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Master's Thesis
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Role of Atg8 family proteins during autophagy
following 2-deoxy-D-glucose treatment
in adult hippocampal neural stem cells.

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ABSTRACT

The autophagy related gene 8 (Atg8) family proteins play a central role at various steps of autophagy, including autophagosome initiation, elongation, maturation and lysosome fusion, as well as cargo targeting. However, details regarding the distinct roles of individual Atg8 members during autophagy is not much known.

The objective of this project is to determine the roles of each Atg8 family protein and understand their intracellular signaling and molecular effects during autophagy induced by 2-deoxy-D-glucose (2-DG) treatment in adult hippocampal neural stem (HCN) cells. Especially, we will be focusing on three proteins: microtubule-associated protein 1 light chain 3 beta (LC3B), GABA type A receptor associated protein like 1 (GABARAPL1), and 2 (GABARAPL2). HCN cells with stable knockdown of individual Atg8 family member proteins will be treated with 2-DG to induce autophagy and downstream signaling pathways will be examined.

Through these experiments, we will elucidate the effects of Atg8 family proteins and further understand the relevance of Atg8 proteins during autophagy in HCN cells following 2-DG treatment.

Keywords: Atg8, 2-deoxy-D-glucose, autophagy, hippocampal neural stem cells

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1. Introduction

1.1 General introduction to autophagy.

Autophagy is a cellular process, which enables cells to breakdown unnecessary or dysfunctional cellular components. Through autophagy, cells are able to recycle cellular materials, control the quality of proteins and organelles, and adapt to stressful conditions [1]. Once autophagy is induced, a phagophore encloses the targeted cellular components and develops into a autophagosome. The autophagosome then conjugates with a lysosome and evolves into an autolysosome. Within the autolysosome, the encapsulated cellular components are broken down for recycling (Figure 1). Therefore, autophagy has been widely accepted as a mechanism incorporated by cells for cellular survival as well as viability. For instance, autophagy is incorporated in various biological processes including cell development, differentiation, and maintenance, while dysregulation of autophagy may lead to diseases including cancer, heart and liver damage, infection, as well as neurological diseases [2].

1.2 Adult hippocampal neural stem cells (HCN cells)

HCN cells are neural stem cells located at the dentate gyrus of the sub-granular zone within the hippocampus, which is one of the two restricted sites for adult neurogenesis in the mammalian brain [6].

Since adult neurogenesis generates new functional neurons, which are crucial for proper brain function and cognitive ability [6,7], the study of autophagy and ACD in HCN cells is of great significance. ACD in neural stem cells has been reported in a previous study, which discovered that adult hippocampal neural stem (HCN) cells undergo autophagic cell death upon insulin withdrawal despite their sufficient capability for apoptosis [8], which increases curiosity and significance in studying HCN cells.

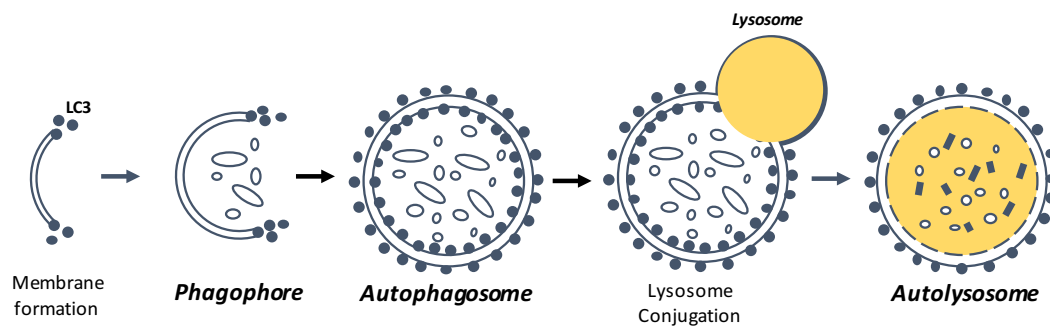


Figure 1. The autophagic process.

The autophagosome conjugates with a lysosome and evolves into an autolysosome. Within the autolysosome, the encapsulated cellular components are broken down for recycling (Figure 1).

1.3 ATG8 family members.

Autophagy-related protein 8 (Atg8) is a protein central to autophagy processes, including autophagosome initiation, elongation, maturation and fusion with lysosome, as well as cargo targeting. Recently, mammalian homologs of yeast Atg8 such as microtubule-associated protein 1 light chain 3 (LC3), GABA type A receptor associated protein like 1 (GABARAP L1), and 2 (GABARAP L2) have been reported [2]. Among the various types of Atg8 family proteins, seven distinct Atg8 family members are said to be expressed in humans and each member participates in distinct processes during autophagy (Table 1). However, the exact and distinct roles of individual Atg8 family members during autophagy is still yet to be fully studied.

