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ORIGINAL ARTICLE

Chromogranin B: intra- and extra-cellular mechanisms to regulate catecholamine storage and release, in catecholaminergic cells and organisms

Kuixing Zhang,*,†,[1] Nilima Biswas,*,†,[1] Jiaur R. Gayen,*,† Jose Pablo Miramontes-Gonzalez,*,† C. Makena Hightower,*,† Maja Mustapic,*,† Manjula Mahata,*,† Chun-Teng Huang,*,† Vivian Y. Hook,*,† Sushil K. Mahata,*,† Sucheta Vaingankar*,† and Daniel T. O'Connor*,†

*Departments of Medicine and Pharmacology, and Institute for Genomic Medicine (IGM), University of California at San Diego, La Jolla, California, USA

†VA San Diego Healthcare System, La Jolla, California, USA

Abstract

Chromogranin B (CHGB) is the major matrix protein in human catecholamine storage vesicles. *CHGB* genetic variation alters catecholamine secretion and blood pressure. Here, effective Chgb protein under-expression was achieved by siRNA in PC12 cells, resulting in ~ 48% fewer secretory granules on electron microscopy, diminished capacity for catecholamine uptake (by ~ 79%), and a ~ 73% decline in stores available for nicotinic cholinergic-stimulated secretion. *In vivo*, loss of *Chgb* in knockout mice resulted in a ~ 35% decline in chromaffin granule abundance and ~ 44% decline in granule diameter, accompanied by unregulated catecholamine release into plasma. Over-expression of *CHGB* was achieved by transduction of a *CHGB*-expressing lentivirus, resulting in ~ 127% elevation in CHGB protein, with ~ 122% greater abundance of secretory granules, but only ~ 14% increased uptake of catecholamines, and no effect on nicotinic-triggered secretion. Human CHGB protein and its proteolytic fragments inhibited nicotinic-stimulated catecholamine release by ~ 72%. One conserved-region CHGB peptide inhibited nicotinic-triggered secretion by up to ~ 41%, with partial blockade of cationic signal transduction. We conclude that bi-directional quantitative derangements in CHGB abundance result in profound changes in vesicular storage and release of catecholamines. When processed and released extra-cellularly, CHGB proteolytic fragments exert a feedback effect to inhibit catecholamine secretion, especially during nicotinic cholinergic stimulation.

Keywords: chromogranin B, hypertension, adrenal, chromaffin, catecholamines.

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The sympathetic branch of the autonomic system exerts minute-to-minute control over the circulation. Sympathoadrenal catecholamine secretion is exocytotic (all-or-none), releasing not just catecholamines but also the proteins with which catecholamines are co-stored. The chromogranins/secretogranins comprise a family of acidic, soluble proteins that are widely stored in secretory granules with hormones, transmitters, and neuropeptides throughout the endocrine and nervous systems (Huttner *et al.* 1991; Taupenot *et al.* 2003).

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Address correspondence and reprint requests to Daniel T. O'Connor, Sucheta Vaingankar, or Sushil K. Mahata, Department of Medicine and IGM, UCSD School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0838, USA. E-mails: doconnor@ucsd.edu; svaingankar@ucsd.edu; smahata@ucsd.edu

[1]These authors contributed equally to this study.

Abbreviations used: BP, blood pressure; CHGA, chromogranin A; CHGB, chromogranin B; cPPT, central polypurine tract; DBP, diastolic blood pressure; HBSS, Hank's balanced salt solution; hCHGB, human CHGB; hPGK, human phosphoglycerate kinase; HPLC-ECD, high-performance liquid chromatography coupled to an electrochemical detection; HR, heart rate; IPTG, isopropylβ-D-1-thiogalactopyranoside; KO, knock-out; LC-MS/MS, C-18 reverse-phase liquid chromatography, followed by tandem mass spectroscopy; LDCV, large dense-core vesicle; LTR, long terminal repeat; MOI, multiplicity of infection; PC12, rat pheochromocytoma cell line; PCR, polymerase chain reaction; QTL, quantitative trait locus; SBP, systolic blood pressure; siRNA, small interfering RNA; T2A, 2A peptide, self-cleavage; TEM, transmission electron microscopy; UTR, untranslated region; VSV, vesicular stomatitis virus; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

Chromogranin B (CHGB), first described in 1980s, (Falkensammer *et al.* 1985; Rosa *et al.* 1985) seems to be the quantitatively most abundant matrix protein in the core of human catecholamine storage vesicles (O'Connor *et al.* 1984; Schober *et al.* 1987). Expression studies in chromaffin cells suggest that CHGB plays a functional role in secretory vesicle biogenesis (Huh *et al.* 2003).

CHGB may have both extra-cellular and intra-cellular roles in the neuroendocrine system. Extracellular roles for CHGB are dependent on its proteolytic processing within secretory granules (Gill *et al.* 1991) to form smaller peptides; such peptides may have a role in the neuroendocrine/sympathoadrenal stress response to systemic infection (Taupenot *et al.* 2003), and CHGB fragments may also play an autocrine inhibitory role on the release of co-stored hormones, such as insulin (Karlsson *et al.* 2000). Within chromaffin cells and sympathetic axons, CHGB functions in sorting and trafficking of peptide hormone and neuropeptide precursors to secretory granules (Natori and Huttner 1996), perhaps as triggers to secretory granulogenesis (Huh *et al.* 2003).

Hypertension is a complex trait in which deranged autonomic control of the circulation may be an early etiological culprit (Binder 2007). Expression of *CHGB* may mark the action of quantitative trait locus (including the *CHGB* locus itself) influencing exocytotic sympathoadrenal activity (Greenwood *et al.* 2004, 2006). *CHGB* is over-expressed in rodent models of genetic (Schober *et al.* 1989; O'Connor *et al.* 1999) as well as acquired (Takiyyuddin *et al.* 1993) hypertension, thus suggesting augmented sympathoadrenal activity in the pathogenesis of these syndromes. Therefore, *CHGB* gives rise to early, pathogenic 'intermediate phenotypes' (Lillie and O'Connor 2006) for exploration of sympathoadrenal activity in human essential hypertension. Indeed, we reported that two common *CHGB* promoter SNPs, A-296C (rs236140) and A-261T (rs236141), are strongly associated with hypertension in the population (Zhang *et al.* 2009, 2010), while in *CHGB* knockout (*CHGB*[−/−]) mice, we observed substantial elevations in both systolic blood pressure and diastolic blood pressure (Zhang *et al.* 2009). In this report, we have studied how *CHGB* quantitative variation (both over- and under-expression) affects not only secretory granulogenesis, but also catecholamine uptake, storage, and exocytotic release. We also describe the effects of novel CHGB fragments upon catecholamine release and its nicotinic cholinergic signal transduction pathway.

Methods

Chromaffin cell biology

Rat pheochromocytoma (PC12) cells were cultured in a medium containing Dulbecco's modified Eagle's medium with high glucose (cat#: 11965; Invitrogen, Carlsbad, CA, USA), 10% horse serum, 5% fetal bovine serum, and 1× penicillin/streptomycin/glutamine. The cells were cultured in an incubator with 6% CO_2 at 37°C, and cell passage number (since initiation of the line) was between 10 and 25 in these experiments.

Secretion of catecholamines from chromaffin cells

Norepinephrine secretion from PC12 cells was assayed as described previously (Biswas *et al.* 2008). After washing, cells treated with nicotine (60 μM; for nicotinic cholergic stimulation) or KCl (55 mM; for membrane depolarization) in physiological secretion medium. Each experiment was repeated three times.

Transmission electron microscopy

Transmission electron microscopy on cells/tissues was conducted as previously described (Courel *et al.* 2006).

Silencing *CHGB* expression by small interfering RNA (siRNA) in PC12 cells

We used a rat *CHGB* siRNA siGENOME 'smart pool' (Cat# M-099320-00; Dharmacon). One day before transfection, PC12 cells were grown on poly-L-lysine-coated 12-well plates and transfected for 48 h with 30, 15, or 7.5 nmol/well siRNA using the Transfectin reagent (Bio-Rad Laboratories, Hercules, CA, USA). Silencing of *CHGB* expression was evaluated by immunoblotting at the protein level and real-time PCR at the mRNA level. A control siRNA (Cat#: D-001210-01-05; Dharmacon) pre-designed to minimize off-target effects was used as a transfection negative control. The effect of reduced expression of *CHGB* on dense-core secretory granule abundance was examined by transmission electron microscopy (EM). The anti-CHGB antibody (C-19; catalog #: sc-1489; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was a goat polyclonal IgG (provided at 200 μg/mL, and used at 1 : 3000) for detecting CHGB of rat or human origin.

