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Neuronatin Gene Expression in Brain Stem Thalamic Circuit Involving Energy Homeostasis

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Abstract:Regulated gene expression in neurons within the gut-brain axis plays an important role in energy balance.I sought to explore the expression of neuronatin in relation to brain circuits that are involved in energy metabolism.I screened through Allen Brain connectivity and in situ hybridization databases,and observed the projections of GABAergic,glycinergic,and glutamatergic neurons and found that only glycinergic neurons project out of the nucleus of the solitary tract(NTS)into the zona incerta(ZI).My analyses showed that both glycinergic NTS neurons and their target neurons within the ZI are both neuronatin expressing cells.Taken together,my findings reveal that regulated expression of neuronatin in NTS and ZI neurons is necessary for the proper development of feeding behavior and metabolic processes.A better understanding of neuronatin expression and neurons expressing neuronatin may pave the way for new and improved therapeutic targets to help delay the onset and progression of metabolic associated conditions such as diabetes and obesity.

1. Introduction

Neuronatin is a gene that involves in mammalian development and regulates metabolism.At the cellular level,neuronatin is required for the regulation of energy metabolism and the synthesis of glycogen[1].Recent studies have shown that neuronatin plays a crucial role in energy expenditure and diet-based obesity.The deletion of the *neuronatin* gene in rodents causes lowered energy expenditure and becomes more susceptible to diet-based obesity.Other studies have also shown that the alteration of the *neuronatin* gene in humans may cause several disorders in organ development and metabolic processes[1],[2].The expression of neuronatin in cells promotes energy metabolism by regulating the control of intracellular signaling pathways.In fact,neuronatin plays a pivotal role in the governance of food intake and energy homeostasis.Interference with neuronatin expression will cause malfunctions throughout mammalian adulthood health.

The fidelity of energy balance,including food and water intake,is crucial for survival in all animals.Acids and enzymes residing in the stomach digest food to nutrients for absorption in the intestines.The carbohydrates,however,are broken down into a six-carbon sugar known as glucose,which goes through the process of adenosine triphosphate(ATP)synthesis to produce ATP.The exothermic reaction of removing a phosphate group provides the organism with energy[3].The NTS,a major feeding center in the brain,plays a crucial role in maintaining energy balance.Multiple systems within the body,including the respiratory,cardiovascular,and gastrointestinal systems are controlled by the NTS.As the gastrointestinal tract breaks down food,it signals the central nervous system(CNS),especially the nucleus of the solitary tract(NTS),through the vagus nerve[4].At the NTS,it sends different neurotransmitters,such as glutamate and GABA to the hypothalamus for appetite control and energy expenditure.The leptin receptor expressing NTS neurons send projections to the hypothalamus to mediate hunger and satiety.The disruption to the NTS increases food intake in mice and induces the onset of obesity[5].

The NTS is known to make bidirectional connections to several brain regions,such as the ZI,that mediate gastric regulatory functions.Lesion studies have shown that mice without ZI suffer from satiety and satiation malfunctions,such as early termination of food and water intake,and reduced meal frequency,ultimately impairing an organism's ability to survive[6],[7].However,the molecular identity of neurons within the NTS-ZI circuit is not well understood.

In recent years,the development of two-photon microscopes has enabled us to examine the brain with a higher resolution and at a greater depth.The theory behind this imaging technology relies on two photons with at least half the wavelength of its emitted light to be projected onto a targeted spot within the sample and emits light as it returns to its ground state.This enables the laser to mark spots that are perfectly suited for the emitted wavelength,thus creating high resolution and accurate projection images[8],[9].Other state of the art technologies such as Cre-*lox* and ISH have allowed us to modify genes and view expression results within the brain.

Cre-*lox* recombination is a genetic manipulation tool used *in vivo* for controlling gene expressions.This technique utilizes the Cre recombinase enzyme and the DNA sequence *loxP*.A Cre recombinase is an enzyme derived from the P1 bacteriophage that creates recombinases by cutting out DNA sequences between two *loxP* sites.The recombinase is composed of the amino acids,Arginine 173,Histidine 289,Arginine 292,Tyrosine 324,and Tryptophan 315.This composition allows the Cre recombinase to bind to its DNA substrates.The *loxP* sequence consists of a 34 base pair sequence made up of two 13 base pair palindromic recognition sites at each end,with an 8 base pair sequence as the spacer,which dictates the direction of the sequence.Due to the asymmetric nature of the *loxP* sites,when the Cre recombinase binds to these sites,it can initiate either deletion,inversion,or translocation between *loxP* sites.The orientation of the *loxP* sequence determines what can happen to the gene.The *loxP* sites that are sequenced in the same orientation result in a deletion,*loxP* sites in opposing orientations result in an inversion,and interchromosomal recombinations of the sites can result in translocation[10].

