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The role of chromogranin B in an animal model of multiple sclerosis

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Abstract

Chromogranin B (CGB) is a high capacity, low affinity calcium binding protein in the endoplasmic reticulum (ER) that binds to the inositol 1,4,5 trisphosphate receptor (InsP3R) and amplifies calcium release from ER stores. Recently, it was discovered that levels of CGB-derived peptides are decreased in the cerebrospinal fluid of multiple sclerosis (MS) patients. One of the mechanisms by which neurodegeneration in MS is thought to occur is through increased levels of intra-axonal calcium. The combination of excess intracellular calcium and dysregulated levels of CGB in MS led us to hypothesize that CGB may be involved in MS pathophysiology. Here, we show in a mouse model of MS that CGB levels are elevated in neurons prior to onset of symptoms. Once symptoms develop, CGB protein levels increase with disease severity. Additionally, we show that elevated levels of CGB may have a role in the pathophysiology of MS and suggest that the initial elevation of CGB, prior to symptom onset, is due to inflammatory processes. Upon development of symptoms, CGB accumulation in neurons results from decreased ubiquitination and decreased secretion. Furthermore, we show that calpain activity is increased and levels of InsP3R are decreased. From these results, we suggest that the elevated levels of CGB and altered InsP3R levels may contribute to the axonal/neuronal damage and dysregulated calcium homeostasis observed in MS. Additionally, we propose that CGB can be a biomarker that predicts the onset and severity of disease in patients with MS.

Keywords

Chromogranin B (CgB); Inositol 1,4,5 trisphosphate receptor (InsP3R); Intracellular calcium signaling; Multiple Sclerosis (MS)

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Introduction

Multiple sclerosis (MS) is a devastating disease of the central nervous system (CNS) that is characterized by demyelination, inflammation, and neurodegeneration. It affects more than 2.5 million people worldwide and those who are affected begin developing symptoms around 30 years of age (Fox et al., 2006).

One of the prevailing theories by which neurodegeneration occurs in MS is through dysregulation of intracellular calcium. Studies have suggested that under pathological conditions, excess calcium may enter the axon via numerous channels (Kornek et al., 2001; Craner et al., 2004a; Craner et al., 2004b). Excessive intra-axonal calcium activates damaging cascades involving proteases such as calpains, which degrade major components of the cytoskeleton (Shields et al., 1999; Guyton et al., 2005). Pharmacological blockade of calcium flux into the cytoplasm via these channels was found to reduce axonal damage (Brand-Schieber and Werner, 2004; Waxman, 2005).

Recently, it was shown that levels of chromogranin B (CGB) derived peptides are decreased in the cerebrospinal fluid of multiple sclerosis (MS) patients (Mattsson et al., 2007). CGB is a high capacity, low affinity calcium binding protein expressed in the endoplasmic reticulum (ER), large dense core vesicles and the nucleus (Taupenot et al., 2003). In the ER, CGB acts both as a buffer for calcium and a binding partner for the inositol 1,4,5-trisphosphate receptor (InsP3R), an intracellular calcium channel spanning the ER membrane (Berridge, 1993). Upon binding to the InsP3R, CGB amplifies calcium release from the ER stores. The combination of excess intracellular calcium and dysregulated levels of CGB in MS, led us to hypothesize that CGB may be involved in MS pathophysiology. Additionally, the fact that CGB peptides are decreased in MS patients indicates that it may be a potential biomarker for MS.

Due to these observations, we decided to examine the role of CGB in MS using a mouse model of MS, experimental autoimmune encephalomyelitis (EAE) mice. In EAE mice, we show that CGB protein and mRNA levels are elevated in neurons prior to onset of symptoms. Upon development of symptoms, CGB protein levels remain increased and were found to correlate with disease severity. These results, along with the data from prior studies (Mattsson et al., 2007), indicate that CGB is a potential biomarker that can be used to (1) predict the onset and severity of MS prior to development of symptoms, and (2) to monitor disease progression.

Furthermore, we demonstrate that elevated CGB may have a role in the pathophysiology of MS. We are able to show that the initial elevation in CGB, prior to symptom onset, may be connected to inflammatory processes such as increased TNF-alpha production by infiltrating T-cells and active microglia. Upon development of symptoms, CGB accumulation in neurons results from decreased ubiquitination and defective secretory processes. Additionally, we observed that there was an increase in calpain activity that likely causes an increase in the cleavage of InsP3R. The elevated CGB levels and altered InsP3R levels, along with decreased plasma membrane calcium ATPase levels (Nicot et al., 2005), eventually leads to a buildup of intracellular calcium that could cause the neuronal/axonal damage and pathophysiology observed in MS.

Materials and Methods

Animal use and treatment

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol

was approved by the Institutional Animal Care & Use Committee at Yale University. All surgical procedures were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

EAE induction

Female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice (4–8 weeks old) were immunized with 200 µg of myelin oligodendrocyte glycoprotein peptide (35–55) (MOG) emulsified with Complete Freud's Adjuvant (CFA, containing 400 µg of H37RA mycobacteria) (DIFCO BD Diagnostics, Sparks, MD) via subcutaneous injection on day 0. Pertussis toxin (PTX), 250 ng (List Biologicals Laboratories, Campbell, CA), was injected intravenously on day 0 and again on day 2 (Nichols et al., 2009). Control mice were immunized with CFA and received a subcutaneous injection of 250 ng of PTX on days 0 and 2. EAE was scored as grade 0, no abnormal motor symptoms; grade 1, tail paralysis; grade 2, weakness of hind limbs with an altered gait; grade 3, hind limb paralysis; grade 4, tetraparalysis; grade 5, death.

