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# PC2/CPE mediated neuropeptide processing and *in vitro* stressing response of RGC-5 cell

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## ABSTRACT

PC2 and CPE are two major members of the pro-protein processing convertases which involve in the maturation of neuropeptide. The RGC-5 cell differentiated in 0.1μM staurosporine for 24h was stressed by different OGD injuries. The acute or chronic OGD-inducing cell death rates were obtained by using PI or TUNEL staining. By using the Western blot method and artificial PC2 substrate, the PC2, CPE and preproNPY protein levels in the ischemic RGC-5 cells and conditioned medium were analyzed. The results showed that the ischemia caused substantial cell death in an OGD dose-dependent manner. In the cells, proPC2 and preproNPY protein levels gradually increased whereas proCPE gradually decreased. After the OGDs, the PC2 activities were decreased. In the medium, proPC2 and PC2 proteins gradually decreased whereas proCPE, CPE, and preproNPY proteins gradually increased. The experimental results demonstrated that OGD inhibits the neuropeptide pro-protein processing system by reducing PC2 activity and the maturation of proPC2. The aggregation of the pro-proteins and the increase of the active CPE excision adversely exacerbate the cell injury. These results demonstrate that PCs mediated processing system plays a critical role in the ischemic stress of RGC-5 cells.


## KEYWORDS

Neuropeptide pro-protein processing system;
Retina ganglion cell-5;
*in vitro*;
Oxygen glucose deprivation;
Pro-protein convertase-2;
Carboxypeptidase-E;
Preproneuropeptide-Y.

## INTRODUCTION

*Subtilisin*-like pro-protein convertases (PCs) are the major proteolytic processing enzymes that mediate the biosynthesis of a great variety of secreted and membrane proteins. These processed proteins have the ranges from embryogenesis, gene expression, cell cycle, programmed cell death, intracellular protein targeting and endocrine/neural functions[1,2]. These precursor molecules need to undergo a series of pro-protein processing steps that involve in the multi-protein family of mammalian serine proteases[3,4]. They are expressed together or separately in functional cells in both vertebrates and invertebrates. The mammalian PCs have been demonstrated to involve in several diseases, i.e. HIV, hepatitis B, severe acute respiratory syndrome (SARS), anthrax, cancer, Alzheimer's disease, arthritis, stroke, glucauma, and diabetes[5,10].

Pro-protein convertase-2 (PC2) and carboxypeptidase-E (CPE) are expressed widely in neuro-endocrine tissues and play a major role in the proteolytic processing[11]. S.Tanaka et al.[12] reported that proPC2

with 638 amino acid residues (74 kDa) automatically activates into mature PC2 with 529 amino acid residues (64 kDa) in adequate conditions, such as 5.0 pH and higher $Ca^{+2}$. The soluble CPE (sCPE) is an exopeptidase that cleaves neuroendocrinopeptides with C-terminal basic amino acids and produces active forms of peptide hormones and neuropeptides[13,14]. The membrane-bound CPE (mCPE) functions as a sorting receptor in the trans-Golgi network (TGN) and facilitates the sorting of pro-hormones into the regulated secretary pathway[15,16]. N.Brakch et al.[17] and R.Miller et al.[18] reported that the maturation of preproNPY peptide needs the participation of PC2 and CPE.

The profile of neuro-endocrinopeptide in functional tissue may determine their response to ischemic stress, apoptosis, and necrosis. In the paper, the ischemia-induced changes in the PC system were observed in the RGC-2 cells. Our long term goal is to understand PC molecular mechanisms in some diseases, which will help diagnosis and treatment to the diseases.