In addition to techniques that enable the visualization and manipulation of cells,ISH is an important technique that allows the mapping of gene expression in cells via *in vitro* labeling of genetic sequences.This process involves using complementary DNA or RNA to localize a specific gene of interest[11],[12].These complementary sequences contain antigens that can bind to antibodies-conjugated to enzymes that produce color reactions for visualization upon the addition of a substrate.The enzyme that is linked to the antibody is alkaline phosphatase,which reacts to the substrates,nitroblue tetrazolium(NBT)and 5-bromo-4-chloro-3-indolyl phosphate(BCIP).The alkaline phosphatase would convert the BCIP into an indoxyl intermediate that would further react with NBT to form a purplish

dye.The dye would indicate if the specific mRNA is present in the location that is being studied[13].All of these technologies have been able to create high-resolution images for more accurate data analysis.These ensure the accuracy and reproducibility of the experimental results.

In this paper,I focused on studying the neural mechanisms of neuronatin expressing NTS neurons in energy metabolism.To explore how neuronatin NTS neurons relay signals derived from the gut to the rest of the brain,I investigated three neurotransmitter NTS neuronal subtypes,namely,the GABAergic,glycinergic,and glutamatergic neurons by studying neurons that express Cre recombinase under the gene promoter *Gad65*,*GlyT2*,and *vGlut3*,respectively.Gad65 is an enzyme that catalyzes the synthesis of GABA,while GlyT2 and vGluT3 are transporters that transport glycine and glutamate neurotransmitters,respectively,to the synapse[14],[15].Two-photon microscopy was used to detect the projections of Gad65,GlyT2,and vGlut3 expressing neurons,and ISH data were used to characterize the molecular identity of neurons in the NTS-ZI circuit.This research would further our understanding of neuronatin involvement in neural circuits that control energy metabolism.

2. Materials and Methods

2.1 In Situ Hybridization

8 week old adult C57BL/6J male mice were used throughout the study.Prior to freezing,the mice were anesthetized with 0.5%isoflurane and then dissected and frozen,and stored at-80ºC.The frozen brains were then sliced into 25µm on Leica 3050 S cryostats[11].

With the standard procedure,the mice were then treated with hydrogen peroxide to block peroxidase activity and were treated with the enzyme,proteinase K to increase the permeability of brain tissues.After treatment,the mice brain tissues were incubated for 5.5 hours at 63.5ºC.Removing samples from incubation,it was rinsed with decreasing salt concentration solutions to reduce any background miRNA that may interfere with results[11].

Before running through *in situ* hybridization(ISH),horseradish peroxidase(HRP)conjugated anti-digoxigenin antibody and Tyramide Signal Amplification(TSA)was used to increase the sensitivity of ISH imaging.Following,the tissue samples were then incubated in a biotin-coupled tyramide,which is a highly reactive intermediate that can bind rapidly to HRP-linked probes.This amplification technique allows more biotin up for detection.The biotins then bind to neutravidin-alkaline phosphatase(AP)and a colorimetric reaction allows the neutravidin to cleave a phosphate from BCIP converting it to NBT.The NBT will produce a purplish dye at sites where the probes are bound to brain tissues.As a final step,the tissues were treated with ethylenediaminetetraacetic acid(EDTA)and then with a 4%PFA to stop the colorimetric reaction[11].

When ready for ISH imaging,both positive and negative controls were used.The positive controls contain an antisense *Drd1a* probe that intensifies the dye stain from the previous step.The negative controls were not treated with any probe and were used to assess any background staining[11].

I screened the ISH database athttps://mouse.brain-map.org/for the mapping of gene expression in mice brain tissues for *neuronatin* and *GlyT2* in the NTS and ZI[11].I interpreted the dye density created by ISH to analyze the gene expressions.

2.2 Cre-Lox

Cre viral tracing was used in C57BL/6J(wild-type)adult mice.With injection sites at the NTS,the projection of neuronal subtypes can be traced throughout the brain.These Cre-expressing engineered mice were then injected with a fluorescent rAAV vector to label the projection to different regions of the brain.These transgenic mice had the *Cre recombinase* genes inserted after the promoter.Due to the fact that this promoter can only be expressed in certain cell types,the Cre recombinase expression can be limited[9],[10].