Animal perfusion and tissue preparation

Mice were sacrificed and perfused intracardially with ice-cold 0.1M phosphate buffer (PB, pH 7.4). For western blot and quantitative RT-PCR, spinal cords were immediately dissected, snap-frozen in liquid nitrogen and kept at –80°C until further processing. For immunofluorescence, the animals were perfused with 4% (w/v) paraformaldehyde (PFA, pH 7.4) before dissection. Lumbar spinal cords and brains were postfixed in PFA and cryoprotected in two steps using 20% and 30% PB-sucrose solution, where each step was maintained overnight at 4°C. The tissues were embedded in O.C.T compound (Sakura Finetek, Baltimore, MD) and cut as 40 µm thick free-floating sections.

Western Blot and immunoprecipitation

Tissue was sonicated in Cytobuster (Novagen, Billerica, MA) and centrifuged at 4°C at 10,000×g. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were loaded onto Tris-HCl gels (Bio-Rad/Life Science, Hercules, CA) and transferred onto PVDF membranes. For immunoprecipitation, lysates were incubated with ubiquitin (PD41, Santa Cruz, Santa Cruz, CA) antibody. The immunoprecipitates were boiled and resolved by SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% milk and 0.1% Tween-20 in PBS for 1 hour, followed by incubation with antibodies diluted in blocking buffer. Visualization was performed by chemiluminescence (Amersham, Freiburg, Germany). The signal was captured on X-ray film, which were then scanned. Antibodies used for probing western blots were: Chromogranin B (1:1000, BD Biosciences, San Jose, CA), InsP3R I (1:5000, (Johanning et al., 2002)), GAPDH (1:500, Abcam, Cambridge, MA), and beta-actin (1:10,000, Abcam, Cambridge, MA).

RNA isolation, reverse transcription and RT-PCR

RNA was isolated from frozen spinal cord and cortex tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and RT-PCR was performed on ABI Prism 7500 using TaqMan Gene Expression Master Mix and premade assays for CGB (Mm00483287_m1) and Gapdh (4352932E), all purchased from Applied Biosystems. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method and expressed relative to GAPDH.

Immunofluorescence

The following primary antibodies and dilutions were used: mouse anti chromogranin B (CGB, 1:250, BD Biosciences), rabbit anti InsP3RI (1:1000, (Johanning et al., 2002)), rabbit anti calnexin (1:250, Stressgen, Plymouth Meeting, PA), rabbit anti myelin basic protein (MBP, 1:50, Santa Cruz, Santa Cruz, CA), rat anti MBP (1:200, Abcam, Cambridge, MA), rabbit anti glial fibrillary acidic protein (GFAP, 1:200, DAKO, Glostrup, Denmark), rat anti mouse CD11b (1:200, Abcam, Cambridge, MA), rabbit anti activated caspase-3 (Casp3, 1:200, Abcam, Cambridge, MA).

The sections were extensively washed in phosphate buffer, permeabilized with 0.3% Triton X-100 for 2h and transferred to blocking solution (5% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% L-lysine and 0.3% Triton X-100 in PB, pH 7.4) for 1h at room temperature (RT). The sections were then incubated with primary antibodies for 24–36h at 4°C, followed by the fluorochrome-coupled secondary antibodies Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-rabbit or Alexa Fluor 633 goat anti-rat (Molecular Probes Invitrogen, 1:500) for 2h at RT. In some sections nuclei were stained by incubating the sections with TO-PRO3 (1:400 in PBS, Molecular Probes, Grand Island, NY) for 45 min at RT. Sections were mounted in Vectashield Mounting Medium or Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Negative controls, where primary antibodies were omitted, showed no staining.

Activity Assays

Spinal cords were lysed in Cytobuster and used for the Calpain-Glo Protease Assay, Proteasome-Glo Trypsin-Like Cell-Based Assay, and the DUB-Glo Protease Assay (Promega, Madison, WI) according to manufacturer's protocol. Activity was measured with luminescence and the relative luminescence was averaged over 10s, background-subtracted and normalized to the amount of protein in the lysate.

Microscopy and image analysis

Confocal laser scanning microscopy was carried out on a LSM 510 and LSM 510 NLO (Zeiss) in multitrack configuration. Images were captured using 10x, 20x, 40x and 63x water or oil immersion objectives. Fluorophores or dyes were excited with an argon laser 488nm (Alexa 488), a krypton laser 568nm (Alexa 568), a HeNe laser 633nm (Alexa633 and TOPRO-3) and a tunable MaiTai Sapphire Laser for DAPI excitation. Fluorophore excitation spectra were filtered as follows: Alexa 488, BP 505–530nm; Alexa 568, BP 585–615nm; TOPRO-3, LP 650nm; DAPI, KP 685nm. 4–8 frames were averaged to get an image with reduced noise. For imaging tissue whose size extended the spatial range of the 10x objective, the 'stage-function' was used to automatically compose single images into one. Stack images were made by imaging serial slices with 1µm thickness and 3D reconstruction was done using Volocity (PerkinElmer, Waltham, MA). For quantification of CGB-stained neurons, spinal cord segments showing cellular infiltration (highly intense DAPI-staining) into the grey matter and normal appearing adjacent areas were selected. A 200µm × 200µm region of interest (ROI) was randomly placed into these areas and CGB-stained neurons were counted. 3–5 ROIs per section and 2–4 sections per animal were analyzed.

