology compared to the ventral brain which hardly develops Aβ plaques (Fig. 2).

3.3. Correlation of QC and TRH mRNA expression in distinct brain regions

Data from QC and TRH mRNA quantification were analyzed regarding a possible correlation between the enzyme and its substrate. For the correlation analyses, values of wild type or Tg2576 littermates aged between 4 and 17 months (n = 24) were combined and displayed with respect to the distinct brain areas ventral brain, neocortex and hippocampus (Fig. 3).

For the ventral brain, containing hypothalamus with well-described expression of QC and TRH, a strong correlation between QC and TRH mRNA levels was observed, whereas in neocortex no such correlation was detected (Fig. 3). Interestingly, in the hippocampus of Tg2576 mice, but not of wild type littermates, the expression of QC and TRH mRNA was significantly correlated (Fig. 3). Thus, regarding the development of Aβ pathology there is evidence for a role of QC and its substrate TRH in the hippocampus.

3.4. Reactive astrocytes around Aβ deposits in Tg2576 mice are QC and TRH immunoreactive

Since the quantification of QC and TRH mRNA provided evidence for a regulation of TRH by Aβ pathology, we asked whether the hippocampal expression of TRH on the protein level might also be altered.

Interestingly, TRH immunohistochemistry of Tg2576 mice aged between 4 and 21 months revealed astrocytic TRH immunoreactivity in close proximity to Aβ plaques of older animals. In cortical and in hippocampal areas of Tg2576 mice Aβ plaques are detectable at about 10 and 13 months of age, respectively. The appearance of TRH-positive astrocytes correlated with the age-dependent development of Aβ plaques in neocortex (10 months) and hippocampus (13 months) of these human APP-transgenic mice (Fig. 4A). Furthermore, TRH-immunoreactive

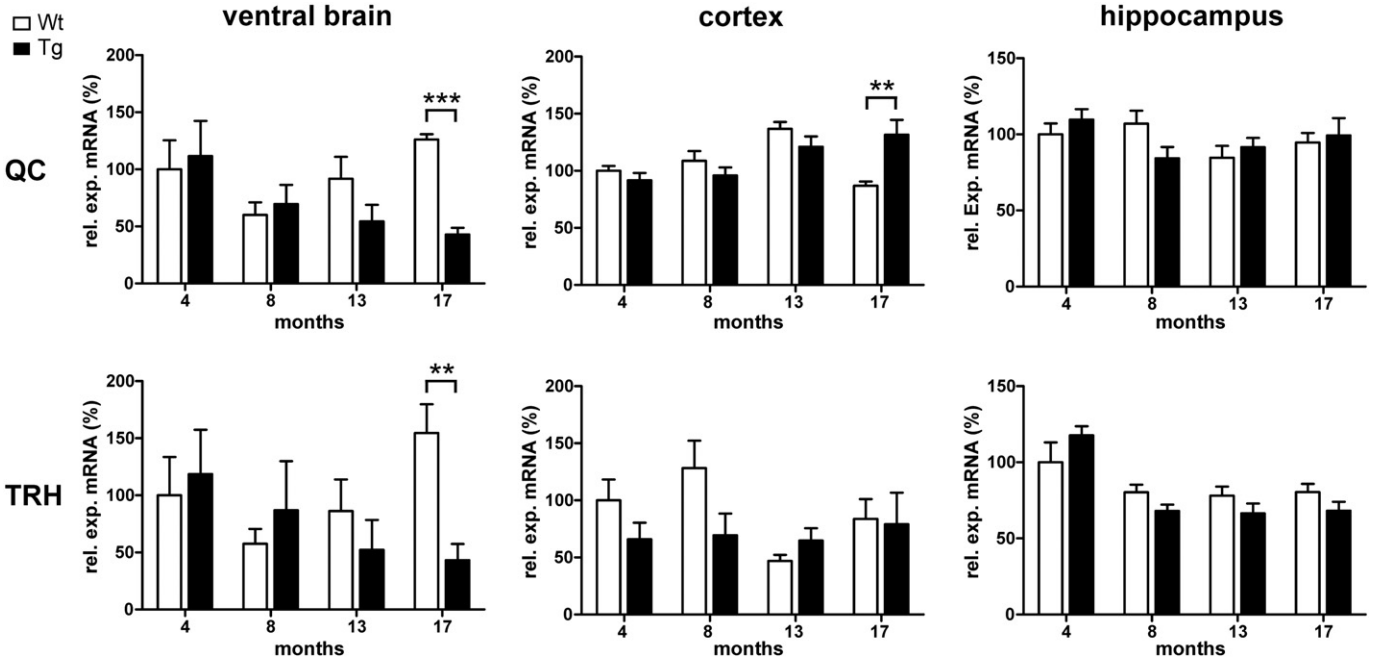

Fig. 2. QC and TRH mRNA quantification in distinct brain regions of Tg2576 mice aged between 4 and 17 months. Expression of QC and TRH mRNA was analyzed in ventral brain (containing hypothalamus), neocortex and hippocampus of Tg2576 mice and their wild type littermates. Mice aged between 4 and 17 months were used in order to study the expression of QC and TRH in the course of aggravating Aβ pathology. With regard to the expression of QC and TRH mRNA in the ventral brain an opposing trend in Tg2576 and wild type littermates was observed. QC and TRH mRNA levels were increasing from 8 months onwards in wild type mice. In contrast, in Tg2576 mice we observed from 4 months to 17 months of age a continuous decrease of both investigated gene transcripts resulting in a significant lowered expression at the age of 17 months compared to their wild type littermates. On the contrary, in neocortex of 17 months old Tg2576 mice with distinct Aβ pathology the expression of QC mRNA was significantly increased and the expression of TRH mRNA displayed a stable level from 4 months to 17 months without significant variants. Also in the hippocampus of the investigated Tg2576 and their wild type littermates the expression profile of QC and TRH mRNA showed a constant level. Mean values (n = 6) are indicated as ratio of 4 months wild type (= 100%, arbitrary units (a. u.)), statistical differences were analyzed using Student's t-test and are indicated by asterisks (*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$).