The most widely researched Atg8 family member is microtubule-associated protein 1 light chain 3 beta (LC3B), which is reported to aid in the process of autophagy substrate selection and autophagosome biogenesis. Shortly after LC3 is translated, it follows a multistep process, during which the structure of LC3 is modified and transferred to phosphatidylethanolamine (PE), a phospholipid backbone in biological membranes (Figure 2). The LC3-PE complex is incorporated into autophagosome membranes and upon completion of autophagy, LC3 is recycled [3]. Due to its extensive involvement and pronounced protein level flux during autophagy, LC3 is often used as the universal autophagy marker.

Atg8 homologs of LC3, GABARAP and GABARAP L1/L2 are also expected to play a role in autophagosome synthesis and carry out autophagy. In fact, it has been reported that LC3 are involved in phagophore membrane elongation, whereas, GABARAP subfamilies are involved in later stages of autophagosome maturation [4]. In addition to autophagy related roles, different GABARAP subfamilies have been reported to be involved in non-autophagy related activities and are expressed at different levels in different regions of the body [5]. Such variations shown among Atg8 homolog proteins increase the need for further study on this topic.

Autophagy is a crucial process directly linked to the survival and viability of cells including those of the nervous system. Therefore, further studies on Atg8 family members and elucidating their role holds significance in the field of neuroscience and neurodegenerative diseases and in large, important for the survival of an organism.

Subfamily	Gene Names	Process	Participating Member
LC3	MAP1LC3A	Nucleation	GABARAP/GABARAPL1
	MAP1LC3B	Carto targeting	LC3/GABARAPL1
	MAP1LC3B2	Elongation	LC3
	MAP1LC3C	Phagophore Closure	LC3/GABARAPL2
GABARAP	GABARAP	Autophagosome-Lysosome Fusion	GABARAP/GABARAPL2/LC3
	GABARAPL1		
GATE-16	GABARAPL2	Target trafficking	GABARAP/GABARAPL1

Table 1. List of Atg8 family members and associated functions.

Mammalian homologs of yeast Atg8 such as microtubule-associated protein 1 light chain 3 (LC3), GABA type A receptor associated protein like 1 (GABARAP L1), and 2 (GABARAP L2) have been reported. Among the various types of Atg8 family proteins, seven distinct Atg8 family members are said to be expressed in humans and each member participates in distinct processes airing autophagy.

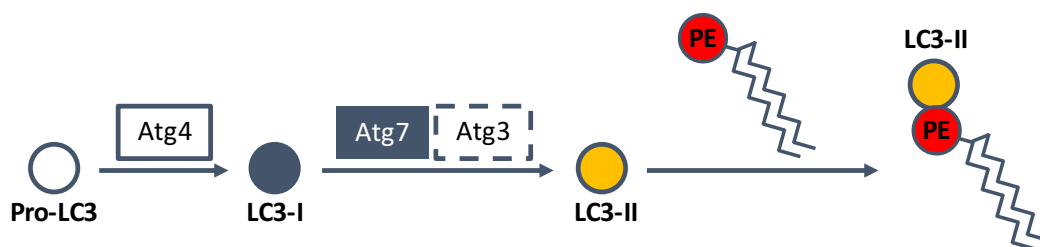


Figure 2. The process of the LC3 conjugation system.

Shortly after LC3 is translated, it follows a multistep process, during which the structure of LC3 is modified and transferred to phosphatidylethanolamine (PE), a phospholipid backbone in biological membranes, and forms LC3-II (type 2). Conjugated LC3-II is incorporated during autophagosome formation, hence the reason why LC3-II is used to detect autophagy rate.

1.4 2-deoxy-D-glucose (2-DG).

2-deoxy-D-glucose (2-DG) is a glucose analogue, which is reported to block glycolysis and growth in various cells and mammalian tissues [9]. Compared to glucose molecules, 2-DG molecules have the 2-hydroxyl group replaced by hydrogen, which prevents further glycolysis (Figure 3). Despite the structural difference, glucose transporters uptake 2-DG molecules as if they were normal glucose molecules; however, since 2-DG cannot be further incorporated into glycolysis, which hinders energy production and eventually, cell growth [10].

Ongoing research in our lab has shown 2-DG treatment to induce cellular autophagy, but not autophagic cellular death. In addition to previous studies on insulin deprivation, our study on 2-DG induced autophagy and the different roles taken by Atg8 family members during 2-DG induced autophagy may further elucidate the relevance of Atg8 proteins in HCN cells. Our project will include observations made on the molecular effects of 2-DG on Atg8 knockdown HCN cells as well to further understand how lacking certain Atg8 members may alter the cell signaling pathways.

