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Developmental expression and subcellular localization of glutaminyl cyclase in mouse brain

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ABSTRACT

Glutaminyl cyclase (QC) converts N-terminal glutaminyl residues into pyroglutamate (pE), thereby stabilizing these peptides/proteins. Recently, we demonstrated that QC also plays a pathogenic role in Alzheimer's disease by generating the disease-associated pE-Abeta from N-terminally truncated Abeta peptides *in vivo*. This newly identified function makes QC an interesting pharmacological target for Alzheimer's disease therapy. However, the expression of QC in brain and peripheral organs, its cell type-specific and subcellular localization as well as developmental profiles in brain are not known. The present study was performed to address these issues in mice. In brain, QC mRNA expression was highest in hypothalamus, followed by hippocampus and cortex. In liver, QC mRNA concentration was almost as high as in brain while lower QC mRNA levels were detected in lung and heart and very low expression levels were found in kidney and spleen. In the developmental course, stable QC mRNA levels were detected in hypothalamus from postnatal day 5 to 370. On the contrary, in cortex and hippocampus QC mRNA levels were highest after birth and declined during ontogenesis by 20–25%. These results were corroborated by immunocytochemical analysis in mouse brain demonstrating a robust QC expression in a subpopulation of lateral and paraventricular hypothalamic neurons and the labeling of a significant number of small neurons in the hippocampal molecular layer, in the hilus of the dentate gyrus and in all layers of the neocortex. Hippocampal QC-immunoreactive neurons include subsets of parvalbumin-, calbindin-, calretinin-, cholecystokinin- and somatostatin-positive GABAergic interneurons. The density of QC labeled hippocampal neurons declined during postnatal development matching the decrease in QC mRNA expression levels. Subcellular double immunofluorescent analysis localized QC within the endoplasmatic reticulum, Golgi apparatus and secretory granules, consistent with a function of QC in protein maturation and/or modification. Our results are in compliance with a role of QC in hypothalamic hormone maturation and suggest additional, yet unidentified QC functions in brain regions relevant for learning and memory which are affected in Alzheimer's disease.

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1. Introduction

Glutaminyl cyclase (QC; EC 2.3.2.5) is a Zn⁺⁺-dependent acyl transferase with a mixed α -helix/ β -sheet structure catalyzing the intramolecular N-terminal cyclization of glutaminyl residues into pyroglutamate (pE; Schilling et al., 2003a, 2005; Booth et al., 2004; Huang et al., 2005). QC has initially been described as a component of

papaya latex (Messer and Ottesen, 1964) and was found subsequently in different mammalian tissues with highest QC mRNA and protein levels being present in brain (Pohl et al., 1991; Böckers et al., 1995; Sykes et al., 1999). Although mammalian QCs share a high degree of homology, there is no protein sequence homology between mammalian and plant QCs suggesting different evolutionary origins of these QC families (Song et al., 1994; Dahl et al., 2000; Schilling et al., 2003b). The pE modification of N-terminal glutamine occurs preferentially at a mildly basic pH (Schilling et al., 2004) and affects a number of neuropeptides and peptide hormones such as orexin A, gastrin, gonadotropin-, thyrotropin- and corticotropin-releasing hormones, and neurotensin (Fischer and Spiess, 1987; Busby et al., 1987). This modification results in the stabilization and reduced proteolysis of these hormones, thus extending their biological half-life and period of action. In the case of

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BSA, bovine serum albumin; DAB, 3,3'-diaminobenzidine; GFAP, glial fibrillary acidic protein; PB, phosphate buffer; PBS, phosphate-buffered saline; QC, glutaminyl cyclase.

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gonadotropin- and thyrotropin-releasing hormones, the pE modification is even essential for the biological activity of these peptides *per se* (Folkers et al., 1970; Seprodi et al., 1978). Based on the presence of QC substrates in pituitary and hypothalamic nuclei, previous investigations on the expression of QC in brain focussed on these brain regions (Pohl et al., 1991; Böckers et al., 1995). In pituitary of bovine brain Böckers et al. (1995) demonstrated neuronal QC expression within hypothalamic nuclei and colocalization with somatotropins, but not with corticotropins.

We have recently demonstrated that QC is also involved in pathogenic cascades resulting in the development of Alzheimer's disease (AD; Schilling et al., 2008a,b). In particular, it has been shown that QC also accepts N-terminal glutamate residues as substrates in a mildly acidic milieu thus catalyzing – *inter alia* – the pE modification of Abeta peptides (Schilling et al., 2004), the principle constituents of AD amyloid plaques. This particular pE-Abeta peptide species displays very fast aggregation and fibril formation kinetics and longer biological half-life as well as higher neurotoxic characteristics compared to unmodified Abeta peptides (He and Barrow, 1999; Russo et al., 2002). Additionally, pE-Abeta was shown to act as a seed for aggregation and co-aggregation of non-modified Abeta peptides (Schilling et al., 2006; Schlenzig et al., 2009; D'Arrigo et al., 2009). The pE-Abeta modification can be prevented by QC inhibitors in cell cultures, resulting in faster Abeta clearance and reduced neurotoxicity (Cynis et al., 2006). Consistently, long-term pharmacological QC inhibition in different transgenic mouse models and in a *Drosophila* model of AD reduced pE-Abeta formation and also the total Abeta accumulation (Schilling et al., 2008b). Moreover, QC inhibition reduced gliosis and improved the performance of mice in contextual fear and Morris water maze paradigms (Schilling et al., 2008b). These observations and the demonstration of QC overexpression in brains of AD patients identify QC as a novel, rational target for causative AD therapy.

In this perspective, it is crucial to reveal the expression of QC in brain regions important for learning and memory which are affected in AD. These structures include neocortex and hippocampus as well as hypothalamic nuclei which have a modulatory role in cognition (Atwood et al., 2005; Magri et al., 2006). The identification of the cell type-specificity, subcellular localization and developmental profile of QC expression may, therefore, explain some AD-underlying mechanisms, help to identify novel QC substrates *in vivo* and to estimate possible side-effects of QC inhibition in AD patients. In order to specifically demonstrate QC immunoreactivity in different brain regions and to reveal intracellular QC localization we used brains from wild type and from QC knock-out mice as controls, respectively. Moreover, the ontogenetic profile of QC expression was studied in neocortex, hippocampus and hypothalamus by quantitative RT-PCR.

2. Experimental procedures

2.1. Experimental animals

Wild type C57Bl6 mice and QC knock-out mice were used for this study. For the quantification of QC mRNA in different organs and in defined brain regions during ontogenesis, wild type mice at the postnatal age of 5, 20, 42 and 370 days were used ($N = 7\text{--}8$ each). For the immunohistochemical analysis of QC expression wild type mice (aged 5, 20, 42 and 370 days; $N = 4$ each) and QC knock-out mice as controls were used. QC knock-out mice were generated on the basis of classical homologous recombination approach. Cre-mediated excision of exons 5 and 6 was confirmed by PCR and RT-PCR. The constitutive deletion of these exons resulted in an additional frame shift and thus in a complete loss of the C-terminal part of the protein (not shown).