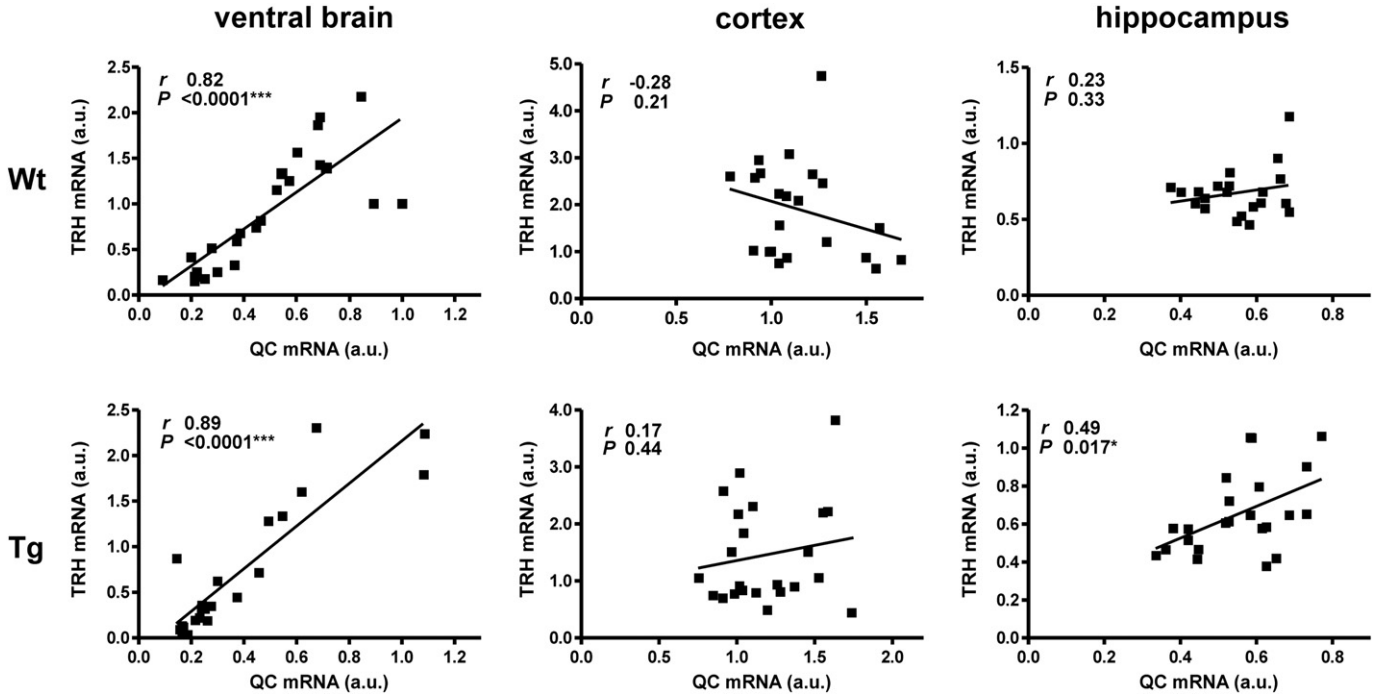

Fig. 3. Correlation of QC and TRH mRNA expression in distinct brain regions of Tg2576 mice. Correlation analysis revealed a strong interdependence of QC and TRH mRNA expression in the ventral brain of, both, Tg2576 mice and their non transgenic littermates as well as a significant correlation in the hippocampus of Tg2576 but not of wild type animals. No correlation was measured in the neocortex. For correlation analysis mRNA values of all mice of the indicated genotype aged between 4 and 17 months were combined (n = 24 each). Pearson correlation coefficient r and p values (*p < 0.5; **p < 0.01; ***p < 0.001) are indicated in the diagrams.

glia-like cells surrounded Aβ plaques and showed typical morphological characteristics of activated astroglia such as enlarged somata and processes (Fig. 4A). This distinct glia-like TRH staining was not observed distant from Aβ plaques.

3.5. QC and TRH are co-localized in activated astrocytes in Tg2576 mice

Double immunofluorescent labelings for GFAP and pGlu-Aβ in hippocampal brain slices of 17 months old Tg2576 mice confirmed the activation of astrocytes around Aβ plaques (Fig. 4B). Reactive astrocytes in proximity to plaques but not in astrocytes away from Aβ deposits were found to be pGlu-Aβ immunoreactive (Fig. 4B). Similarly, Aβ plaque-associated reactive astrocytes also displayed TRH immunoreactivity (Fig. 4C). Both products of the enzymatic activity of QC, TRH and pGlu-Aβ, were found to be co-localized with QC in Aβ plaque-associated reactive astrocytes (Fig. 4D). Thus, there is a distinct induction of QC, TRH and pGlu-Aβ in plaque-associated reactive astrocytes.

3.6. Expression of QC and TRH in cultures of primary astrocytes upon Aβ treatment

As described above, we observed activated astrocytes surrounding Aβ plaques of Tg2576 mice which show co-expression of QC and TRH. In order to reveal whether Aβ peptides directly mediate the induction of QC and TRH in reactive astrocytes we used a primary mouse astrocyte cell model. Primary astrocytes were stimulated with Aβ or a general pro-inflammatory mixture of LPS/IFN-γ and the expression of QC and TRH mRNA as well as immunoreactivity for QC and TRH protein was studied after a 48 h stimulation period. Compared to vehicle-treated astrocytes, the expression of QC mRNA was increased by 23% ($p < 0.05$) upon Aβ stimulation, whereas the 15% increase in TRH mRNA levels was not statistically significant (Fig. 5A). Immunocytochemical labeling of QC and TRH also suggested increased protein levels (Fig. 5B). Quantitative image analysis demonstrated increased QC (+65%; $p < 0.01$) and for TRH (+57%; $p < 0.05$) protein levels (Fig. 5A). This indicates that a primary Aβ-induced increase in QC mRNA and protein expression results in the stabilization and, therefore, increased concentration of the QC substrate TRH without significant change in its mRNA level. In contrast, a general pro-inflammatory stimulation of astrocytes with LPS/IFN-γ did not induce QC or TRH mRNA or protein levels (not shown).

4. Discussion

In the 1990s, new Aβ peptide variants were identified comprising N-terminally truncated and pGlu-modified species. These Aβ variants display altered biochemical, biophysical and cell biological properties leading to enhanced neurotoxicity and resistance to proteolytical degradation. The enzyme QC was demonstrated to catalyze the pGlu modification of Aβ and pharmacological QC inhibition in Tg2576 mice resulted in a reduction of Aβ plaque burden [12]. However, when using QC inhibition as pharmacological tool for AD treatment, it is important to understand the physiological function of QC expression.

In a previous report, we already mapped regional and cellular QC expression in wild type mouse brain and identified QC-immunoreactive interneurons in distinct layers of the hippocampus [13]. The neurohormone TRH is known to be an important physiological QC substrate in diencephalon where both, enzyme and substrate, are expressed by neurons of the paraventricular nucleus of the hypothalamus. Studies concerning an alternative TRH function as neurotransmitter and neuromodulator in the hippocampus [25,28,34,35] prompted us to relate TRH to QC expression in this brain region.

