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理學博士 學位論文

에너지 항상성 조절에 대한 시상하부 미세아교세포의 역할에 대한 연구

Study for role of hypothalamic microglia on control of energy homeostasis

蔚 山 大 學 校 大 學 院

生 命 科 學 科

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Study for role of hypothalamic microglia on control of energy homeostasis

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CONTENTS

Study for role of hypothalamic microglia on control of energy homeostasis

CHAPTER 1

P2Y12 receptor in the microglia affects the hypothalamic neuronal activation through regulation of the microglia morphology

Abstract

Morphology change of the microglia by P2Y12 receptor performs surveillance and phagocytosis functions in the various brain conditions, and these roles are involved in maintaining brain homeostasis. The P2Y12 receptor activation is affected by the generated adenosine 5’-triphosphate (ATP) in the mitochondria of the neurons, and this activation is involved in the regulation of the microglia process. In particular, change in hypothalamic microglial morphology through the P2Y12 receptor plays a crucial role in the energy homeostasis mechanism of the microglia that is important in regulation of neuronal functions by releasing cytokines and chemokines. However, it is unclear whether the morphological change of microglia through the P2Y12 receptor is affected by energy starvation. Therefore, in this study, I investigated the morphological change of hypothalamic microglia by overnight fasting. In the hypothalamic arcuate nucleus (ARC), process length and P2Y12 receptor activation in microglia were decreased by overnight fasting. Administration of P2Y12 agonist and antagonist affected the morphological parameters such as process length and branching of the hypothalamic microglia. Interestingly, the P2Y12 agonist and antagonist also affected neuronal activation in the hypothalamic ARC. These observations suggest that the P2Y12 receptor of hypothalamic microglia is a crucial mediator of microglial morphology and neuronal activation.

Introduction

Microglia are brain-resident macrophages and play important functions such as neuronal development and phagocytosis in the central nervous system (CNS) (1-3). During the past decade, a great deal of attention has been paid to investigating the microglial morphology associated with various brain environments, such as inflammation and development (4-6). Microglia morphology plays an important role in surveying the surrounding environment, sensing energy sources, and controlling physiological or pathological conditions in the brain (7-9). Previous reports showed that microglia morphology was affected by pathological environments such as brain injury and diet-induced obesity (DIO) (10, 11). However, recent reports have shown increasing evidence that microglia morphology is modulated by the P2Y12 receptor and ATP in the physiological environment (12, 13). Thus, the importance of understanding the morphological changes in microglia caused by the P2Y12 receptor is emphasized in a physiological environment.

The P2Y12 receptor is one of the ADP-dependent purinergic receptors that mediate the important intracellular signaling pathways of actin cytoskeleton reorganization in microglia (14-16). The activation of P2Y12 receptors in the CNS is regulated by ADP that is generated by the hydrolysis of ATP produced in the brain, and it has been shown that this regulation changes the morphology of microglia (15). ATP is a neurotransmitter that is generated in the mitochondria of neurons and contributes to the functional regulation of neurons and glial cells as well as the regulation of brain energy balance (17, 18). From the point of view of ATP generation, hypothalamic

neurons show decreased ATP generation during energy starvation, and hypothalamic neurons and glial cells show increased ATP generation during over-nutrient states such as high-fat diet (HFD) (19, 20). In addition, recent reports show that the microglia processes were decreased through P2Y12 receptor knockout and inhibition of P2Y12 receptor activation in mice, which affected the inhibition of microglia surveillance and contact with neurons. Microglia surveillance and contacts with neurons are important for the regulation of inflammatory responses, neuronal functions, and energy homeostasis (13). However, it is unclear whether microglia morphology and regulation of neuronal functions by microglia are affected by energy starvation. Thus, in this study, I investigated whether energy starvation changes the morphology of microglia in the hypothalamus.

Materials and Methods

Animals and experiment design

In this study, all animal experiments were conducted as required by the Regulations of the University of Ulsan for the Care and Use of Laboratory Animals. Eight-week-old male C57BL/6 mice (bodyweight 23–27 g, Korea) were used. Mice were maintained in temperature- and humidity-controlled rooms with a 12 h light - 12 h dark cycle, with the lights on from 7:00 a.m. to 7:00 p.m. and ad libitum and given free access to tap water. For the food deprivation experiments, food was removed for 18 hours (from 4:00 p.m. to 10:00 a.m.).

Cannulation and administration of P2Y12 receptor agonist and antagonist

For intracerebroventricular (i.c.v) cannula implantation, mice were anesthetized by i.p injection of tribromoethanol (250 mg/kg, Sigma-Aldrich) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The cannula (26 gauge) was implanted into the right lateral ventricle (1.0 mm lateral, 0.3 mm posterior, and 2.4 mm ventral to the bregma) according to the Stereotaxic Mouse Brain Atlas (Paxinos G and Franklin KBJ, 2001, Academic Press, San Diego, CA, USA) and secured to the skull with dental cement. After 7 days of recovery, mice were injected with vehicle (saline) or with 2ME-sADP (100 μmol/2 μl, Tocris, Minneapolis, NE, USA) after overnight fasting. In addition, the normal fed mice were injected with PSB 0739 (3ug/ul, Tocris, Minneapolis, NE, USA). For immunohistochemistry analysis, mice were sacrificed 1 h after the injection of the 2ME-sADP, and the mice were sacrificed 3h after the injection of the PSB 0739.

Micropunch dissection and ATP analysis

After overnight fasting, at 10 am the next day, mice were sacrificed by decapitation. The mice brains were quickly removed and obtained the around 1mm3 tissue surrounding the ARC with a 1mm-diameter micro punch by using anatomical landmarks from the mouse brain atlas with the micro-punching set (Stoelting, Wood Dale, IL). Micro-punched ARC was stored in microcentrifuge tubes at -80 ℃.

The ATP measurement was using the Enlitenk ATP assay system with a bioluminescence detection kit (FF2200, Promega). Briefly, micro-punched ARC samples were neutralized to pH7.4 with a 10ul 4M Tris and this 10ul is transferred new tube with 90ul ATP-free water. Before the measured ATP concentration by the luminometer added the luciferase reagent. ATP concentration was calculated compared with the ATP standard curve.

Immunohistochemistry (IHC)

Animals were deeply anesthetized with tribromoethanol and transcardially perfused with phosphate buffer (PB, 0.1 M, pH 7.4), followed by a fresh fixative of 4% paraformaldehyde in PB. Brains were post-fixed overnight at 4°C, sliced to a thickness of 50 μm using a vibratome (VT1000P; Leica Microsystems, Wetzlar, Germany), and then washed several times in PB. Coronal brain sections containing the hypothalamic arcuate nucleus (ARC) were preincubated with 0.3% Triton X-100 (T8787, Sigma-Aldrich) in PB for 30 min to permeabilize the tissues and cells. After further washing with PB, the sections were treated with primary antibodies overnight at 4°C; as follows rabbit anti-Iba1 antibody (1:500; 019-19741, Wako, Osaka, Japan), rabbit anti-P2Y12

(1:1,000; 848002, Biolegend, CA, USA) and mouse anti-c-fos antibody (1:1,000; sc-166940, Santa Cruz, Dallas, TX, USA). On the next day, sections were washed in PB. For immunofluorescence staining, sections were incubated with the following secondary antibodies for 3 h at room temperature: goat anti-rabbit Alexa Fluor 488 (1:500; A11008, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit Alexa Fluor 594 (1:500; A11012, Invitrogen) and goat anti-mouse Alexa Fluor 488 (1:500; A11001, Invitrogen). Stained brain sections were imaged using an FV-1200 confocal laser-scanning microscope (Olympus America, Inc., Center Valley, PA, USA).