## MATERIOLS AND METHODS

### 1. Cell culture and *in vitro* simulated ischemia

Retina Ganglion Cell-5 (RGC-5) cell line was transformed from rat retina ganglion cell (RGC). The cells were cultured in DMEM medium containing 10% fetal calf serum (FCS) (Invitrogen Co. USA), 20 mM L-glutamine, penicilin / streptomycin, and G-418 at $37^0$C in a humidified atmosphere equilibrated with 5% $CO_2$. Differentiation was achieved by incubating cells in the DMEM-FCS medium containing 0.1μM staurosporine (Sigma Co. USA) for 24 h at $37^0$C. To investigate biosynthesis of PC2, CPE, and preproNPY under normal and ischemic conditions, Simulated ischemia of the differentiated RGC-5 cells was induced by oxygen and glucose deprivation (OGD). For OGD, cells were placed in an anaerobic chamber (Forma Scientific Co. USA) equilibrated with 85% $N_2$/5% $CO_2$/10% $H_2$ and incubated in glucose/serum/glutamine-free medium containing 25 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 135 mM NaCl, 5 mM KCl, 1X Essential Amino Acid without L-Glu (Invitrogen Co.) and 20 mM 2-deoxyglucose (2-DG) (Sigma Co.). Propidium iodide (PI) was purchased from Sigma Co. and TUNEL staining kit was purchased from Boehringer Mannheim Co. (USA). The acute OGD dose-effect cell model and the chronic OGD dose-effect cell model were used to observe the relationship between PC2/CPE- mediated neuropeptide processing and OGD.

### 1.1 The acute OGD dose-effect cell model

In order to early observe the PCs gene expressions and processing to their substrate after OGD. The acute and chronic OGD cell models were used in our experiments. The acute OGD cells were obtained by collecting them after the cells were incubated 0, 1, 2, 4, 6h in an OGD chamber.

### 1.2 The chronic OGD dose-effect cell model

In order to lately observe the PCs gene expressions and processing to their substrate after OGD. The chronic OGD cells were obtained by collecting them after the cells were incubated 0, 1, 2, 4, 6h in an OGD chamber and subsequently incubated for 24h in a cell culture incubator.

### 2. Analysis of death rate of the differentiated RGC-5 cells after OGD

According to our preliminary data, PI staining is better for the acute OGD cells whereas TUNEL staining is better for the chronic OGD cells. So the death rates of the acute or chronic OGD-inducing RGC-5 cells were obtained by using PI or TUNEL staining. PI staining was referred to the method[19]. Briefly, 1 ml of PBS containing 1μl of PI ethanol solution (10 mg/ml) was added in dishes for 3 min at room temperature in which grew the differentiated RGC-5 cells. After washed 2×5 min with PBS, 1 ml of 10% formalin buffered by PBS was added in the dishes for 15 min at room temperature and subsequently washed one time for 5 min with PBS. 1 % Triton X-100 was added in the dishes for 10 min at room temperature. After washed one time for 5 min with PBS, the cells in the dishes were mounted with the mounting medium DABI. The TUNEL staining method was referred to the company protocol. Briefly, after fixed with 10 % formalin buffered in PBS for 15 min and subsequently permeated with 1 % Triton X-100 for 10 min, the dishes in which grew RGC-5 cells were added 50μl of the enzyme-substrate mixture (1:9 ratio) and covered with parafilm. After wrapped with tinfoil to keep in darkness and subsequently incubated for 1 hours at $37^0$C in an incubator, the dishes were washed with PBS 3×5 min and mounted with the DABI

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medium.

### 3. Western blot analysis

Proteins were extracted from cultured RGC-5 cells with the following protocol. To avoid the contamination of serum in medium in the Western blot assay, Cells in dishes were softly washed one time with warm PBS and subsequently softly suspended in cold PBS. After the cell suspensions were centrifuged at 6000 rpm for 6min, the cell pellets were collected. Proteins were extracted from the cell pellets with the same buffer as the following PC2 activity. After the cell pellets were subjected to 3 cycles of freeze-thaw-vortex to further destruct cellular structures, they were centrifuged at 10,000g for 10 min at $4^0$C and supernatant was collected. Protein concentrations of the supernatants were determined by the Bradford method (Sigma Co.). Proteins (50μg per gel lane) were fractionated by SDS-PAGE (150V for 2h), blotted onto a PVDF membrane (Millipore Co. USA) (166mA for 2h), and probed with an appropriate primary antibody. The antibody bound to the membrane was detected with the enzyme-catalyzed chemiluminescence (ECL) method (NEN Life Science Co). The membrane was cut for ECL according to the molecular weights of the detected proteins. To verify the equality of protein loadings among different samples to be compared, the blotted membrane was detected with β-actin protein as control. For protein analysis in the conditioned medium, after the acute OGD, 5 ml of conditioned medium each OGD dose was collected and centrifuged. The supernatant was added trichloroacetic acid (TCA) to 20 % and calm stayed in ice for 20 min. After centrifuged 10000×g for 20 min, the tube bottom pellet was carefully collect and wholly applied to the SDS-PAG lane for the Western blot analysis. Rabbit serum against PC2 (amino acids 611-638) (donated by Dr An Zhou) at a dilution of 1:1,000 or antibodies against preproNPY (amino acids 68-97) at a dilution of 1:2,000 and CPE at a dilution of 1:2,000 were used. The antibody against CPE or preproNPY was purchased from Research Diagnostics Inc (USA). The polyclonal rabbit anti-actin antibody (1:1000 used) was purchased from Sigma-Aldrich Co.