Here, we report for the first time co-localization of QC and TRH in a neuronal subpopulation of the hippocampus in adult wild type mice. However, since not all QC expressing neurons were positive for TRH immunoreactivity, it is very likely that other, yet unknown QC substrates exist in the hippocampus. Our finding confirms previous reports that demonstrated intrinsic TRH expression in the hippocampus, albeit most hippocampal TRH is supposed to derive from extrinsic sources [26]. When pathological amounts of Aβ peptides are present in AD, QC

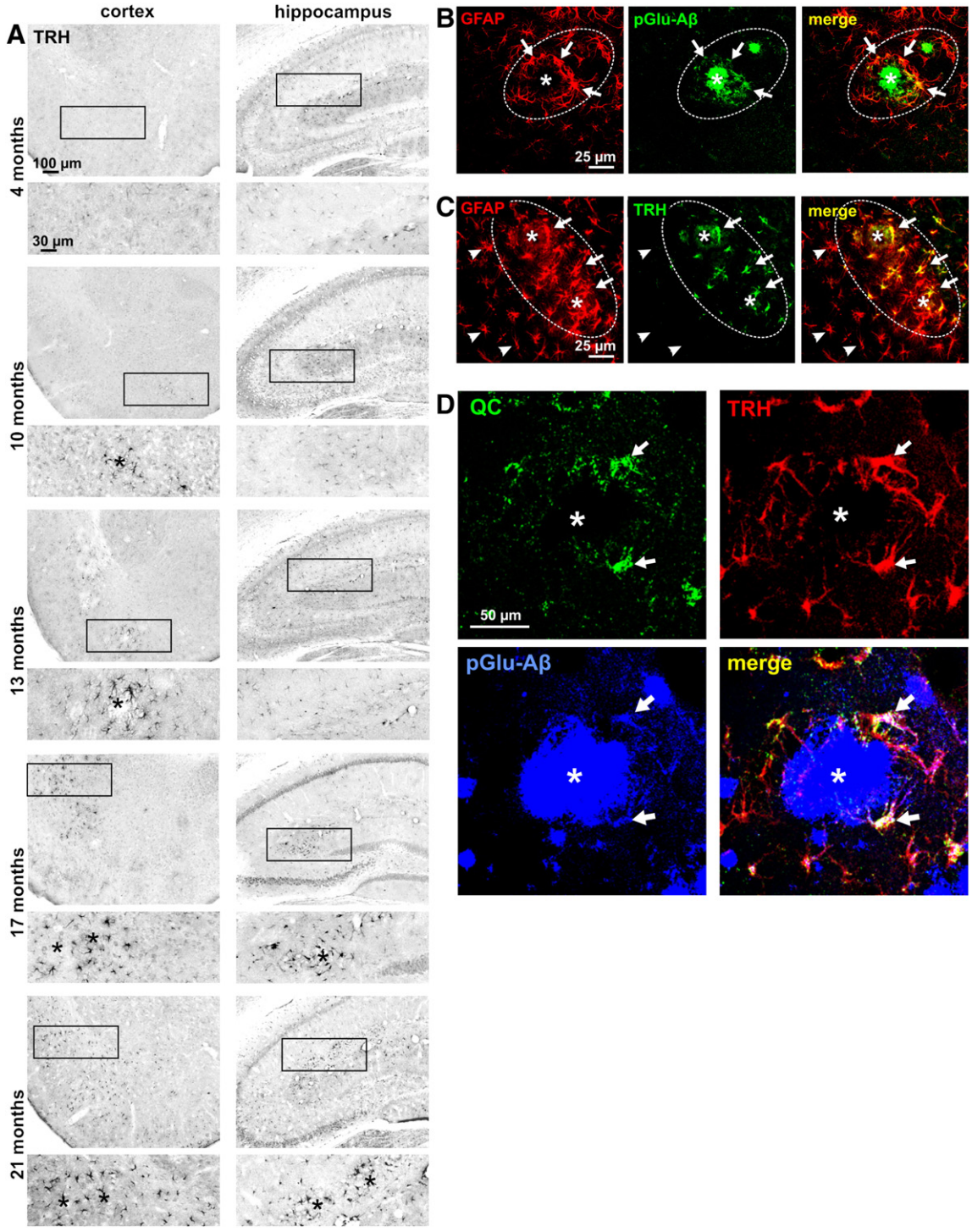

Fig. 4. Age-dependent increase of astrocytic QC and TRH immunoreactivity in the vicinity of Aβ-deposits in the neocortex and hippocampus of Tg2576 mice. Tg2576 mice develop deposits of aggregated Aβ in cortex and hippocampus from 10 and 17 months onwards, respectively. For analysis of TRH immunoreactivity Tg2576 mice aged between 4 and 21 months were examined. (A) Immunohistochemistry revealed TRH staining in astrocytes that encircle Aβ deposits in cortex and hippocampus of Tg2576 mice at 10 and 17 months, respectively (asterisks). The increasing number of TRH-positive astrocytes was associated with age and thereby the quantity of Aβ deposits. Tg2576 mice without apparent Aβ aggregates (4 months old) were devoid of TRH-positive astrocytes. (B) The density of GFAP-immunoreactive astrocytes (red) around pGlu-Aβ deposits (green, asterisk) is increased and their morphology is altered to a reactive phenotype. Some of the Aβ plaque-associated astrocytes (within the white circle) display pGlu-Aβ immunoreactivity (arrows), as indicated by yellow/orange color. Astrocytes distant from Aβ deposits (outside the white circle) do not display pGlu-Aβ immunoreactivity. (C) Aβ plaque-associated astrocytes (red) are also immunoreactive for TRH (green, arrows). The location of Aβ deposits is marked by asterisks. Astrocytes distant from Aβ deposits, outside the circle, are not TRH-immunoreactive (arrowheads). (D) Both products of the enzymatic activity of QC, TRH (red) and pGlu-Aβ (blue, asterisk), and QC itself (green) are co-localized in Aβ plaque-associated reactive astrocytes (arrows) as indicated by white color in the overlay channel.

expressing neurons might be at particular risk of degeneration due to the intracellular modification of Aβ resulting in highly neurotoxic pGlu-Aβ. Such a degeneration of QC expressing interneurons could result in the release of pGlu-Aβ acting as a seed for further Aβ aggregation [9] and to a decrease of TRH concentration in the hippocampus as it was demonstrated in *post mortem* brains of AD [41]. Regarding the neuroprotective ability of TRH to counteract Aβ-induced neurodegeneration [39,40] as well as excitotoxicity in cell culture models [31–33], the