In this study,3 specific transgenic Cre mouse lines were selected.The Gad2-IRES-Cre for the projection of *Gad65*,Slc6a5-Cre_KF109 for the projection of *GlyT2*,and Slc17a8-IRES2-Cre for the projection of *vGluT3*[9].

Through the Allen Brain Connectivity database athttps://connectivity.brain-map.org/,I analyzed the projection of the three neuronal subtypes,specifically the projection of *Gad65*,*GlyT2*,and *vGluT3* in the NTS and ZI[9].

2.3 Two-Photon Microscopy

Two-photon microscopy is an imaging technique used to view images of fixed tissue samples.This requires two photons with a wavelength that is half the wavelength of the emitted light.The two photons must simultaneously be excited to a higher energy state and as the photons return to their ground state,green light will be given off.Light will only be given off at regions where the laser is most tightly focused,allowing for a more accurate image[8].

3. Results

3.1 Glycinergic Nts Neuronal Axons Target the Zona Incerta

The NTS consists of heterogeneous neuronal populations characterized by distinct gene expressions.To identify which NTS subpopulations are essential for communicating with the rest of the brain,I investigated the projections of three different neurotransmitter expressing neurons.The three neuronal types studied were glycinergic(*GlyT2*-positive)neurons,GABAergic(*Gad65*-positive)neurons,and glutamatergic(*vGluT3*-positive)neurons.I analyzed the efferent projections of these neuronal types by screening an online database(Allen Brain Institute:Allen brain atlas).The database contains an array of neuronal tract-tracing experiments that utilize the Cre-*lox* system.

In my study,I investigated three different transgenic mice expressing Cre recombinase enzyme driven by *Gad65*,*GlyT2*,and *vGluT3* gene promoters,with localized transduction of Cre-dependent fluorescent reporter expressing virus in the NTS.This system enables the expression of reporter fluorescence throughout the entirety of the neurons,thus allowing us to visualize both the cell body and axon,under a microscope with light emitted at the appropriate excitatory wavelength.I observed that *GlyT2*-expressing neurons form axons projecting into the ZI(Figure 1D and 1E).Whereas,there was neither *Gad65*-nor *vGluT3*-expressing axons detected within the ZI(Figure 1F-1O).These results indicate that excitatory NTS neurons are local interneurons,and only glycinergic but not GABAergic or glutamatergic NTS neuron types connect the NTS to the zona incerta.

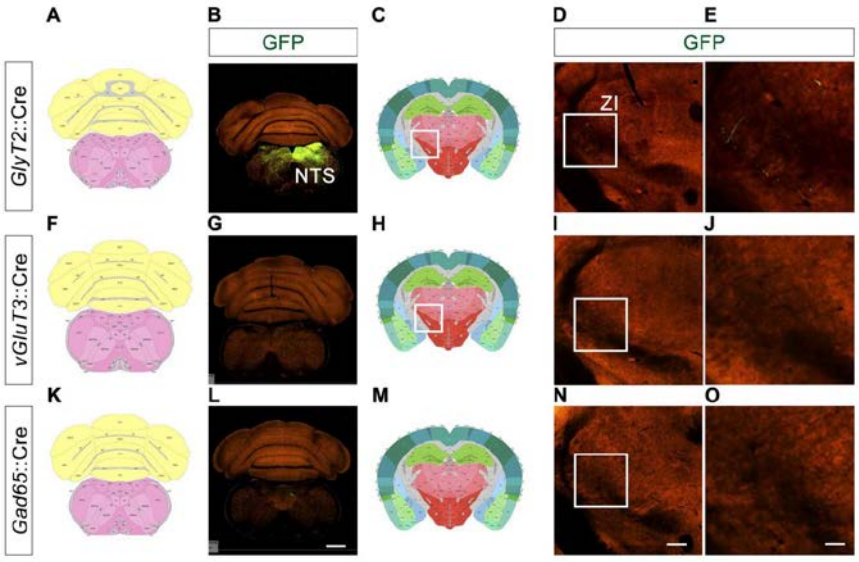

Fig.1 Glycinergic Neurons in the Nts Projects to the Zona Incerta

(A)A schematic of a coronal plane of the hindbrain containing the NTS.

(B)A representative image of virus transduction in the NTS of a *GlyT2* Cre-knock-in mouse brain,in green(GFP).

(C)A schematic of a coronal plane of the hindbrain containing the ZI.

(D)A representative image of the thalamus derived from the plane identified in the schematic,white box,C.

(E)A representative image of the ZI,a zoom in of white box,D.