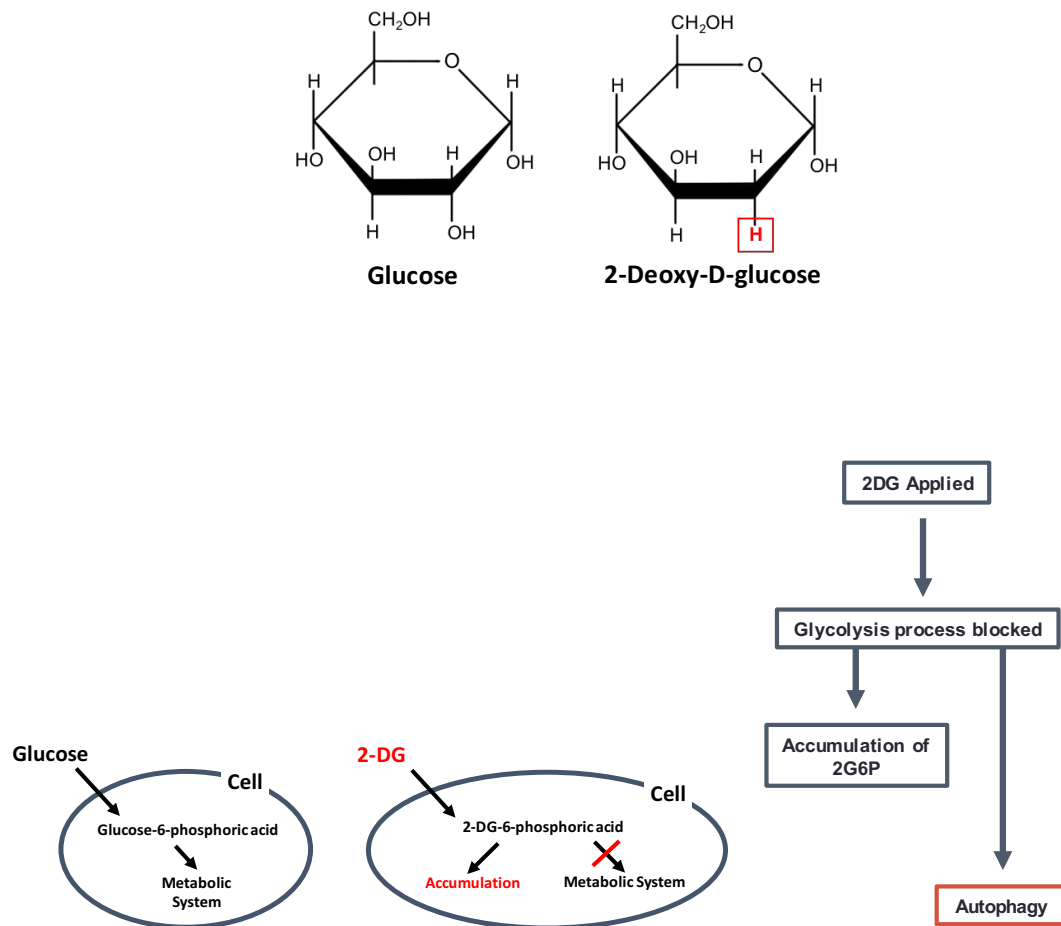


Figure 3. Structural difference between 2DG and glucose induces difference cellular response.

Compared to glucose molecules, 2-DG molecules have the 2-hydroxyl group replaced by hydrogen, which prevents further glycolysis and causes cellular accumulation of 2-DG in the cell.

2. Materials & Methods

2.1 Cell culture & Atg8 member knockdown cell

HCN cells from 2-month-old Sprague Dawley rats were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media (Invitrogen) supplemented with insulin (1 μ l/ml, Sigma), sodium selenite (6 μ l/L, Sigma), progesterone (20 μ l/L, Sigma), putrescin (16 mg/L, Sigma), apo-transferrin (100 mg/L, Sigma), and bFGF (0.2 μ l/ml, Invitrogen) then incubated in 37 °C, as previously reported [11]. Knockdown cells were stocked in -80 °C and thawed for experiments. Atg8 family member knockdown adult hippocampal neural stem cells (HCN cells) were provided by Hye Young Ryu. Knockdown stable cell lines were constructed using lenti viruses. pLKO.1 puro with shRNA construct was used for the transfer vector. Additionally, VSV-G vector was used as the envelope vector and delta 8.2 vector was used for the packaging vector. Cell were stocked in -80 °C and thawed to revive when needed for experiments. The cells were selected with puromycin.

2.2 Antibodies and Chemicals

Primary antibodies were used as follows: phosphorylated Akt (S473), phosphorylated GSK-3 β (S9), p62, phosphorylated AMPK (T172), phosphorylated mTOR (S2448), phosphorylated Ulk1 (S757) from Cell

Signaling Technology; β -actin from Santa Cruz; and LC3 from Novus. 2-deoxy-D-glucose is from Sigma Aldrich (D8375).

2.3 Cell Death Assay

Atg8 member knockdown cells and control cells were cultured in 96 well culture plates and checked for cell death rates. Cells were stained with Hoechst-Propidium iodide (Ho/PI) double staining (1% volume of media in the well, 1/1000 final dilution) and observed under a fluorescent microscope (Axiovert 40 CFL, Carl-Zeiss Micro Imaging GmbH). Cells were counted and cell death rates were calculated as (*Cell death rate* = $PI \text{ stained cells} / \text{Hoechst stained cells} * 100$).