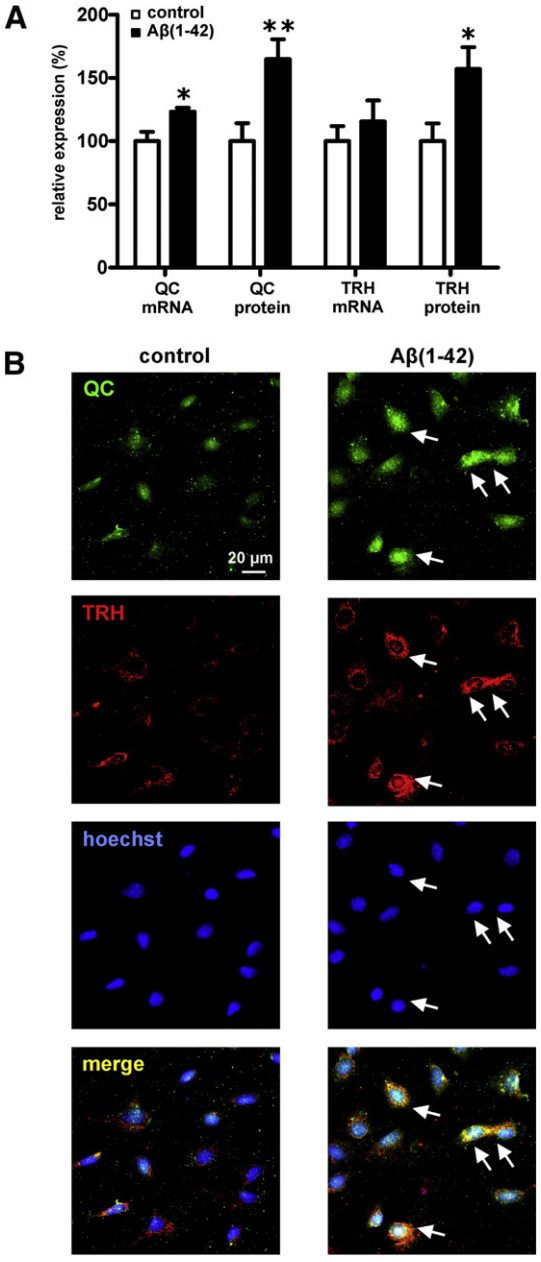

Fig. 5. Expression of QC and TRH in primary astrocytes. Cultured primary astrocytes were activated by incubation with Aβ (5 μM) for 48 h. (A) Quantitative RT-PCR revealed an increase in QC mRNA in Aβ-stimulated astrocytes (black bars) by 23% ($p < 0.05$), whereas TRH mRNA levels remained unchanged ($n = 6$). Moreover, protein levels for QC and TRH were increased by 65 and 57%, respectively as revealed by quantitative immunocytochemistry. Mean values for treated astrocytes are given as per cent of untreated value (=100%). Statistical differences were analyzed using Student's t-test and are indicated by asterisks (*$p < 0.5$). (B) Examples of immunocytochemical fluorescent labelings of astrocytes for QC (green) and TRH (red) under control conditions and after Aβ stimulation. Note the robust increase in the staining intensity after Aβ stimulation in a high proportion of the astrocytes (arrows). Cellular nuclei are stained with Hoechst dye (blue).

degeneration of hippocampal TRH-expressing neurons could aggravate pathological processes in AD. Also, in Parkinson's disease [36,37] and in the context of CNS trauma [38,39] neuroprotective effects of TRH were reported.

In order to study the regional expression of QC and TRH with regard to Aβ pathology, we compared Tg2576 mice with wild type littermates at different postnatal ages using quantitative PCR as well as immunohistochemistry. We detected a significantly higher expression of QC mRNA only in the neocortex of 17-month-old transgenic mice with substantial Aβ pathology which could account for the enhanced formation of pGlu-Aβ deposits as revealed by immunohistochemistry. In contrast, unchanged QC mRNA levels in the hippocampus of Tg2576 mice could be related to the delayed development of Aβ pathology in this brain region. In the hippocampus as well as in the neocortex we did not observe significant differences in the expression of TRH mRNA in Tg2576 mice compared to wild type littermates. In the light of the induction of QC and TRH protein in reactive astrocytes of aged Tg2576 mice (see below), increased levels of these transcripts might be expected. This discrepancy may be explained by a dissociation between the regulation of mRNA and protein expression and/or a reduction of neuronal QC and TRH mRNA expression in parallel with the induction of these transcripts in reactive astrocytes.

On the other hand, diminished TRH protein levels were reported for hippocampus in AD [41]. Since we observed constant hippocampal TRH mRNA levels and an induction of TRH protein in reactive astrocytes of aged Tg2576 mice, our findings point towards differences between the transgenic animal model and the human disease. For example, human brain samples analyzed always reflect the final stage of the disease, the post mortem delay is much longer than in animal studies and the mouse model used only mimics aspects of the human disease.

A correlation analysis revealed a significant correlation between QC and TRH mRNA in the hippocampus of Tg2576 mice but not of wild type mice pointing to an effect of developing Aβ pathology on the expression of QC and TRH.

To study the expression of QC and TRH in a part of the brain, which is less affected by Aβ pathology, we used tissue of the ventral brain including the hypothalamus amongst other subcortical nuclei. In this part of the brain, the mRNA expression of both, QC and its substrate TRH, was significantly decreased in 17-month-old Tg2576 mice compared to wild type littermates. Most TRH is synthesized in the paraventricular nucleus of the hypothalamus, which projects — apart from the pituitary gland — also to the hippocampus [44], brain stem [45] and spinal cord [46]. A decrease in the release of hypothalamus-derived TRH could therefore impair TRH-mediated neuromodulation as well as HPT axis, the latter being affected in AD patients as reported by Yong-Hong et al. [47]. *Vice versa*, hippocampal Aβ pathology may in a retrograde manner affect hypothalamic afferents. In Tg2576 mice devoid of hypothalamic Aβ pathology, hypothalamic metabolic and hormonal dysfunction have been reported [48,49] which may by the basis for the effects observed here on TRH and QC mRNA expression.

Since determination of mRNA expression is not sufficient to gain insight in cellular expression, immunohistochemical stainings were performed. In order to obtain more information about pronounced state of pathology in hippocampus, we decided to use brain slices of Tg2576 mice aged between 4 and 21 months for immunohistochemistry, because qPCR experiments revealed significant differences in mRNA expression levels particularly in 17-month-old mice with distinct Aβ pathology.