(F)A schematic of a coronal plane of the hindbrain containing the NTS.

(G)A representative image of virus transduction in the NTS of a *vGluT3* Cre-knock-in mouse brain,in green(GFP).

(H)A schematic of a coronal plane of the hindbrain containing the ZI.

(I)A representative image of the thalamus derived from the plane identified in the schematic,white box,H.

(J)A representative image of the ZI,a zoom in of white box,I.

(K)A schematic of a coronal plane of the hindbrain containing the NTS.

(L)A representative image of virus transduction in the NTS of a *Gad65* Cre-knock-in mouse brain,in green(GFP).

(M)A schematic of a coronal plane of the hindbrain containing the ZI.

(N)A representative image of the thalamus derived from the plane identified in the schematic,white box,M.

(O)A representative image of the ZI,a zoom in of white box,N.

Scale bars:1000μm(B,G,L),250μm(D,I,N),100μm(E,J,O).

3.2 Neuronatin is a Molecular Component of the Nts-Zi Connection

Neuronatin is an important gene that is implicated in various metabolic diseases,and studies in both humans and rodents have shown that the NTS and ZI are canonical brain regions that control food

intake.Is neuronatin an essential protein for the NTS and ZI connectivity to influence energy balance?To determine whether GlyT2 projecting neurons within the NTS express neuronatin,I investigated the gene expression of NTS neurons for *GlyT2* and *neuronatin* by screening the Allen Brain ISH database.Consistent with my previous observation that *GlyT2* is a gene promoter that is capable of driving the expression of Cre recombinase(Figure 1A-1E),I found that GlyT2 is highly expressed within the entire NTS(NTSm,NTSl,NTSce)(Figure 2D-2F).Next,I examined the gene expression of neuronatin within the NTS using the same approach.Interestingly,neuronatin is specifically expressed within the medial region of the NTS,and not in the surrounding NTS nuclei(Figure 2A-2C).Lastly,I analyzed the zona incerta for *neuronatin* gene expression,and I observed neuronatin expressing neurons located in a region which we have shown to receive glycinergic NTS axonal input(Figure 2G and 2I).Together,my findings suggest that regulated *neuronatin* gene expression within the NTS-ZI circuit is potentially necessary for metabolic homeostasis.

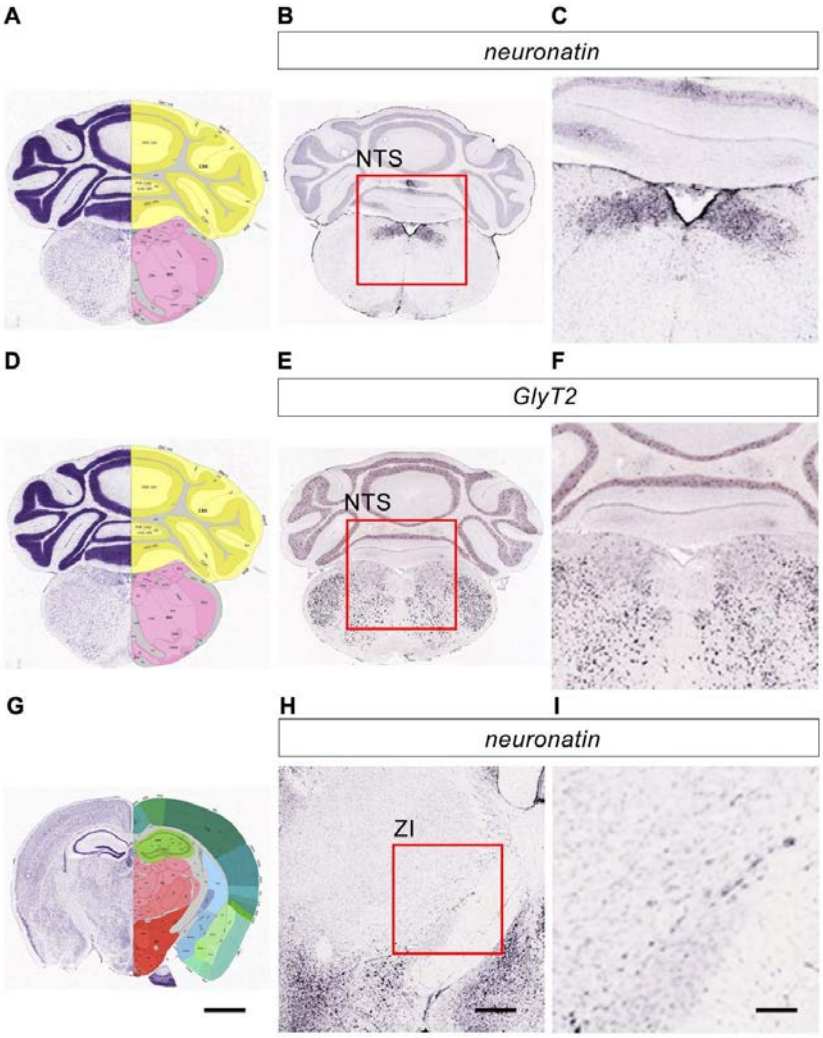

Fig.2 The Nts-Zi Circuit Consists of Neuronatin Expressing Neurons

(A)A schematic of a coronal plane of the hindbrain containing the NTS.
(B)A representative image of chromogenic *in situ* hybridization of *neuronatin* in mouse brains.

(C)A representative image of the NTS expressing *neuronatin*,a zoom in of red box,B.

(D)A schematic of a coronal plane of the hindbrain containing the NTS.

(E)A representative image of chromogenic *in situ* hybridization of *GlyT2* in mouse brains.

(F)A representative image of the NTS expressing *GlyT2*,a zoom in of red box,E.

(G)A schematic of a coronal plane of the hindbrain containing the ZI.

(H)A representative image of chromogenic *in situ* hybridization of *neuronatin* in the ZI in mouse brains.

(I)A representative image of the ZI expressing *neuronatin*,a zoom in or red box,H.

Scale bars:1000μm(A,B,D,E,G),250μm(C,F,H),100μm(I).

4. Discussion

Neuronatin has been shown to express in NTS and ZI neurons.The expression of the neuronatin correlates with the regulation of energy metabolism,glycogen synthesis,and development.Some studies have revealed that the deletion of the neuronatin gene restricts growth potential and leads to the onset of obesity.In humans,the alteration of this crucial gene may induce disorders related to energy metabolism in adulthood.Though researchers have shown the association between *neuronatin* and organ development and obesity by the deletion of *neuronatin* in rodents[2].The deletion of the entire neuronatin gene does not distinguish the gene's mechanism behind the metabolic deficit[1].One way to elucidate the role of neuronatin could be to delete the neuronatin gene from specific parts of the body,such as the NTS,ZI,pancreaticβ-cells,adipocytes,or other regions that may express neuronatin.This would allow researchers to pinpoint the exact location of neuronatin expressing tissues in the body that causes metabolic deficits.

Neuronatin's involvement in energy expenditure is vital to homeostasis[2].As mentioned earlier,the deletion or alteration of this gene in rodents and humans causes an imbalance in energy expenditure and may lead to many long term negative effects in maintaining energy homeostasis[1].From my research,I have shown that *neuronatin* is expressed in both the NTS and ZI.This relay between the two brain regions is critical in sustaining proper energy homeostasis.The NTS receives and responds to various signals from the gastrointestinal system to increase energy expenditure.While the ZI enhances food and water intake behavior to replenish the energy spent by the NTS.Together,the NTS-ZI circuit balances how nutrients are acquired and expended as energy on a daily basis.

Neuronatin's role in the NTS-ZI circuit stabilizes energy metabolism to ensure normal bodily function[16].When food is in the gut,it sends signals to the NTS via the vagus nerve.The glycinergic neurons in the NTS would be activated to relay an inhibitory signal to the ZI,thus,inhibiting ZI GABAergic neuron population,which is associated with binge-like eating behavior[5],[7].Overall,the feedback loop between the NTS and ZI maintains healthy homeostasis via energy metabolism and reducing energy intake.Though the *neuronatin* gene has been shown to regulate metabolism,its role in intracellular calcium ion signaling in relation to energy regulation and gastrointestinal functions remains unclear[2].In a broader picture,a reduction in food intake reduces caloric intake,therefore,reduces energy intake[16].On the contrary,when the *neuronatin* gene is dysfunctional,the organism would be more susceptible to diet-based obesity due to an increase in food intake,which upsets energy balance[2].New advancements in the field would open new doors to therapeutic treatments for patients with diabetes,obesity,and other metabolic associated diseases.

5. Conclusion

Neuronatin is a crucial component of the NTS-ZI circuit involving energy metabolism.By activating intracellular signaling and promoting glycogen synthesis,neuronatin aids in the maintenance of energy balance.New findings regarding the brainstem thalamic circuit would allow new treatments for diabetic and obese patients,as well as those with poor metabolic regulations.

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