CHGB ablation *in vivo*: knockout mice, transmission EM, and catecholamine release

Mouse studies were conducted with the approval of the UCSD Animal Subjects Committee, and the ARRIVE guidelines were followed. *CHGB* knockout mice were generated from mouse embryonic stem cells by homologous recombination, as previously described (Zhang *et al.* 2009; Diaz-Vera *et al.* 2010). The effect of *CHGB* knockout on dense-core secretory granule abundance was examined by transmission EM. Plasma catecholamines were investigated by high-performance liquid chromatography coupled to an electrochemical detection (HPLC-ECD) system.

Over-expression of CHGB in PC12 cells by transduction of a *CHGB* lentivirus

Human cDNA clone h*CHGB*-pCMV6XL5 (Cat#: SC119004; Origene, Rockville, MD, USA) was used as template. The 5′ primer, 5′- agcCCCGGGatgcagccaacgctgcttct -3′, includes a *Sma*I site (capitalized), while the 3′ primer, 5′- caaGTCGACtcagccccttggctgaatt -3′, includes a *Sal*I site (capitalized). The 2034 bp PCR product was digested with *Sma*I and *Sal*I (New England Biolab), purified (Cat#: 28074; Qiagen, Valencia, CA, USA), and ligated into corresponding site of SIN18-human phosphoglycerate kinase (hPGK)-tdTomato-2A peptide, self-cleavage (T2A) vector to generate SIN18-hPGK-tdTomato-T2A-hCHGB

lentiviral vector plasmid (Figure S1). The SIN18-hPGK-tdTomato is used as control lentiviral vector.

The standard operation procedure of lentivirus production is as described (Tiscornia *et al.* 2006). Briefly, three plasmids (the lentiviral vector plasmid, the packaging plasmid pCMVDR8.74, and the vesicular stomatitis virus glycoprotein expression plasmid pMD.G) are mixed in a ratio of 3 : 2 : 1 for transient transfection into HEK293T cells using the calcium phosphate method. Unconcentrated viral supernatant is collected every 24 h up to 4 days post-transfection, and a commercially available serum-free ultraculture medium (Bio-Whitttaker #12-725F) with 1 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin is used to refeed the transfected plate. The pooled viral supernatant is filtered through 0.22 μm pores, followed by concentration and purification by 20% sucrose gradient centrifugation at 21 000 rpm for 2 h. The viral lysate is resuspended in 1× Hank's Balanced Salt Solution, followed by flow cytometry titration in 293T cells.

For lentiviral transduction, concentrated viral supernatant was added into PC12 cells at a multiplicity of infection of 0.5, and incubated at 37°C overnight. Cells were subjected to culture, experiment, or analysis 72 h post-transduction.

Flow cytometry

PC12 cells grown on 100-mm plates and infected with the lenti-hPGK-tdTomato-T2A-h*CHGB* or lenti-hPGK-tdTomato-T2A control viruses were suspended in Accutase detachment solution (Innovative Cell Technologies) at a concentration of 2×10^6 cells/mL. Cells (7×10^6/sample) were analyzed for tdTomato fluorescence using a MoFlo cell sorter (DakoCytomation). Fluorescence-activated cell sorting achieved the isolation of lentivirus-infected cells. An aliquot of each cell sample was processed for immunocytochemistry (anti-HA monoclonal antibody) to evaluate the expression of *CHGB*, and the remaining cells were processed for characterization by biochemistries and imaging by transmission electron microscopy.

Prokaryotic expression and purification of CHGB: pET-hCHGB-6-His

Human *CHGB* cDNA encoding the mature protein (minus the 20-residue signal peptide) was excised by PCR from the eukaryotic expression plasmid h*CHGB*-pCMV6XL5 (Cat#: SC119004, Origene) as template. The 5′ primer, 5′-gtcCATATGatgccagtggataacaggaa-3′ is preceded by a *Nde*I site (capitalized) and the 3′ primer, 5′-tgaGTCGACgccccttggctgaatttct-3′, is preceded by a *Sal*I site (capitalized). The PCR product was digested with *Nde*I and *Sal*I (New England Biolab) and gel-purified (Cat#: 28074; Qiagen). The resulting fragment of 1974 base pairs was directionally cloned into the expression vector, pET-21a-d (Novagen, Madison, WI, USA). The final construct was designated to encode the mature (i.e., minus signal peptide) human CHGB (657 residues) with addition of the residues VDKLAAALEHHHHHH at the carboxy-terminus (thus a total of 682 residues for the final recombinant protein). The nucleotide sequences of the PCR products and the junction regions between the insert and the vector were verified from both strands by direct dideoxy-capillary sequencing.

BL21(DE3)pLysS (Novagen) competent E.coli were transformed with the pET-(hCHGB-6-His) plasmid. Cells were induced with isopropyl β-D-thiogalactopyranoside; 1 mM, and grown in LB broth containing ampicillin (50 μg/mL) at 37 °C until an A_{600} of ~ 0.8 was reached. The recombinant protein was purified as described previously using its 6-His affinity tag (Taylor *et al.* 2000). Protein concentration was determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories). Aliquotted protein was stored at −70°C until further use.

Endoproteolytic cleavage of CHGB

Plasmin is serine protease found in chromaffin cells that can process chromogranin A (CHGA) to its bio-active catestatin fragment (Conlon *et al.* 1992; Parmer *et al.* 2000; Jiang *et al.* 2001). In order to liberate a 'catestatin-like' peptide from CHGB, we thus turned to plasmin as a protease. Purified human recombinant CHGB protein (10 μmol/reaction) was incubated with human plasmin (EC_3.4.21.7, 2 μmol/reaction; Calbiochem catalog #527624; specific activity 10 U/mg protein) in digestion buffer (10 mM Tris-HCl, pH 8.0; 0.15 M NaCl) at 37°C for 30 min. The reaction was terminated by the addition of aprotinin (2.5 μM). For secretion assay, the cells were treated with nicotine (60 μM) in secretion medium, either alone or in combination with recombinant CHGB (3.0 μM) full-length, or pre-digested by plasmin. The digestion reaction (1 h) contained CHGB (10 μM) and plasmin (2 μM) in a reaction volume of 120 μL of 10 mM Tris-Cl, pH 8.0 and NaCl 150 mM. After digestion, peptides were purified (and intact plasmin removed) by C18 cartridge (Waters, Milford, MA, USA), lyophilized, and suspended in water.

LC–MS/MS

In one experiment, plasmin-digested human CHGB (without added aprotinin) was subjected to LC-MS/MS, for identification of cleavage sites. Plasmin-digested peptides were analyzed by HPLC coupled to tandem mass spectroscopy (LC-MS/MS) using nanospray ionization, in a Triple TOF 5600 hybrid mass spectrometer (AB-SCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10 cm-100 micron ID glass capillary packed with 5-μm C-18 ZorbaxTM beads (Agilent Technologies, Santa Clara, CA, USA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–60%) of acetonitrile (ACN) at a flow rate of 250 μL/min for 1 h. The buffers used to create the ACN gradient were: Buffer A (2% ACN, 0.2% formic acid, and 0.005% TFA, in H_2O) and Buffer B (0.2% formic acid, and 0.005% TFA in ACN). MS/MS data were acquired as follows: MS-1 data were obtained for 250 ms at m/z of 400–1250 Da, while MS-2 data spanned m/z of 50–2000 Da. The independent data acquisition parameters were as follows: MS-1 TOF 250 ms, followed by 50 MS-2 events of 25 ms each, and independent data acquisition criteria of over 200 counts (threshold) and charge state +2 to +4, with a 4 s exclusion. Finally, collected data were analyzed by MASCOT® (Matrix Sciences) and Protein Pilot 4.0 (AB-SCIEX) for peptide sequence identification, with reference to the human CHGB amino acid sequence as template (UniProt P05060, NCBI NP_001810).

CHGB peptide synthesis

Five peptides were synthesized by solid-phase F-moc chemistry, with molecular mass verified by electrospray mass spectrometry (Mimotopes, Minneapolis, MN, USA). CHGB regions were selected

for a preponderance of sequence features bearing similarity to the catecholamine release-inhibitory CHGA fragment catestatin (human CHGA[352-372]) (Mahata *et al.* 1999; Mahapatra *et al.* 2005): basic isoelectric point (pI), basic amino acids (Arg, Lys; including dibasic or tribasic sites), hydrophobicity or aromaticity (Leu, Ile, Val, Phe, Tyr, His); amphiphilicity (alternating hydrophobic and cationic residue side chains); and relative abundance of the product after cleavage out of the CHGB precursor in chromaffin granules *in vivo* (Gupta *et al.* 2010; Hook *et al.* 2010; Wegrzyn *et al.* 2010). The synthetic sequences were as follows: hCHGB[60–67], KFEVRLLR; hCHGB[259–272], TRPRHHHGRSRPDR; hCHGB[337–354], LEWERYRGRGSEEYRAPR; hCHGB[389–419], RGLEPGKG-RHHRGRGGEPRAYFMSDTREEKR; and hCHGB[480–497], RFQDKQ YSSHHTAEKRKR.