### 5. PC2 activity analysis

Analysis of PC2 activity followed a modulated protocol according to Berman et al. [20]. The whole reaction volume was 100μl. Cells were homogenized with a buffer consisting of 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 10% glycerol and a cocktail of protease inhibitors (aprotinin 1μM, PMSF 1 mM, benzamidine 1 mM) (Sigma Co.). To control the detergent concentration in the reaction buffer, 10μl of protein supernatant was incubated with 200μM L-pyroglutamyl-Arg-Thr-Lys-Arg-7-amino-4-methyl-coumarin in 100 mM sodium acetate (pH 5.0), and 1 mM $CaCl_2$ in the presence of the inhibitors. All incubations were carried out at $37^0$C for 4 h. In parallel incubations, 2μM CT peptide (SVNPYLQGKRLDNVVAKK), a PC2-specific inhibitor derived from the C-terminus of 7B2 protein. The release of 7-amino-4-methylcoumarin was measured by using a Spectra Max GEMINI spectrofluori meter (Molecular Devices Co, USA; $\lambda_{ex}$ = 360 nm; $\lambda_{em}$ = 480 nm). The amount of product formed was calculated by using free 7-amino-4-methylcoumarin as a standard. The activity inhibited by CT peptide was taken as PC2 activity.

## RESULTS

### 1. Morphology of the differentiated and stressed RGC-5 cells

RGC-5 cells were induced differentiation in 0.1μM staurosporine. The non-differentiated cells are round and spindle shape for light microscope. There is not dendrite on the body and granule in cells (Figure 1A). The differentiated cells are polygonal shape and has many dendrites on the body. There is much granule in the body and dendrite. The dendrites among different cells connect with each other (Figure 1B). The shape of the OGD-stressed RGC-5 cells is similar to that of the differentiated RGC-5 cells. but the cells gather in clusters. A little granule was observed in cells and dendrites (Figure 1C).

### 2. Analysis of the death rates of the OGD-inducing RGC-5 cells

### 2.1. Death rates of the acute OGD-inducing RGC-5 cells

The death rates were obtained by using 0, 2, 4, 6h OGD dose and subsequent PI staining. The results showed that the OGD caused substantial cell death in an OGD dose-dependent manner ($P<0.01$) (Figure 2).

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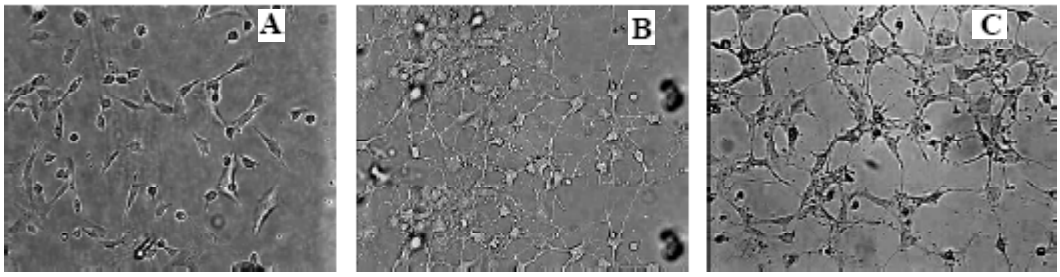


**Figure 1: Morphology of the differentiated and stressed RGC-5 cells (Mag.15×10 times for light microscope). (A): The non-differentiated cells. (B): The differentiated cells. (C) : The differentiated RGC-5 cells stressed by a 2.5-h OGD**