2.2. Preparation of tissue for qRT-PCR

Mice were sacrificed by decapitation at the postnatal age indicated, the brains were removed from the skull and hippocampus, neocortex and hypothalamus were prepared and snap-frozen in liquid nitrogen. Additionally, approximately 100 mg

tissue of liver, lung, heart, kidney and spleen were collected, snap-frozen in liquid nitrogen and stored at -80°C pending RNA isolation.

2.3. RNA isolation and reverse transcription

One hundred milligram of frozen tissue was homogenized mechanically with 1 ml Trizol reagent (Invitrogen, Karlsruhe, Germany), incubated for 5 min at room temperature (RT) and then mixed thoroughly with 0.2 ml CHCl₃. After further incubation for 2–3 min at RT, samples were centrifuged and the aqueous phase containing RNA was transferred into a fresh tube and mixed with 0.5 ml isopropanol to pellet RNA by centrifugation at 12,000 $\times g$ (10 min, 4 °C). The pellet was washed twice with 1 ml ethanol (75%, v/v), the supernatant was removed and the air-dried pellet was re-solubilized with 30 μl sterile water. The RNA concentration was determined photometrically and total RNA (2 μg), N6-random hexamer primers (2 μM) and superscript II reverse transcriptase (1 μl ; Invitrogen) were used for reverse transcription. The reaction was performed for 60 min at 37 °C and terminated by heating to 90 °C for 3 min.

2.4. Quantitative RT-PCR

One microlitre of the obtained cDNA was amplified by real-time PCR using *Taq* PCR Core Kit (Qiagen, Hilden, Germany) and 5 pmol each of the specific forward and reverse primers. QC-specific primers were designed using the pearl primer program and chosen on the basis of selectivity, exon-exon overlap, and resulting product length. Primers of the following sequences were synthesized by BioTez (Berlin, Germany): QC forward 5'-GCCACGGATTCAGCTGTGC-3', QC reverse 5'-GAATGTTGGATTGCTGCTC-3', yielding a PCR product of 303 bp. SYBR-Green I dye (Invitrogen, Karlsruhe, Germany) was added to the reaction to detect and quantify the formation of PCR products.

In order to generate a standard curve either a pcDNA3.1 plasmid containing the mouse QC cDNA sequence or a given QC cDNA in different dilutions were co-amplified. The relative concentration of QC mRNA in the tissue samples was calculated from the amplification of these standards. Quantitative RT-PCR (qRT-PCR) was performed using the following amplification protocol: hot start at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, elongation at 72 °C for 45 s; and final elongation at 72 °C for 7 min. The fluorescence signals were recorded during the elongation periods and the relative concentration of cDNA in tissue samples was calculated from CT values and initial amount of template.

The identity of the specific PCR product was revealed by (i) a melting curve of the PCR product and (ii) digestion of the PCR fragment with 0.5 μl BgII at 37 °C for 45 min, resulting in the generation of 204 and 99 bp fragments (not shown). As negative control, PCR was performed without template.

2.5. Preparation of brain samples for immunohistochemistry

C57Bl6 wild type mice and QC knock-out mice were anaesthetized with pentobarbital and perfused transcardially with 25 ml phosphate-buffered saline (PBS, 0.01 M; pH 7.4) followed by perfusion with 25 ml 4% paraformaldehyde in phosphate buffer (PB, 0.1 M; pH 7.4). The brains were removed from the skull and postfixed by immersion in the same fixative overnight at 4 °C. After cryoprotection in 30% sucrose in 0.1 M PB 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 PB.

2.6. QC immunohistochemistry

Immunohistochemistry to detect QC was performed using the rabbit antiserum Ab1301 at a dilution of 1:500. After inactivation of endogenous peroxidase with 0.6% H₂O₂ in 0.1 M TBS for 15 min and blocking of unspecific binding sites with 5% of the appropriate normal serum in TBS containing 0.3% Triton-X 100, brain sections were incubated in the same solution with the primary antibody at 4 °C overnight in a humid chamber on a shaker. The following day they were incubated with secondary biotinylated goat anti-rabbit antibodies (Dianova; 1:400) in TBS-2% BSA for 60 min at room temperature followed by the ABC method which comprised incubation with complexed streptavidin-biotinylated horseradish peroxidase (Invitrogen). Incubations were separated by washing steps (3 times 5 min in TBS). QC-immunoreaction was visualized by incubation with 4 mg 3,3'-diaminobenzidine (DAB) and 2.5 μl H₂O₂ per 5 ml Tris buffer (0.05 M, pH 7.6) for 1–2 min.

2.7. Double immunofluorescent labeling procedures

In order to reveal the identity of hippocampal QC-immunoreactive neurons and the intracellular localization of QC, the rabbit anti-QC antiserum 1301 was used in combination with monoclonal antibodies from mice or a polyclonal antiserum, from goat or chicken, respectively, directed against neuron-, astrocyte- and compartment-specific marker proteins (see Table 1). Brain sections were incubated with cocktails of primary antibodies overnight at 4 °C. On the next day, sections were washed 3 times with TBS and were then incubated with biotinylated goat anti-rabbit, 1:400 and Cy3-conjugated goat anti-mouse, 1:200, followed by Cy2-conjugated streptavidin 1:100 (Dianova) for 60 min at room temperature. In the case of labeling with rabbit anti-QC antibody combined with goat anti-chromograninA antibody as well as with chicken

Table 1

Cell type- and compartment-specific antibodies used for double immunofluorescent localization of QC.

	Antibody to	Host	Source	Dilution
Neurons	NeuN	Mouse	Chemicon	1:1000
Astrocytes	GFAP	Mouse (clone GA5)	Sigma	1:500
GABAergic interneuron marker	Parvalbumin	Mouse	Swant	1:4000
GABAergic interneuron marker	Calbindin	Mouse	Swant	1:1000
GABAergic interneuron marker	Calretinin	Goat	Swant	1:1000
GABAergic interneuron marker	Somatostatin	Goat	St. Cruz	1:200
GABAergic interneuron marker	Cholecystokinin	Goat	St. Cruz	1:100
Mitochondria	ATP synthase β chain	Mouse	Chemicon	1:100
Early endosomes	EEA-1	Mouse	Abcam	1:100
Late endosomes	Mannose-6-Ph-receptor	Mouse	Abcam	1:100
Recycl. endosomes	Rab 11	Mouse (clone 47)	BD Sciences	1:50
ER membrane	Calreticulin	Chicken	Abcam	1:200
Golgi apparatus	Syntaxin-6	Mouse (clone 3D10)	Abcam	1:100
Secretory granules	ChromograninA	Goat	St. Cruz	1:50

Abbreviations: GFAP: glial fibrillary acidic protein, EEA-1: early endosome antigen-1, ER: endoplasmatic reticulum.

anti-calreticulin antibody, secondary antibodies from donkey (Dianova) were used at the same concentrations.