Nicotinic cholinergic signal transduction: $^{45}Ca^{2+}$ uptake

PC12 cells were seeded onto poly-L-lysine–coated six-well polystyrene culture dishes 2 days before study. Cells were rinsed with 1 mL of secretion buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4) every 15 min for 1 h at 37°C. Then $^{45}Ca^{2+}$ was added (2 μCi/mL, 14.95 mCi/mg, DuPont-NEN) to Ca^{2+}-free buffer (secretion buffer without 2 mM $CaCl_2$), and drugs were to the labeled buffer at 37°C. The cells were incubated with labeled buffer under different conditions for 5 min at room temperature. Calcium uptake was stopped simultaneously in all six wells by inversion of the plate, so all wells were decanted together, followed by prompt addition of ice-cold release buffer containing 2 mL of 1 mmol/L $LaCl_3$ for termination of further uptake of extracellular labeled calcium. The culture dishes were then rinsed twice with ice-cold release buffer. One mL of cell lysis buffer was added to cells in each well and collected for liquid scintillation counting. The data were expressed as counts per minute per well.

Statistical analyses

Results are expressed as mean ± 1 SEM. The reported '*n*' refers to the number of replications. Data were evaluated by Student's *t*-test or ANOVA, followed by post hoc tests, in Excel (Microsoft, Bellevue, WA, USA) or SPSS-17 (Chicago, IL, USA). *p*-values < 0.05 were considered statistically significant.

Results

Silencing *CHGB* expression in chromaffin cells by siRNA

Introduction of rat *CHGB* siRNA into PC12 dose-dependently decreased CHGB protein expression on immunoblot. *CHGB* siRNA knocked down CHGB protein by 81% ($p = 0.02$) at 7.5 nM and > 95% at 15 nM ($p = 0.01$, Fig. 1a). In the catecholamine storage/synthesis pathway, after *CHGB* siRNA treatment real-time qPCR indicated that *CHGB* mRNA declined by 46% ($p < 0.05$), while these mRNAs increased: *Chga* (by 35% $p < 0.05$); and *Dbh* (by 243%, $p = 0.003$). There were no changes in mRNAs encoding *Scg2* or *Th* (Fig. 1b). The increase in *Dbh* transcription suggests negative feedback control by cellular catecholamine content, but the mechanism of such an effect is not established.

Effect of *CHGB* siRNA knockdown on catecholamine storage, uptake, or secretion in chromaffin cells

After *CHGB* siRNA, transmission electron micrographs indicated a ~ 48% less of dense core granule abundance in *CHGB* siRNA treated PC12 cells compared with control cells (Fig. 1c). There were 25.4 ± 2.7 granules per 100 μm^2 in control PC12 cells and 13.3 ± 2.5 granules per 100 μm^2 in *CHGB* siRNA PC12 cells ($p = 0.006$, Fig. 1d left). Average granule diameter was 63.4 ± 2.75 nm after *CHGB* siRNA, versus 92.8 ± 1.87 nm in control cells, thus declining by ~ 32% during knockdown ($p < 0.001$, Fig 1d right). Uptake of exogenous catecholamines in *CHGB* siRNA-silenced cells declined by ~ 78% compared to control ($p < 0.0001$, Fig. 1e). Secretion of catecholamines during nicotinic cholinergic stimulation also declined by ~ 75% ($p = 0.005$, Fig. 1e). Basal secretion of catecholamine was also diminished in *CHGB* siRNA-silenced cells ($p = 0.023$, Fig. 1e).

CHGB targeted ablation *in vivo*: *CHGB* knockout mice

Morphology (transmission EM of the adrenal medulla)

Transmission EM indicated that dense core granules in the adrenal medulla of *CHGB*(−/−) mice displayed both loss of abundance and decline in size. In *CHGB* knockout mice, the number of granules was 9.1 ± 0.55 per μm^2 in *CHGB*(+/+) mice versus 5.9 ± 0.59 per in *CHGB*(−/−) mice, thus declining by ~ 35% ($p < 0.001$, Fig. 2a). The average diameter of dense core granules was 173.1 ± 6.3 nm in *CHGB*(+/+) mice, declining by ~ 44% to 97.4 ± 3.6 in *CHGB*(−/−) mice ($p < 0.001$, Fig. 2a).

Catecholamine secretion in vivo

Here, we found that plasma concentrations of both norepinephrine and epinephrine were significantly higher in *CHGB* knockout mice, with norepinephrine increasing from 22.6 ± 3.4 to 36.9 ± 4.2 ng/mL in *CHGB*(−/−) mice, and epinephrine from 8.1 ± 0.9 to 14.7 ± 2.2 ng/mL: increments of ~ 1.6-fold for norepinephrine and ~ 1.7-fold for epinephrine (each $p < 0.05$, Fig. 2b).

Over-expression of *CHGB* in chromaffin cells by lentivirus transduction

After transduction by *CHGB*-lentivirus, over-expressing PC12 cells were selected by flow cytometry based on the tdTomato photoprotein red fluorescence (Figure S2). Transduction caused an increase of CHGB protein by ~ 127% compared to control cells (Fig. 3a), while the abundance of dense core granules increased from 22.2 ± 4.4 per high-power field (2.0×10^{-10} m^2) in control cells to 50.3 ± 8.3 in virus-infected cells ($p = 0.01$, Fig. 3b and c). The diameter of granules was unchanged, at 92.8 ± 1.87 nm in control cells versus 90.9 ± 2.98 nm in *CHGB* over-expressing cells. Over-expression of CHGB caused increased exogenous catecholamine uptake by ~ 14% ($p = 0.019$),