Interestingly, we did not observe any obvious alteration in the number of QC and TRH positive neurons (not shown), but detected induction of strong TRH labeling of astrocytes in close proximity to Aβ plaques. Furthermore, QC, the enzyme which converts TRH into its bioactive state, was co-localized with its substrate in activated astrocytes. We consider this as being strong evidence for an intracellular production of biologically active TRH in astrocytes. However, we cannot completely rule out the possibility that the observed immunoreactivity is caused by endocytosis of neuronally secreted TRH. Although the expression and processing of neuropeptides by astroglia is still controversially discussed, there is some evidence supporting this hypothesis. For example, astrocytes were shown to express enzymes that are involved in pro-TRH processing like carboxypeptidase C/D [50,51] and peptidyl-glycine alpha-amidating monooxygenase [52]. Recently, spinal astrocytes were demonstrated to process prodynorphin and release dynorphin [53] indicating that astrocytes are principally able to process pro-peptides. In addition, a glioblastoma cell line was shown to release TRH [54] and with respect to cultured astrocytes, earlier publications already

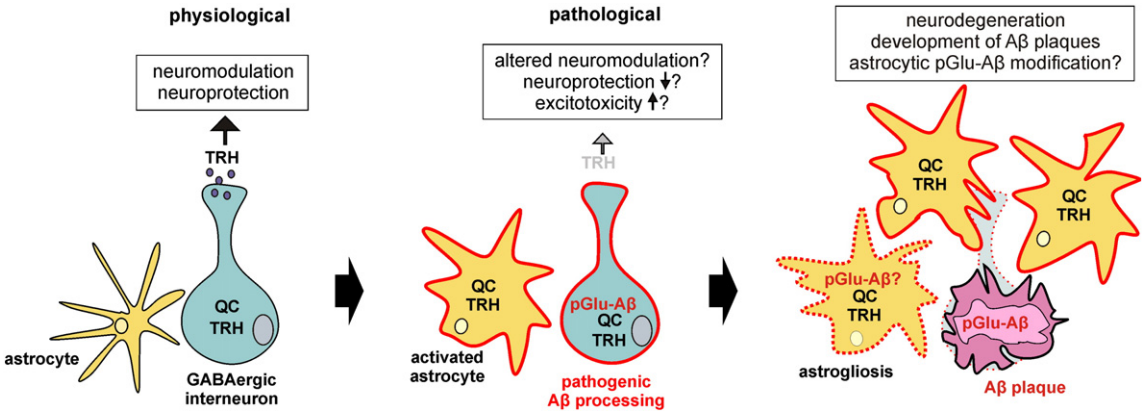

Fig. 6. Potential relevance of hippocampal QC and TRH under physiological and pathological conditions. Under physiological conditions hippocampal interneurons co-express QC and TRH and contribute to LTP modulation and neuroprotection by releasing TRH. However, under pathological conditions in AD, QC-catalyzed pGlu-Aβ generation induces degeneration of these interneurons. This results in diminished release of neuroprotective TRH and presumably in altered neuromodulation and increased excitotoxicity. Furthermore, the formation of Aβ plaques as well as neurodegeneration induces the activation of astrocytes. The co-induction of QC and TRH in reactive astrocytes could contribute to TRH-mediated neuroprotection as well as to further neurodegeneration by generation of astrocyte-derived pGlu-Aβ.

mentioned an increased phosphoinositol turnover after administration of TRH in medium [55].

Furthermore, in performing qPCR on mouse primary astrocytes we detected substantial expression of TRH mRNA and QC mRNA. The transcript levels of QC and protein concentrations of QC and TRH were increased upon stimulation with Aβ. The elevated TRH protein concentration without induction of the corresponding mRNA suggests post-translational pGlu modification and, thereby, stabilization of TRH by QC. On the other hand, the stimulation of cultured astrocytes with LPS and IFN-γ did not modulate QC or TRH mRNA or protein expression. This is strong evidence for Aβ peptides themselves — and not other factors present in or around Aβ deposits — being triggers for astrocytic QC and TRH expression. Since we also observed strong TRH immunoreactivity in astrocytes enclosing Aβ plaques in Tg2576 mice, it is conceivable, that it is due to a chronic, Aβ-mediated activation of astrocytes in these AD model mice.

The finding of QC and TRH in activated astrocytes might be of importance with regard to a role of astroglia contributing to AD pathogenesis. Since astrocytes were demonstrated to express the β-secretase BACE1 [56,57], the additional expression of QC provides the possibility of astroglial derived toxic pGlu-Aβ leading to astrogliosis and the formation of Aβ plaques. Moreover, astrocytes are involved in maintaining a stable extracellular milieu for proper neuronal functioning and excitability. For example, astrocytes express specific glutamate transporters to avoid excessive extracellular concentrations of glutamate that could induce excitotoxicity [58]. In this context, a function of TRH is discussed in reducing the neuronal release of glutamate [34], thereby modulating neuronal activity and presumably diminishing excitotoxicity. Astrocytes were also shown to express receptors for leptin [59,60] which is well-known to increase TRH expression [61]. For leptin, a role in AD pathology has been discussed by influencing the composition of membrane lipid rafts and thereby the activity of BACE1 [62–64].

5. Conclusions

This is the first demonstration of (i) a neuronal co-localization of QC and TRH in hippocampus, (ii) distinct differences in QC and TRH mRNA expression in brain regions with and without Aβ pathology in Tg2576 mice and (iii) astroglial co-expression of QC and TRH in the vicinity of Aβ plaques in the hippocampus. Functionally, the expression of QC in astrocytes could play a role in neuroprotection by the activation and release of TRH, thereby reducing excitotoxicity but also in neurodegeneration due to the formation of pGlu-Aβ (Fig. 6). With regard to QC as a therapeutical target for pharmacological inhibition in AD, our results suggest that a potential decrease of a neuroprotective TRH effects would be compensated by the benefits of a reduction in Aβ pathology.