2.4 2DG drug preparation and treatment.

2DG was dissolved in filtered DW and stocked as 1M concentration solutions in -20 °C until use. 250 μ M 2DG was treated for 6 h and 12 h to control HCN cells to observe Atg8 protein member response. 500 μ M 2DG was treated for 8 h to scramble and Atg8 knockdown cells to observe the effects of lack of Atg8 family members. 2DG was administered to I(+) media and then treated to the cells via media change.

2.5 Western Blot

Atg8 member knockdown cells and control cells were harvested and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, and 1% Triton X-100) to obtain proteins. BCA protein assay reagent (Thermo Scientific) was used to measure lysate protein concentration. Samples were prepared and loaded into SDS gels and transferred to polyvinylidene difluoride (PVDF) membrane with semi-dry transfer cell (Bio-Rad, Richmond, CA). Membranes were blocked with 5 % skim-milk (Sigma Aldrich) at room temperature and then incubated with primary antibodies overnight at 4 °C. Incubated membranes were washed with TBST and incubated at room temperature in peroxidase-conjugated secondary antibody diluted in blocking solution. After incubation, proteins of interest were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo scientific).

3. Results

3.1 Effects of Atg8 knockdown on HCN cell survival.

Among the various types of Atg8 members, we have decided to focus on three Atg8 family member proteins, which are microtubule-associated protein 1 light chain 3 beta (LC3B), GABA type A receptor associated protein like 1 (GABARAP L1), and 2 (GABARAP L2). In order to first understand the significance of the three different Atg8 members (LC3B, GABARAP L1, and GABARAP L2) during autophagy and how they impact HCN cells, we observed how the cells respond to cellular stress in the absence of each three Atg8 members. Upon checking whether each strain of HCN cells had their corresponding Atg8 members properly knocked down (**Figure 4**), we deprived control and Atg8 knockdown HCN cells of insulin for 24 h, 48 h, and 72 h, which is reported to induce autophagic cell death in HCN cells [8], and observed cell death rates (**Figure 5**).

Insulin deprived conditions were achieved by using media that did not include any insulin in its composition. Atg8 knockdown cells and control cells were cultured in 96 well culture plates and checked for cell death rates. Cells were stained with Hoechst-Propidium iodide (Ho/PI) double staining (1 % volume of media in the well, 1/1000 final dilution) and observed under a fluorescent microscope (Axiovert 40 CFL, Carl-Zeiss MicroImaging GmbH). Cells were counted and cell death rates were calculated as (*Cell death*

*rate=PI stained cells / Hoechst stained cells * 100).*

Comparing the cell death rates of control and Atg8 knockdown cells upon autophagic cell death induced by insulin withdrawal, not only confirmed that GABARAP L1, GABARAP L2, and LC3B are all actively involved during autophagy in HCN cells, but also revealed an intriguing observation suggesting which molecules might be more tightly involved and induce the longest effect on HCN cells during autophagy compared to other molecules.

We have observed that GABARAP L1 knockdown HCN cells had the highest and longest lasting survival rate against autophagic cell death induced by insulin withdrawal compared to those of the other Atg8 knockdown strains. Thus, we conclude that GABARAP L1 is the most tightly involved during insulin deprivation induced autophagy.

3.2 Responses of Atg8 family members upon 2DG treatment.

Upon observing that GABARAP L1 knockdown in HCN cells decreased the rate of cell death, we have deliberated that GABARAP L1 may play a significant role during autophagy in HCN cells. In order to further investigate the responses of Atg8 members during other forms of autophagy in HCN cells, we decided to examine the cellular responses upon treating 2-deoxy-D-glucose (2DG), which showed to induce

autophagy, but not autophagic cell death during ongoing research in our lab. During the past years, various ways have been implicated by researchers to induce and observe autophagy in cells. Among various autophagy inducers, 2DG has been shown to induce autophagy through hindering proper glycolysis, eventually resulting in energy stress in cells.

The significance of investigating Atg8 family member responses following 2DG induced autophagy is due to the major difference between insulin deprivation induced autophagy and 2DG induced autophagy. While insulin deprivation autophagy leads to cellular death in HCN cells, 2DG induced autophagy does not induce cell death in HCN cells. Therefore, the major difference lies in that 2DG induced autophagy is meant as a survival mechanism within HCN cells. To understand the role of Atg8 members in HCN cells during 2DG induced autophagy, we treated HCN cells with 2DG and observed the molecular effects (**Figure 6**).

Following 2DG treatment, we once again observed increased response in GABARAP L1 compared to those of other Atg8 family members. Compared to the responses of other Atg8 family members, significant increase was shown in GABARAP L1 type 2, which is the membrane bound form of GABATAP L1 and also an indicator of increased autophagosome production. Once again, we have seen that GABARAP L1 shows the most robust reaction to 2DG treatment in HCN cells and thus, concluded that GABARAP L1 may play a crucial role in survival autophagy.

3.3 Effects of Atg8 knockdown in HCN cells upon 2DG treatment.

In the previous sections, GABARAP L1 knockdown HCN cells were observed to have the highest and longest lasting survival rate during autophagic cell death induced by insulin deprivation. Additionally, GABARAP L1 showed the most robust reaction following 2DG induced autophagy. The results from the cell death assay and 2DG treatment both lead us to realize the possibility that GABARAP L1 may play an unexpected, significant role during both cell death causing autophagy as well as cell survival autophagy.

In order to further investigate the role of Atg8 family member proteins, especially GABARAP L1, during autophagy following 2DG treatment in HCN cells, we treated the Atg8 knockdown cells with 2DG and observed Atg8 family member proteins as well as other nutrient-deficiency or glucose starvation responder proteins (**Figure 7**). Upon 2DG treatment, scramble cells as well as shLC3 and shL2 cells showed an increase in pAMPK; however, shL1 cells did not show a clear change in pAMPK. Additionally, shL1 cells did not show a clear increase in LC3 upon 2DG treatment, while they did show increase in GABARAP L1 type 2 despite having GABARAP L1 knocked down and also showed increase in GABARAP L2 type 1. shL2 cells showed increase in both GABARAP L1 and LC3. While shLC3 cells did not show clear change in either GABARAP L1 or GABARAP L2, they did show increase in GABARAP L1 type 1.

Observations made following 2DG treatment in HCN cells showed accumulation of GABARAP L1 type 1 in shLC3 cells, increased both types of GABARAP L1 and accumulation of GABARAP L2 type 1 in

shL1 cells, and increase in both LC3 type 2 and both types of GABARAP L1 in shL2 cells. Collectively, at this stage, it seems that lacking LC3 hinders that conjugation of GABARAP L1 with PE and lacking GABARAP L1 hinders the conjugation of GABARAP L2 with PE.

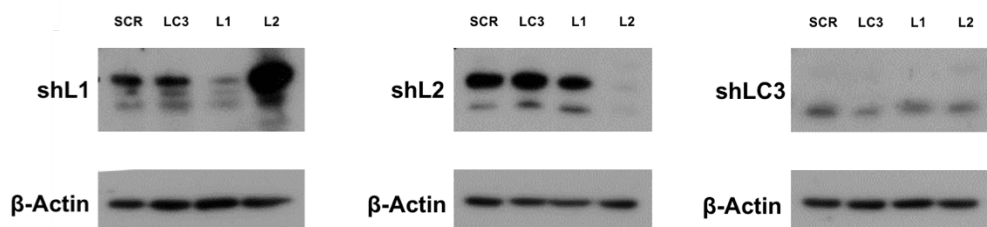
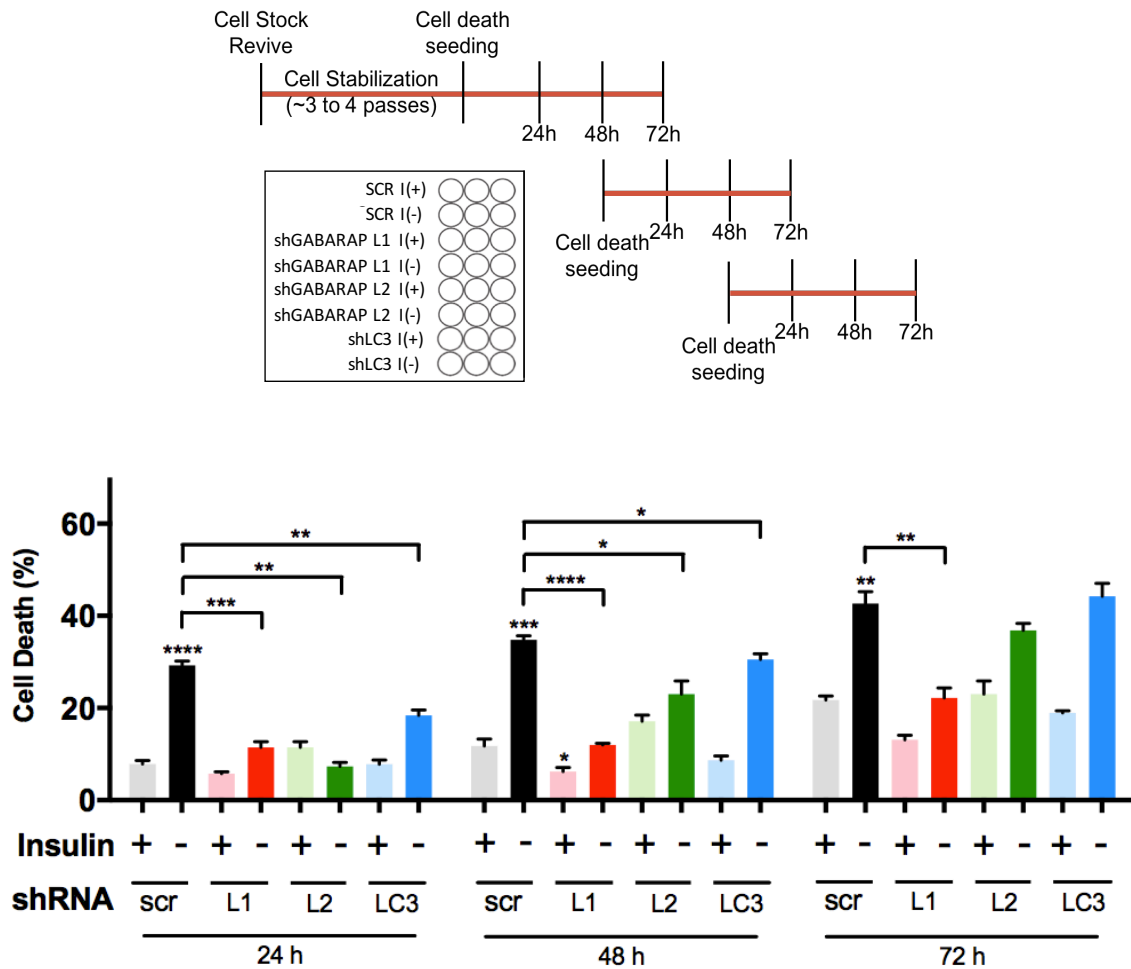


Figure 4. Western blot shows knockdown of Atg8 family members in HCN cells.

Atg8 knockdown HCN cell strians were checked before experimental use. All three Atg8 family members were properly knocked down, which was confired with western blot. Cells were stocked in -80 °C and thawed before use.



< Cell death analysis following 24 h, 48 h, 72 h of I(-) treatment >

Figure 5. Cell death analysis following 24h, 48h, 72h of insulin withdrawal in Atg8 knockdown HCN cells indicate GABARAP L1 to show highest cell survival rate against insulin deprivation derived cell death. Following the experimental scheme, HCN cells were seeded in 96 well plates, treated with insulin deprived media and observed for cell death rate.

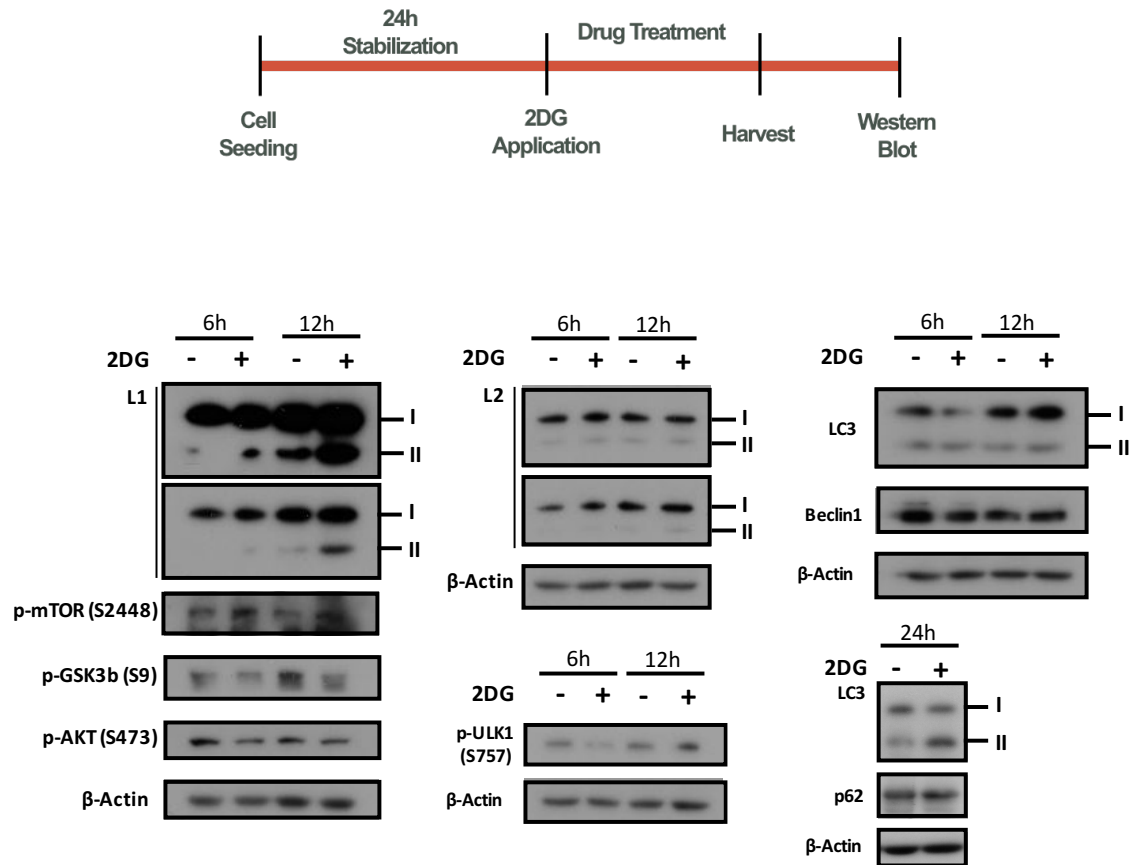


Figure 6. Western blot shows responses of Atg8 family members upon 2DG treatment in HCN cells.

HCN cells were treated with 250 μ M 2DG and western blot was conducted to observe how Atg8 family members as well as other proteins reacted to 2DG induced autophagy. Atg8 family members as well as other nutrient-deficiency or glucose starvation responder proteins were observed.

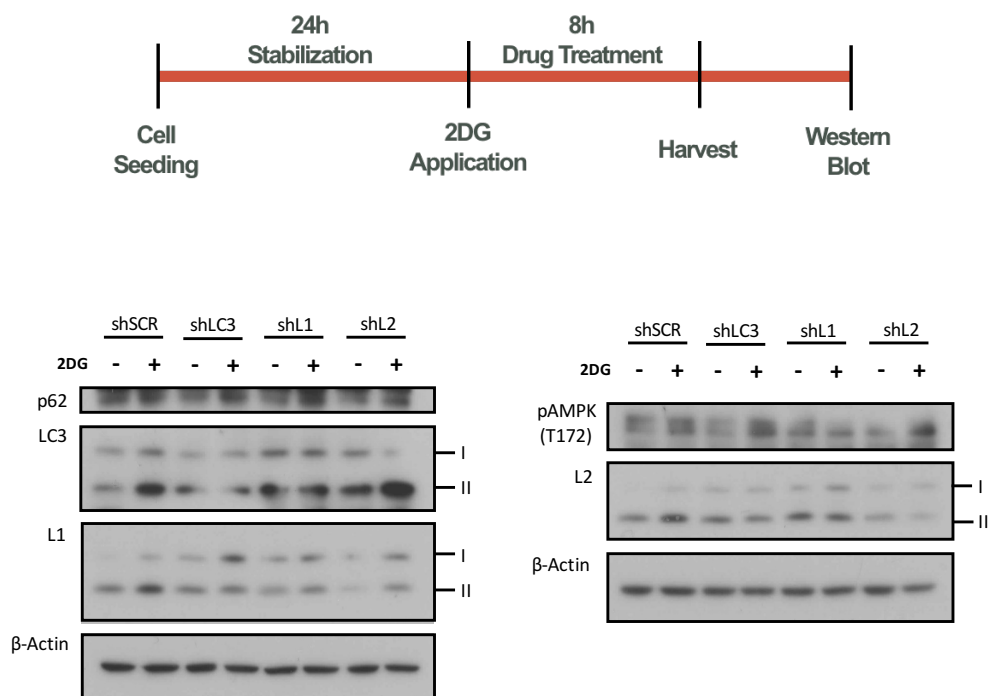


Figure 7. Western blot shows responses of Atg8 knockdown HCN cells following 2DG treatment.

Atg8 knockdown HCN cells were treated with 500 μM 2DG for 8 h and western blot was conducted to observe how Atg8 family members as well as other proteins reacted to 2DG induced autophagy. Atg8 family members as well as other nutrient-deficiency or glucose starvation responder proteins were observed.

4. Discussion

GABARAP L1 knockdown HCN cells were observed to have the highest and longest lasting survival rate against autophagic cell death induced by insulin withdrawal. This suggests that GABARAP L1 is highly involved in insulin deprivation induced autophagy in HCN cells. Interestingly, there was a distinct difference in the degree and length of cell survival rates depending on which Atg8 family member was knocked down. Although shL2 and shLC3 cells do not have survival rates as high and as long lasting as those of shL1 cells, shL2 cells do shows significant survival rate during 24hrs, which is also shown moderately in shLC3 cells. Further investigation will be needed to answer whether the distinct difference in the degree and length of cell death protection holds a further meaning.

Upon 2DG treatment, GABARAP L1 protein levels increased more rapidly than that of GABARAP L2 and LC3. This suggests that GABARAP L1 may be more sensitive to autophagy inducing conditions and may show a more robust reaction compared to GABARAP L2 or LC3. We have already concluded that lacking GABARAP L1 resulted in decreased cell death, 2DG induced autophagy resulted in most increase in GABARAP L1 type 2, and GABARAP L1 being speculated to be influenced by LC3 and influence GABARAP L2 leads to the idea that GABARAP L1 may also be an intermediate factor that may play a role in the link or switch between autophagic cell death and cell survival via autophagy. However, in order to clarify the exact relationship, we would need a more in-depth study that shows molecular changes following

whole pathways and also thoroughly observe double knockdown cells as well.

Some questions may be brought upon unexpected protein expression patterns such as the lack of change in pAMPK in shL1 cells and the increase in p62 upon 2DG treatment in all cell strains. At a glance, it is easy to assume that 2DG did not work in shL1 cells; however, our 2DG administration method ensures that all cells are treated with equal concentrations of 2DG. Additionally, shL1 cells do show increase in GABARAP L1 despite being knocked down and also shows increase in p62, which is shown in HCN cells upon 2DG administration. Thus, it is hard to believe that the lack of change in pAMPK levels in shL1 cells was due to imprecise drug administration. The lack of change in pAMPK in shL1 cells may suggest that lacking GABARAP L1 somehow hinders the molecular signaling that initiates glucose starvation induced AMPK activation in HCN cells, but once again, this study does not provide sufficient information to confirm the speculation. As for p62, the increase in p62 protein expression following 2DG administration is quite puzzling. Generally, p62 is observed to measure the rate of autophagic flux; p62 is incorporated during autophagy and is degraded upon autophagy completion. Therefore, we expected the level of p62 to decrease upon autophagy, which is clearly shown in insulin deprived conditions; however, not shown in 2DG induced autophagy. Although insulin deprivation induced autophagy and 2DG induced autophagy are completely different types of autophagy, the former causing cell death and later aiding cell survival, either conditions

are reported to show active autophagy. Thus, it is quite interesting that p62 increases upon 2DG treatment in HCN cells. Although this study cannot provide further explanation for the exact reason why p62 increases in HCN cells upon 2DG induced autophagy, further investigation may be able to discover a distinct and unique characteristic of HCN cells.

In conclusion, shL1 cells showed highest survival rate during insulin deprivation induced autophagy and knocking down different Atg8 family members resulted in different cell death rates. This opens up a possibility that Atg8 family members may play distinctive roles during autophagy in HCN cells. As we hypothesized, GABARAP L1 seem to be indeed involved in 2DG induced autophagy, However, the degree of sensitivity towards 2DG was less than expected. This, as I mentioned, may be due to different cellular characteristics and conditions. GABARAP L1 was shown to be crucial during both autophagic cell death and survival autophagy, along with type 1 accumulations seen in L1 and L2. Finally, lacking L2 did not affect the increase in LC3 and GABARAP L1 action upon 2DG treatment and this may open up a wide possibility of roles and relationships between atg8 members. LC3, L1, and L2 may be linked in the conjugation process or, the seemingly unaffected protein expression patterns of L1 and LC3 in shL2 cells may be due to the lack of L2 being compensated with increase of LC3 and L1. Although at this step, this study may not provide sufficient information to address all the points made in the discussion, and may not

be able to fully answer all the questions, it may serve as an initial starting point for further investigations, which will help further understand autophagy in HCN cells and aid in finding the clue for neurodegenerative diseases in the brain.

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요약문

2-deoxy-D-glucose 약물 처리 후, 성체 해마 줄기세포내 Atg8 family 단백질의 역할.

자가포식 관련 유전자 8 (Atg8)은 오토파고솜 형성 개시, 신장, 성숙 뿐 아니라, 라이소솜과의 결합, 그리고 운반 표적 선정을 포함한 자가포식 과정 전반에 걸쳐 주요한 역할을 한다. 그러나, 자가포식 과정에서 개별 Atg8 단백질이 어떠한 역할을 하는지 아직 완벽히 밝혀지지 않았다.

본 논문을 통해, 우리는 2-deoxy-D-glucose (2DG) 약물을 처리한 성체 해마 줄기세포에서 개별 Atg8 단백질의 역할 및 세포내 신호 전달과 분자 효과를 관찰하고자 한다. 특히, 우리는 쥐의 성체 해마 줄기세포에서 Atg8 family 단백질 중 세가지 단백질: LC3B, GABARAPL1, 그리고 GABARAPL2 에 집중을 할 것이며, 개별 Atg8 이 knockdown 된 성체 해마 줄기세포에 2DG 를 처리하여 자가포식을 유도한 후, 그에 따른 하위 신호 전달 경로와 관련 단백질 발현의 변화를 관찰 할 것이다.

위의 실험을 통해, 우리는 Atg8 family 단백질들의 효과 뿐 아니라, 성체 해마 줄기세포에 2DG 를 처리하여 세포생존성 자가포식을 유도하였을 때, 자가포식과 관련하여 Atg8 단백질들의 추가적인 역할을 알아내고자 한다. 궁극적으로, 우리는 이 결과가 개별 Atg8 단백질의 역할에 대한 이해를 더욱 높이고, 나아가, 자가포식 관련 신경변성질환에 대한 이해에 기여 할 수 있기를 바란다.

핵심어: Atg8, 2-deoxy-D-glucose, 자가포식, 성체 해마 줄기세포