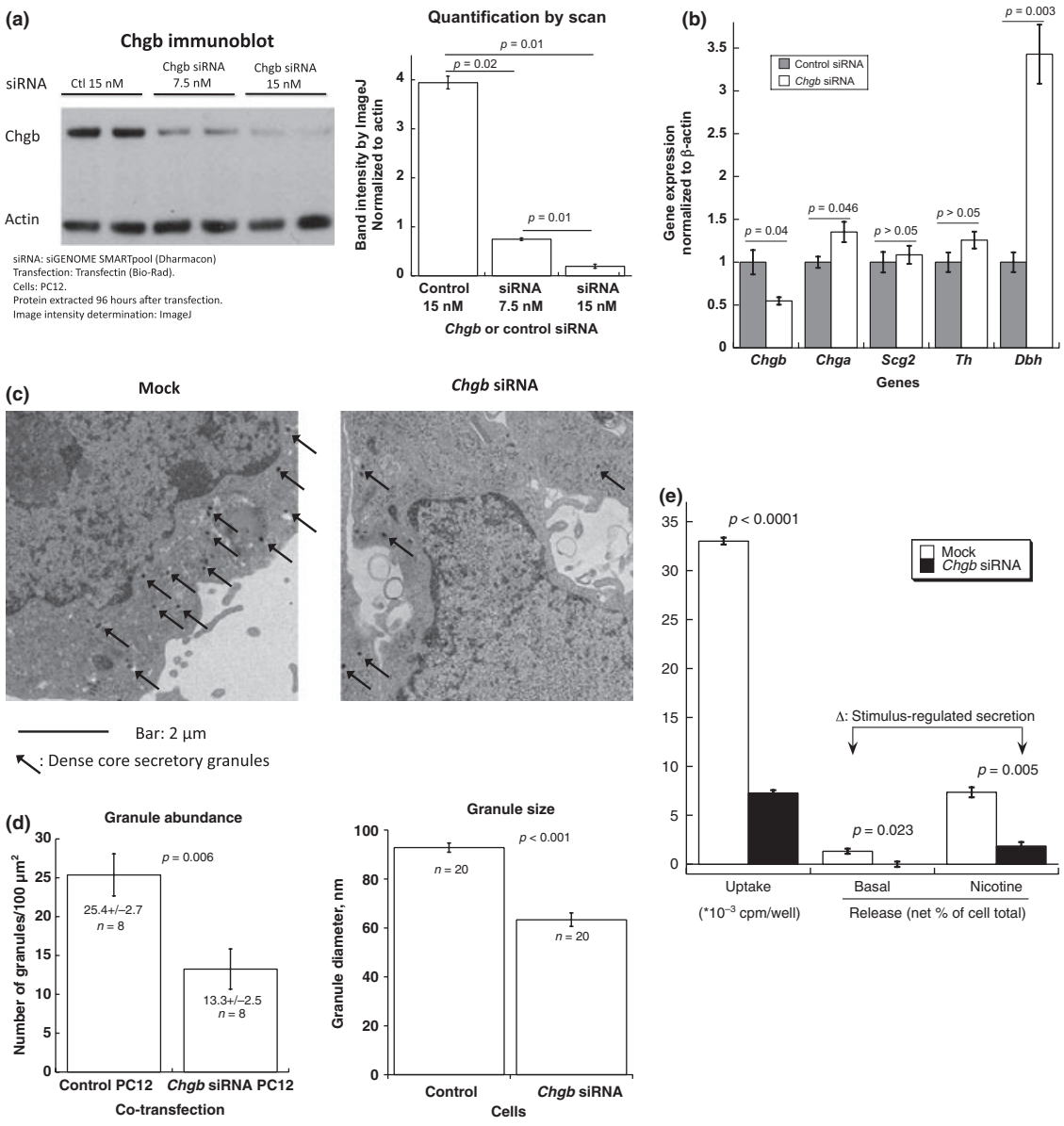

Fig. 1 Under-expression of *CHGB* by siRNA in chromaffin cells. (a) Left: Immunoblot of chromogranin B (CHGB) protein in siRNA-transfected PC12 cells. The rat *CHGB* siRNA siGENOME 'smart pool' was transfected into PC12 cells and silencing of *CHGB* expression was evaluated by immunoblotting at the target protein level. ~ 95% knockdown of *CHGB* expression was achieved by 15 nM siRNA. Right: Quantification of CHGB in the blot. Band intensity was scanned and scored by ImageJ. (b) Expression of catecholamine pathway (storage, synthesis) genes in *CHGB* siRNA-silenced PC12 cells. The rat *CHGB* siRNA siGENOME 'smart pool' was transfected at 15 nM into PC12 cells and transcript expression was scored by real-time qPCR, and normalized to the signal for β-actin. Each condition was repeated four times. During knockdown, the *CHGB* transcript was under-expressed, while *Chga* and *Dbh* were over-expressed, but *Scg2* and *Th* were unchanged. (c) Visualization of dense-core granules in *CHGB* siRNA-silenced PC12 cells by transmission electron microscopy (EM). PC12 cells were transfected with the rat *CHGB* siRNA siGENOME 'smart pool' at 15 nM and visualized by Zeiss EM10B electron microscope. CHGB siRNA-silenced PC12 cells display fewer dense-core granules than control (mock-transfected) cells. Arrowheads: Dense core secretory granules. Bar: 2 μm. (d) Quantification of dense-core chromaffin granule abundance after *CHGB* silencing by siRNA. The data come from (c). Left, granule number, $n = 8$ cells were counted for each condition. Right, granule diameter, $n = 20$ granules were measured for each condition. (e) Effect of *CHGB* silencing on exogenous catecholamine uptake and secretagogue-triggered release. PC12 cells were labeled with [^{3}H]-L-norepinephrine and treated with nicotine at 60 μM. The supernatant and cell lysates were collected to measure radioactivity. The uptake was expressed as counts in cell lysate. The percent (%) secretion was expressed as [amount released/(amount released + amount in cell lysate)] × 100. Each condition was repeated three times. CHGB-silenced PC12 cells displayed diminished uptake of exogenous catecholamine, as well as reduced release catecholamine when stimulated by nicotine.

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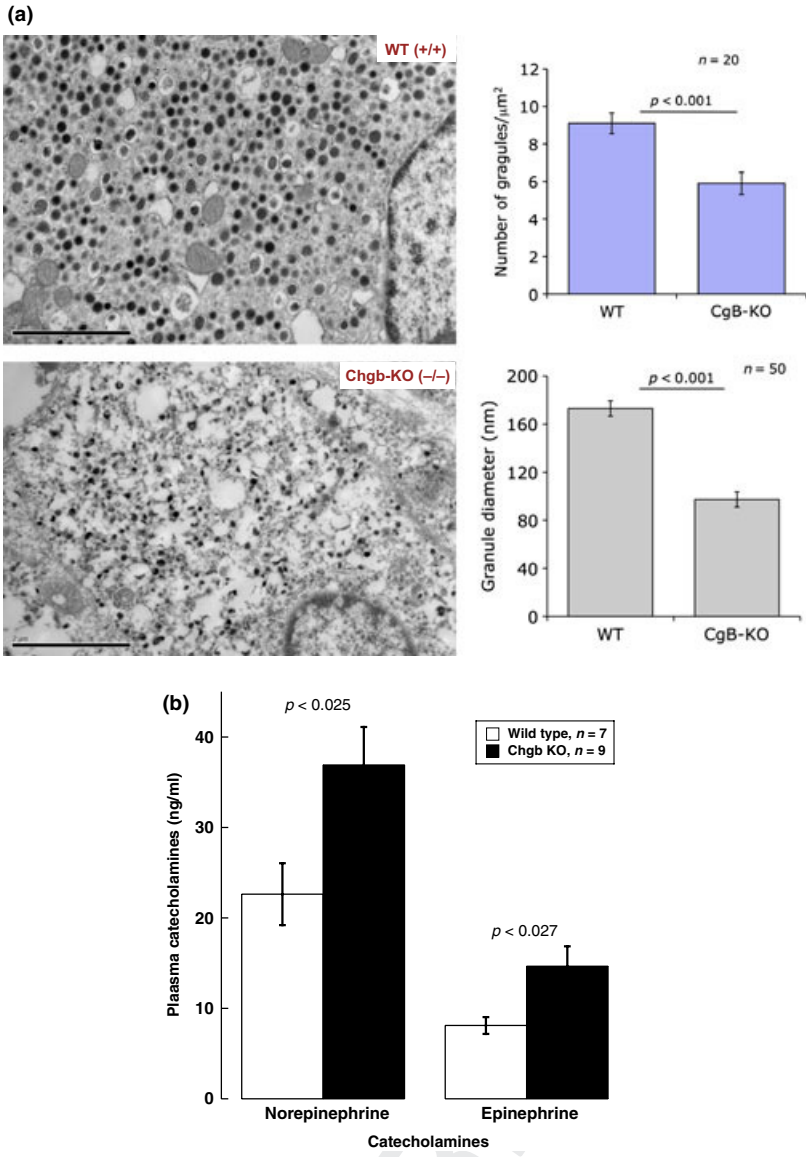

Fig. 2 Effects of *CHGB* targeted ablation *in vivo*: The *CHGB* ablation (–/–) mouse. (a) Left: Morphology of the adrenal medulla by transmission electron microscopy (EM). Visualization of dense-core granules in *CHGB* (+/+; top) versus *CHGB* (–/–; bottom) mice. Bars: 2 μm. Right: Quantification and diameter determination of dense-core chromaffin granule abundance in *CHGB* (+/+) versus *CHGB* (–/–). Both the number (n = 20 cells were counted for each condition) and size of granules (n = 20 granules were measured for each condition) declined in *CHGB* (–/–) mice. (b) Catecholamine secretion during *CHGB* ablation *in vivo*. Mouse plasma sample were collected and catecholamines were measured by high performance liquid chromatography coupled to an electrochemical detection (HPLC-ECD) system. Both norepinephrine and epinephrine in the circulation increased after *CHGB* ablation. (n = 7 for wild type and n = 9 for Chgb knockout).

but did not alter nicotinic cholinergic stimulation of catecholamine release ($p < 0.05$, Fig. 3d).

Extra-cellular effects of CHGB and its proteolytic fragments: catecholamine release from chromaffin cells

Both intact CHGB itself and its plasmin-digested fragments inhibited nicotinic cholinergic-stimulated catecholamine release, with ~ 53% reduction by CHGB itself and ~ 72% reduction by its fragments ($p < 0.01$, Fig. 4a, left). In contrast, CHGB or its fragments only moderately inhibited membrane depolarization-stimulated catecholamine release: ~ 23% reduction by CHGB and ~ 32% by its proteolytic fragments ($p < 0.01$, Fig. 4a, right).

Screening CHGB peptides for inhibition of nicotinic cholinergic secretion

The 5 synthetic CHGB peptides displayed a spectrum of inhibitory effects on nicotinic cholinergic secretion, with the shortest fragment CHGB[60–67] (KFEVRLLR) exerting the most prominent effect, reducing nicotinic stimulation's effect by ~ 41%. As a positive control, human catestatin (CHGA [352–372]) completely abrogated nicotine's effect (Fig. 4b, left). In contrast, synthetic CHGB peptides had little or no effect on catecholamine release when stimulated by membrane depolarization (Fig. 4b, middle), suggesting an element of nicotinic pathway specificity for the secretory effect.