Data and statistical analysis

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was done for multiple group comparisons and the students' t-test was used when two groups were compared. Values were considered significantly different if $p < 0.05$.

Results

CGB protein levels are increased in neurons in EAE mice. The elevated CGB levels correlate with disease severity

Western blots were performed on isolated spinal cords to compare total CGB levels in EAE mice (score 2 to 3, 15–34 days post immunization (DPI)) to control mice. Immunoblot analysis showed that levels of full-length CGB and its cleaved fragments were elevated in the spinal cord in diseased mice (n=12) when compared to controls (n=6) (Figure 1a). C57BL/6-EAE mice were used in our studies due to the predominance of C57BL/6 as the genetic background for knockout and knock-in mice. MOG was used to induce EAE in our mice, due to the fact that myelin basic protein (MBP) and proteolipid proteins (PLP) are unable to induce EAE in C57BL/6 mice (Kuerten et al., 2007).

The liver, an organ expressing CGB but with little relevance to MS pathophysiology, was collected from EAE and control mice and subjected to western blotting. Levels of CGB from the livers of EAE mice were not significantly different from that of the control mice, indicating that elevated CGB protein levels are specific to the CNS (Figure 1b).

Immunohistochemistry of spinal cord from EAE mice showed that CGB was elevated in neurons (Figure 1c) and not in astrocytes (Figure 1d). CGB elevation in neurons was present in all cellular compartments (soma, dendrites, and axons) and the nucleus. As expected, CGB colocalized with the InsP3R type I (Figure 1e).

To determine if there was a relationship between CGB elevation and demyelination, spinal cord sections from EAE mice at 12 DPI were immunostained for CGB and myelin basic protein (MBP); DAPI was used to identify nuclei. All animals demonstrated at least hind limb weakness (grade 2 or higher), which is indicative of ongoing demyelination and inflammation in the lumbar spinal cord. Peripheral myelin damage and overall cellular infiltration into the parenchyma of white and grey matter were found in specific areas of the spinal cord, as indicated by the arrows (Figure 1f, panels 1–3). Neurons with elevated CGB were approximately 20-fold more abundant at these pathologic areas (26.6 ± 3.0 cells per ROI, n=4) when compared to adjacent, normal appearing areas (1.1 ± 0.4 cells per ROI, n=4). MBP did not colocalize with CGB indicating that, similar to the astrocytes (Figure 1d), oligodendrocytes contain low basal levels of CGB (Figure 1f, panel 4).

Overall, protein levels of CGB were elevated in EAE mice. The elevated CGB was located primarily in neurons and near areas of demyelination and inflammation. Additionally, the increased levels of CGB correlate significantly with increasing disease severity. These results suggest that CGB elevation is involved in the disease progression of EAE.

CGB protein levels are increased in certain mice prior to onset of symptoms – demonstrating that CGB may be a biomarker that predicts the severity and onset of disease symptoms. CGB protein levels are also increased in relation to disease severity in symptomatic mice – demonstrating the ability of CGB as a biomarker for monitoring disease progression

We observed that CGB protein levels are already increased when symptoms manifest in EAE mice at 12 DPI (Figure 1c) and that once symptoms manifest, levels of CGB were elevated (Figure 1a). However, it is not known if CGB is elevated prior to symptom onset.

To determine the time course of CGB elevation and its relationship to EAE development, we isolated spinal cords from mice 1 to 12 DPI. Immunoblot analysis of CGB showed that CGB protein levels were increased in several EAE mice 1 and 2 DPI, when compared to their same-day controls (Figure 2). No differences in protein levels were observed in EAE mice 3

and 4 DPI (Figure 2). At 5 and 6 DPI, however, CGB levels were increased in more EAE mice and continued to increase throughout the duration of the study (Figure 2). At 11 and 12 DPI, when mice began to develop symptoms and when disease scores can be assigned, increased CGB protein levels in EAE mice correlated with increased disease severity. EAE mice with no clinical symptoms, a score of 0, had no obvious changes in CGB levels from their same-day controls. In contrast, mice with a score of 2 or above had a dramatic increase in CGB protein levels, when compared to same-day control mice. It is interesting to note that in the days before development of symptoms, 1 to 10 DPI, not all EAE mice had elevated CGB levels. For instance, at 1 and 2 DPI, 5 out of 10 EAE mice had increased protein levels relative to control mice. Of the 5 mice with elevated CGB, there was a large range in protein expression (Figure 2).

The variability observed – in the early stages before symptom development and in the later stages after symptom development – is likely due to the fact that not all mice get sick to the same degree (mimicking MS observed in patients). Although we are unable to perform the same correlation in mice 1–10 DPI that we conduct in mice that are symptomatic (11–12 DPI), the fact that only some mice show elevated CGB levels prior to onset of symptoms (and that later, these elevated levels are observed to correlate with disease severity) indicate that CGB may predict the severity and onset of disease symptoms. Additionally, when symptoms manifest at in EAE mice 11–12 DPI, we were able to observe that CGB levels correlated with disease severity.

Elevation of CGB protein levels after onset of symptoms does not result from altered mRNA levels

The elevated protein levels of CGB could be due to numerous factors. To determine if the elevation in CGB is due to increased mRNA levels, RT-PCR was performed on spinal cord tissue harvested from control and EAE mice 1 to 12 DPI. mRNA levels were significantly increased in EAE mice 5 and 7 DPI, when compared to their same-day controls. These elevated levels of mRNA dropped back down to levels similar to that of same-day controls at 8 DPI, and decreased significantly at 11 and 12 DPI (Figure 3a). The drop in mRNA in EAE mice at 11 and 12 DPI was found to correlate significantly with disease severity (Figure 3b). mRNA levels in EAE mice with a score of 2 and above, had $56 \pm 4\%$ less mRNA when compared to same-day controls, whereas mRNA levels in EAE mice with a score of 0 showed no significant difference to same-day controls. When levels of CGB mRNA from individual EAE mice were plotted relative to their same-day controls, mRNA levels were noticeably variable (Figure 4b). For instance, EAE mice 7 DPI had increases in mRNA that ranged from 20% to 47% when compared to their same-day control (Figure 3c). However, after onset of symptoms (11–12 DPI), elevated levels of CGB are due to factors other than increased mRNA levels.

Elevated CGB protein and mRNA levels in EAE mice prior to onset of symptoms are likely due to inflammatory processes

Inflammatory cytokines play a large role in the development of MS symptoms. One of these inflammatory cytokines is tumor necrosis factor-alpha (TNF-alpha) – a cytokine that is known to be involved in the induction of oligodendrocyte apoptosis and demyelination, and whose expression parallels the disease course in EAE mice (Sharief and Hentges, 1991; Imitola et al., 2005). Additionally, TNF-alpha is activated by the transcription factor, CRE-binding protein (CREB) (Gustin et al., 2004), and CGB transcription is regulated by CREB (Mahapatra et al., 2006). As a result, TNF-alpha may regulate CGB transcription, and the elevated mRNA levels in EAE mice could be the result of increased TNF-alpha secretion. To test this, we treated SHSY5Y cells, a human-derived neuroblastoma cell line, with TNF-alpha and found that CGB protein levels were upregulated (Figure 4a). This indicates that

TNF-alpha does have a role in regulating CGB protein levels and that the increase in CGB mRNA may be the result of increased production of TNF-alpha, among other cytokines.

However, to understand the relationship between CGB and inflammation, we looked at the pathology of EAE. The histopathological hallmarks of MS and EAE are demyelination and inflammation and result, in part, from the excess TNF-alpha produced by autoreactive, infiltrating T-cells and activated microglia (Gold et al., 2006). To test if there was a relationship between increased CGB levels and the onset of inflammatory lesions, and if this change was cell-type-specific, we immunostained sections from lumbar spinal cord of control and EAE mice at 12 DPI. In EAE mice, lumbar spinal cords had multiple active lesions, along with prominent parenchymal infiltration by CD11b⁺ microglia, macrophages, and CD3⁺ T-cells (Figure 4b,c and Figure 4d); levels of CGB were also elevated. None of this was observed in the control mice or mice prior to disease onset.

The infiltration of microglia and macrophages, which leads to inflammation, is consistent with the pathophysiology seen in MS. Interestingly, these cells also showed strong immunoreactivity for CGB at this stage (Figures 4b, c, and d). We also detected enriched CGB levels in neurons that were in close proximity to microglia accumulation and T-cell infiltration, as indicated by the arrows (Figures 4b, c, and d). These results demonstrate that CGB levels are increased near areas of inflammation, suggesting that the initial increase in CGB protein levels is due to elevated TNF-alpha and that the later increase in CGB protein results from factors other than TNF-alpha.

Elevated CGB levels in symptomatic EAE mice results from decreased ubiquitination

The elevated protein levels of CGB after symptom onset does not result from increased mRNA; in fact levels of mRNA were decreased in relation to disease severity (Figure 3a). One of the potential mechanisms by which CGB protein levels may be elevated is through decreased ubiquitination.

To determine if CGB is ubiquitinated, we treated SHSY5Y cells with increasing concentrations of MG132, a proteasome inhibitor. We observed that levels of CGB increased with increasing concentrations of MG132 (Figure 5a), indicating that CGB is, in fact, ubiquitinated. After ubiquitination of CGB was established, we then assessed whether the elevated CGB protein levels in EAE mice were the result of decreased ubiquitination. Immunoblot analysis of spinal cord lysates from EAE mice 11 and 12 DPI showed that global ubiquitination was not altered relative to same-day control mice (Figure 5b). However, when ubiquitin was immunoprecipitated from spinal cord lysates of EAE mice 11 and 12 DPI, less CGB was co-immunoprecipitated when compared to same-day control mice (Figure 5c). Additionally, when spinal cord lysates from control mice and EAE mice 11 and 12 DPI were subjected to a deubiquitinating enzyme (DUBs) activity assay, we observed that there was increased DUBs activity with increasing disease severity (Figure 5e). No significant difference was observed in EAE mice 1 to 10 DPI (Figure 5d). Increased DUBs activity has been linked to decreased ubiquitination (Kowalski et al., 2011). These results suggest that CGB is less ubiquitinated and therefore contributes to the accumulation of CGB in EAE mice.