2.8. Confocal laser scanning microscopy

Confocal laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) was performed to reveal co-localization of QC and intracellular compartment markers. For Cy2-labeled antigens (QC; green fluorescence), an argon laser with 488 nm excitation and 510 nm emission was used applying a low-range band pass (505–530 nm). For Cy3-labeled antigens (compartment markers; red fluorescence), a helium-neon-laser with 543 nm excitation and 570 nm emission was used applying high-range band pass (560–615 nm).

2.9. Statistical analysis

Data from quantification of QC mRNA concentrations are given as the mean \pm SEM. Statistical significance of differences between aging groups was determined by ANOVA followed by Student's *t*-test. Differences between data were considered statistically significant when $P < 0.05$.

3. Results

3.1. QC mRNA expression in different organs and brain regions

In order to reveal the expression of QC mRNA in peripheral organs and in brain regions with known expression of QC – such as hypothalamus – and with a potential pathogenic role of QC – such

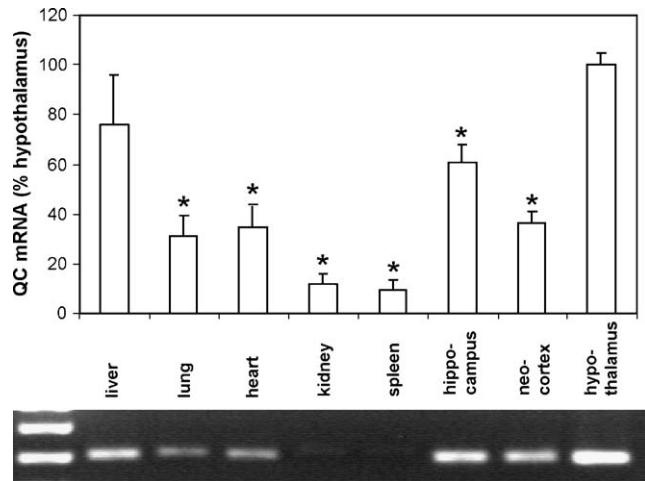


Fig. 1. Quantification of QC mRNA levels in different brain regions and peripheral organs. QC mRNA levels were quantified by real-time RT-PCR and were expressed as percentage of the QC mRNA level present in hypothalamus. Brain QC mRNA levels (hippocampus, neocortex, hypothalamus) are generally higher than those of peripheral organs, with the exception of liver. Data are mean \pm SEM ($^*P < 0.05$ versus hypothalamus, one-way ANOVA followed by Student's *t*-test). The insert at the bottom of the figure shows an example of PCR products run on an agarose gel.

as hippocampus and neocortex – qRT-PCR was performed. The highest level of QC transcripts was detected in hypothalamus (Fig. 1). Comparable amounts of QC mRNA were detected in liver (76% of hypothalamus), but significantly lower QC mRNA concentrations were observed in all other peripheral organs tested reaching 31% (lung), 34% (heart), 12% (kidney) and 9% (spleen) of

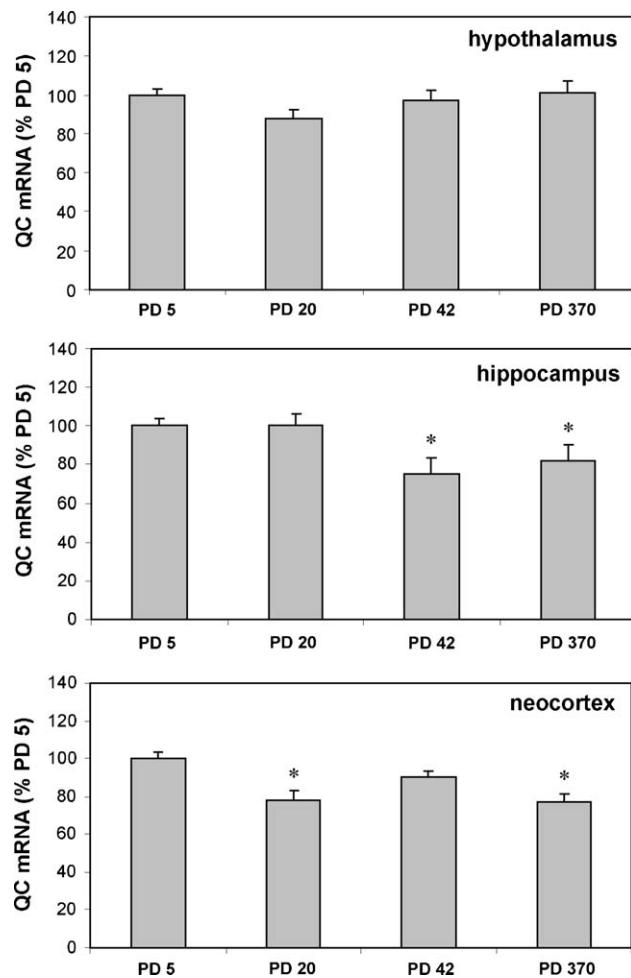


Fig. 2. Developmental profile of QC mRNA levels in different brain regions. A differential postnatal development of QC mRNA expression in different brain regions was observed. While QC mRNA levels remained at a constant level in hypothalamus at all postnatal ages investigated, there was a 20–25% decline compared to PD 5 in QC mRNA levels in neocortex and hippocampus. This decline was detectable earlier in neocortex (on PD 20) than in hippocampus (on PD 42). Data are mean \pm SEM ($^*P < 0.05$ versus PD 5, one-way ANOVA followed by Student's *t*-test).

the QC mRNA levels present in hypothalamus (Fig. 1; $P < 0.05$). QC mRNA levels in hypothalamus were also significantly higher compared to those present in hippocampus and neocortex (61% and 36% of hypothalamus, respectively; Fig. 1; $P < 0.05$).

3.2. Developmental profile of QC mRNA expression in mouse brain

In order to detect changes in QC mRNA expression in mouse brain during ontogenesis, qRT-PCR with tissue from hypothalamus, hippocampus and neocortex was performed at the age of 5, 20, 42 and 370 postnatal days (PD). In hypothalamus, stable QC mRNA concentrations were detected during the entire developmental profile investigated (Fig. 2). In contrast, hippocampal QC mRNA levels were significantly reduced on PD 42 (by 25%) and on PD 370 (by 18%) compared to PD 5 (Fig. 2; $P < 0.05$). A comparable QC mRNA expression pattern in the developmental course was

observed in neocortex. Here, compared to PD 5, QC transcript levels were already reduced on PD 20 (by 22%) and remained at this level during the entire postnatal period investigated (Fig. 2; $P < 0.05$).

3.3. Immunohistochemical QC localization in mouse brain

The expression in telencephalic and diencephalic brain regions, cellular origin and subcellular localization of the QC protein was determined by immunohistochemistry. We observed neuronal QC labeling in brain sections of wild type mice, which was absent in tissue derived from QC knock-out mice (Fig. 3). A pronounced amount of QC protein was present in a relatively low number of small-sized interneurons dispersed across all neocortical layers (Fig. 3). Similarly, in the hippocampal formation, we mainly detected small non-pyramidal QC-immunoreactive cells, which

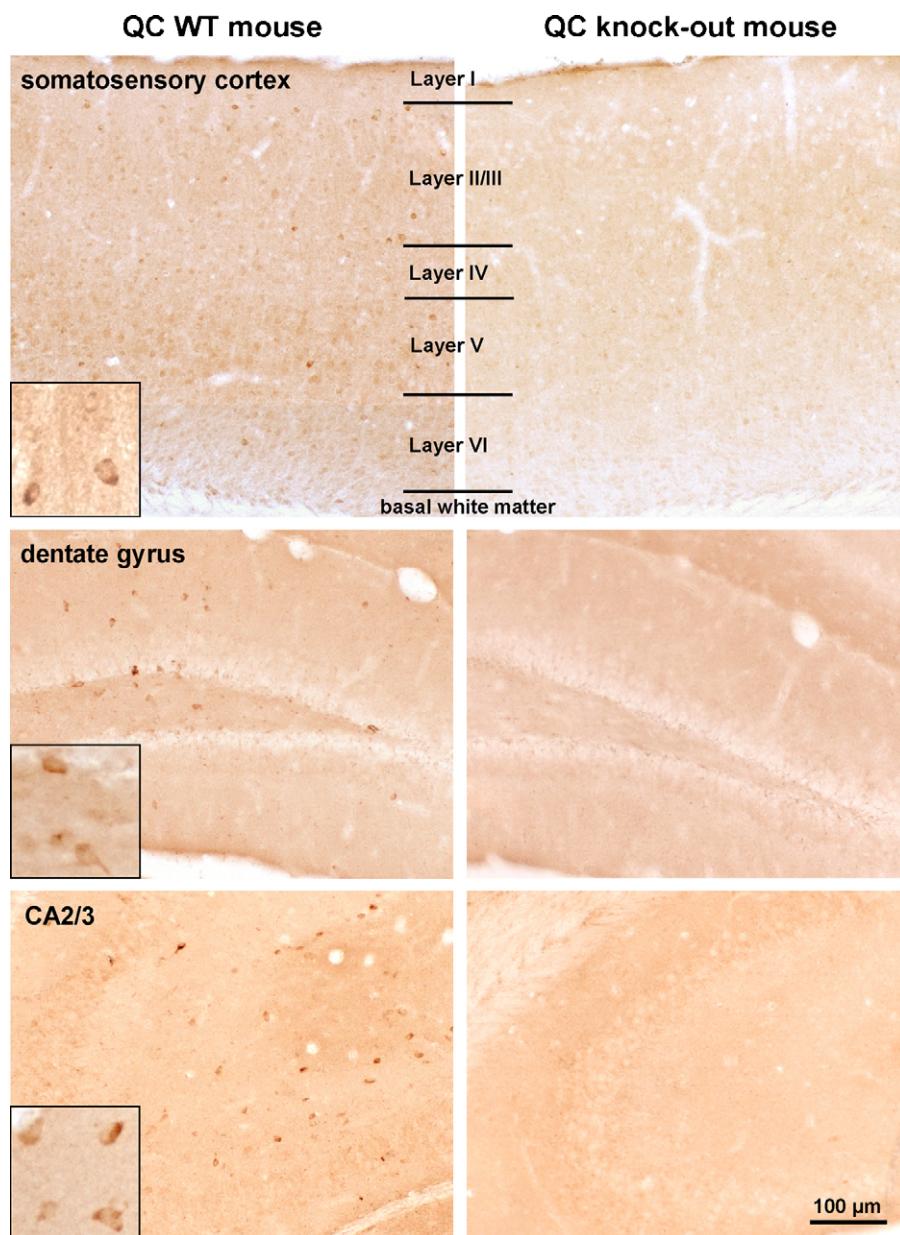


Fig. 3. Immunohistochemical detection of QC in different brain regions. The specificity of QC protein detection in brain sections of wild type mice (left) was demonstrated by comparison to brain sections from QC knock-out mice (right). Neurons with distinct QC expression can be detected in all layers of neocortex and hippocampus, as well as in lateral and paraventricular hypothalamus as indicated. Insets show examples of the cytoplasmic QC staining in all brain regions at higher magnification. Additional tel- and diencephalic brain regions with significant QC expression are indicated in Table 2.

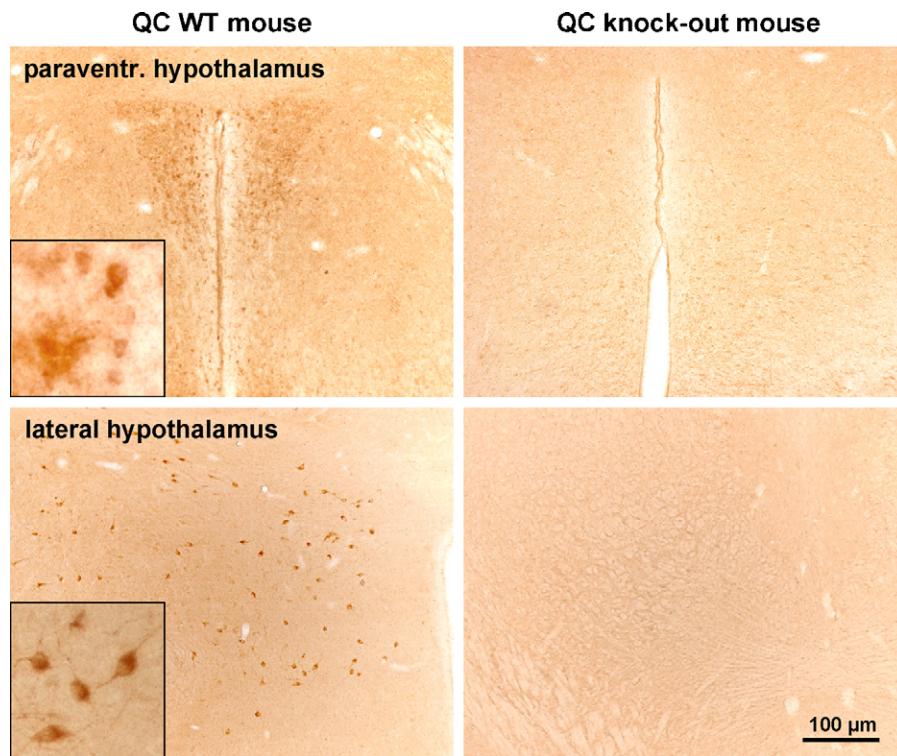


Fig. 3. (Continued).