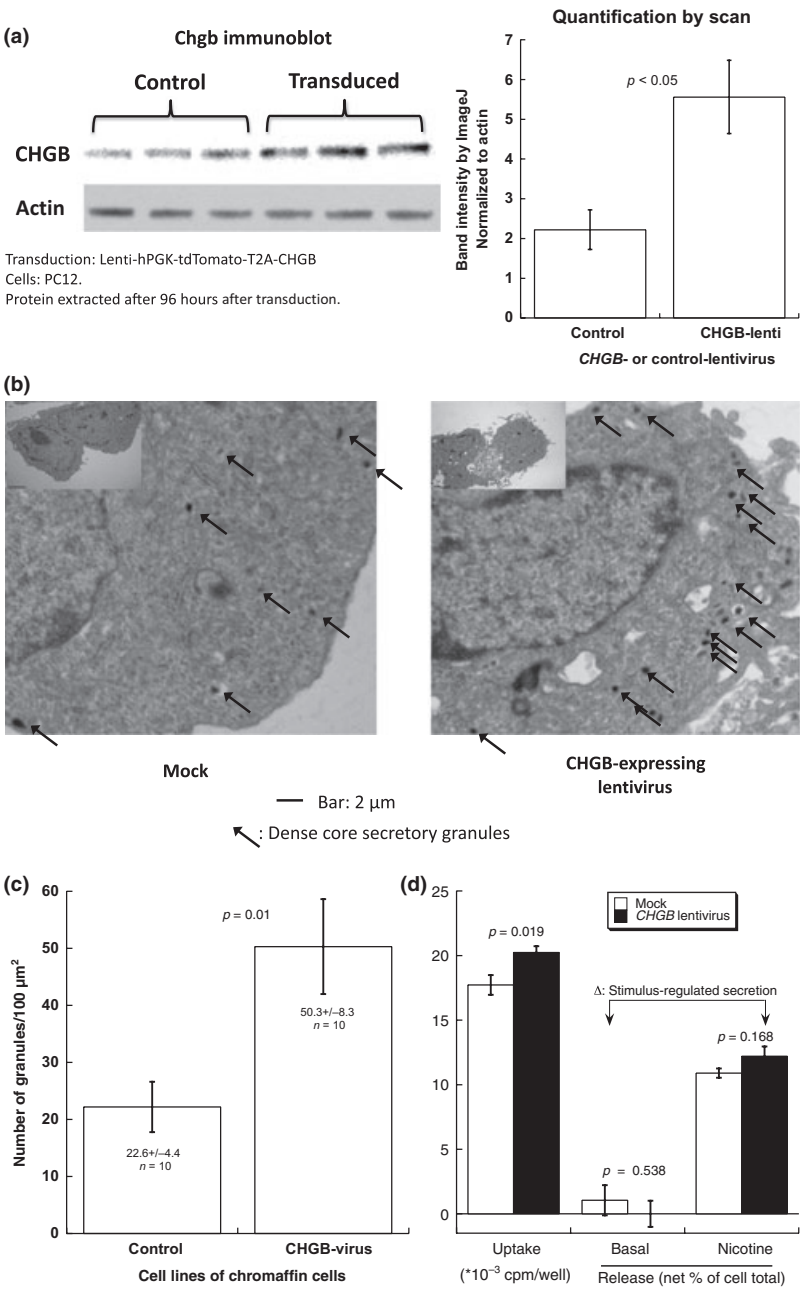

Fig. 3 Over-expression of *CHGB* by lentiviral transduction in chromaffin cells. (a) Left: Immunoblot of CHGB in human *CHGB*-lentivirus transduced PC12 cells. Right: Quantification of chromogranin B (CHGB) by scan. After 48 h of transduction, CHGB was quantified by immunoblot with goat polyclonal IgG (sc-1489; Santa Cruz Biotechnology). Actin was used as a housekeeping probe. A ~ 150% increase of CHGB protein was achieved by this transduction. Band intensity was scored by ImageJ. (b) Visualization of dense-core granules in *CHGB*-lentivirus-transduced PC12 cells, by transmission electron microscopy (EM). The main/larger image magnifications are 1/12 of the originals (insets shown in upper left corners). The magnification of the original image (inset) was 10 000×. The scale bar in the original (inset) images is 2 µm. Arrowheads: Dense core secretory granules. (c) Quantification of dense-core chromaffin granule abundance in h*CHGB*-lentivirus-transducted PC12 cells. The h*CHGB* transducted PC12 cells display more granules than control PC12 cells ($p = 0.01$, $n = 10$ cells were counted for each condition). Granule diameter was similar in both cell lines, at 92.8 ± 1.87 nm in control cells and 90.9 ± 2.98 nm in *CHGB* over-expressing cells. (d) Effect of CHGB over-expression on catecholamine uptake and release in PC12 cells. Each condition was repeated three times. After the transduction of CHGB-lentivirus, the uptake of exogenous catecholamine increased by ~ 14%, but nicotine-stimulated secretion did not change.

Potency and efficacy of CHGB peptide CHGB[60–67] (KFEVRLLR) to inhibit nicotinic cholinergic-stimulated catecholamine secretion, as well as its cationic signal transduction in chromaffin cells

The inhibitory effect of CHGB[60–67] on nicotinic cholinergic secretion was dose dependent in PC12 cells. The potency (IC[50]) was 4 µM for CHGB[60–67], versus 0.45 µM for catestatin, while the efficacy (maximal effect, at 10 µM peptide) was ~ 49% inhibiton for CHGB[60–67] versus ~ 100% for catestatin (Fig. 4b, right). Nicotine stimulated uptake of $^{45}Ca^{2+}$, an effect strongly (~ 82%) inhibited by catestatin, and moderately (~ 22%) inhibited by CHGB[60–67] ($p < 0.0001$, Fig. 4c). The inhibitory peptide sequence, CHGB[60-67] (K_{60}FEVRLLR$_{67}$), was 100% (i.e., perfectly) conserved across human (P05060), rat (O35314), and mouse (P16014) CHGB protein sequences (UniProt identifiers <http://www.uniprot.org>). This oligopeptide region is located within an amphiphilic domain (similar to the CHGA fragment catestatin) (Kennedy *et al.* 1998), and more specifically within an amphiphilic helical (or coiled-coil) patch, as previously described for both CHGA and CHGB (Mosley *et al.* 2007).