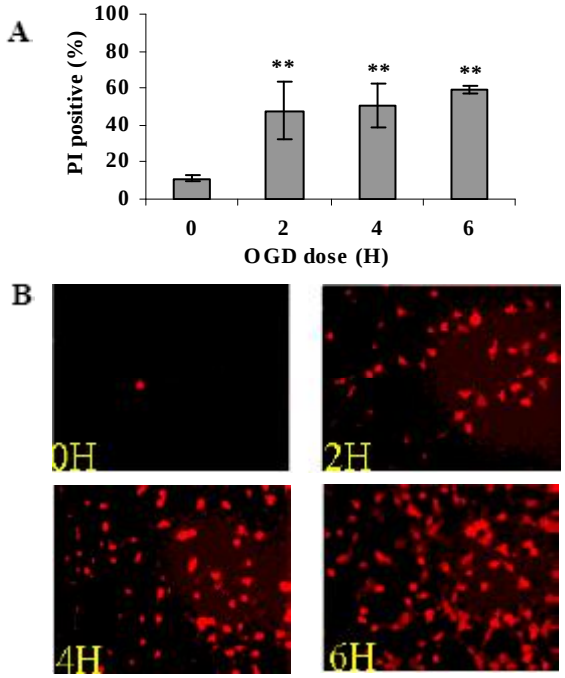


**Figure 2: Analysis of the death rates of the acute OGD-inducing RGC-5 cells (n=2). (A): the statistical results of the death rates. **P<0.01 vs the result at the 0H-OGD in $X^2$ test; (B): The PI staining pictures of the acute OGD dose-dependent death (Mag.10×10 times for light microscope). The numbers on the pictures were OGD doses**

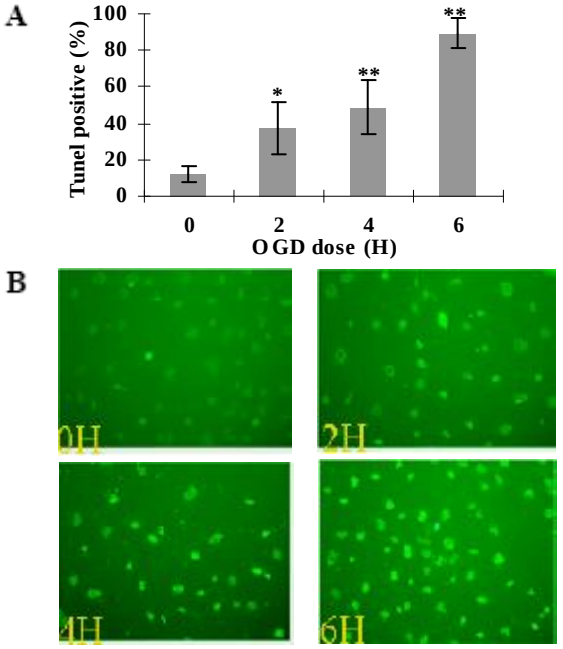


**Figure 3: Analysis of the death rates of the chronic OGD-inducing RGC-5 cells (n=2). (A): the statistical results of the death rates. * P<0.05 or ** P<0.01 vs the result at the 0H-OGD in $X^2$ test; (B): The TUNEL staining pictures of the chronic OGD dose-dependent apoptosis (Mag.10×10 times for light microscope). The numbers on the pictures were OGD doses**

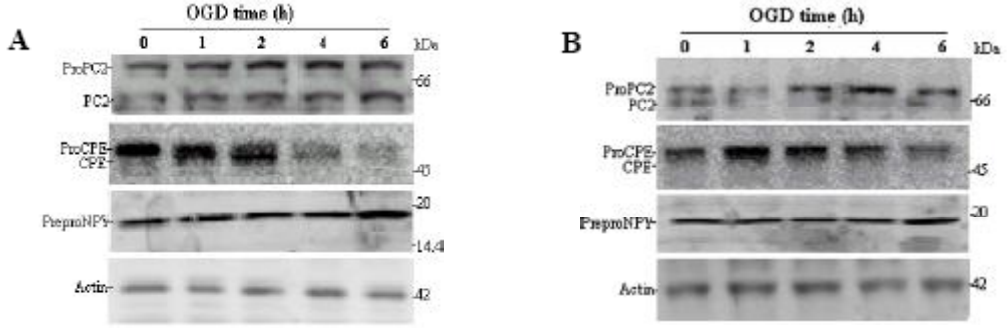


**Figure 4: Western blot analysis of PC2, CPE, and preproNPY protein expressions in the acute (A) or chronic (B) OGD cells. 7.5% SDS-PAG and the protein markers 14.4-220 kDa for PC2 and CPE; 15% SDS-PAG and protein markers 45-3.5 kDa for preproNPY and actin. The internal control wasβ-actin protein**