IHC image analyses

The microglia morphology analysis, the length of processes, processes branching, and terminal points were measured by using the semi-automatic filament plug-in in the Imaris 3D software (Bitplane, Zurich, Switzerland). The IMARIS automatic filament tracer reconstructs the quantified morphological parameters such as process length, branch point and terminal points in the confocal image stack of microglia. Algorithms of filament tracer use the no-loops approach based on local intensity contrast and use large and small seed points, which were extracted from soma and process. Automatically extracted unnecessary morphological features were deleted by manual editing by unbiased observers.

The intensity of P2Y12 and soma size were measured using ImageJ V 1.50 software (National Institutes of Health, Bethesda, MD) in the ARC. Region of interest (ROI) within an image was manually selected with the Mouse Brain Atlas for ARC (ARC: between -1.46 and -1.82 mm from bregma). The images were converted to 8-bit images and a threshold was applied. The images were binarized to separate the

immuno-positive cells from the background. The number of immune-positive cells in the hypothalamus was counted by unbiased observers.

Statistical analyses

Statistical analyses were performed in GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± SEM. The statistical significance between two groups was analyzed by unpaired Student's t-test.

Results

Food deprivation affects the microglial process in the hypothalamic arcuate nucleus.

It has been well established that microglia are crucial for the maintenance of energy homeostasis through sensing the energy sources in the brain. To determine whether energy deprivation affects hypothalamic microglia morphology, I investigated changes in microglia morphology in the hypothalamic arcuate nucleus (ARC) of fed and fasted mice, using an antibody for iba-1 as a marker for microglia (Fig 1A). Interestingly, fasted mice had decreased total dendrite length, branch points, and terminal points than fed mice, but soma size did not differ between the two groups of mice (Fig 1B-E). In addition, I measured the number of microglia in the hypothalamic ARC to see whether the change in microglia morphology is affected by the number of microglia in the hypothalamic ARC. The number of hypothalamic ARC microglia had not been changed in mice fasted overnight compared to normally fed mice (Fig 1F). These findings suggest that fasting affects the morphology of hypothalamic ARC microglia.

Food deprivation has no effect on the microglial process in any other region of the brain than the hypothalamus.

Feeding behavior is closely associated with various hypothalamic nuclei such as the paraventricular nucleus (PVN), dorsomedial (DMH) and ventromedial (VMH) hypothalamus. Therefore, I next investigated whether microglia morphology of the PVN, VMH and DMH is affected by food deprivation. The iba-1 was observed in the PVN, VMH and DMH (Fig 2A, E, I). The fasted mice did not show a difference in

morphological parameters (process length, branch points and terminal points) in PVN (Fig 2B-D), VMH (Fig 2F-H) and DMH (Fig 2J-L) microglia compared to fed mice. Since the hippocampus and cortex, also reveal food deprivation-induced energy metabolism and neurogenesis, in addition to the hypothalamus, I next investigated whether microglia morphology in the hippocampus and cortex is affected by food deprivation (Fig 2M, Q). I analyzed changes in microglia morphology parameters in the hippocampus and cortex of fasted mice compared to fed mice. Fasting did not cause any difference in morphological parameters in the hippocampus (Fig 2N-Q) and cortex (Fig 2R-V) compared to fed mice. Thus, the food deprivation induced morphological changes of the microglia only in the hypothalamic ARC.

The hypothalamic ATP levels by energy state affect the activity of microglia P2Y12 receptors.

The P2Y12 receptor is involved in the change of the microglia process. In particular, the P2Y12 receptor activation is affected by generated ADP through hydrolysis of extracellular ATP. To further identify whether the change in microglia morphology is affected by the P2Y12 receptor, I investigated the change of ATP level in the hypothalamic ARC by energy state. The fasted mice showed decreased ATP levels in the hypothalamic ARC compared to fed mice (Fig 3A). Accordingly, I observed the P2Y12 receptor expression in the hypothalamic ARC by energy state. The fasted mice showed significantly decreased P2Y12 receptor intensity in hypothalamic ARC (Fig 3B-C). Collectively, these observations suggest that the decreased microglia process in fasting status is closely related to P2Y12 receptor activation by ATP.

The P2Y12 receptor agonist has an effect on microglia process recovery.

It has been well established that the P2Y12 receptor activation is involved in the morphological changes of microglia in the central nervous system. Thus, to determine whether the decreased P2Y12 receptor activation influences the microglia process, mice were administered with 2MEsADP, a P2Y12 receptor agonist, during overnight fasting. 2MEsADP induced an increase in P2Y12 receptor intensity in the hypothalamic ARC during overnight fasting (Fig 4A, B). In addition, 2MEsADP increased microglia process length, branch points and terminal points in the hypothalamus (Fig 4C-F). Thus, these results suggest that the regulated microglia process in the fasting state is affected by the P2Y12 receptor activation.

Effects of P2Y12 antagonist administration on the microglia process in the normal condition.

In the fasted condition, I observed a decrease in the microglia process and P2Y12 receptors (Fig 1A, 3C). Thus, I investigated whether the inhibition of the P2Y12 receptor activation in the normally fed mice affects the change in microglia morphology. Mice were administered with PSB 0739, a P2Y12 receptor antagonist, to normally fed mice and P2Y12 receptor activation was observed in the hypothalamic ARC (Fig 5A). PSB 0739 significantly decreased P2Y12 receptor activation in the hypothalamic ARC. In addition, morphological parameters of hypothalamic microglia such as total process length, branch points and terminal points were also decreased (Fig 5C-F). Thus, these results indicate that the energy state regulates microglia morphology and that these morphological changes are associated with P2Y12 receptor activation by the energy state.

Effect of P2Y12 receptor activation on the regulation of hypothalamic neuronal activation.

It has been well established that the P2Y12 receptor is involved in the morphological changes of microglia as well as contact with neurons. In addition, in the normal condition, microglia contact with neurons is associated with inhibition of neuronal activation. Therefore, I examined whether change in P2Y12 receptor activation by 2MEsADP and PSB0739 is involved in the regulation of hypothalamic neuronal activation (Fig 6A, C). First, the increased neuronal activation by overnight fasting is decreased by the 2MEsADP (Fig 6B). Second, in the normally fed mice, hypothalamic neuronal activation is increased by the PSB 0739 (Fig 6D). Collectively, the hypothalamic microglia P2Y12 receptor activation affects regulation of the microglia process and decreased P2Y12 receptor activation results in decreased microglia contact with neurons, which induces to increase in neuronal activation in the hypothalamus.