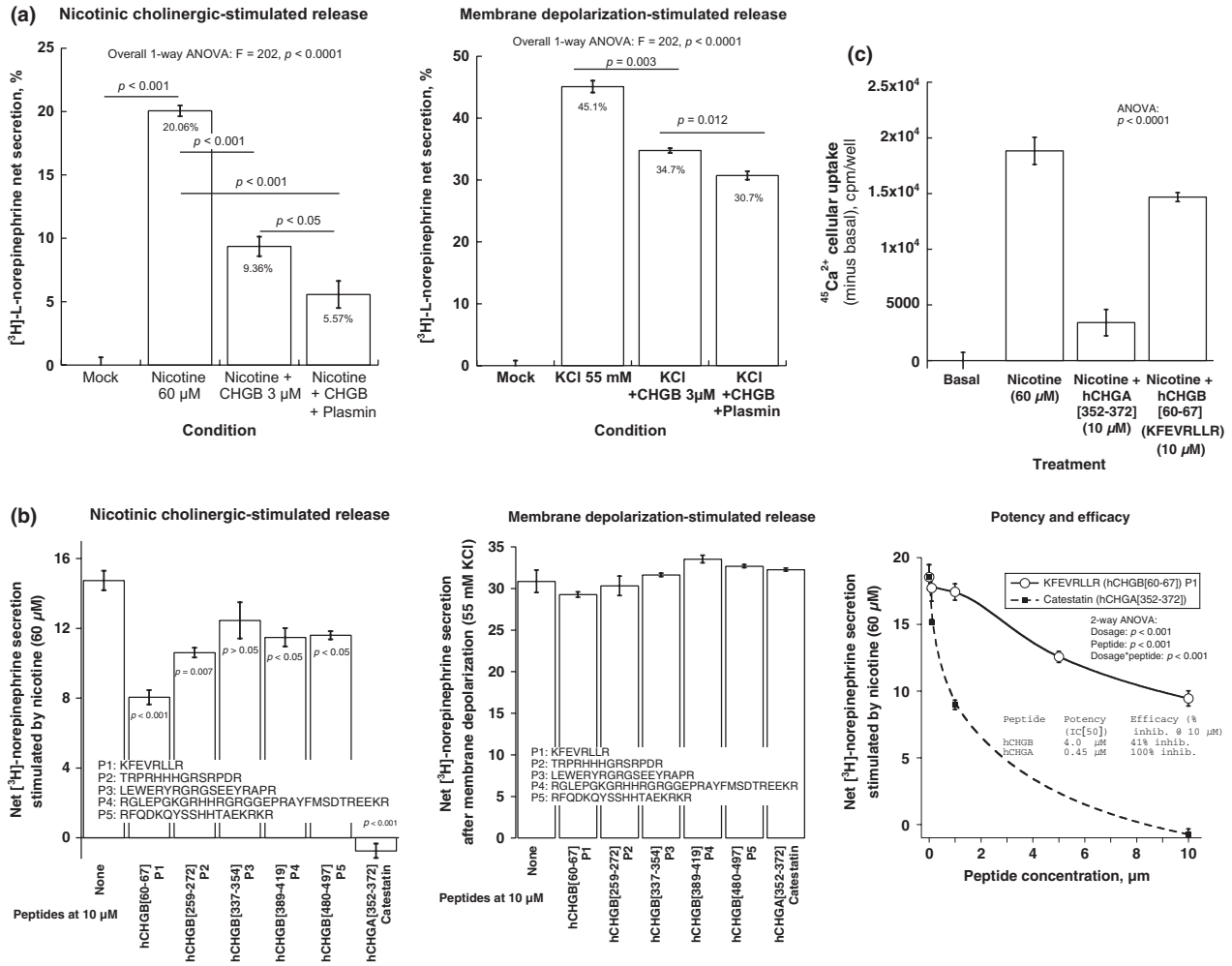

Fig. 4 Extracellular chromogranin B (CHGB) effects on catecholamine release. Each experiment was repeated three times. (a) Human CHGB protein or its proteolytically digested fragments on nicotinic cholinergic versus membrane depolarization (KCl) -stimulated catecholamine release. CHGB protein or its plasmin-digested fragments inhibited predominantly nicotine-stimulated catecholamine release. (b) Left: Synthetic CHGB peptides on nicotinic cholinergic-stimulated catecholamine release. Catestatin (human CHGA[352–372]) served as a positive inhibitory control. Peptide human CHGB[60–67] displayed the greatest inhibitory effect among these peptides. *p*-value is displayed for each peptide compared with negative control. Middle: Synthesized Human CHGB peptides on membrane depolarization (KCl)- stimulated catecholamine release. Catestatin (human CHGA[352-372]) served as a positive inhibitory control. No inhibitory effect was observed for these peptides ($p < 0.05$ for each peptide). Right: Potency and efficacy of CHGB[60–67] on nicotinic cholinergic-stimulated catechalmine release. Catestatin (human CHGA[352–372]) served as a positive control. Potency and efficacy of CHGB[60–67] are displayed. (c) CHGB peptide effect on nicotinic cholinergic cationic (Ca^{2+}) signal transduction in chromaffin cells. PC12 cells were treated with $^{45}Ca^{2+}$ at 2 μCi/mL and incubated under different conditions including basal, nicotine alone at 60 μM, and nicotine with hCHGA or hCHGB peptides. Catestatin (human CHGA[352–372]) served as a positive control inhibitor. Human CHGB[60–67] moderately (by ~ 22%) inhibited nicotine-triggered uptake of ^{45}Ca.

Endoproteolytic cleavage of CHGB in the region of the active secretory peptide

We analyzed plasmin-liberated human CHGB peptides by LC-MS/MS, to position the cleavage sites for this chromaffin cell endoproteolytic system. In the region of the active peptide (Table 1a), extensive cleavage was noted, primarily at monobasic sites (i.e., R or K), though also at dibasic site $R_{46}K_{47}$. As a control to explore endoprotease cleavage targets elsewhere in CHGB, we also evaluated a region ($CHGB_{565–596}$) containing three dibasic sites – $K_{566}R_{567}$, $K_{578}R_{579}$, and $R_{595}K_{596}K_{597}$ – that bound two putative peptides for liberation ($CHGB_{568–577}$ and $CHGB_{580–594}$); each of these three dibasic sites was utilized by plasmin, and the expected peptides were formed (Table 1b).

Table 1 Tandem MS on human CHGB after endoproteolytic cleavage by plasmin

Identified	Sequence	No. Obs.
(a) CHGB amino-terminal region harboring the active secretory peptide		
Peptide_1	--VLKTS*RK*DVKDKETTENENTKFEVR----------	1
Peptide_2	-----TS*RK*DVKDKETTENENTKFEVR----------	19
Peptide_3	-----TS*RK*DVKDKETTENENTKFEV----------	6
Peptide_4	-QVLKTS*RK*DVKDKETTENENTKFEVR----------	1
Peptide_5	---------DVKDKETTENENTKFEVR----------	4
Peptide_6	----------DKETTENENTKFEV----------	6
Peptide_7	----------DKETTENENTKFEVR----------	3
Peptide_8	----------DKETTENENTKFEVRL----------	6
Peptide_9	-----TS*RK*DVKDKETTENENTKFEVRL----------	10
Peptide_10	-----TS*RK*DVKDKETTENENTKFEVRLLRDPADASEAHESSSRGEAGAPGEEDIQGPTK-	2
CHGB_template	CRQVLKTS*RK*DVKDKETTENENTKFEVRLLRDPADASEAHESSSRGEAGAPGEEDIQGPTKAD	–
	| | | | | |	
Residue	40 50 60 70 80 90	
(b) Small CHGB carboxy-terminal region with three nearby dibasic sites		
Peptide_1	---LARVPKLDL*KR*QYDRVAQLDQLLHY---	3
Peptide_2	---LARVPKLDL*KR*QYDR----------	1
Peptide_3	-*R*NLARVPKLDL*K*----------	3
Peptide_4	--NLARVPKLDL----------	6
Peptide_5	--NLARVPKLDL*K*----------	13
Peptide_6	--NLARVPKLDL*KR*----------	1
Peptide_7	--NLARVPKLDL*KR*QYDR----------	4
Peptide_8	--NLARVPKLDL*KR*QYDRVAQLDQLLHY---	5
Peptide_9	--NLARVPKLDL*KR*QYDRVAQLDQLLHY*R*--	3
Peptide_10	---LARVPKLDL----------	3
Peptide_11	----------*R*QYDRVAQLDQLLHY---	7
Peptide_12	----------QYDRVAQLDQLLHY---	2
Peptide_13	----------QYDRVAQLDQLLHY*R*--	5
Peptide_14	----------*R*QYDRVAQLDQLLHY*R*--	8
CHGB_template	*KR*NLARVPKLDL*KR*QYDRVAQLDQLLHY*RKK*	–
	| | |	
Residue	570 580 590	

Alignments by Clustal Omega (1.2.0).

Legend: *Italics* = Paired basic residues; Underlined = Active peptide tested; No. Obs. = Number of times that peptide was observed in spectra.

Discussion

Overview

The chromogranin/secretogranin acidic proteins are mainly located within neuroendocrine secretory vesicles such as chromaffin granules and large dense-core vesicles of monoaminergic neurons (Huttner *et al.* 1991; Taupenot *et al.* 2003). In these organelles, chromogranins constitute the main protein component of the intravesicular matrix. An important property of chromogranins is their ability to bind solutes, thereby allowing large dense-core vesicles to accumulate large amounts of catecholamines while maintaining their osmotic stability (Helle *et al.* 1985; Videen *et al.* 1992), an effect essential for the maintenance of neurosecretory activity. Several functions have been proposed for chromogranin B (CHGB), including granule biogenesis and sorting (Natori and Huttner 1996; Huh *et al.* 2003), Ca^{2+} sequestration and release (Yoo and Jeon 2000), and a source of bioactive peptides (Strub *et al.* 1995; Winkler *et al.* 1998). Alterations in CHGB expression accompany several human diseases of disordered neurotransmitter storage or release, such as hypertension (Zhang *et al.* 2009, 2010, 2011), schizophrenia (Landen *et al.* 1999; Marksteiner *et al.* 2000), and Alzheimer disease (Marksteiner *et al.* 2002). While previous studies of CHGB abundance suggested alterations in storage granule abundance, quantitative studies of physiological processes underpinning catecholamine synthesis, uptake, storage, and secretagogue-triggered release have been lacking.