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References

[1] D.J. Selkoe, D. Schenk, Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 545–584, http://dx.doi.org/10.1146/annurev.pharmtox.43.100901.140248.
[2] H.W. Querfurth, F.M. LaFerla, Alzheimer's disease, N. Engl. J. Med. 362 (2010) 329–344, http://dx.doi.org/10.1056/NEJMra0909142.
[3] E.R. Kandel, The molecular biology of memory storage: a dialogue between genes and synapses, Science 294 (2001) 1030–1038, http://dx.doi.org/10.1126/science.1067020.
[4] K. Blennow, M.J. de Leon, H. Zetterberg, Alzheimer's disease, Lancet 368 (2006) 387–403, http://dx.doi.org/10.1016/S0140-6736(06)69113-7.
[5] T.C. Saido, T. Iwatsubo, D.M. Mann, H. Shimada, Y. Ihara, S. Kawashima, Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques, Neuron 14 (1995) 457–466, http://dx.doi.org/10.1016/0896-6273(95)90301-1.
[6] T.C. Saido, W. Yamao-Harigaya, T. Iwatsubo, S. Kawashima, Amino- and carboxyl-terminal heterogeneity of beta-amyloid peptides deposited in human brain, Neurosci. Lett. 215 (1996) 173–176.
[7] W. He, C.J. Barrow, The A beta 3-pyroglutamyl and 11-pyroglutamyl peptides found in senile plaque have greater beta-sheet forming and aggregation propensities *in vitro* than full-length A beta, Biochemistry 38 (1999) 10871–10877, http://dx.doi.org/10.1021/bi990563r.
[8] C. Russo, E. Violani, S. Salis, V. Venezia, V. Dolcini, G. Damonte, U. Benatti, C. D'Arrigo, E. Patrone, P. Carlo, G. Schettini, Pyroglutamate-modified amyloid beta-peptides-AbetaN3(pE)-strongly affect cultured neuron and astrocyte survival, J. Neurochem. 82 (2002) 1480–1489, http://dx.doi.org/10.1046/j.1471-4159.2002.01107.x.
[9] S. Schilling, T. Lauber, M. Schaupp, S. Manhart, E. Scheel, G. Böhm, H.U. Demuth, On the seeding and oligomerization of pGlu-amyloid peptides (*in vitro*), Biochemistry 45 (2006) 12393–12399, http://dx.doi.org/10.1021/bi0612667.
[10] D. Schlenzig, S. Manhart, Y. Cinar, M. Kleinschmidt, G. Hause, D. Willbold, S.A. Funke, S. Schilling, H.U. Demuth, Pyroglutamate formation influences solubility and amyloidogenicity of amyloid peptides, Biochemistry 48 (2009) 7072–7078, http://dx.doi.org/10.1021/bi900818a.
[11] S. Schilling, T. Hoffmann, S. Manhart, M. Hoffmann, H.U. Demuth, Glutaminyl cyclases unfold glutamyl cyclase activity under mild acid conditions, FEBS Lett. 563 (2004) 191–196, http://dx.doi.org/10.1016/S0014-5793(04)00300-X.
[12] S. Schilling, U. Zeitschel, T. Hoffmann, U. Heiser, M. Francke, A. Kehlen, M. Holzer, B. Hutter-Paier, M. Prokesch, M. Windisch, W. Jagla, D. Schlenzig, C. Lindner, T.

