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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