CHGB under- and over-expression in catecholaminergic cells, as well as *in vivo*

In PC12 cells, si-RNA silencing successfully diminished CHGB protein expression, with consequent reduction of chromaffin granule abundance, uptake of exogenous

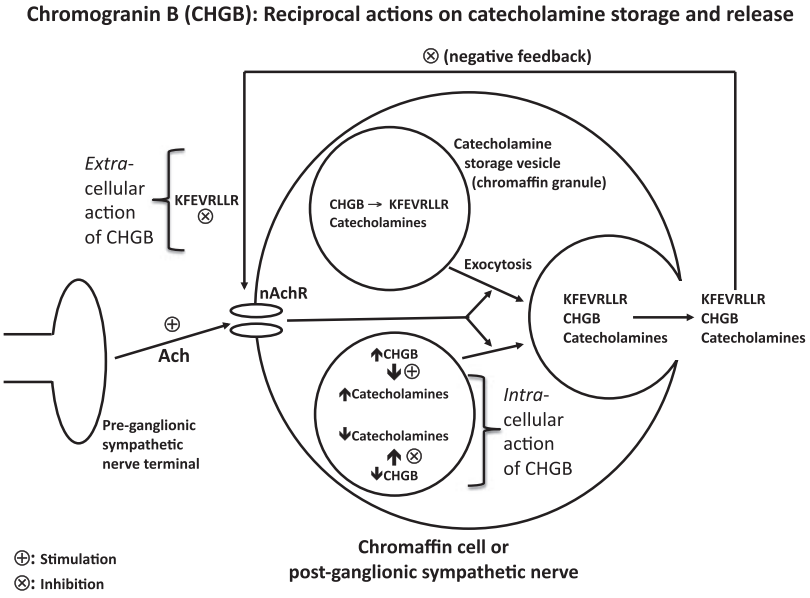

Fig. 5 Schema: Reciprocal actions of chromogranin B (CHGB) on catecholamine storage (stimulation) and release (inhibition). The figure synthesizes the consequences of our CHGB results for these physiological processes. Within chromaffin cells, CHGB participates in assembly of catecholamine secretory vesicles (dense-core granules), and governs the secretory capacity of these granules under the nicotinic stimulation. After proteolytic cleavage and release into the extracellular space, CHGB (and particularly its peptide hCHGB[60–67]) seems to exert a negative feedback effect to inhibit the secretory response to acetylcholine.

catecholamines, and secretagogue-stimulated catecholamine release (Fig. 1e). Conversely, expression viral transduction successfully increased CHGB expression, with an increase in chromaffin granule abundance, but only minor changes in cellular ability to uptake catecholamines or respond to secretory stimulation (Fig. 3d). Thus, the range of CHGB concentrations between physiological and zero have profound consequences for catecholamine metabolism, while elevation of CHGB beyond the physiological range does influence granule morphogenesis, but seems to have fewer physiological effects, suggesting a sufficient or saturating CHGB concentration under basal circumstances.

Both we (Fig. 1b) and Diaz-Vera *et al.* (2010) found changes in expression of other chromaffin cell mRNAs during diminution of *CHGB* expression, especially overexpression of *Chga* mRNA. We speculate that such an increase may have dampened the already impressive consequences of CHGB depletion (Fig. 1e). Diaz-Vera *et al.* (2010) noted diminished adrenal medullary storage of epinephrine and norepinephrine, consistent with our finding of decreased catecholamine uptake capacity (Fig. 1e).

In vivo (targeted ablation of the mouse *CHGB* locus), we found similar changes in granule morphology (Fig. 2a), with apparently unregulated catecholamine release (Fig. 2b), which may underlie the substantially elevated blood pressure observed in the *CHGB*(−/−) mouse (Zhang *et al.* 2009). Of note, we previously observed similar catecholamine release and blood pressure effects in the *Chga* knockout mouse (Mahapatra *et al.* 2005).

Using an expressed frog CHGA (fCHGA) cDNA and a non-chromaffin cell line (COS-7), Montero-Hadjadje, *et al.* demonstrated that CHGA has the intrinsic capacity to induce formation of mobile secretory granules and to promote the sorting and release of peptide hormones (Montero-Hadjadje *et al.* 2009). Our finding of a role for CHGB on the biogenesis of dense core granules (in chromaffin and PC12 cells) indicates that these two homologous proteins exert similar functions.

CHGB peptide regions on catecholamine release

The CHGA peptide catestatin (human CHGA[352–372]) inhibits the physiological (nicotinic cholinergic) pathway of catecholamine release in chromaffin cells (Mahata *et al.* 1999; Mahapatra *et al.* 2005) and polymorphisms in the human catestatin region are associated with risk of hypertension (Rao *et al.* 2007). Here, we observed a similar (though less potent) inhibitory effect on nicotinic-stimulated secretion for CHGB and its proteolytic fragments (Fig. 4a). We then synthesized several peptides that potentially mimic the effects of catestatin, and found that such peptides can inhibit nicotinic cholinergic-stimulated catecholamine release (Fig. 4b left) and cationic signal transduction (Fig. 4c) in chromaffin cells, albeit at lower potency and efficacy than catestatin. The complete lack of effect of CHGB synthetic peptides on catecholamine secretion triggered by membrane depolarization (Fig. 4b center) reinforces the specificity of such peptides for the nicotinic cholinergic pathway.

The active human CHGB region is completely conserved across mammalian species (human, rat, mouse), and the target region is extensively cleaved by the chromaffin cell plasmin endoproteolytic system (Table 1a), though we have not yet positioned the precise boundaries of the active peptide as formed *in vivo*; as a control, the same system exerts the expected cleavage pattern on multiple dibasic sites in the human CHGB substrate (Table 1b). A more extensive synthetic peptide scan across the entire CHGB protein (or its

highly conserved regions) could better establish the active region(s) of the protein.

Conclusions and perspectives

Within chromaffin cells, CHGB participates in assembly of catecholamine secretory vesicles (dense-core granules), and governs the secretory capacity of these granules under the nicotinic stimulation. After proteolytic cleavage and release into the extracellular space, CHGB (and particularly its peptide hCHGB[60–67]) seems to exert a negative feedback effect to inhibit the physiological secretory response to acetylcholine. The autocrine effects of released/processed CHGB on secretion (Fig. 5) would be most likely to occur on chromaffin cells, whose chromaffin granules (or large dense core vesicles) typically contain both chromogranins and catecholamines, though even sympathetic nerve termini which contain substantial numbers of small dense core vesicles, without chromogranins also harbor some chromogranin-bearing large dense core vesicles (O'Connor *et al.* 1991).

Derangements of CHGB expression may thus lead to impaired catecholamine storage, with consequently elevated constitutive catecholamine release, eventuating in systemic hypertension. Our data provide direct bi-directional evidence implicating CHGB in vesicular storage of catecholamines, and also novel evidence implicating released CHGB in control of exocytosis. Thus, CHGB emerges as a multi-functional protein/peptide, with both intra-cellular and extracellular activities (Fig. 5), whose novel properties may contribute to a spectrum of catecholaminergic disease states.

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Conflicts of interest

None to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Structure of the CHGB over-expression lentiviral vector.

Figure S2. PC12 cells transduced by *CHGB* lentivirus: Visualization of expression by red fluorescence microscopy, detecting the photoprotein.

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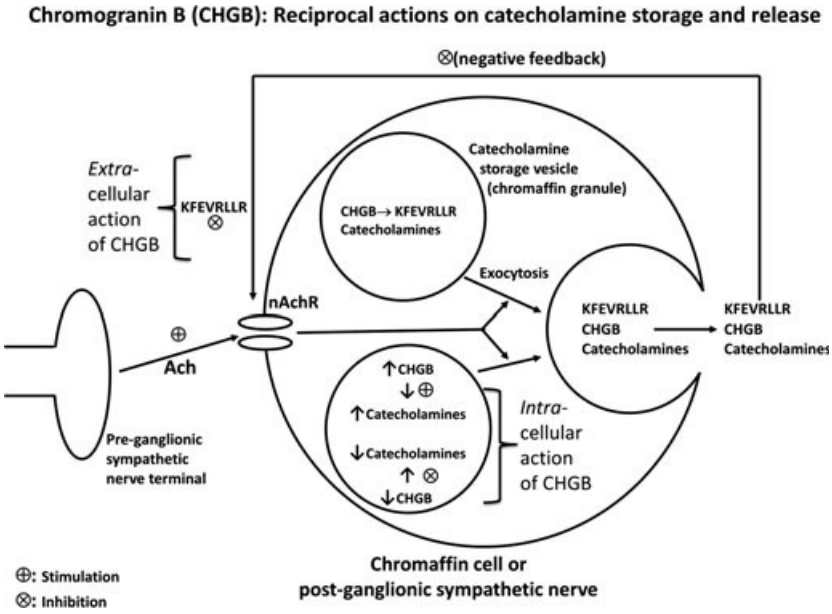

Here, we show reciprocal actions of Chromogranin B (CHGB) on catecholamine storage (stimulation) and release (inhibition). The figure synthesizes consequences of experimental results. Within chromaffin cells, CHGB participates in assembly of catecholamine secretory vesicles, and governs their secretory capacity under nicotinic stimulation. After cleavage and release into the extracellular space, CHGB [and its peptide hCHGB[60–67](KFEVRLLR)] exerts negative feedback effects to inhibit the secretory response to acetylcholine (ACh).