Figures

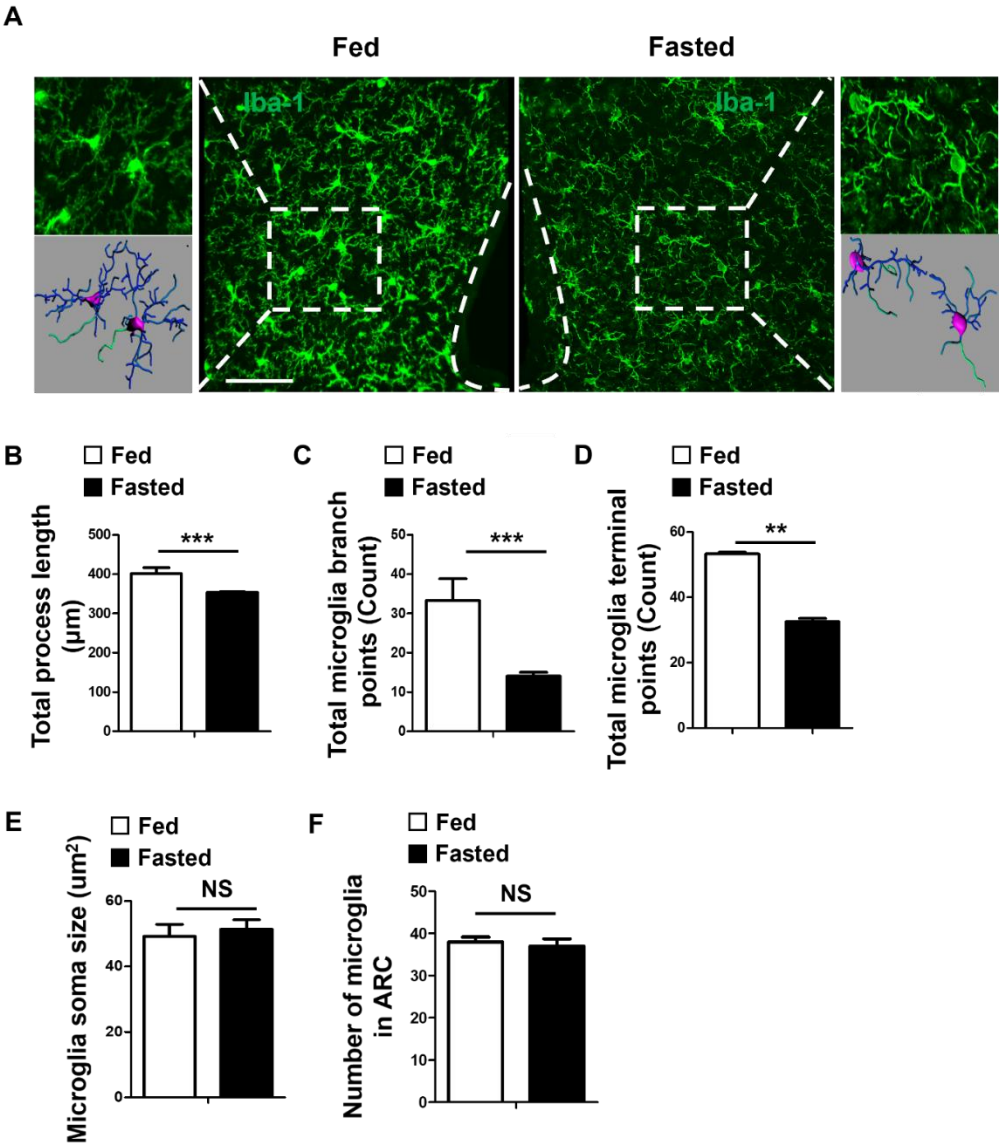

Figure. 1 Morphological change of the hypothalamic microglia in the fasting status

Analysis of morphological changes in hypothalamic microglia using 3D reconstruction. Representative immunohistochemical images (A) and calculated graphs show that the total process length (B), branch points (C) and terminal points (D) were decreased in 18 h fasted mice compared to normal fed mice. Hypothalamic microglia soma size (E)

and number (F) were not different in each group of mice. Data are presented as mean ± SEM. (n=3 sections of 4 mice/group). **p<0.01and ***p<0.001. ns, not significant. Scale bar = 100 μm.

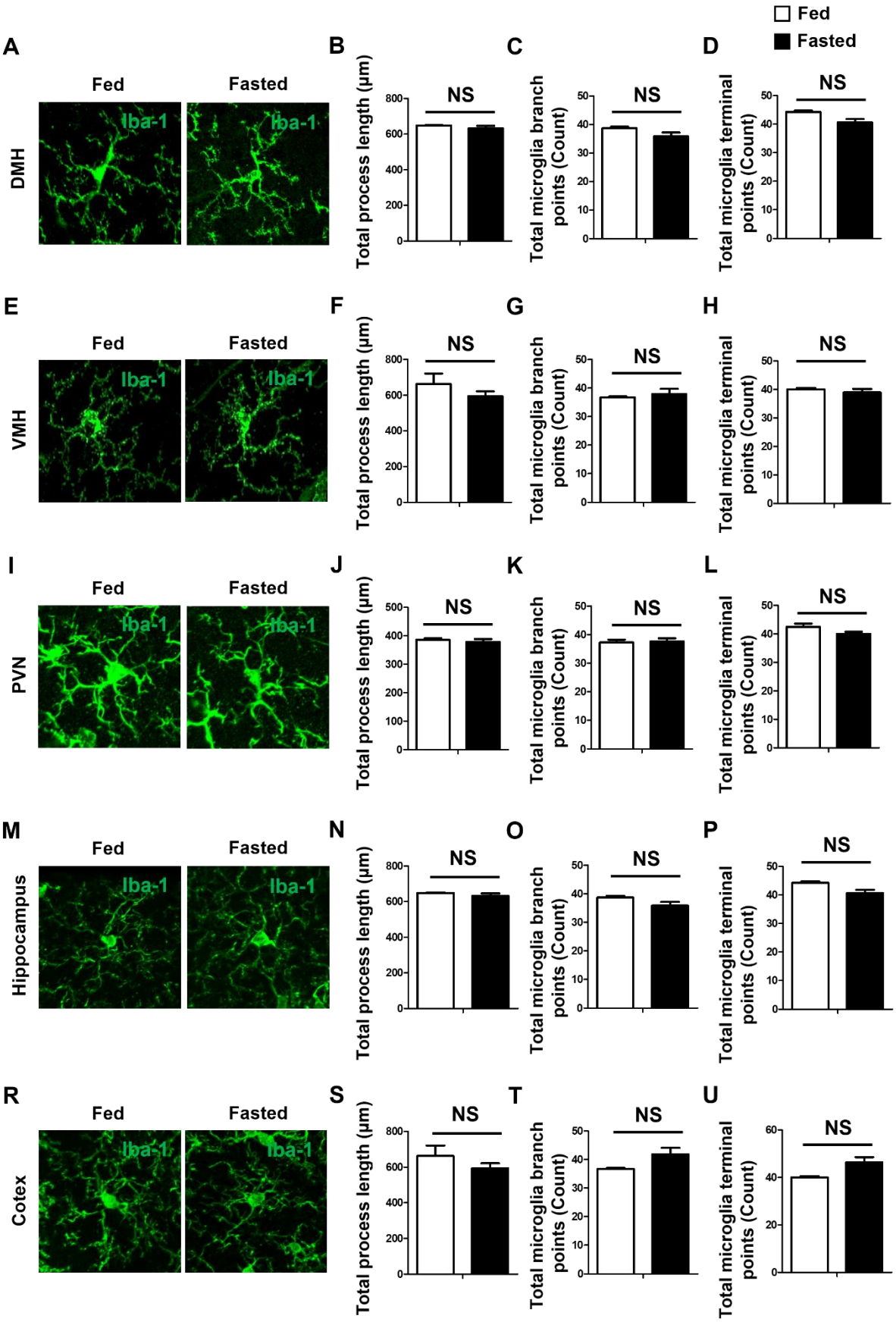
A
B
C
D
Fed
Fasted
DMH
Iba-1
Total process length (µm)
Total microglia branch points (Count)
Total microglia terminal points (Count)
NS
E
F
G
H
VMH
I
J
K
L
PVN
M
N
O
P
Hippocampus
R
S
T
U
Cotex

Figure. 2 Morphological changes of the microglia in the various brain regions in fasting status.

Morphology of the microglia does not change in various hypothalamic nuclei and other brain regions. To analyze the hypothalamic nuclei and other brain regions, morphology of iba-1 positive microglia was measured in hypothalamic nuclei (DMH, VMH and PVN), hippocampus and cortex of fasted and fed mice. (A-L) Iba-1 positive morphology did not change in DMH (A-D), VMH (E-H) and PVN (I-L) of fasted mice compared to control mice. In addition, microglia morphology in the hippocampus and cortex were not changed in fasted mice compared to control mice (M-T). Data are presented as mean ± SEM (n=3 sections of 4 mice/group). ns, not significant.

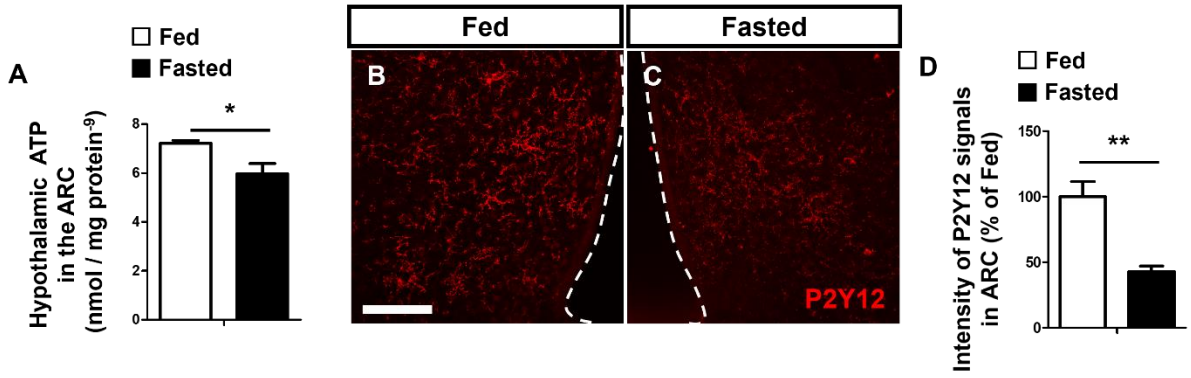

Figure. 3 P2Y12 receptor activation of microglia is affected by ATP level of the hypothalamus.

Overnight fasting-induced P2Y12 receptor intensity, ATP and ADP level were measured in the hypothalamic ARC. ATP and ADP contents are measured by luminescence assay in the hypothalamic ARC. (A) ATP content was significantly decreased in the 18 h fasted mice compared to control mice. (n=4 mice/group). (B-D) Representative immunohistochemical images (B, C) and calculated graph (D) show decreased P2Y12 intensity in the fasted mice compared to control mice in the hypothalamic ARC (n=3 sections of 4 mice/group). Data are presented as mean ± SEM. *p<0.05 and **p<0.01. Scale bar = 100 μm.

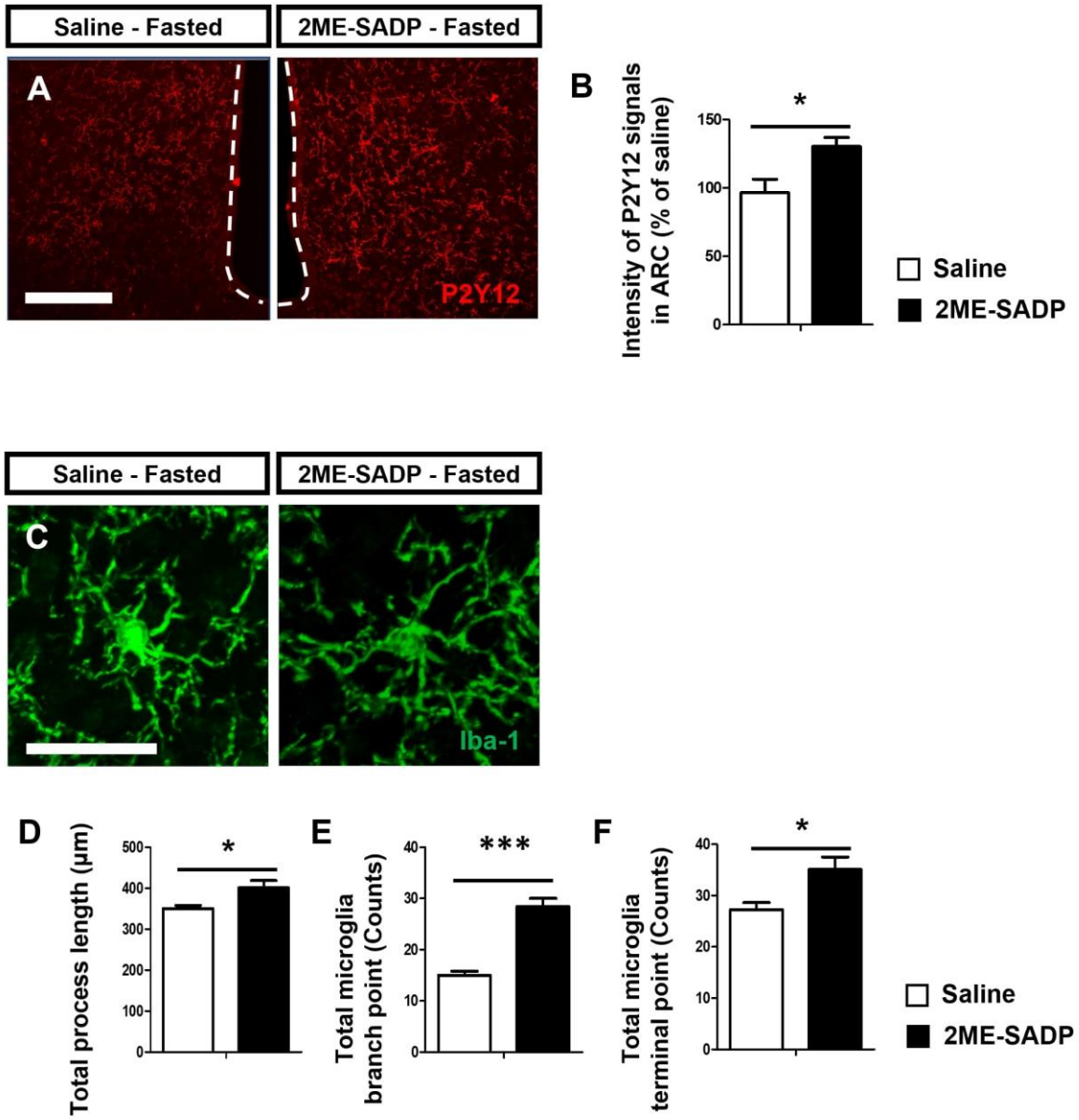

Figure. 4 The P2Y12 receptor activity is affected the hypothalamic microglia morphology in the fasting status.

P2Y12 receptor agonist-induced increase of P2Y12 receptor intensity increased microglia process after 18 h fasting in the hypothalamic ARC. To identify effect of the P2Y12 receptor agonist, overnight fasted mice were icv injection of P2Y12 receptor

agonist 2ME-SADP (100 μmol/2 μl), and then microglia (Iba-1) and P2Y12 (P2Y12) were analyzed in hypothalamic ARC using immunohistochemistry. (A-B) Representative images (A) and calculated graph (B) show an increase in the P2Y12 receptor intensity after 18 h fasting by the 2MEsADP compared to control mice. (C-F) Representative images (C) and calculated graphs (D-F) show an increase in microglia morphological parameters such as total process length (D), branch points (E) and terminal points (F) by the 2MEsADP after 18 h fasting compared to control mice. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). *$p<0.05$ and ***$p<0.001$. Scale bar = 100 μm.

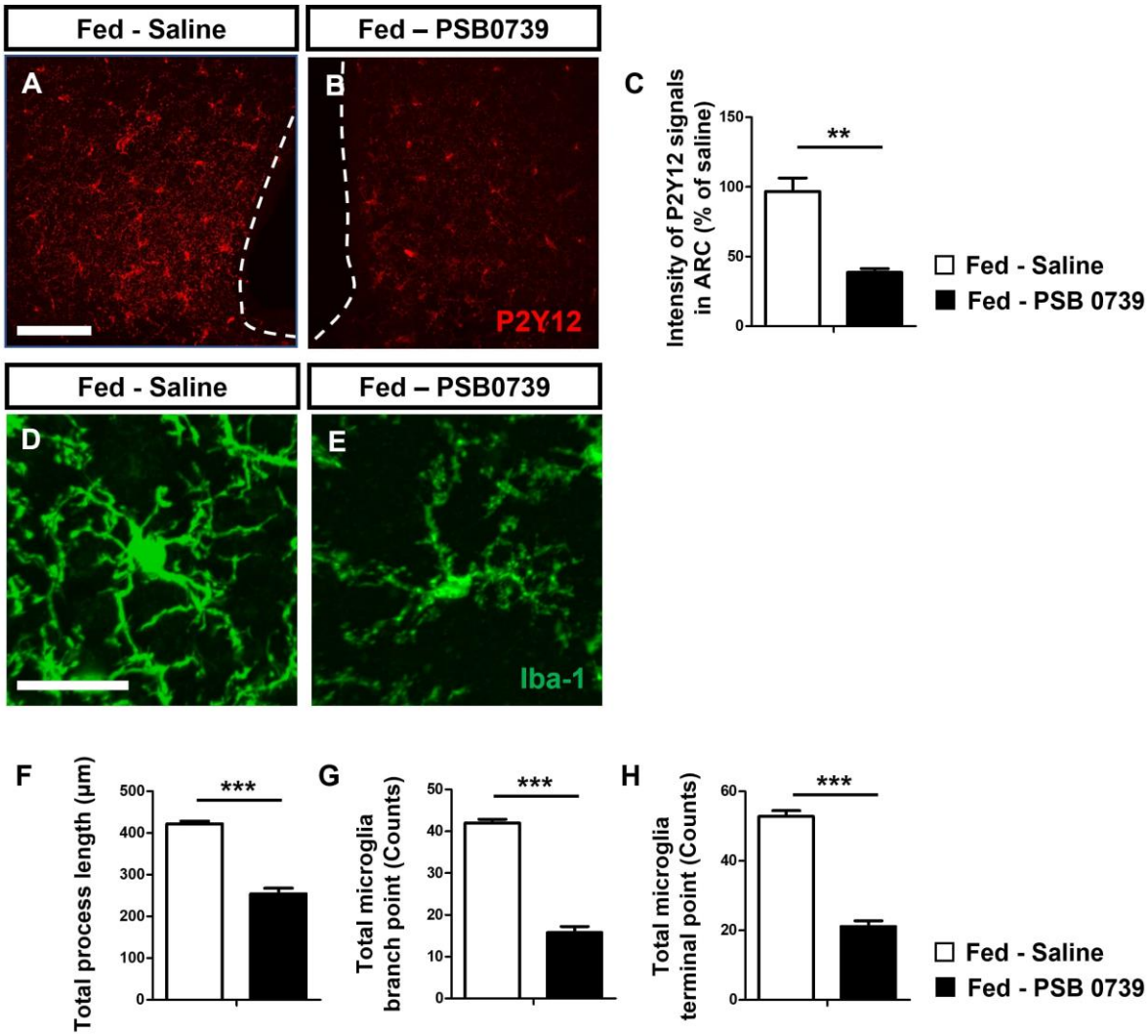

Figure. 5 Effect of the P2Y12 receptor antagonist on the microglia morphology in fed mice

P2Y12 receptor antagonist-induced decrease of the P2Y12 receptor intensity resulted in a decrease in microglia process in the hypothalamic ARC. To identify effect of the P2Y12 antagonist, normally fed mice were icv injected with of PSB0739 (3ug/ul), and then microglia morphology (iba-1) and P2Y12 (P2Y12) were analyzed in the hypothalamic ARC using immunohistochemistry. (A, B) Representative images (A) and calculated graph (B) show a decrease in the P2Y12 receptor intensity by the PSB0739

in normally fed mice compared to control mice. (C-F) Representative images (C) and calculated graphs (D-F) show a decrease in microglia morphological parameters such as total process length (D), branch points (E) and terminal points (F) by the PSB0739 in normally fed mice compared to control mice. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). **p<0.01 and ***p<0.001. Scale bar = 100 μm.

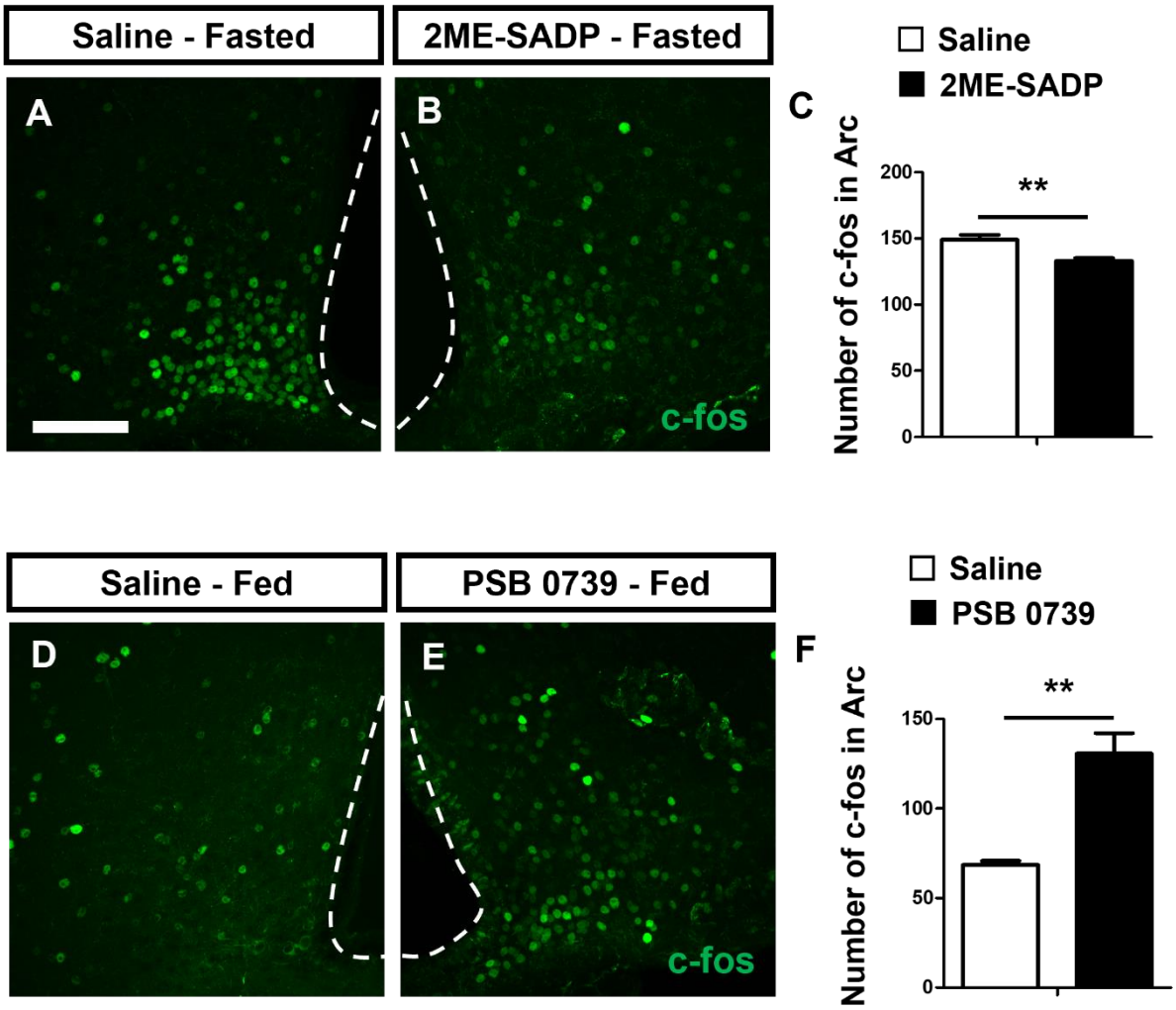

Figure. 6 Effect of the P2Y12 receptor activity on the neuronal activation in the hypothalamus.

Hypothalamic neuronal activation was decreased in the hypothalamic ARC by the P2Y12 receptor agonist after overnight 18h fasting, also in normal fed mice hypothalamic neuronal activation is increased by the P2Y12 receptor antagonist. To identify the effect of the P2Y12 receptor agonist and antagonist, overnight fasted mice were icv injection of 2MEsADP and normal fed mice were icv injection of PSB0739,

and then c-fos, a neuronal activation marker, were analyzed in the hypothalamic ARC using immunohistochemistry. (A-B) Representative images (A) and calculated graph (B) show a decrease in the neuronal activation by the 2MEsADP in the overnight fasted mice. (C, D) Representative images (C) and calculated graphs (D) show an increase in the neuronal activation by the PSB 0739 in normally fed mice. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). **p<0.01. Scale bar = 100 μm.

Discussion

In this study, I found that the decreased process in the microglia of the hypothalamus was caused by the decreased expression of the P2Y12 receptors in the fasted state. In addition, changes in P2Y12 receptor activation are closely related to hypothalamic neuronal activation by energy state. These results show that the morphology of the microglia by P2Y12 receptor in the normal environments is an important regulator of the hypothalamic neuronal activity by energy state.

Microglia are present overall in the brain and perform surveillance on surrounding neurons through continuous morphological changes such as process extension and retraction (21). The ramified and amoeboid microglia are representative morphologies that are affected by physiological and pathological conditions such as inflammation and brain injury (3, 4). The P2Y12 receptor, which is a homeostatic microglia marker, is an important indicator in the morphological changes in microglia (23). Microglia P2Y12 receptors are activated by extracellular ADP in the brain, which affects the microglia process extension or retraction (14-16). Mice with controlled P2Y12 receptor activation had decreased microglial processes as well as decreased direct interaction with neurons (13). These previous results suggest that physiological conditions exerting change in ATP levels in the brain may induce a morphological change of the microglia through P2Y12 receptor signaling, which affects the microglial interaction with neurons. In this study, I observed that microglia process length and P2Y12 receptor expression are decreased in the hypothalamic ARC during overnight fasting.

This change suggests that the change of microglia morphology in the hypothalamic ARC is caused by decreasing the P2Y12 receptor expression.

Previous reports showed that the mitochondria of the hypothalamic AgRP and POMC neurons undergo fission during overnight fasting, which results in a decrease in ATP generation (19, 20). Thus, I examined whether overnight fasting affects ATP, ADP and P2Y12 receptor levels in hypothalamic ARC. The content of ATP and ADP decreased dramatically in the hypothalamic ARC. Moreover, this food deprivation also caused a decreased expression of P2Y12 in the ARC. Thus, I administered 2MEsADP, a P2Y12 receptor agonist, to mice that had fasted overnight and analyzed P2Y12 receptor expression and morphological parameters of microglia. After administration of the 2MEsADP, the P2Y12 receptor and microglia process length were increased compared to fasted mice. In association with these, administration of PSB 0739, a P2Y12 receptor antagonist, causes the P2Y12 receptor and microglia process length to decrease in normal fed mice.

The present results show that microglia morphology is regulated in hypothalamic ARC by the P2Y12 receptor during overnight fasting. These microglia morphologies and P2Y12 expression changes are closely associated with contact with neurons. From this point of view, I examined whether hypothalamic neuronal activation is regulated by the 2MEsADP and PSB 0739 in fed and fasted mice. Hypothalamic neuronal activation is decreased by 2MEsADP in fasted mice. However, the neuronal activation of normally fed mice is increased by PSB 0739. Microglia contact with neurons is correlated with a decrease in neuronal activation. It is well known that hunger signals induce AgRP neuronal activity and AgRP is responsible for compensating for the

energy deficiency in the body (ref). Therefore, it is an interesting further question whether fasting-induced changes in microglia morphology are closely related to AgRP neuronal activity.

Overall, the present data show that the microglia morphology was affected by overnight fasting, which shows that changes in microglia are associated with P2Y12 receptors and hypothalamic ATP levels. In addition, the change of hypothalamic neuronal activation is affected by microglia morphology, which is regulated by P2Y12 receptor activation.

References

1. Brown GC, Neher JJ: Microglial phagocytosis of live neurons. Nat Rev Neurosci. 2014;15:209-216.

2. Thion MS, Ginhoux F, Garel S: Microglia and early brain development: An intimate journey. Science. 2018;12:185-189.

3. Spittau B: Aging microglia-phenotypes, functions and implications for agr-related neurodegenerative. Front Aging Neurosci. 2017;14:9-194.

4. Walker FR, Nilsson M, Jones K: Acute and chronic stress-induced disturbances of microglial plasticity, phenotype and function. Curr Drug Targets. 2013;11:1262-1276.

5. Lecours C, Bordeleau M, Cantin L, Parent M, Paolo TD, Tremblay ME: Microglial Implication in Parkinson's Disease: Loss of Beneficial Physiological Roles or Gain of Inflammatory Functions?. Front Cell Neurosci. 2018;30:282.

6. Mosser CA, Baptista S, Arnoux I, Audinat E: Microglia in CNS development: Shaping the brain for the future. Prog Neurobiol. 2017;149-150:1-20.

7. Bernier LP, York EM, Kamyabi A, Choi HB, Weilinger NL, MacVicar BA: Microglial metabolic flexibility supports immune surveillance of the brain parenchyma. Nat Commun. 2020;11:1559

8. Arcuri C, Mecca C, Bianchi R, Giambanco I, Donato R: The Pathophysiological Role of Microglia in Dynamic Surveillance, Phagocytosis and Structural Remodeling of the Developing CNS. Front Mol Neurosci. 2017;10:191

9. Leon S, Nadjar A, Quarta C: Microglia-Neuron Crosstalk in Obesity:: Melodious

Interaction or Kiss of Death?. Int J Mol Sci. 2021;22:5243

10. Valdearcos M, Robblee MM, Benjamin DI, Nomura DK, Xu AW, Koliwad SK: Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. Cell Rep. 2014;6:2124-38

11. Taj SH, Blon DL, Hoornaery C, Daans J, Quarta A, Praet J, Linden AVD, Ponsaerts P, Hoehn M: Targeted intracerebral delivery of the anti-inflammatory cytokine IL13 promotes alternative activation of both microglia and macrophages after stroke. J Neuroinflammation. 2018;15:174

12. Badimon A, Strasburger HJ, Ayata P, Chen X, Nair A, Ikegami A, Hwang P, Chan AT, Graves SM, Uweru JO, Ledderose C, Kutlu MG, Wheeler MA, Kahan A, Ishikawa M, Wang YC, Loh YHE, Jiang JX, Surmeier DJ, Robson SC, Junger WG, Sebra R, Calipari ES, Kenny PJ, Eyo UB, Colonna M, quintana FJ, Wake H, Grandinaru Vm Schaefer A: Negative feedback control of neuronal activity by microglia. Nature. 2020;7829:417-423

13. Cserep C. Posfia B, Lenart N, Fekete R, Laszlo ZI, Lele Z, Orsolit B, Molnar G, Heindl S, Schwarcz AD, Ujvari K, Kornyei Z, Toth K, Szabadits E, Sperlagh B, Baranyi M, Csiba L, Hortobagyi T, Magloczky Z, Martinecz B, Szabo G, Erdeyi F, Szipocs R, Tamkun MM, Gesierich B, Duering M, Katona I, Liesz A, Tamas G, Denes A: Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science. 2020;6477:528-537

14. Whitelaw BS, Matei EK, Majewska AK: Phosphoinositide-3-Kinase γ Is Not a Predominant Regulator of ATP-Dependent Directed Microglial Process Motility or Experience-Dependent Ocular Dominance Plasticity. eNeuro. 2020;6:ENEURO.0311

15. Madry C, Attwell D: Receptors, ion channels, and signaling mechanisms underlying microglial dynamics. J Biol Chem. 2015;20:12443-50

16. Das R, Chinnathambi S: Actin-mediated Microglial Chemotaxis via G-Protein Coupled Purinergic Receptor in Alzheimer's Disease. Neuroscience. 2020;488:325-336

17. Guo X, Wu L, Wang W, Medeiros DM, Clarke S, Lucas E, Smith B, Chowanadisai W, Lin D: Hypothalamic mitochondria in energy homeostasis and obesity. Intergr Mol Med. 2016;3;590-599

18. Yu SB, Pekkurnaz G: Michanisms Orchestrating Mitochondrial Dynamics for Energy Homeostasis. J Mol Biol. 2018;430;3922-3941

19. Haigh JL, New LE, Filippi BM: Mitochondrial Dynamics in the Brain Are Associated With Feeding, Glucose Homeostasis, and Whole-Body Metabolism. Front Endocrinol. 2020;11:580879

20. Kurita H, Xu KY, Maejima Y, Natake M, Dezaki K, Santose P, Yang Y, Arai T, Gantulga D, Muroya S, Lefor AK, Kaker M, Watanabe E, Yada T: Acuate Na+, K+-ATPase senses systemic energy states and regulates feeding behavior through glucose-inhibited neurons. Am J Physiol Endocrinol Metab. 2015;4:E320-33

21. Bernier LP, Bohlen CJ, York EM, Choi HB, Kamyabi A, Olesen LD, Hefendehl JK, Collins HY, Stevens B, Barres BA, MacVicar MA: Nanoscale Surveillance of the Brain by Microglia via cAMP-Regulated Filopodia. Cell Rep. 2019;10:2895-2908

22. Koizumi S, Ohsawa K, Inoue K, Kohsake S: Purinergic receptors in microglia: functional modal shifts of microglia mediated by p2 and p1 receptors. Glia.