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### 2.2. Death rates of the chronic OGD-inducing RGC-5 cells

The death rates were obtained by using TUNEL staining after 0, 2, 4, 6h OGD and subsequent 24-h recovery time. The results showed that the OGD caused substantial cell apoptosis in an OGD dose-dependent manner ($P<0.05$ or $P<0.01$).

### 3. Western blot analysis of PC2, CPE, and prepro NPY protein expression in the ischemic cells and medium

### 3.1.PC2, CPE, and prepro NPY protein expression in the acute OGD cells

The results (Figure 4A) indicated that after the cells were stressed by the acute OGD, PC2 and preproNPY protein levels in the cells gradually increase in an OGD dose-dependent manner, whereas proCPE gradually decrease. At the lower OGD doses (0 and 2h), mature CPE protein gradually increases. At the higher OGD doses (4 and 6h), CPE expressions obviously reduced.

### 3. 2. PC2, CPE, and preproNPY protein expression in chronic OGD cells

After the chronic OGD, the up-regulations of PC2 and preproNPY protein levels in the cells were still observed in an OGD dose-dependent manner. Comparing with CPE result at the time point of 0h-OGD, proCPE protein gradually decreased in an OGD dose-dependent manner. At the time points of 1 and 2h OGD, mature CPE occurred (Figure 4B).

### 3.3. PC2, CPE, and preproNPY proteins in the conditioned medium

Medium samples were collected after the acute OGD incubations. The tube bottom pellet was collect by precipitating with 20% TCA and subsequently centrifuging. The Western blot results indicated that in an OGD dose-dependent manner, proPC2 and PC2 gradually decreased whereas proCPE, CPE, and prepproNPY gradually increased.

### 4. PC2 activity analysis

PC2 activity analysis of the acute or chronic OGD-inducing RGC-5 cells (Figure 6A and 6B) showed, with the increasing of the OGD dosage, the PC2 activities were decreased ($P<0.01$ or $P<0.05$).

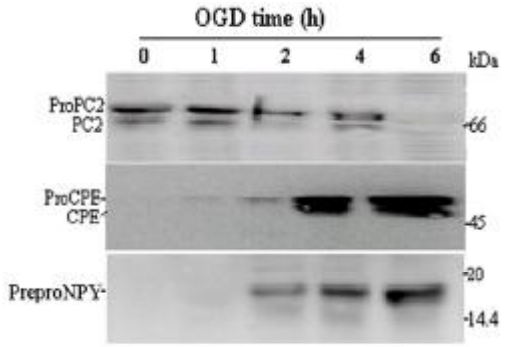
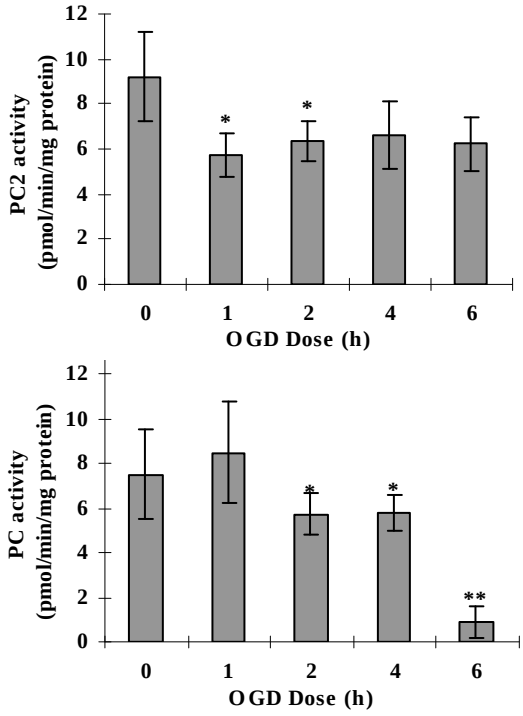



**Figure 5: Western blot analysis of PC2, CPE, and preproNPY protein in the conditioned medium. 7.5% SDS-PAG and the protein markers 14.4-220 kDa for PC2 and CPE; 15% SDS-PAG and protein markers 45-3.5 kDa for preproNPY**




***P<0.05 or **P<0.01 vs Result at the 0h-OGD in t test**

**Figure 6**

## CONCLUSION

In brain cells, the production of active neuropeptides relies on the presence and proper function of a set of neuropeptide processing enzymes including, but not limited to, proprotein convertase 2 and CPE. Little is known about the neuropeptide processing system in the retinal ganglion cells which are vulnerable to ischemic injury and may play a critical role in the pathology of retina disorders. In this study, a series of experiments

were carried out to establish: 1) the presence of an active PC2 protease in RGC-5 cells, 2) the impact of ischemic stress on expression levels of PC2, prepro NPY, and CPE in RGC-5. Cells ***in vitro***

PC2 is a major endoproteolytic enzyme and CPE is an C-terminal exoproteolytic one. When precursor molecule is endoproteolyzed by PCs, the soluble CPE recognizes the specific basic residue at the C-terminus and processes it. PreproNPY molecule is one of the most abundant peptides found in the mammalian tissue[24,25]. Its maturation is subjected to the processing of PC2 and CPE. PreproNPY has 97 amino acid residues in mouse, human and 98 amino acid residues in rat. The isoelectric point (pI) of preproNPY is 11.1. The Western blot result of preproNPY indicated that the specific band of preproNPY was close to 17 kDa, which is because the higher pI of the peptide forms a larger molecule by combining with more SDS molecules. The electric mobility was very similar with the GHRH analogs we ever reported[26].

In our many preliminary experiments, we discovered that pc2 was a whole-process expression gene during OGD. It was regulated from 10 min to 6h OGD dose and no matter what a cold stimulation or heavy ischemia is. According to these results, I developed the acute OGD dose-effect cell model (early gene expression) and the chronic OGD dose-effect cell model (late gene expression).

In the experiments of the death rates of the OGD-inducing RGC-5 cells: PI staining or TUNEL staining was used to measure the acute or chronic cell death rates, because the acute OGD cause a higher permeability to PI dye so that the dye molecules enter and combine with DNA. TUNEL staining is better to the chronic OGD cells, because TUNEL staining is used to measure apoptotic cells with DNA fragments that occurred lately.

The Western blot results of PC2, CPE, and prepro NPY protein expression in the ischemic cells indicated that PC2 and preproNPY protein levels gradually increase in the OGD dose-dependent manner whereas the proCPE gradually decrease, which was because OGD caused aggregations of proPC2 and preproNPY by inhibiting PC2 activity and the maturation of proPC2. The gradual increase of proPC2 may be to compensate for the insufficiency of the active PC2. The Western blot results of the conditioned medium indicated that the gradual decrease of proPC2 and PC2 and the gradual increase of preproNPY & CPE stood for the alteration of the cell secretary function, which revealed that PC2 may be very important to rescue ischemic injury. In order to increase PC2 quantity in cells, the decrease of the PC2 excision and the increase of the proPC2 expression are necessary. We have known that the over-expression of pro-protein is toxic to cell function. The aggregation of preproNPY protein in cells may induce an increase of excision of preproNPY molecule. We guess that high PC2 may rescue or diminish ischemic injury.

In the medium, proCPE and CPE protein gradually increased, but in the cells they gradually decreased, which suggested that the production and transformation of proCPE were normal. The increase of CPE excision at the higher OGD doses may be appropriate for the decrease of PC2 activity. In the experiments, proPC2 and proCPE showed adverse regulations during OGD, which may be another unknown OGD event.

After glucose oxygen deprivation, the exhaustion of energy or ATP is the first event. The maturation, moving and secretary of the vesicle containing PCs and substrates need a lot of ATP. In a word, the cascades of OGD→ATP block→ vesicle maturation block may lead to inhibition of transformation of proPC2 into PC2.

## ACKNOWLEDGMENTS

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### Abbreviations

PC, prohormone convertase; OGD, oxygen glucose deprivation; PC2, proprotein convertase 2; CPE, carboxypeptidase E; preproNPY, preproneuropeptide Y; CT peptide, SVNPYLQGKRLDNVVAKK; RGC-5, Retina ganglion cell-5; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling.

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