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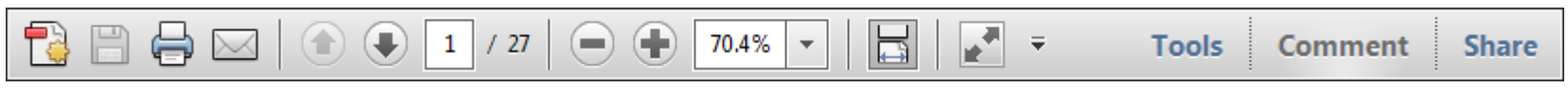
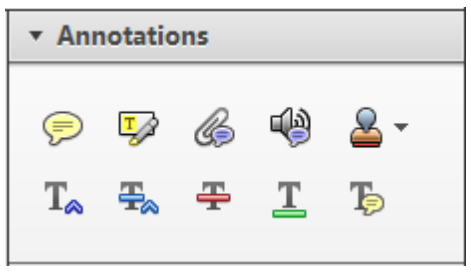
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

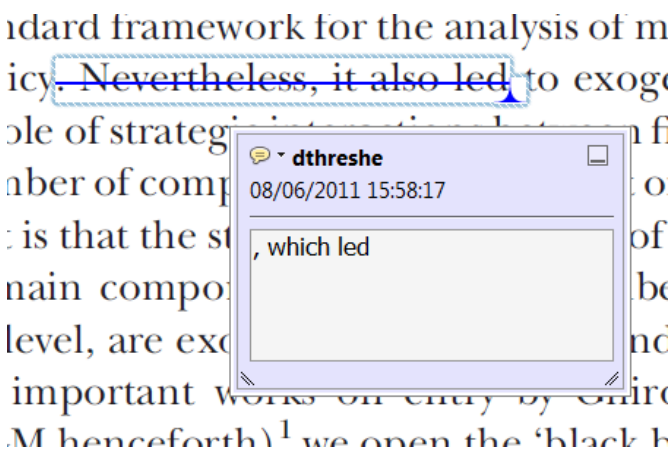


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

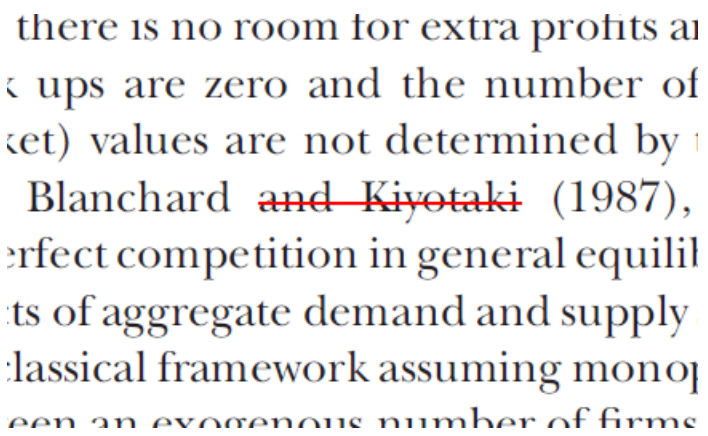


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

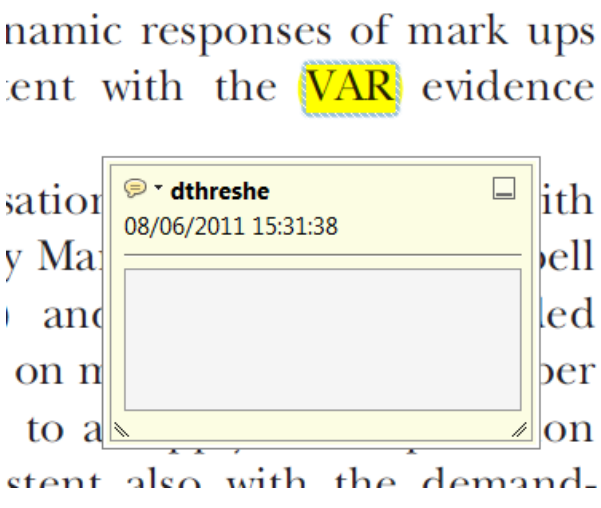


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

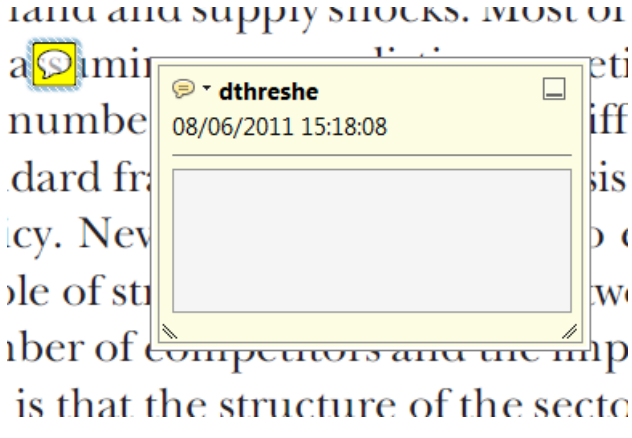


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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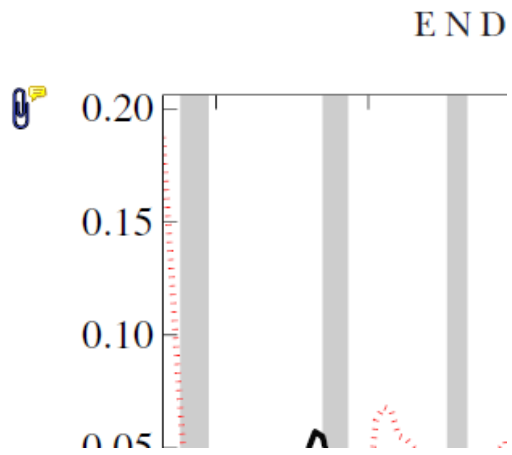
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

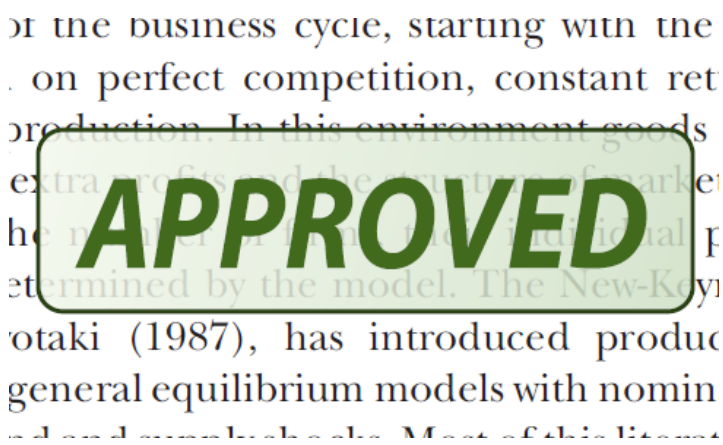


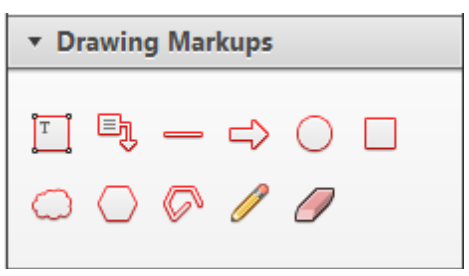
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

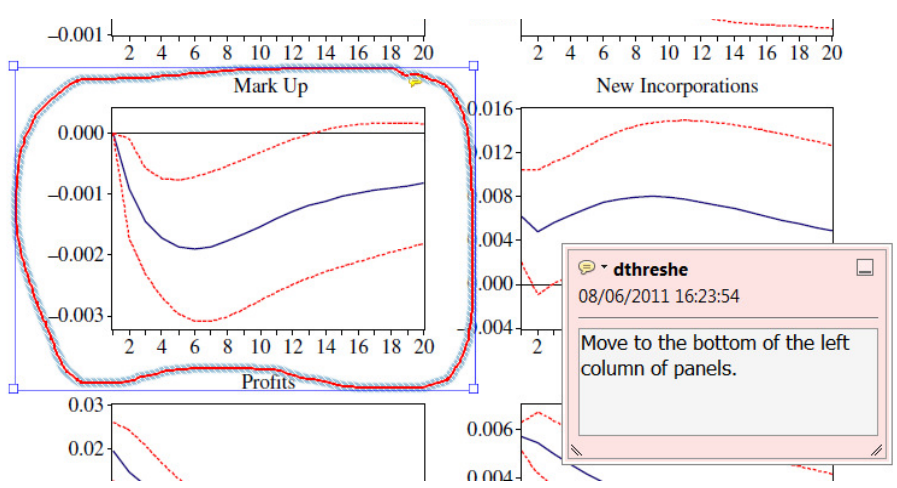
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: