Neurochemical phenotype of cytoglobin-expressing neurons in the rat hippocampus

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Abstract. Cytoglobin (Cygb), a novel oxygen-binding protein, is expressed in the majority of tissues and has been proposed to function in nitric oxide (NO) metabolism in the vasculature and to have cytoprotective properties. However, the overall functions of Cygb remain elusive. Cygb is also expressed in a subpopulation of brain neurons. Recently, it has been shown that stress upregulates Cygb expression in the brain and the majority of neuronal nitric oxide synthase (nNOS)-positive neurons, an enzyme that produces NO, co-express Cygb. However, there are more neurons expressing Cygb than nNOS, thus a large number of Cygb neurons remain uncharacterized by the neurochemical content. The aim of the present study was to provide an additional and more detailed neurochemical phenotype of Cygb-expressing neurons in the rat hippocampus. The rat hippocampus was chosen due to the abundance of Cygb, as well as this limbic structure being an important target in a number of neurodegenerative diseases. Using triple immunohistochemistry, it was demonstrated that nearly all the parvalbumin- and heme oxygenase 1-positive neurons co-express Cygb and to a large extent, these neuron populations are distinct from the population of Cygb neurons co-expressing nNOS. Furthermore, it was shown that the majority of neurons expressing somastostatin and vasoactive intestinal peptide also co-express Cygb and nNOS. Detailed information regarding the neurochemical phenotype of Cygb neurons in the hippocampus can be a valuable tool in determining the function of Cygb in the brain.

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Introduction

The hippocampus is of paramount importance as it controls a wide range of physiological processes, including learning and memory, spatial navigation and neurological diseases, such as Alzheimers's disease (AD), depression, schizophrenia and stroke, which often target the hippocampus and have profound effects on physiology (1-4). The function of the hippocampus has been extensively studied from a behavioral, biochemical and neuroanatomical perspective, establishing the hippocampus as an excellent model for studying the function of a protein. Cytoglobin (Cygb) was identified as the fourth vertebrate heme-globin in 2002 (5,6). Cygb has, despite low sequence homology with the canonical hemoglobin and myoglobin, retained the classical globin fold and can reversibly bind oxygen and other diatomic gasses (7). Cygb is expressed in a number of tissues and is also found in neurons of the brain where Cygb is localized in the soma, neuronal processes and nuclei (8,9). Within the mouse brain, Cygb is expressed in distinct areas with large regional differences in the expression levels. The areas with pronounced Cygb expression are the hippocampus, reticular thalamic nucleus (RT), habenula, laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus (8,10,11). The Cygb expression patterns in the mouse brain were recently determined to be identical in the rat and human brain, demonstrating that the rodent brain can be used as a translational model for studying Cygb in humans, at least at the anatomical level (12,13). The function of Cygb remains largely unknown, but several studies have linked Cygb to reactive oxygen/nitrogen species (RNS) nitric oxide (NO) scavenging (14-20). Furthermore, Cygb overexpression protects against ischemic cell death *in vivo* (21), although not when expressed at endogenous levels (22). In our previous studies, Cygb-immunoreactivity (ir) was shown to be highly co-localized with one of the enzymes producing NO, namely neuronal nitric oxide synthase (nNOS), in the mouse brain (9) and found that the majority of the nNOS-ir neurons of the rat hippocampus co-localized with Cygb-ir (13). However, due to the larger number of Cygb-ir cells in the hippocampus, the majority of Cygb-ir cells remain uncharacterized (13).

The aim of the present study was to extend our previous study (13) in the rat hippocampus by providing a detailed neurochemical phenotype of the Cygb-ir neurons in

association with the subpopulation co-expressing nNOS. Knowledge regarding the neurochemical phenotypes of neurons expressing the protein of interest can be a valuable tool, as it will allow the investigator to determine if the protein of interest is primarily co-localized with one other protein or groups of proteins associated with known specific functions or pathways. This information can then be used to test functional hypotheses regarding the protein of interest by investigating whether affecting the functions/pathways of the co-expressing proteins will also affect the protein of interest.

Materials and methods

Animals. Six male Wistar rats (250 g) from Taconic (Denmark), were used in the experiment. All the rats were perfusion-fixed in 4% paraformaldehyde and the brains were dissected and post-fixed in the same fixative for 24 h at 4˚C. The brains were cryo-protected with 30% sucrose in phosphate-buffered saline for five days and stored at -80˚C until required. The brains were cryo-sectioned in 40-μm coronal sections in a series of five sections. Animal care and all the experimental procedures were conducted in accordance to the principles of Laboratory Animal Care (Law on Animal Experiments in Denmark, publication 1306, November 23, 2007) and approved by the Faculty of Health, University of Copenhagen (Copenhagen, Denmark).

Immunohistochemistry (IHC). The IHC protocol has been described previously (23). The primary antibodies employed for IHC were: i) Rabbit anti-Cygb [in-house, code# 5092/6, 1:3,000 dilution and characterized previously (9,12,13)]; ii) sheep anti-nNOS [Dr Emson, University of Cambridge (Cambridge, UK), 1:3,000 dilution and characterized previously (24,25)]. nNOS produces the gas-neurotransmitter NO, which is involved in a number of physiological and pathological processes, including vasodilatation and RNS-mediated damage. iii) Goat anti-somastostatin (SOMA) [code# sc-7819, 1:1,000 dilution and characterized previously (26,27); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA]. SOMA is a neuroendocrine peptide hormone and regulates a number of secondary hormones via its G protein-coupled receptors. iv) Rabbit anti-vasoactive intestinal peptide (VIP) [in-house, code# 291E-3, 1:15,000 dilution and characterized previously (28)]. VIP is a major regulatory peptide in the brain and is involved in a number of processes, including circadian and neuroendocrine control. v) Rabbit anti-parvalbumin (PV) [code# PV25, 1:40,000 dilution and characterized previously (29,30); Swant, Swizerland]. PV is a calcium-binding protein involved in numerous physiological processes. vi) Rabbit anti-heme oxygenase 1 (HO-1) [code# ADI-SPA-895, 1:60,000 dilution and characterized previously (10,31); Enzo Life Sciences, AH Diagnostics AS, Aarhus, Denmark]. HO-1 is an enzyme that catalyzes the degradation of heme groups to produce biliverdin, iron and the gas-neurotransmitter carbon monoxide. The primary antibodies were detected with either a donkey anti-sheep Alexa-488 or 568 (code# A11015 and A21009, 1:800 dilution; Life Technologies, Carlsbad, CA, USA), donkey anti-rabbit Alexa-594 or 647 (code# A21207 and A31573, 1:800 dilution; Life Technologies) and donkey anti-goat Alexa-649 (code# 705-606-147, 1:300 dilution; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). When two rabbit primary antibodies were used in continuation, a previously described protocol (23) was used. Briefly, following the initial block of endogen peroxide with H_2O_2 treatment, the first primary antibody, which was highly diluted, was detected with a biotin-conjugated donkey anti-rabbit (code# 711-066-152, 1:800 dilution; Jackson Immunoresearch Laboratories, Inc.) followed by the avidin-biotin complex (code# PK-6100; Vector Laboratories, Peterborough, UK), the Tyramide Signal Amplification system (code# NEL700001KT; Perkin-Elmer, Waltham, MA, USA) and visualized with a strepavidin-conjugated Cy2 or Cy5 antibody (code# 016-220-084 and 016-170-085, 1:800 dilution; Jackson Immunoresearch Laboratories, Inc.). The second primary antibody was detected with a donkey anti-rabbit Alexa-594.

Image analyses. Images were captured using an iMIC confocal microscope (FEI Munich GmbH, Germany) equipped with the appropriate filter settings for detecting 4',6-diamidino-2-phenylindole and CY2/Alexa-488, and CY3/Alexa-594 and CY5/Alexa-640. For the overview images captured in the x10 wide-field section of the microscope and 9x3 images were stitched together using the LA-stitch module of Fiji (ImageJ 1.47 64-bit, National Institutes of Health, Bethesda, MD, USA). The image analysis in the higher magnification, the images were captured by the spinning disk confocal section of the microscope. The Z-stacks of ~60-70 images were separated in the Z-level by 0.5 μm and deconvoluted in AutoQuant X (version 3.02; Media Cybernetics, Inc., Rockville, MD, USA) and the localization of dendritic processes and cell bodies were further analyzed using the co-localization module of IMARIS (version 7.6.4; Bitplane USA, South Windsor, CT, USA). Finally, the images were corrected for brightness and contrast in Adobe Photoshop CS5 (Adobe Systems, Mountain View, CA, USA), and extended and mounted into plates using Adobe Illustrator CS5 (Adobe Systems).

Results

Cygb localization and co-localization. Intense Cygb-ir was observed throughout the rostral-caudal extend and in the majority of the hippocampal structures, with the highest density in the dentate gyrus (DG) and hilus (Fig. 1A). In the pyramidal cell layer (Py), in fields CA1-CA3, evident Cygb-ir was detected in the cell soma, nuclei and processes of the pyramidal cells (Fig. 1A). Intense nNOS-ir neurons were observed in the DG and CA1-CA3 (Fig. 1B). PV-ir interneurons and processes were observed in the same areas as Cygb-ir. The vast majority co-stored Cygb-ir, and a subset also co-stored nNOS-ir (Fig. 1B and D). The majority of the stratum radiatum (Rad) nNOS-ir cells did not contain Cygb-ir (Fig. 1D). Intense HO-1-ir cells were observed in the DG, as well as in a few cells of the CA1-CA3 (Fig. 2B), where almost all the cells co-stored Cygb-ir and a small subset also co-stored nNOS-ir (Fig. 2D). A few medium-intensity stained VIP-ir cells were observed to be scattered in DG and CA1-CA3 (Fig. 3B), where the majority of cells co-expressed Cygb-ir and nNOS-ir (Fig. 3D). SOMA-ir was highly expressed throughout the hippocampus (Fig. 4B) and was found in the

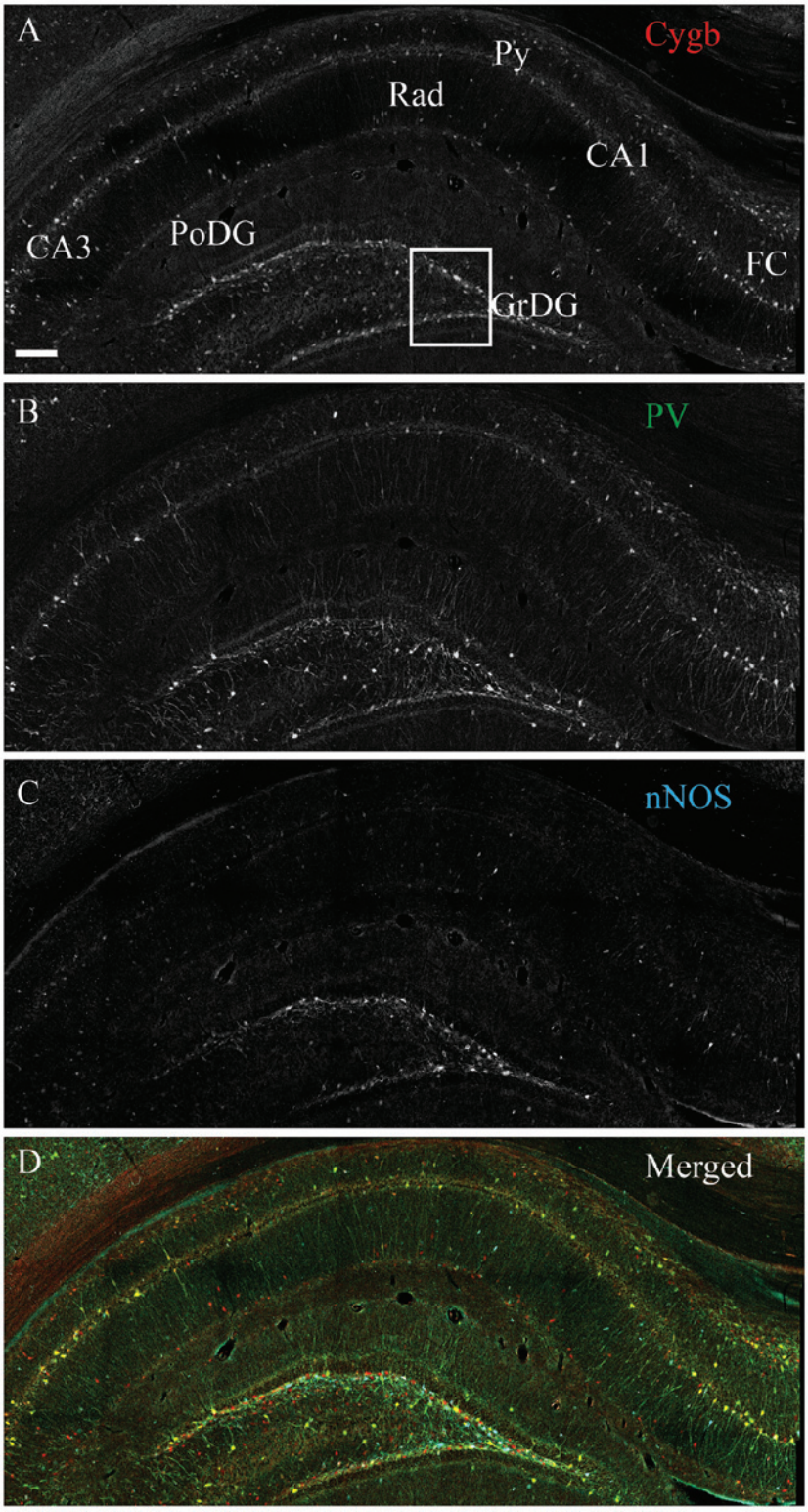

Figure 1. Cytoglobin (Cygb), parvalbumin (PV) and neuronal nitric oxide synthase (nNOS) expression in the rat hippocampus. Immunohistochemical staining of (A) Cygb, (B) PV and (C) nNOS. (D) The merged image of A-C is shown. A high degree of co-localization was observed between Cygb-immunoreactivit y (ir) (red) and PV-ir (green) throughout the hippocampus and the majority of the PV-ir neurons did not co-localize with nNOS-ir (cyan). The area within the white box is magnified in Fig. 6. CA1-3, corun ammonis 1-3; FC, fasio larumcinereum; GrDG, striatum granulosum of dentate gyrus; PoDG, polymorph layer of DG; Py, pyramidal cell layer; Rad, stratum radiatum. (A-D) Scale bar, 200 μm.

majority of the cells that also expressed Cygb-ir and nNOS-ir, although a subset only co-stored Cygb-ir (Fig. 4D). Outside the hippocampus, such as the RT, a high degree of co-localization between Cygb-ir and the neurochemical marker, PV, was also observed (Fig. 5).

Image analysis. The Z-stack image analysis of the areas within the white squares in Figs. 1-4 confirmed the co-localization between Cygb-ir and the neurochemical markers, and confirmed that the majority of the cells co-expressing Cygb-ir/PV-ir and Cygb-ir/HO-1-ir did not express nNOS-ir

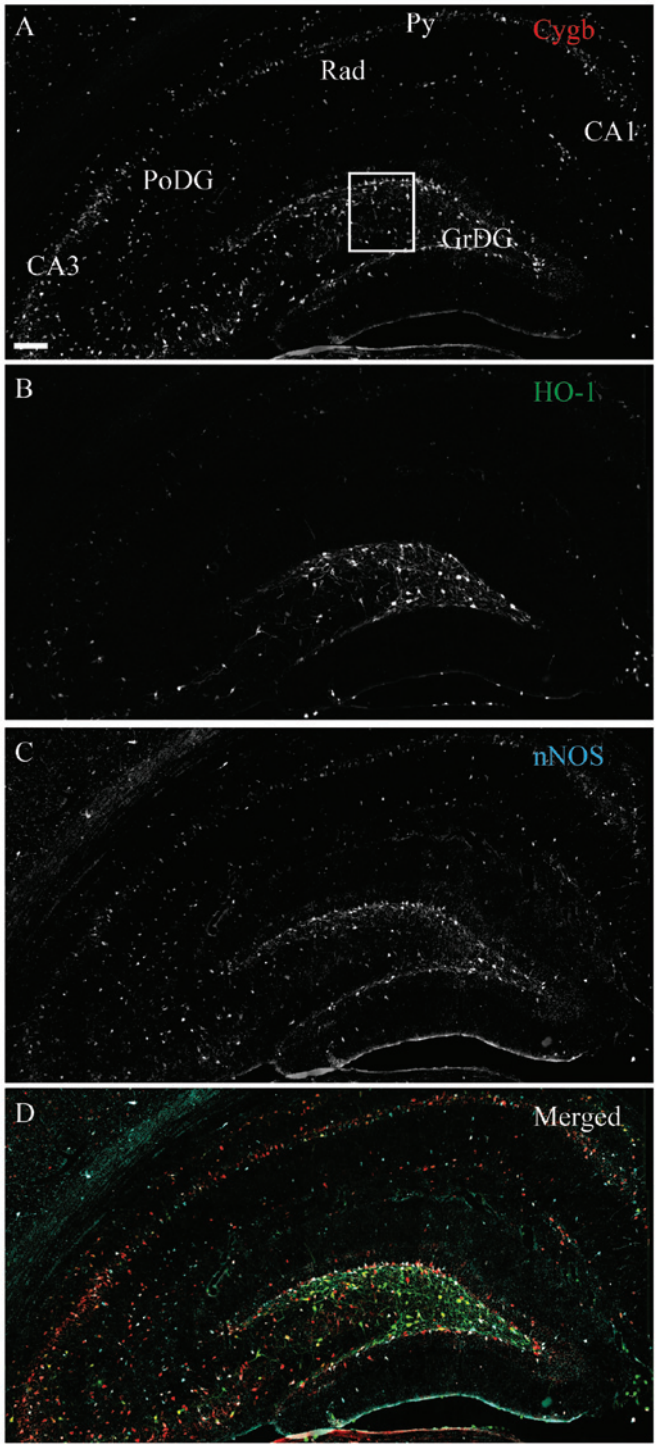

Figure 2. Cytoglobin (Cygb), heme-oxygenase-1 (HO-1) and neuronal nitric oxide synthase (nNOS) expression in the rat hippocampus. Immunohistochemical staining of (A) Cygb, (B) HO-1 and (C) nNOS. (D) The merged image of A-C is shown. A high degree of co-localization is shown between Cygb-immunoreactivity (ir) (red) and HO-1-ir (green) in the DG. The majority of nNOS-ir (cyan) neurons did not co-localize with HO-1-ir. The area within the white box is magnified in Fig. 6. CA1-3, corun ammonis 1-3; GrDG, striatum granulosum of dentate gyrus; PoDG, polymorph layer of DG; Py, pyramidal cell layer; Rad, stratum radiatum. (A-D) Scale bar, 200 μm.

(Fig. 6A-H). Similarly, the high degree of co-localization between VIP-ir, SOMA-ir and Cygb-ir was also confirmed (Fig. 6I-L and M-P, respectively).

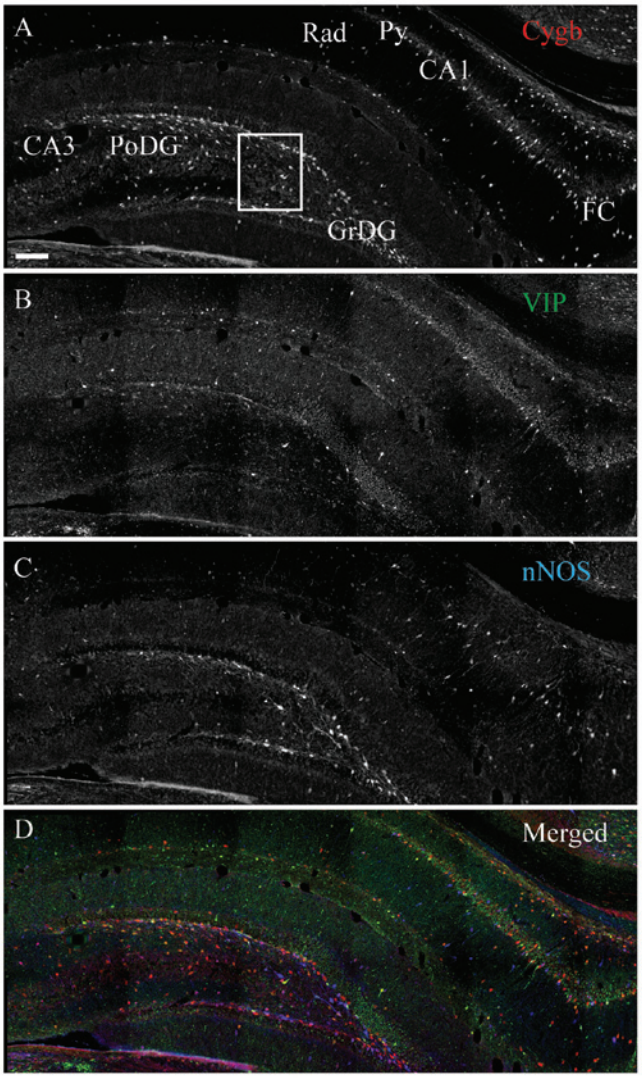

Figure 3. Cytoglobin (Cygb), vasoactive intestinal peptide (VIP) and neuronal nitric oxide synthase (nNOS) expression in the rat hippocampus. Immunohistochemical staining of (A) Cygb, (B) VIP and (C) nNOS. (D) The merged image of A-C is shown. VIP-immunoreactivity (ir) (green) neurons were found to co-localize with the Cygb-ir (red) and nNOS-ir (cyan) neurons. The area within the white box is magnified in Fig. 6. CA1-3, corun ammonis 1-3; FC, fasio larumcinereum; GrDG, striatum granulosum of dentate gyrus; PoDG, polymorph layer of DG; Py, pyramidal cell layer; Rad, stratum radiatum. (A-D) Scale bar, 200 μm.

Discussion

Despite intensive research (32) over the past 12 years, the function of Cygb remains an enigma. To the best of our knowledge, the present study is the first detailed characterization of the neurochemical phenotype of Cygb-ir neurons in the rat hippocampus and can, in combination with other methods, contribute to an improved understanding of the Cygb function. The study was performed in the rat model as the employed antibodies produced an optimal immunohistochemical staining, as opposed to the mouse.

Clarifying the function of a protein in the brain is challenging due to the high complexity and the interconnections of the brain structures. Therefore, having tools that minimize the complexity and provide a clearer functional readout are highly advantageous. We have previously used neurochemical phenotyping to obtain

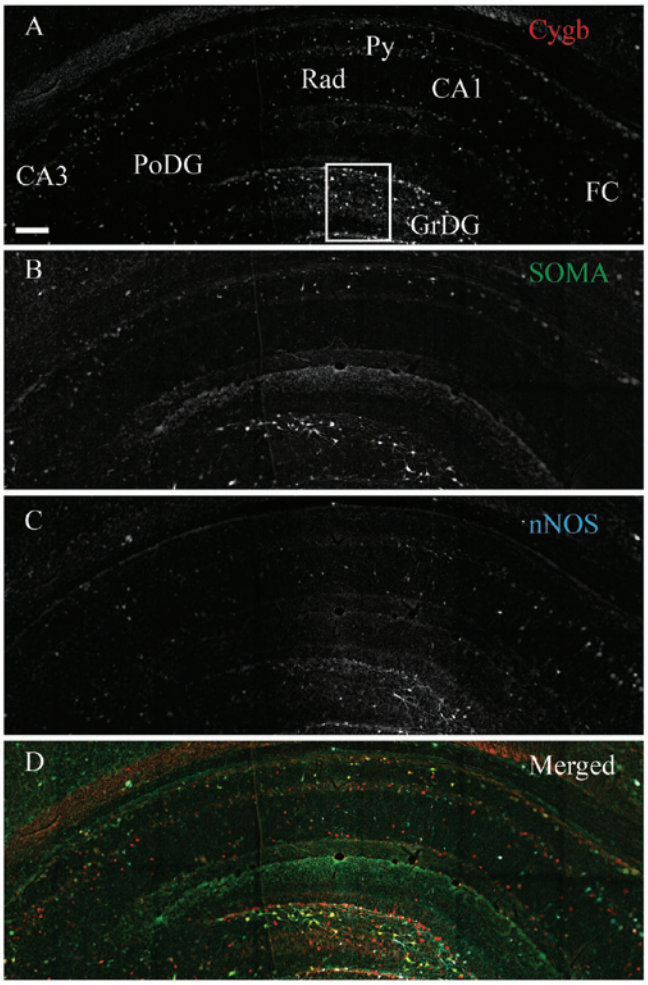

Figure 4. Cytoglobin (Cygb), somastostain (SOMA) and neuronal nitric oxide synthase (nNOS) expression in the rat hippocampus. Immunohistochemical staining of (A) Cygb, (B) SOMA and (C) nNOS. (D) The merged image of A-C is shown. As for vasoactive intestinal peptide, SOMA-immunoreactivity (ir) (green) neurons primarily belonged to the sub-population of Cygb-ir (red) neurons, also co-localizing nNOS-ir (cyan). The area within the white box is magnified in Fig. 6. CA1-3, corun ammonis 1-3; FC, fasio larumcinereum; GrDG, striatum granulosum of dentate gyrus; PoDG, polymorph layer of DG; Py, pyramidal cell layer; Rad, stratum radiatum. Scale bar, 200 μm.

surrogate markers of the protein of interest in knock-out mice in order to study the fate of neurons that would have expressed the protein of interest. This enabled the study of whether these neurons were more prone to hypoxia-induced immediate early gene expression and cell death (33).

The principal novel finding of the present study is the high degree of co-localization between Cygb and the two markers, PV and HO-1, in the hippocampus of which only a subset of neurons also co-express nNOS-ir. The high degree of Cygb-ir/PV-ir co-expression therefore shows for the first time that Cygb-expressing neurons are primarily inhibitory interneurons. The high degree of co-expression with HO-1 is notable as HO-1 is an oxidative stress-inducible protein and enzymatic degradation of heme leads to the production of iron, biliverdin (an antioxidant) and CO (34). Cygb may therefore either be a heme substrate for HO-1 or alternatively, Cygb may, in these HO-1-expressing neurons, regulate the level of the gas-neurontransmitter CO produced by HO-1 in a similar way to that proposed for NO (18,19,21).

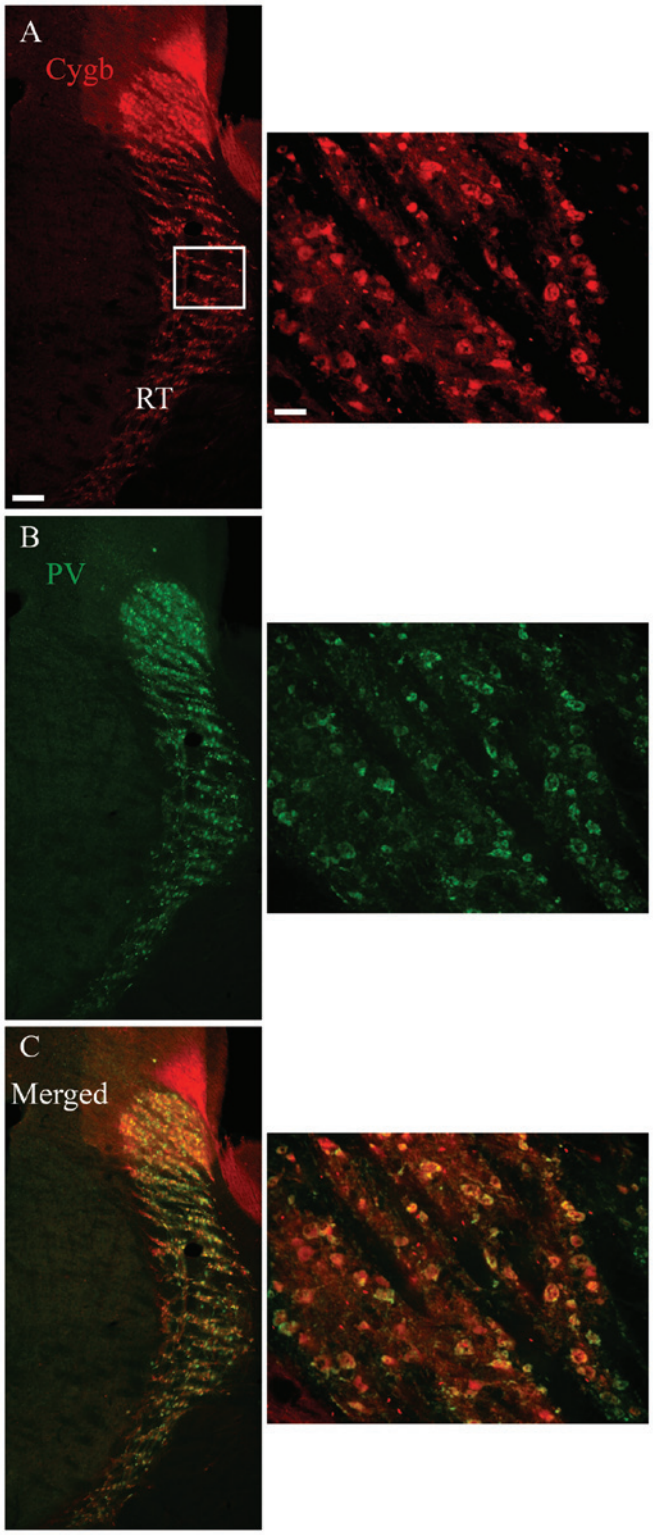

Figure 5. Co-localization of cytoglobin (Cygb)-immunoreactivity (ir) and parvalbumin (PV)-ir in the reticular thalamic nucleus. (A) Intense Cygb-ir (red) and (B) PV-ir (green) were observed in the reticular thalamic nucleus (RT) of the rat brain. (C) All the PV-ir neurons co-expressed with Cygb-ir. The images on the right side are higher magnifications of the area within the white square in (A). Scale bar, (A-C) 200 μm and 25 μm in high magnifications.

Notably, in association with the putative role of Cygb in neuroprotection, a selective reduction of PV-ir neurons were observed in the hippocampus of the AD patients and transgenic animal models of AD (35-38). Of note, HO-1-ir

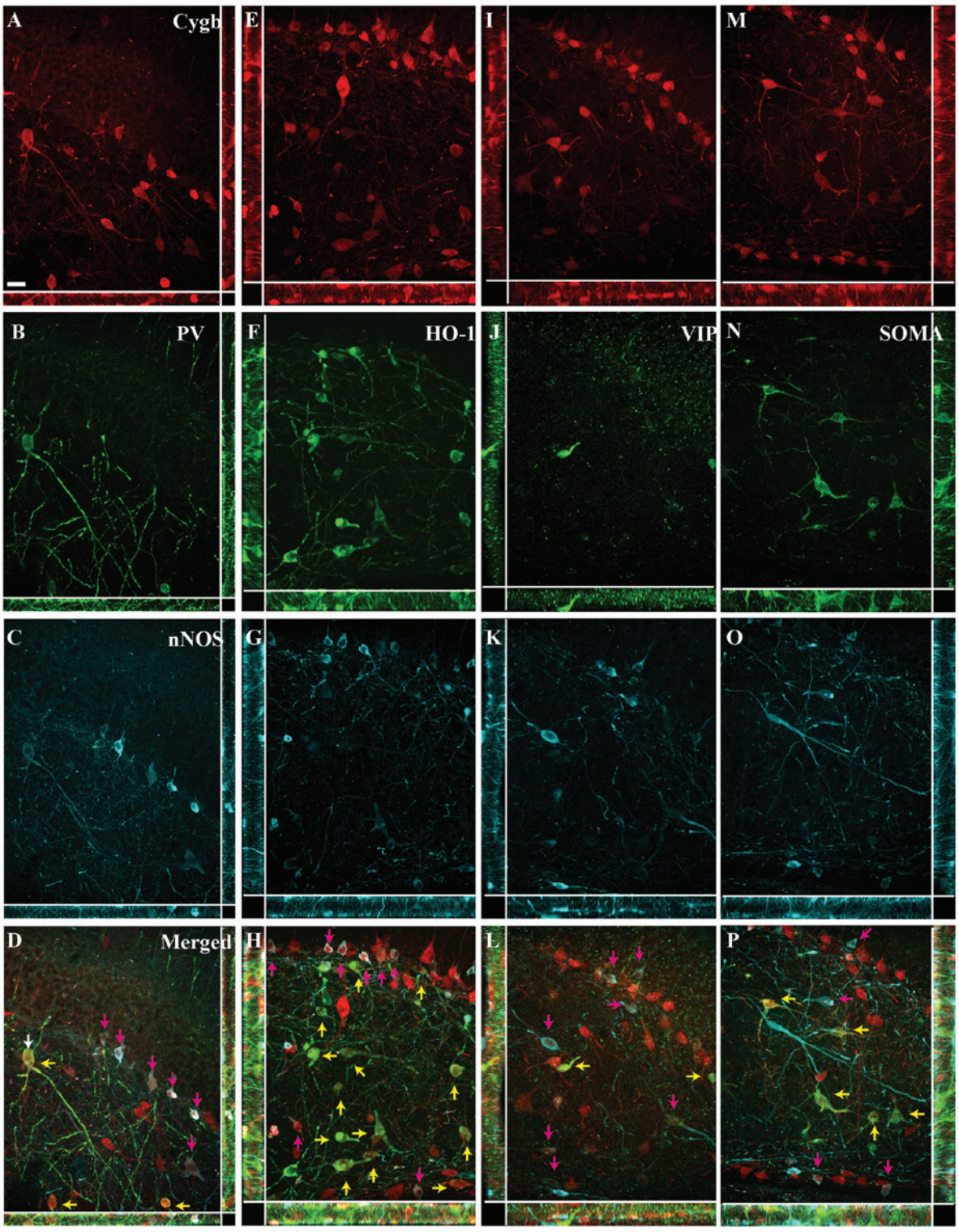

Figure 6. High magnification images of the white squares in Figs. 1D-4D. (A-D) A Z-stack image of dentate gyrus neurons expressing cytoglobin (Cygb) (red), parvalbumin (PV) (green) and neuronal nitric oxide synthase (nNOS) (cyan). The yellow arrows (D) indicate that certain cells co-store Cygb and PV, the purple arrows indicate the cells co-storing Cygb and nNOS and the white arrow show a cell co-expressing all three. Similarly, (E-H) a Z-stack image of co-expression with heme-oxygenase-1 (HO-1) (green). The yellow arrows (H) indicate that certain cells co-store Cygb and HO-1 and the purple arrows indicate the cells co-storing Cygb and nNOS. (I-L) Co-localization with vasoactive intestinal peptide (VIP) (green) and (M-P) with somastostain (SOMA) is shown. The white vertical and horizontal lines indicate the Y-Z and X-Z axis, respectively. Scale bar, 25 μm.

has been shown to be upregulated in hippocampal neurons of the AD patients (39) and to contribute to the pathology by excessive production of free iron (40-42). The high degree of co-expression with Cygb indicates that Cygb could be a heme substrate for HO-1 enzymatic activity and thereby a source for excessive iron production, which contributes to the pathology of AD. Taken together, this indirectly indicates that expressing Cygb alone does not provide neurons with a selective

protection against neurodegenerative cell death, which is in line with the lack of selective sparring of Cygb-expressing neurons following brain ischemia (22).

Cygb-ir was also co-expressed in SOMA-ir and VIP-ir neurons to a high degree. These neurons, however, belonged primarily to the neurons that also expressed nNOS-ir. VIP and SOMA have a broad range of functions in the central nervous system, however, in association with neuronal protection, a reduced number of viable SOMA neurons were found in human AD brains (43,44), whereas VIP have been shown to exert a level of protection in neurodegenerative disorders (45-47). These observations indirectly show that despite SOMA, neurons also express Cygb and they are still not spared in neurodegenerative disorders, thus questioning if Cygb at endogenous levels functions in neuronal protection.

Future studies using Cygb null mice (20,48) would be highly beneficially for studying the fate/function of neurons expressing PV, HO-1, SOMA and VIP. These studies will show whether a lack of Cygb affects neuronal survival or normal cell physiology by using these four proteins as markers. Based on the large expression of Cygb in the hippocampus, it is highly likely that Cygb has an important function in hippocampal normal physiology. The results in the present study will be a significant aid for future studies in elucidating any functional roles.

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