Elevated CGB levels in symptomatic EAE mice also result from defective CGB processing and secretion

In addition to its role as a calcium binding protein and InsP3R binding protein, CGB is also proteolytically cleaved and secreted from cells (Laslop et al., 1998). Furthermore, decreased CGB peptides were found in the CSF of MS patients (Mattsson et al., 2007). The increased accumulation of CGB and its cleaved fragments (Figure 1a) indicate that CGB processing

and secretion are defective. These data demonstrate that, in addition to decreased ubiquitination, defective secretion of CGB may be occurring in symptomatic EAE mice.

To determine if decreased secretion is occurring, we used a trypsin-like activity assay. Trypsin-like activity has been found to play a role in regulating secretion in cells. Increased trypsin-like activity has been shown to decrease secretion of cytokines (Steffen et al., 2000), and increased trypsin-like activity has been found to decrease synaptic vesicle pools (Willeumier et al., 2006) and release of neurotransmitters (Rinetti and Schweizer, 2010). We subjected spinal cord lysates from control and EAE mice 11 and 12 DPI, to a trypsin-like activity assay to determine if decreased secretion leads to elevated levels of CGB. We found that trypsin-like activity increased by $69 \pm 24\%$ (Figure 6b) in EAE mice with a score greater than 2 when compared to controls, demonstrating that decreased secretion of CGB occurs with increasing disease severity. These results suggest that in symptomatic EAE mice, decreased secretion of CGB contributes to the accumulation of CGB in neurons.

Elevated CGB levels in EAE mice could contribute to the neuronal and axonal damage observed in EAE mice

Dysregulation of intracellular calcium is hypothesized to lead to neurodegeneration in MS. The InsP3R is the predominant intracellular calcium channel in neurons that regulates calcium signaling (Bezprozvanny and Ehrlich, 1995). It was previously found that in areas with little InsP3R, CGB levels are elevated to compensate for the decreased InsP3R expression (Nicolay et al., 2007). Additionally, subcellular changes in intracellular CGB expression lead to significantly altered calcium release kinetics and calcium signal initiation sites (Choe et al., 2004; Jacob et al., 2005; Schmidt et al., 2011). For instance, overexpression of CGB causes an increase in InsP3R mediated-calcium release. Due to these results, it is likely that the accumulation of CGB leads to elevated cytosolic calcium levels.

To test if InsP3R expression is altered to compensate for elevated CGB, we performed RT-PCR and western blotting on spinal cord tissue harvested from control and EAE mice 1 to 12 DPI. InsP3R type I mRNA levels were increased by $46 \pm 13\%$ in EAE mice at 5 DPI when compared to same-day controls (Figure 7a). mRNA levels then began to decrease at 6 to 10 DPI, with a significant decrease of $40 \pm 7\%$ at 9 DPI (Figure 9a). We next looked at InsP3R type I protein levels in spinal cord tissue isolated from control mice and EAE mice 1 to 12 DPI. Immunoblot analysis of InsP3R type I in EAE mice showed that InsP3R type I protein levels increased significantly by $90 \pm 16\%$ at 3 DPI (Figure 7b). At 11 and 12 DPI, when mice developed symptoms and when disease scores can be assigned to symptomatic mice, InsP3R type I protein levels in EAE mice correlated with increased disease severity (Figures 7c and 7d). EAE mice with no clinical symptoms, a score of 0, had no significant change in InsP3R type I levels when compared to their same-day controls. In contrast, mice with a score of 2 or above had a decrease of $81 \pm 17\%$ when compared to their same-day controls. It is interesting to note that InsP3R protein levels are inversely correlated with CGB protein levels, which supports results obtained from prior studies (Nicolay et al., 2007).

When symptoms develop in EAE mice at 11 and 12 DPI, a decrease in InsP3R type I protein levels is observed even though mRNA levels remain unaltered. This decrease in InsP3R protein levels may be the result of increased cleavage of the InsP3R by calpain, whose activity has been shown to be elevated in EAE mice (Guyton et al., 2005). The cleaved InsP3R isoform can then create a calcium leak in the ER (Kopil et al., 2011), and thus contribute to EAE disease progression by increasing cytosolic calcium levels. To determine if calpain activity is elevated in EAE mice upon disease onset, we subjected spinal cord lysates from control and EAE mice 11 and 12 DPI to a calpain activity assay. We observed that calpain activity increased by $16 \pm 4\%$ in EAE mice with a score greater than 2 when

compared to controls (Figure 8b). We also noted that calpastatin levels increased significantly by $354 \pm 118\%$ in EAE mice with a disease score greater than 2 when compared to control mice (Figures 8c and 8d). EAE mice with a score of 0 showed a decrease of $56 \pm 16\%$ in calpastatin levels when compared to controls (Figures 8c). The increase in calpastatin follows with the increase in calpain activity, as increased calpain turns on calpastatin (Averna et al., 2003). These results suggest that the elevated calpain activity leads to increased cleavage of the InsP3R in symptomatic EAE mice. The cleaved InsP3R isoform can then create a calcium leak that would contribute additional calcium to the already increased cytosolic calcium concentration of a diseased EAE neuron.

Discussion

This study identifies CGB as a potential biomarker that can be used to (1) predict the onset and severity of MS prior to development of symptoms, and (2) to monitor disease progression. Additionally, we show that elevated CGB may have a role in the pathophysiology of MS. Using a mouse model of MS, we show that CGB protein levels are elevated in neurons prior to onset of symptoms, and that once symptoms develop CGB protein levels increase with disease severity. We also demonstrate that the initial elevation in CGB, prior to symptom onset, is linked to inflammatory processes such as increased TNF- α production by infiltrating T-cells and active microglia. Upon development of symptoms, CGB accumulation in neurons result from decreased ubiquitination and decreased secretion. mRNA levels, on the other hand, do not correlate with the elevated CGB levels and are decreased in relation to disease severity. Furthermore, we found that InsP3R type I protein levels correlate negatively with CGB protein levels and disease severity.

Although many biomarkers have been identified to monitor the progress of MS, there are currently no biomarkers that can predict the onset and severity of MS. The identification of a predictive biomarker is particularly important for those who are at high-risk for developing MS, as early preventative strategies could thwart the course of disease progression. With the use of a predictive biomarker, one could intervene prior to onset of disease symptoms and could ensure favorable long-term outcomes in patients with MS (Harris and Sadiq, 2009).

We have identified CGB as a potential biomarker for predicting the onset and severity of MS and for monitoring disease progression of MS. Before symptoms even develop in EAE mice, CGB protein levels and mRNA are elevated significantly. However, even though all mice were equally induced to develop EAE, only some mice show altered CGB protein or mRNA levels. This is important to note, as in this model of EAE, not all EAE mice develop symptoms at the same rate or of equal severity. When mice do develop symptoms, the mice with a score of 0 have CGB protein and mRNA levels similar to that of control mice. Mice with a score of 2 and greater, however, had obvious increases in protein levels and significant decreases in mRNA levels.

Although we are not able to correlate symptoms with CGB levels at the pre-symptomatic stage of disease development, it is very likely, based on data from the symptomatic stage, that altered CGB levels at the early stages correlate with progression and onset of disease. As a result, CGB is a potential biomarker for predicting onset and severity of MS and for monitoring disease progression, due to the fact that: (1) prior to onset of symptoms, CGB protein and mRNA levels are altered in only a few EAE mice, (2) only symptomatic EAE mice have altered CGB protein and mRNA levels correlating with disease severity, and (3) decreased levels of CGB peptides were found in CSF of MS patients. Moreover, the fact that CGB is proteolytically processed and secreted from cells (Laslop et al., 1998) makes CGB an ideal biomarker; especially since the cleavage products of CGB have already been shown

to be easily detected in the serum of patients with pheochromocytomas (Stridsberg and Husebye, 1997) and in the CSF of MS patients (Mattsson et al., 2007).

Aside from its role as a biomarker, elevated CGB protein levels may also have a role in the pathophysiology of MS. As mentioned earlier, one of the factors leading to neuronal/axonal degeneration in MS is dysregulation of cytosolic calcium levels. Our study demonstrates that in symptomatic EAE mice, a dramatic accumulation of CGB occurs. This increase in CGB likely contributes to the elevation of cytosolic calcium levels, as demonstrated by prior studies (Choe et al., 2004; Jacob et al., 2005; Schmidt et al., 2011). The elevation in cytosolic calcium then turns on calcium dependent proteases, namely, calpain. Calpain is then able to cleave a variety of proteins, one of them being the InsP3R type I. Cleavage of InsP3R creates a calcium leak in the ER and further contributes to the already elevated cytosolic calcium levels. Additionally, we show that prior to onset of symptoms, the elevated CGB levels may be due to inflammatory processes induced by cytokines such as TNF-alpha. Later, when symptoms develop, CGB accumulation results from decreased ubiquitination and dysregulation of secretory processes. We can therefore propose a potential model of how acute neuronal/axonal degeneration may occur in MS using data from this study and supporting literature.

In a healthy neuron (Figure 9a), CGB facilitates the formation of secretory vesicles (Chanat et al., 1993; Schmidt et al., 2011), is ubiquitinated, is secreted, and positively modulates the activity of the InsP3R (Choe et al., 2004). CGB also alters cellular calcium kinetics such as peak calcium release, total calcium release, and calcium signal initiation sites (Choe et al., 2004; Jacob et al., 2005; Schmidt et al., 2011).

Upon induction of EAE and prior to onset of symptoms (Figure 9b), inflammatory cytokines are released by activated microglia and infiltrating T-cells (Imitola et al., 2005). TNF-alpha, in particular, is critical for early stages of disease progression as it is proinflammatory during disease initiation but has disease suppressive properties at later stages (Kassiotis and Kollias, 2001). We have shown that in the pre-symptomatic stage of EAE, the inflammatory properties of TNF-alpha may lead to an increase in CGB mRNA and protein levels; the effects of TNF-alpha on CGB mRNA at the later stage of disease is counterbalanced by other molecular mechanisms. Additionally, levels of InsP3R mRNA and protein are increased at the early stages of disease, which is likely due to regulation of InsP3R by TNF-alpha (Park et al., 2009). A trend indicating increased calpain activity (Figure 8a) and decreased trypsin-like activity (Figure 7a) is observed at the early stages of disease as well.

When symptoms do develop (Figure 9c), CGB protein accumulates in the neuron (in relation to disease severity) and InsP3R protein levels decrease (also in relation to disease severity). The increase in CGB results from (1) decreased ubiquitination (2) dysregulated processing/secretion, as observed by a buildup of cleaved CGB fragments in diseased mice (Figure 1a). The defective proteolytic processing and secretion of CGB is caused by an increase in trypsin-like activity, and may also be due to altered expression of CPE. CGB's link to CPE is due to the fact that CGB is proteolytically processed by prohormone convertases 1 and 2 (PC1 and PC2) and CPE (Che et al., 2001). CPE, in particular, regulates vesicular sorting and transport, and its expression was found to decrease as the severity of EAE increases (Ibrahim et al., 2001). Moreover, the proposed decreased secretion of CGB in our EAE mice is supported by the study demonstrating decreased CGB peptides in the CSF of MS patients (Mattsson et al., 2007).

The dramatic increase in CGB protein levels upon symptom onset would lead to an amplification of calcium release from the ER (Choe et al., 2004; Jacob et al., 2005; Schmidt et al., 2011). This is further compounded by an increase in calpain-cleaved isoforms of the

InsP3R, leading to a calcium leak in the ER, and a reduction in levels of plasma membrane calcium ATPase 2 (PMCA2) (Nicot et al., 2005). As calpain only cleaves the InsP3R between residues 1917 and 1918, in the cytoplasmic domain, the transmembrane domain is intact and CgB would still be able to bind to the cleaved insP3R. Such a prolonged increase in intracellular calcium activates calcium dependent proteases, initiates proteolytic degradation of cytoskeletal components, and eventually result in axonal death (Wang et al., 2012).

In summary, this study has identified CGB as a potential biomarker that can predict the onset and severity of MS – a critical discovery, as there are currently no predictive biomarkers for MS. Additionally, we show that once symptoms develop, CGB can be used as a biomarker to monitor disease progression. Furthermore, we elucidated the mechanism by which CGB accumulates in neurons and examine the ramifications that elevated CGB levels would have in the neuronal/axonal degeneration that occurs in MS. The results of this study introduce a novel connection between CGB and MS pathophysiology that has the potential to be used clinically and for the development of better therapeutics for MS.

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Fig. 1. CGB is elevated in the CNS of EAE mice

(a) Representative western blot of spinal cords isolated from control (n=6) and EAE mice probed with CGB (top panel) and Gapdh (bottom panel); Gapdh was used as a loading control. EAE lanes grouped according to disease severity (left to right): 2.0 (n=3), 2.5 (n=5), 3.0 (n=4). Top bands at 105kD (arrowhead) represent the full length CGB protein and multiple lower bands represent cleavage products. (b) No significant change in levels of CGB was observed in the liver. Control, n=6 mice, EAE mice, n=12. Gapdh was used as a loading control. (c) CGB is expressed in the motor neurons of EAE mice, whereas little to none is expressed in control mice. (d) Spinal cord astrocytes do not show detectable levels of CGB in either control or EAE mice. Lumbar spinal cord sections from a control and an EAE 12 DPI mouse immunostained for CGB and the astrocyte marker GFAP. Both groups display no colocalization of CGB with GFAP. Images are representative of 2 independent experiments including 3–5 mice per group. Scale bar: 50µm (e) Cross section of the lumbar spinal cord shows that CGB (green) colocalizes with the InsP3R (red). The nuclei are stained with TO-PRO 3 (blue). Scale bar 50µm. (f) Representative images of longitudinal spinal cord sections from EAE mice at 12 DPI, immunostained for CGB (green), myelin basic protein (MBP, red) and stained for overall nuclei with DAPI (blue). CGB-positive neurons are abundantly found in spinal cord segments with peripheral demyelination and cellular infiltration into the grey matter (arrows) as opposed to neighboring, normal appearing regions. The bottom panel shows axons surrounded by their myelin sheath that contain different amounts of CGB (arrows). The myelin does not colocalize with CGB. Scale bar 5 µm.

Fig. 2. CGB protein is increased before symptoms develop

(a) CGB (105 kDa) is increased in some mice before development of symptoms, as indicated by the representative western blots of spinal cord from EAE mice 1–10 DPI when compared to their day controls. Each lane represents an individual mouse for a total of $n=5$ mice per group. Beta-actin was used as a loading control. CGB is increased when disease develops in EAE mice 11–12 DPI. For the representative western blot, each lane represents an individual mouse for a total of $n=5$ mice per group. Lanes 1–3 in the 11 DPI group and lanes 3 and 5 in the 12 DPI group, are EAE mice that had a disease score of 2 or above. The remaining lanes are mice that had a disease score of 0.

Fig. 3. CGB mRNA is altered in EAE mice

(a) CGB mRNA is increased significantly in EAE mice at day 5 and day 7 relative to their day controls. At day 11 and 12, CGB mRNA decreases. $n=5$ for each EAE and control day group. (b) CGB mRNA decreases significantly in relation to disease severity upon onset of disease symptoms. (c) CGB mRNA levels are variable as indicated when the percent change in mRNA levels in EAE mice were plotted relative to their day controls. mRNA levels were increased significantly in some mice at 1–8 DPI compared to their day controls.