Rudolph, G. Reuter, H. Cynis, D. Montag, H.U. Demuth, S. Roßner, Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology, Nature Med 14 (2008) 1106–1111.
[13] M. Hartlage-Rübsamen, K. Staffa, A. Waniek, M. Wermann, T. Hoffmann, H. Cynis, S. Schilling, H.U. Demuth, S. Roßner, Developmental expression and subcellular localization of glutaminyl cyclase in mouse brain, Int. J. Dev. Neurosci. 27 (2009) 825–835, http://dx.doi.org/10.1016/j.ijdevneu.2009.08.007.
[14] M. Hartlage-Rübsamen, M. Morawski, A. Waniek, C. Jäger, U. Zeitschel, B. Koch, H. Cynis, S. Schilling, R. Schliebs, H.U. Demuth, S. Roßner, Glutaminyl cyclase contributes to the formation of focal and diffuse pyroglutamate (pGlu)-Aβ deposits in hippocampus *via* distinct cellular mechanisms, Acta Neuropathol. 121 (2011) 705–719, http://dx.doi.org/10.1007/s00401-011-0806-2.
[15] W.H. Busby, G.E. Quackenbush, J. Humm, W.W. Youngblood, J.S. Kizer, An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl-peptides. Presence in pituitary, brain, adrenal medulla, and lymphocytes, J. Biol. Chem. 262 (1987) 8532–8536.
[16] W.H. Fischer, J. Spiess, Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutamyl peptides, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 3628–3632.
[17] A.C. Awadé, P. Cleuziat, T. Gonzalès, J. Robert-Baudouy, Pyrrolidone carboxyl peptidase (Pcp): an enzyme that removes pyroglutamic acid (pGlu) from pGlu-peptides and pGlu-proteins, Proteins 20 (1994) 34–51, http://dx.doi.org/10.1002/prot.340200106.
[18] K. Folkers, J.K. Chang, B.L. Currie, C.Y. Bowers, A. Weil, A.V. Schally, Synthesis and relationship of L-glutaminyl-L-histidyl-L-prolinamide to the thyrotropin releasing hormone, Biochem. Biophys. Res. Commun. 39 (1970) 110–113, http://dx.doi.org/10.1016/0006-291X(70)90764-3.
[19] G.N. Abraham, D.N. Podell, Pyroglutamic acid. Non-metabolic formation, function in proteins and peptides, and characteristics of the enzymes effecting its removal, Mol. Cell. Biochem. 38 (1981) 181–190, http://dx.doi.org/10.1007/978-94-009-8027-3_11.
[20] E.A. Nillni, Neuroregulation of ProTRH biosynthesis and processing, Endocrine 10 (1999) 185–199, http://dx.doi.org/10.1007/BF02738618.
[21] V. Monga, C.L. Meena, N. Kaur, R. Jain, Chemistry and biology of thyrotropin-releasing hormone (TRH) and its analogs, Curr. Med. Chem. 15 (2008) 2718–2733, http://dx.doi.org/10.2174/092986708786242912.
[22] E.A. Nillni, Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by neuronal and peripheral inputs, Front. Neuroendocrinol. 31 (2010) 134–156, http://dx.doi.org/10.1016/j.yfrne.2010.01.001.
[23] S. Schilling, S. Kohlmann, C. Bäuscher, R. Sedlmeier, B. Koch, R. Eichentopf, A. Becker, H. Cynis, T. Hoffmann, S. Berg, E.J. Freyse, S. von Hörsten, S. Roßner, S. Graubner, H.U. Demuth, Glutaminyl cyclase knock-out mice exhibit slight hypothyroidism but no hypogonadism: implications for enzyme function and drug development, J. Biol. Chem. 286 (2011) 14199–14208, http://dx.doi.org/10.1074/jbc.M111.229385.
[24] I. Merchenthaler, V. Csernus, C. Csontos, P. Petrusz, B. Mess, New data on the immunocytochemical localization of thyrotropin releasing hormone in the rat central nervous system, Am. J. Anat. 181 (1988) 359–376, http://dx.doi.org/10.1002/aja.1001810404.
[25] T. Hökfelt, Y. Tsuruo, B. Ulfhake, S. Cullheim, U. Arvidsson, G.A. Foster, M. Schultzberg, M. Schalling, L. Arborelius, J. Freedman, Distribution of TRH-like immunoreactivity with special reference to coexistence with other neuroactive compounds, Ann. N. Y. Acad. Sci. 553 (1989) 76–105, http://dx.doi.org/10.1111/j.1749-6632.1989.tb54479.x.
[26] W.C. Low, S.D. Farber, T.G. Hill, A. Sattin, M.J. Kubek, Evidence for extrinsic and intrinsic sources of thyrotropin-releasing hormone (TRH) in the hippocampal formation as determined by radioimmunoassay and immunocytochemistry, Ann. N. Y. Acad. Sci. 553 (1989) 574–578.
[27] W.C. Low, J. Roepke, S.D. Farber, T.G. Hill, A. Sattin, M.J. Kubek, Distribution of thyrotropin-releasing hormone (TRH) in the hippocampal formation as determined by radioimmunoassay, Neurosci. Lett. 103 (1989) 314–319, http://dx.doi.org/10.1016/0304-3940(89)90119-5.
[28] J.E. Morley, Extrahypothalamic thyrotropin releasing hormone (TRH) — its distribution and its functions, Life Sci. 25 (1979) 1539–1550.
[29] Y. Tsuruo, S. Ceccatelli, M.J. Villar, T. Hökfelt, T.J. Visser, L. Terenius, M. Goldstein, J.C. Brown, A. Buchan, J. Walsh, Coexistence of TRH with other neuroactive substances in the rat central nervous system, J. Chem. Neuroanat. 1 (1988) 235–253.
[30] G. Wittmann, T. Füzesi, Z. Liposits, R.M. Lechan, C. Fekete, Distribution and axonal projections of neurons coexpressing thyrotropin-releasing hormone and urocortin 3 in the rat brain, J. Comp. Neurol. 517 (2009) 825–840, http://dx.doi.org/10.1002/cne.22180.
[31] M. Pizzi, F. Boroni, M. Benarese, C. Moraitis, M. Memo, P. Spano, Neuroprotective effect of thyrotropin-releasing hormone against excitatory amino acid-induced cell death in hippocampal slices, Eur. J. Pharmacol. 370 (1999) 133–137, http://dx.doi.org/10.1016/S0014-2999(99)00139-9.
[32] L. Jaworska-Feil, M. Kajta, B. Budziszewska, M. Leśkiewicz, W. Lasoń, Protective effects of TRH and its stable analogue, RGH-2202, on kainate-induced seizures and neurotoxicity in rodents, Epilepsy Res. 43 (2001) 67–73, http://dx.doi.org/10.1016/S0920-1211(00)00178-9.
[33] M.C. Veronesi, M. Yard, J. Jackson, D.K. Lahiri, M.J. Kubek, An analog of thyrotropin-releasing hormone (TRH) is neuroprotective against glutamate-induced toxicity in fetal rat hippocampal neurons *in vitro*, Brain Res. 1128 (2007) 79–85, http://dx.doi.org/10.1016/j.brainres.2006.10.047.
[34] Y. Nie, D.D. Schoepp, J.E. Klaunig, M. Yard, D.K. Lahiri, M.J. Kubek, Thyrotropin-releasing hormone (protirelin) inhibits potassium-stimulated glutamate and aspartate release from hippocampal slices *in vitro*, Brain Res. 1054 (2005) 45–54, http://dx.doi.org/10.1016/j.brainres.2005.06.077.
[35] P.Y. Deng, J.E. Porter, H.S. Shin, S. Lei, Thyrotropin-releasing hormone increases GABA release in rat hippocampus, J. Physiol. 577 (2006) 497–511, http://dx.doi.org/10.1113/jphysiol.2006.118141.
[36] F. Crespi, P.E. Keane, M. Morre, *In vivo* evaluation by differential pulse voltammetry of the effect of thyrotropin-releasing hormone (TRH) on dopaminergic and serotoninergic synaptic activity in the striatum and nucleus accumbens of the rat, Exp. Brain Res. 62 (1986) 329–334.
[37] A. Ogata, K. Nagashima, K. Yasui, T. Matsuura, K. Tashiro, Sustained release dosage of thyrotropin-releasing hormone improves experimental Japanese encephalitis virus-induced parkinsonism in rats, J. Neurol. Sci. 159 (1998) 135–139, http://dx.doi.org/10.1016/S0022-510X(98)00150-6.
[38] A.I. Faden, S. Salzman, Pharmacological strategies in CNS trauma, Trends Pharmacol. Sci. 13 (1992) 29–35.
[39] A.I. Faden, V.A. Movsesyan, S.M. Knoblach, F. Ahmed, I. Cernak, Neuroprotective effects of novel small peptides *in vitro* and after brain injury, Neuropharmacology 49 (2005) 410–424, http://dx.doi.org/10.1016/j.neuropharm.2005.04.001.
[40] L. Jaworska-Feil, D. Jantas, M. Leskiewicz, B. Budziszewska, M. Kubera, A. Basta-Kaim, A.W. Lipkowski, W. Lason, Protective effects of TRH and its analogues against various cytotoxic agents in retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells, Neuropeptides 44 (2010) 495–508, http://dx.doi.org/10.1016/j.npep.2010.08.004.
[41] L. Luo, N. Yano, Q. Mao, I.M.D. Jackson, E.G. Stopa, Thyrotropin releasing hormone (TRH) in the hippocampus of Alzheimer patients, J. Alzheimers Dis. 4 (2002) 97–103.
[42] O. Wirths, T. Bethge, A. Marcello, A. Harmeier, S. Jawhar, P.J. Lucassen, G. Multhaup, D.L. Brody, T. Esparza, M. Ingelsson, H. Kalimo, L. Lannfelt, T.A. Bayer, Pyroglutamate Abeta pathology in APP/PS1KI mice, sporadic and familial Alzheimer's disease cases, J. Neural Transm. 117 (2010) 85–96, http://dx.doi.org/10.1007/s00702-009-0314-x.
[43] F. Löffner, S.M. Lohmann, B. Walckhoff, U. Walter, B. Hamprecht, Immunocytochemical characterization of neuron-rich primary cultures of embryonic rat brain cells by established neuronal and glial markers and by monospecific antisera against cyclic nucleotide-dependent protein kinases and the synaptic vesicle protein synapsin I, Brain Res. 363 (1986) 205–221.
[44] Z. Cui, C.R. Gerfen, W.S. Young, Hypothalamic and other connections with dorsal CA2 area of the mouse hippocampus, J. Comp. Neurol. 521 (2013) 1844–1866, http://dx.doi.org/10.1002/cne.23263.
[45] J.C. Geerling, J.-W. Shin, P.C. Chimenti, A.D. Loewy, Paraventricular hypothalamic nucleus: axonal projections to the brainstem, J. Comp. Neurol. 518 (2010) 1460–1499, http://dx.doi.org/10.1002/cne.22283.
[46] L.W. Swanson, H.G. Kuypers, The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods, J. Comp. Neurol. 194 (1980) 555–570.
[47] L. Yong-Hong, P. Xiao-Dong, H. Chang-Quan, Y. Bo, L. Qing-Xiu, Hypothalamic–pituitary–thyroid axis in patients with Alzheimer disease (AD), J. Investig. Med. 61 (2013) 578–581, http://dx.doi.org/10.231/JIM.0b013e318280aafb.
[48] H. Dong, C.M. Yuede, H.S. Yoo, M.V. Martin, C. Deal, A.G. Mace, J.G. Csernansky, Corticosterone and related receptor expression are associated with increased beta-amyloid plaques in isolated Tg2576 mice, Neuroscience 155 (2008) 154–163, http://dx.doi.org/10.1016/j.neuroscience.2008.05.017.
[49] M. Ishii, G. Wang, G. Racchumi, J.P. Dyke, C. Iadecola, Transgenic mice overexpressing amyloid precursor protein exhibit early metabolic deficits and a pathologically low leptin state associated with hypothalamic dysfunction in arcuate neuropeptide Y neurons, J. Neurosci. 34 (2014) 9096–9106, http://dx.doi.org/10.1523/JNEUROSCI.0872-14.2014.
[50] R.S. Klein, B. Das, L.D. Fricker, Secretion of carboxypeptidase E from cultured astrocytes and from AtT-20 cells, a neuroendocrine cell line: implications for neuropeptide biosynthesis, J. Neurochem. 58 (1992) 2011–2018.
[51] M. Eddleston, L. Mucke, Molecular profile of reactive astrocytes—implications for their role in neurologic disease, Neuroscience 54 (1993) 15–36, http://dx.doi.org/10.1016/0306-4522(93)90380-X.
[52] R.S. Klein, L.D. Fricker, Cultured astrocytes express mRNA for peptidylglycine-alpha-amidating monooxygenase, a neuropeptide processing enzyme, Brain Res. 596 (1992) 202–208.
[53] A. Wahlert, L. Funkelstein, B. Fitzsimmons, T. Yaksh, V. Hook, Spinal astrocytes produce and secrete dynorphin neuropeptides, Neuropeptides 47 (2013) 109–115, http://dx.doi.org/10.1016/j.npep.2012.10.006.
[54] S.I. García, P.I. Porto, V.N. Martinez, A.L. Alvarez, S. Finkielman, C.J. Pirola, Expression of TRH and TRH-like peptides in a human glioblastoma–astrocytoma cell line (U-373-MG), J. Endocrinol. 166 (2000) 697–703.
[55] A.M. McDermott, S.L. Dickinson, G.P. Wilkin, Thyrotropin releasing hormone (TRH) and a degradation stabilized analogue (RX77368) stimulate phosphoinositide turnover in cultured astrocytes in a regionally specific manner, Neurochem. Int. 20 (1992) 307–313, http://dx.doi.org/10.1016/0197-0186(92)90045-S.
[56] M. Hartlage-Rübsamen, U. Zeitschel, J. Apelt, U. Gärtner, H. Franke, T. Stahl, A. Günther, R. Schliebs, M. Penkowa, V. Bigl, S. Roßner, Astrocytic expression of the Alzheimer's disease beta-secretase (BACE1) is stimulus-dependent, Glia 41 (2003) 169–179, http://dx.doi.org/10.1002/glia.10178.
[57] S. Roßner, C. Lange-Dohna, U. Zeitschel, J.R. Perez-Polo, Alzheimer's disease beta-secretase BACE1 is not a neuron-specific enzyme, J. Neurochem. 92 (2005) 226–234, http://dx.doi.org/10.1111/j.1471-4159.2004.02857.x.
[58] A. Verkhratsky, M. Olabarria, H.N. Noristani, C.-Y. Yeh, J.J. Rodriguez, Astrocytes in Alzheimer's disease, Neurotherapeutics 7 (2010) 399–412, http://dx.doi.org/10.1016/j.nurt.2010.05.017.
[59] H. Hsuchou, W. Pan, M.J. Barnes, A.J. Kastin, Leptin receptor mRNA in rat brain astrocytes, Peptides 30 (2009) 2275–2280, http://dx.doi.org/10.1016/j.peptides.2009.08.023.