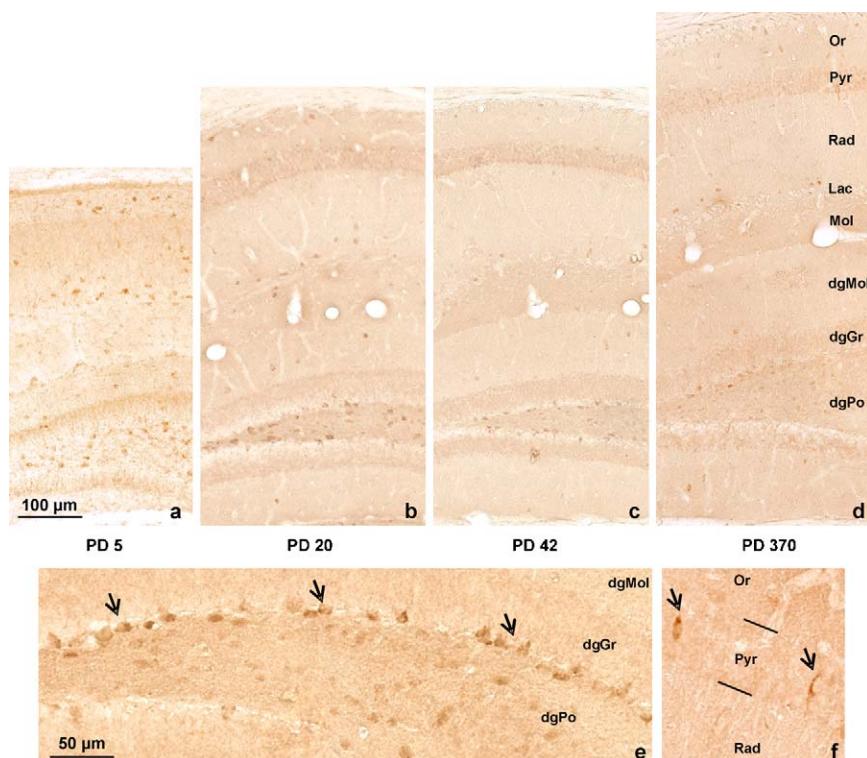


Fig. 4. QC immunoreactivity in the hippocampus during postnatal development. On postnatal day 5 (PD 5) QC-immunoreactive neurons were detected in different layers of the hippocampal formation with highest density being observed in the hilus (polymorph layer) of the dentate gyrus, and strata radiatum and oriens of cornu ammonis (a). At later postnatal ages the number of QC-positive neurons and the staining intensity decreased (see b–d for representative examples). Within the hilus of dentate gyrus, QC-positive neurons often appeared lined up along the subgranular layer whereas granule cells were mostly free of staining (e, PD 20). Likewise, hippocampal pyramidal cells (arrows in f) were only occasionally labeled. Or, Stratum oriens; Pyr, pyramidal cell layer; Rad, stratum radiatum; Lac, stratum lacunosum; Mol, stratum moleculare (all of cornu ammonis); dgMol, stratum moleculare; dgGr, granule cell layer; dgPo, polymorphic layer (all of dentate gyrus).

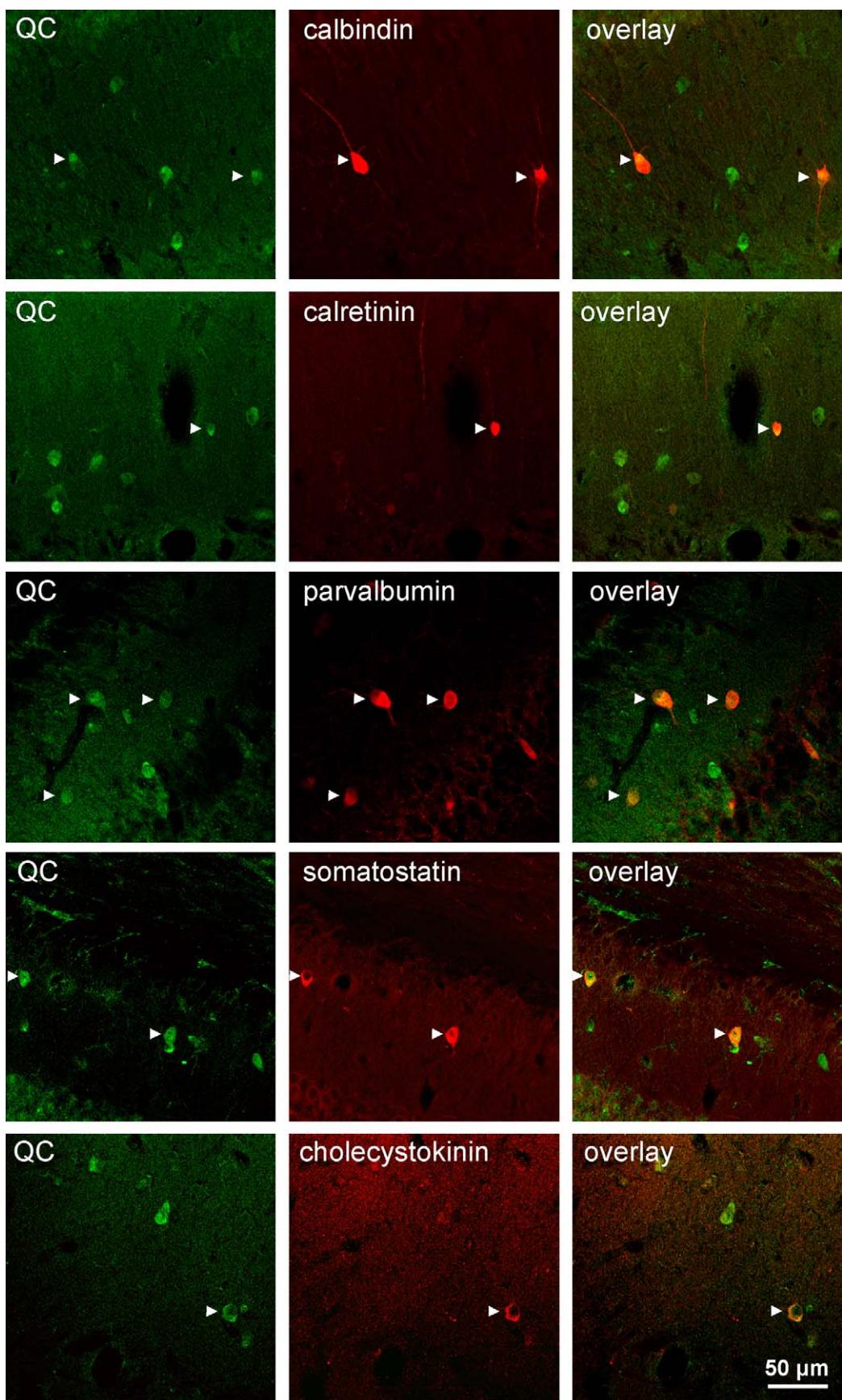


Fig. 5. QC is co-expressed by hippocampal GABAergic interneurons. QC (green immunofluorescence) is expressed in hippocampus by subsets of GABAergic interneurons which co-express the calcium-binding proteins parvalbumin, calbindin and calretinin, respectively as well as the neuropeptides somatostatin and cholecystokinin (red immunofluorescence). Arrowheads point towards neurons with double labeling.

morphologically resembled GABAergic interneurons (Figs. 3 and 4a–d). In cornu ammonis, these neurons were located within the strata radiatum and oriens with the highest density being observed in stratum oriens just below the alveus and in the inner third of stratum radiatum very close to stratum lacunosum. In dentate gyrus, QC immunolabeled neurons were detected in the molecular layer and in the hilus (polymorphic layer; Figs. 3 and 4a–d) where stained neuronal cell bodies often lay within the subgranular zone lining the inner rim of the granular cell layer (Fig. 4e). In contrast, distinct QC expression was only detected in very few neurons within the granular cell layer of the dentate gyrus as well as within the pyramidal layer of the CA1–4 subfields (Fig. 4e and f). During postnatal development, the density of QC immunolabeled neurons in the hippocampus appeared to decrease while the regional distribution remained unaltered (Fig. 4a–d).

The localization of QC-immunoreactive neurons in the hippocampus suggested QC expression by GABAergic interneurons, which are known to co-express cholecystokinin, somatostatin or calcium-binding proteins such as parvalbumin, calbindin and calretinin. In order to demonstrate QC expression by these important modulatory neurons, double immunofluorescent labeling of QC with GABAergic marker proteins was performed. QC was found to be co-expressed by subsets of parvalbumin-, calbindin-, calretinin-, somatostatin- and cholecystokinin-immunoreactive neurons (Fig. 5); without, however, being preferentially co-localized with any of these subpopulations of GABAergic interneurons.

In diencephalic brain regions significant QC expression was mainly found in subsets of neurons in the paraventricular hypothalamus, in the arcuate hypothalamic nucleus and in magnocellular neurons of the lateral hypothalamus (Fig. 3). In this brain region there was no apparent decrease in QC immunoreactivity during postnatal development (not shown). A detailed summary of cortical and hypothalamic brain structures with QC expression is given in Table 2.

3.4. Cell type-specific and subcellular localization of QC

Confocal imaging of double immunofluorescent QC staining with cell-specific markers revealed a strictly neuronal QC expression in normal mouse brain (Fig. 6) and the absence of QC expression in astrocytes (Fig. 7). In QC-immunoreactive

neurons, a dense, perinuclear staining including membranous structures as well as a more diffuse labeling throughout the cytoplasm was observed (Fig. 6).

Considering the known function of QC in protein modification, the cytoplasmatic staining pattern suggested an intracellular localization along the secretory pathway. Therefore, a series of double immunofluorescent stainings of QC with different compartment-specific markers was performed on magnocellular neurons of the lateral hypothalamus (see Table 1). QC was found to be co-localized with markers of the Golgi apparatus, ER and secretory granules (as revealed by combined stainings with antibodies against syntaxin-6, calreticulin and chromograninA, respectively; Fig. 6), but was absent from the cellular nucleus, mitochondria, as well as early and late endosomes (Figs. 6 and 7).

4. Discussion

The present study was conducted to reveal the cell type-specific and subcellular localization as well as the developmental profile of QC expression in mouse brain. Consistent with data in the literature on hypothalamic functions of QC in the pE modification of a number of hormones (Folkers et al., 1970; Seprodi et al., 1978; Fischer and Spiess, 1987; Busby et al., 1987), highest QC mRNA concentration was found in this brain region. However, another pE-modified peptide species, N3(pE)-Abeta, accumulates in neocortex and hippocampus of AD subjects and human amyloid precursor protein (APP) transgenic mice and acts as seed for aggregation and the formation of Abeta plaques (Saido et al., 1995; Miravalle et al., 2005; Piccini et al., 2005). The concentration of these pathogenic peptides in Tg2576 and TASD-41 transgenic mice can be reduced by application of a QC inhibitor *in vivo* (Schilling et al., 2008b), indicating QC expression in these brain regions. Indeed, in the present report we show that QC mRNA levels in parietal cortex and hippocampus reach 40% and 60% compared to the expression level in hypothalamus. This finding is supported by immunocytochemical staining, demonstrating the presence of a number of strongly QC-immunoreactive neurons in hypothalamic regions of the diencephalon, in distinct regions of the hippocampal formation and in the neocortex. In particular, a significant number of strongly QC expressing neurons in the lateral hypothalamic area formed a distinct, albeit dispersed, subpopulation within the magnocellular neuronal population, while the extent

Table 2
QC expression in telencephalic and diencephalic brain regions.

Brain region	Structure	Neuronal population	Signal strength	Estimated proportion of QC-ir neurons
Brain regions with strong QC immunoreactivity				
Neocortex	Cortical layers I–VI	Interneurons	++/+++	0, 5–2%
Hippocampus	CA1–CA3	Pyramidal neurons	+/++	<1%
	Stratum radiatum	Interneurons	+++	1–2%
	Dentate gyrus (dg)	Granule neurons	+/++	<1%
	Hilus of dg (polymorphic layer)	Interneurons	+++	5–10%*
Hypothalamus	Ventromed. preopt. ncl.	Magnocell. neurons	++	Approximately 10%
	Ant. u. lat. hypothalamic area	Magnocell. neurons	++/++++	Approximately 5%
	Magnocellular preoptic ncl.	Magnocell. neurons	++	Approximately 10%
	Periventricular hypothalamic ncl.	Magnocell. neurons	++	10–20%
	Arcuate hypothalamic ncl.	Neurosecr. and projection neurons	++	>20%
	Arcuate hypothalamic ncl. lat.	Neurosecr. and projection neurons	++/+++	40–50%
Brain regions with weak QC immunoreactivity				
Basal forebrain	Medial septum/VDB	Cholinergic/GABAergic neurons	+	Approximately 5%
Hypothalamus	Anteroventr. perivent. ncl.	Cholinergic neurons	+/++	Approximately 10%
	Lat. septal ncl., dorsal	Cholinergic neurons	+	Approximately 10%
	Med. preopt. area	Cholinergic neurons	+	Approximately 5%
	Ncl. basalis magnocellularis	Cholinergic neurons	+	<1%
Mammillary body		Projection neurons	+/++	Approximately 30–40%

* Age-dependent.

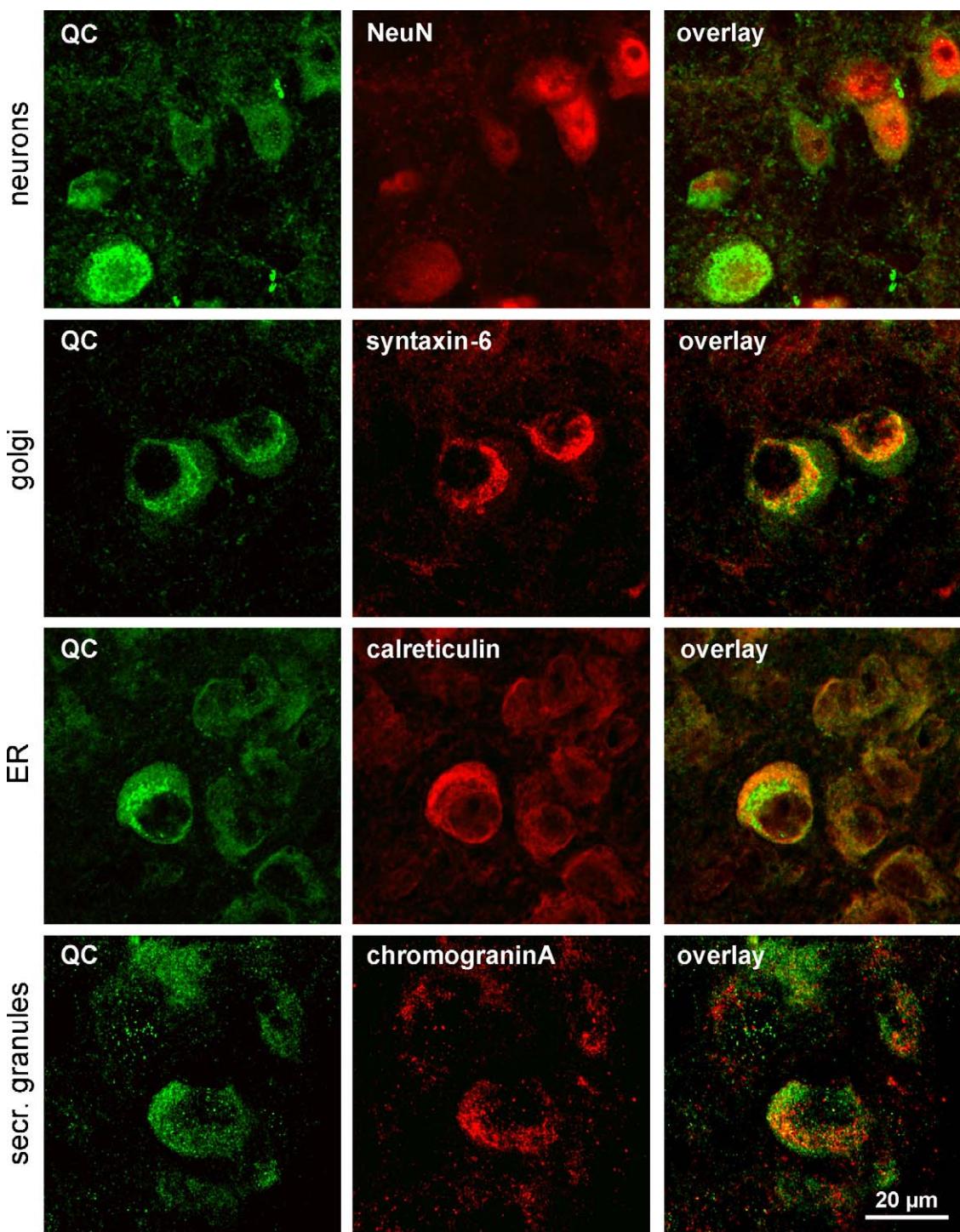


Fig. 6. Cell type-specific and subcellular QC localization in mouse brain. QC (shown in green) is expressed in the cytoplasm of neurons (red fluorescence) in normal mouse brain. Within the cytoplasm, QC is localized to the Golgi apparatus, endoplasmatic reticulum and secretory granules (all labeled by red fluorescence) as indicated by yellow/orange colour in the overlay.

of hypothalamic paraventricular and arcuate nuclei was clearly outlined by immunocytochemical QC staining.

Remarkably, the presence of QC is not limited to structures with known functions of QC – such as hormone maturation in hypothalamic nuclei – but is also detected in brain regions involved in motivation, depression and cognition, i.e. areas affected by AD pathogenesis. In the neocortex, neurons with strong QC expression are relatively rare (<1%) and dispersed across cortical layers. In the hippocampal formation we only occasionally

observed QC-immunoreactive neurons within the granular cell layer of the dentate gyrus and pyramidal cell layers of the hippocampus proper, respectively. Pronounced hippocampal QC expression as detected in the RT-PCR measurements appears to be mostly derived from small-sized neuronal cells, which were identified as different subsets of GABAergic interneurons co-expressing parvalbumin, calbindin, calretinin, somatostatin and cholecystokinin, respectively. The function of QC expression in these neurons is not yet clear. However, the fact that QC expressing

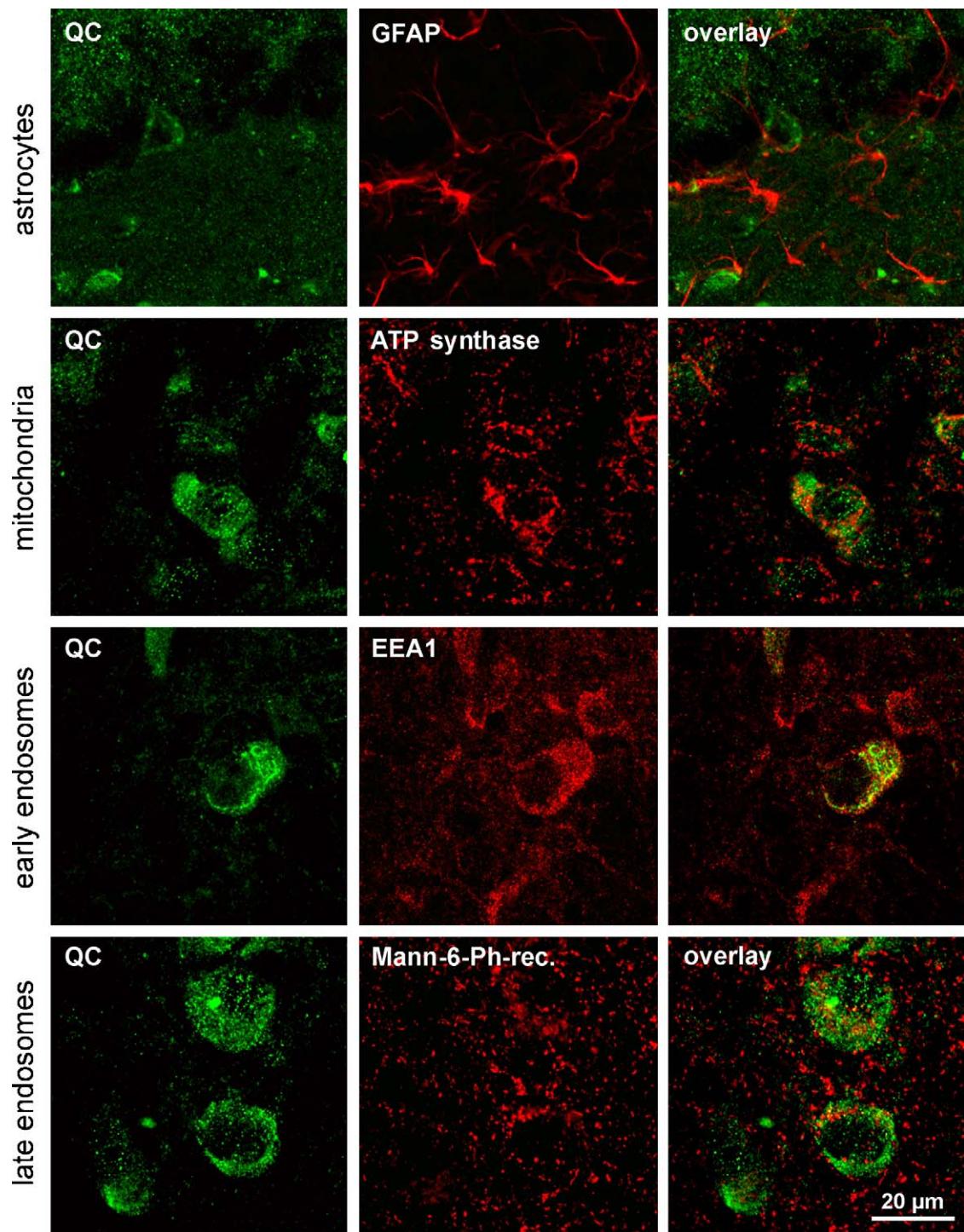


Fig. 7. Absence of QC from astrocytes and neuronal nuclei, mitochondria and endosomes in mouse brain. In the normal mouse brain, QC (green fluorescence) is absent from astrocytes (red fluorescence). Also, QC is not localized to endosomes and mitochondria as indicated by separated green and red colour in the overlay.

neurons in the hilus of the dentate gyrus as well as in the outer third of stratum oriens and the inner third of stratum radiatum of CA1–4 hippocampal subfields tend to form a lamina points to a possible specific function of these cells within the hippocampal interneuronal circuitry. Accordingly, it has been reported that hippocampal GABAergic interneurons at the stratum radiatum–stratum lacunosum moleculare border of the CA1 area, which corresponds to the inner third of the stratum radiatum, establish precise and extremely arborized projections to CA1 pyramidal

neurons as well as projections to perforant pathway termination zones in strata moleculare of CA1 and dentate gyrus (Vida et al., 1998). It is conceivable that possible pathological effects of QC function mediated by N3pE-Abeta peptides might also occur at projection sites of QC expressing neocortical and hippocampal interneurons.

Indeed, the described pattern of QC expression mirrors to some extent the initial stages of Abeta pathology in APP transgenic mouse lines. For example, hippocampal Abeta deposits start to

develop in the hilus of dentate gyrus and in the CA1–4 molecular layer rather than in granular or pyramidal layers (Apelt and Schliebs, 2001; Bianchi et al., 2008; Roßner et al., 2001). The specificity of the newly described QC labeling outside hypothalamic nuclei was demonstrated by the absence of immunolabeling in simultaneously processed brain sections from QC knock-out mice. Moreover, similar results were obtained with a commercially available antibody (AB01; Abcam; not shown).

Neuronal QC expression within different cortical and diencephalic brain regions varied with regard to the intensity of the immunochemical signal, but was comparable with respect to the subcellular localization. In QC-immunoreactive neurons, a distinct perinuclear and cytoplasmic labeling was observed. Double immunofluorescent labeling located QC in the Golgi apparatus, in the ER and in secretory granules, consistent with a function of QC in protein modification within the secretory pathway. Such a physiological QC function is well established for the maturation of hypothalamic hormones (see above) and was recently expanded to the pathogenic pE modification of Abeta peptides by QC in AD brains (Schilling et al., 2004, 2008a,b). Interestingly, QC is present in intracellular compartments with a mildly acidic pH, at which the cyclization of glutamyl precursors – such as truncated Abeta peptides – is favored (Schilling et al., 2004). Thus, it appears that neurons, which functionally express QC in the hormone maturation cascade might be at risk for pE-Abeta induced neurodegeneration under conditions of APP overexpression or increased amyloidogenic APP processing. Since Abeta is predominantly generated along the secretory pathway (Hook and Reisine, 2003; Hook et al., 2008) it is not unlikely that QC converts Abeta as unphysiological substrate into pE-Abeta. Indeed, the dysregulation of hypothalamic hormone function is a well-defined feature of AD and related to Abeta pathology (Casadesus et al., 2005; Meethal et al., 2005). In the present study we focussed on the subcellular QC localization in hypothalamic neurons, where a physiological function of QC is well established. We cannot, however, rule out that the subcellular localization of QC differs in other brain structures or under pathological conditions.

Under basal conditions in the unmanipulated mouse brain studied here, QC expression was restricted to neurons. However, in rat primary cultures QC protein expression was detected in neurons, microglial cells and astrocytes (Apelt and von Hörsten, personal communication), indicating that these cell types, in an activated state, can serve as experimental tools to study the cell type-specific regulation of QC expression. This glial QC expression also appears to play a role under pathological conditions *in vivo* and may, therefore, contribute to pE-Abeta neurotoxicity (Apelt and von Hörsten, personal communication).

During mouse brain ontogenesis stable QC mRNA concentrations were detected in hypothalamus from PD 5 to PD 370. Moreover, no change in the cell type-specific expression or subcellular localization of QC has been observed during normal aging of mice. However, in neocortical and hippocampal brain regions involved in learning and memory we detected a 20–25% decline in QC mRNA concentrations in mice between (PD 20 and PD 42) which was also present at PD 370. It is conceivable that a disturbance of this physiological decline in QC expression resulting in stable or even increased QC levels as shown for the AD brain (Schilling et al., 2008b), may lead to an accumulation of pathogenic QC substrates, compromise neuronal function and induce neuronal cell death. Alternatively, since the significance of QC expression in the neocortex and hippocampus is not yet understood, an additional physiological function of QC during early postnatal development also has to be considered.

Together, our data provide a basis for the understanding of QC function within the secretory pathway in neuronal populations outside hypothalamic nuclei. These structures include brain

regions important for learning and memory that are known to be affected by Abeta pathology in AD.

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