Fig. 4. CGB upregulation in the spinal cord corresponds with the presence of inflammatory factors

(a) CGB is increased after addition with TNF- α in neuroblastoma cells, SHSY5Y. Data are mean $\pm$ SEM; * p <0.5, ** p <0.01, *** p <0.001. (b) Images of lumbar spinal cord sections from control mice, EAE mice at 7 DPI (no disease symptoms), and EAE mice at 12 DPI with grade 3. All sections were stained for CGB (green), microglia (CD11b, red) and overall nuclei (TOPRO-3, blue). CGB levels are low and homogenous in normal appearing white matter of control mice and mice at 7 DPI. In EAE mice at 12 DPI, CGB is increased at small sites of microglia accumulation and parenchymal cell infiltration (arrows). Multiple inflammation sites in the white matter and individual cells in the grey matter show strong CGB staining. (c) High-magnification images of the spinal cord ventral horn from a control and an EAE mouse. Upper panel: resting microglia demonstrate normal morphology of a small cell body and thin, ramified processes in control tissue. Resting microglia do not contain detectable levels of CGB. Lower panel: microglia in EAE spinal cord show a marked upregulation of CD11b and the morphology changes to a rounded, amoeboid appearance consistent with an activated microglial phenotype (magnified box). At this stage, CGB is strongly increased in microglia and nearby neurons. Images are representative of 2

independent experiments, each of which include 3–6 mice per experiment. **(d)** Lumbar spinal cord sections from control mice, mice at 7 DPI (no disease symptoms, and EAE mice at 12 DPI with grade 3, stained for CGB (green) and T-cells (CD3, red). Spinal cords of control mice and mice at 7 DPI display no T-cell infiltration in the white matter and low CGB staining. In the spinal cords of EAE mice 12 DPI extensive parenchymal T-cell infiltration and CGB upregulation is present. Magnified views show colocalization of CGB with CD3, indicating CGB is within the T-cells. Images are representative of 2 independent experiments, each of which include 3–5 mice per experiment. Scale bars – *WT, EAE 7 & 12 DPI*: 500µm (top panels), 250µm (lower panels), *EAE 12 DPI*: 50µm.

Fig. 5. Decreased ubiquitination accounts for CGB accumulation in EAE mice

(a) SHSY5Y cells show accumulation of CGB when treated with increasing concentrations of MG132 – indicating that CGB is ubiquitinated. $n=5$ per group (b) Global ubiquitination is unchanged in EAE mice 11 and 12 DPI relative to controls. $n=5$ per group (c) Immunoprecipitation of ubiquitin from spinal cord of EAE mice 11 and 12 DPI show decreased ubiquitination of CGB relative to controls. (d) DUB activity is elevated slightly in EAE mice 1–2 DPI compared to their day controls, and not significantly altered 3–10 DPI. $n=5$ mice per day. (e) DUB activity in EAE mice increases with increasing disease severity ($n=5$ per group). Data are mean $\pm$ SEM; * $p<0.05$.

Fig. 6. Decreased secretion of CGB likely results from increased trypsin-like activity

(a) Trypsin-like activity is decreased in EAE mice 1–4 DPI and unaltered 5–10 DPI relative to their day controls. $n=5$ mice per day. (b) Trypsin-like activity was increased in EAE mice that had a score greater than 2 ($n=5$). There was no significant change in mice with a score of 0 ($n=5$).

Fig. 7. Levels of InsP3R Type I are decreased in relation to disease severity

(a) InsP3R type I mRNA levels begin to increase in EAE mice 2–3 DPI, are significantly increased 5 DPI, and decreases 9 DPI, when compared to their day controls. $n=5$ mice per day. Gapdh mRNA was used as a control. (b) InsP3R type I protein levels increase in EAE mice 3 DPI, then decreases slightly 5 DPI, with an increase at 9 DPI. $n=5$ mice per day. Data are mean $\pm$ SEM; * $p<0.05$ and ** $p<0.01$. (c) Levels of InsP3R Type I are decreased in EAE mice with a score greater than 2 when compared to controls. No significant difference was observed in mice with a score of 0. Beta-actin was used as a loading control. (d) Representative western blot of spinal cord from EAE mice at 11 and 12 DPI probed with InsP3R Type I and CGB. Beta-actin was used as a loading control. Data are mean $\pm$ SEM; ** $p<0.01$.

Fig. 8. Increased calpain activity leads to decreased levels of InsP3R Type I

(a) Calpain activity is increased in EAE mice 1–4 DPI and decreased 5–10 DPI relative to their day controls. $n=5$ mice per day. (b) Calpain activity was increased in EAE mice that had a score greater than 2 ($n=5$). There was no significant change in mice with a score of 0 ($n=5$). (c) Levels of calpastatin were dramatically increased in EAE mice that had a score greater than 2. There was no significant change in mice with a score of 0. (d) Representative western blot of spinal cord from EAE mice at 11 and 12 DPI probed with calpastatin and CGB. Data are mean $\pm$ SEM; * $p<0.05$.

Fig. 9. Role of CGB in MS pathophysiology

(a) In a healthy neuron, CGB is secreted and ubiquitinated. (b) Prior to onset of symptoms, but when disease begins to manifest, inflammatory processes increase levels of CGB. (c) When symptoms develop, CGB accumulates in the neuron due to decreased ubiquitination and secretion (increased trypsin-like activity). Additionally the decrease in PMCA2 levels, increase in calpain-cleaved isoforms of the InsP3R, and the increase in CGB activation of the InsP3R create an increase in intracellular calcium levels. Excess intracellular calcium leads to axonal and neuronal degeneration.