[60] B. Jayaram, R.S. Khan, A.J. Kastin, H. Hsuchou, X. Wu, W. Pan, Protective role of astrocytic leptin signaling against excitotoxicity, J. Mol. Neurosci. 49 (2013) 523–530, http://dx.doi.org/10.1007/s12031-012-9924-0.
[61] D.L. Carbone, D.G. Zuloaga, A.F. Lacagnina, R.J. Handa, Prepro-thyrotropin releasing hormone expressing neurons in the juxtaparaventricular region of the lateral hypothalamus are activated by leptin and altered by prenatal glucocorticoid exposure, Brain Res. 1477 (2012) 19–26, http://dx.doi.org/10.1016/j.brainres.2012.08.020.
[62] N. Tezapsidis, J.M. Johnston, M.A. Smith, J.W. Ashford, G. Casadesus, N.K. Robakis, B. Wolozin, G. Perry, X. Zhu, S.J. Greco, S. Sarkar, Leptin: a novel therapeutic strategy for Alzheimer's disease, J. Alzheimers Dis. 16 (2009) 731–740, http://dx.doi.org/10.3233/JAD-2009-1021.
[63] S.J. Greco, K.J. Bryan, S. Sarkar, X. Zhu, M.A. Smith, J.W. Ashford, J.M. Johnston, N. Tezapsidis, G. Casadesus, Leptin reduces pathology and improves memory in a transgenic mouse model of Alzheimer's disease, J. Alzheimers Dis. 19 (2010) 1155–1167, http://dx.doi.org/10.3233/JAD-2010-1308.
[64] G. Marwarha, B. Dasari, J.R.P. Prasanthi, J. Schommer, O. Ghribi, Leptin reduces the accumulation of Abeta and phosphorylated tau induced by 27-hydroxycholesterol in rabbit organotypic slices, J. Alzheimers Dis. 19 (2010) 1007–1019, http://dx.doi.org/10.3233/JAD-2010-1298.