2013;1:47-54

CHAPTER 2

Activation of the hypothalamic AgRP neurons is regulated through contact with hypothalamic microglia

Abstracts

Microglia perform an essential role in maintaining brain homeostasis. In addition, microglia metabolic flexibility recognizes changes in the brain environment and performs functions that are suitable for the changing environment through contact with neurons. Microglia contact with neurons, which is mediated by various soluble factors containing ATP as well as contact-dependent mechanisms, is critical for neuronal activation and synaptic development in a healthy brain. In particular, the contact of the ATP of neurons and the P2Y12 receptor of microglia is involved in neuronal inhibition caused by generated adenosine through activated purinergic signaling. However, it is unclear whether the decreased ATP in the hypothalamic arcuate nucleus (ARC) according to energy starvation affects the contact of microglia with AgRP neurons. Moreover, it is unclear whether the activation of AgRP neurons is regulated by adenosine generated by contact with microglia. Interestingly, I observed that microglia contact with AgRP neurons was decreased during overnight fasting and that the contact with AgRP neurons increased when microglia P2Y12 receptor activation was increased. In addition, the increased contact of microglia with AgRP neurons during overnight fasting induces an increase of purinergic signaling mediators such as CD39, vesicular nucleotide transporter (vNUT) and adenosine 1 receptor (A1R) in the hypothalamic ARC. Thus, hypothalamic AgRP neuronal activation is affected by the microglia contact through P2Y12 receptor activation according to the energy state as well as the purinergic signaling in the microglia and AgRP neurons through microglia contact with AgRP neurons.

Introduction

Microglia are representative of immune cells in the central nervous system and are involved in maintaining brain homeostasis by performing phagocytosis and direct contact with neurons in pathological and physiological conditions (1-3). In a healthy brain, microglia are in a state referred to as "ramified microglia" that indicates a longer process length and many branching processes. Ramified microglia contact with surrounding neurons can remove the neurons' nonfunctional synapses and monitor the entire brain environment (4, 5). Thus, recent studies are focused on identifying functions of the microglia in a healthy brain. Recent studies show that the interaction between the generated ATP from mitochondria of neurons and the P2Y12 receptor in microglia is critical for the regulation of contact between microglia and neurons and neuronal activation (6, 7). My previous study showed that the microglia process was decreased in the hypothalamic ARC during energy starvation. In addition, this decrease in the microglia process was affected by the P2Y12 receptor activation according to the ATP level in the brain. However, it is unknown whether hypothalamic neurons cause changes in microglia contact by regulating ATP generation.

From the point of view of mitochondria, many studies have shown that mitochondrial morphology of hypothalamic neurons and glia change according to the energy state (8). ATP generation from the mitochondria of neurons is regulated by mitochondrial fusion and fission (9, 10). In particular, the mitochondria morphology of the hypothalamic AgRP and POMC neurons are changed to fission morphology during energy starvation. However, mitochondria morphology is relatively constant during starvation in glial cells (8). It is suggested that these morphological changes of

mitochondria in AgRP and POMC neurons reflect the decrease of ATP generation during energy starvation. Therefore, it is possible that the decrease of ATP in AgRP and POMC neurons affects the contact of these neurons with microglia through the regulation of the P2Y12 receptor activation.

In the neurons, the generated ATP regulates intracellular homeostasis or is released to extracellular through vesicular nucleotide transporter and various ion channels (11-13). Microglia make contact with neurons by increasing the P2Y12 receptor cluster in response to released ATP. At this time, microglia contact with neurons activates purinergic signaling of microglia that induces degradation of ATP into adenosine by microglia ectonucleotidase CD39 and CD73 (6). Previous studies showed that expression of A1R, which is involved in the inhibition of neurons, was decreased in the hypothalamic AgRP neurons during energy starvation (14). This evidence suggests that the morphology of microglia, which changes depending on energy state, may be involved in AgRP neuron activation by regulating adenosine generation through change of contact with AgRP neurons. Therefore, I investigated whether the microglia contact with AgRP neurons changes by energy state and whether AgRP neuronal activation is regulated by microglia contact with AgRP neurons through interaction between ATP generated by AgRP neurons with microglia P2Y12 receptors.

Materials and Methods

Animals and experiment design

Mice were maintained in temperature- and humidity-controlled rooms with a 12 h light - 12 h dark cycle, with the lights on from 7:00 a.m. to 7:00 p.m. and ad libitum and given free access to tap water. *AgRP-Cre* mice (Stock No. 012899, Jackson Laboratory, Bar Harbor, ME, USA) were crossbred *with Rosa26-lox-stop-lox-tdTomato* mice (*Ai14* reporter mice) to indicate all AgRP-expressing cells with tomato signals (*AgRP-Cre;Ai14* mice). For the food deprivation experiments, food was removed for 18 hours (from 4:00 p.m. to 10:00 a.m.).

Cannulation and administration of P2Y12 receptor agonist and antagonist

For intracerebroventricular (i.c.v) cannula implantation, mice were anesthetized by i.p injection of tribromoethanol (250 mg/kg, Sigma-Aldrich) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The cannula (26 gauge) was implanted into the right lateral ventricle (1.0 mm lateral, 0.3 mm posterior, and 2.4 mm ventral to the bregma) according to the Stereotaxic Mouse Brain Atlas (Paxinos G and Franklin KBJ, 2001, Academic Press, San Diego, CA, USA) and secured to the skull with dental cement. After 7 days of recovery, mice were injected with vehicle (saline) or with 2ME-sADP (100 μmol/2 μl, Tocris, Minneapolis, NE, USA) after overnight fasting. In addition, the normal fed mice were injected with PSB 0739 (3ug/ul, Tocris, Minneapolis, NE, USA). For immunohistochemistry analysis, mice were sacrificed 1 h after the injection of the 2ME-sADP, and the mice were sacrificed 3h after the injection of the PSB 0739.

Immunohistochemistry (IHC)

Animals were deeply anesthetized with tribromoethanol and transcardially perfused with phosphate buffer (PB, 0.1 M, pH 7.4), followed by a fresh fixative of 4% paraformaldehyde in PB. Brains were post-fixed overnight at 4°C, sliced to a thickness of 50 μm using a vibratome (VT1000P; Leica Microsystems, Wetzlar, Germany), and then washed several times in PB. Coronal brain sections containing the hypothalamic arcuate nucleus (ARC) were preincubated with 0.3% Triton X-100 (T8787, Sigma-Aldrich) in PB for 30 min to permeabilize the tissues and cells. After further washing with PB, the sections were treated with primary antibodies overnight at 4°C; as follows rabbit anti-P2Y12 (1:1,000; 848002, Biolegend, CA, USA), mouse anti-c-fos antibody (1:1,000; sc-166940, Santa Cruz, Dallas, TX, USA) rabbit anti-AgRP (1:1000, H-003-57, Phoenix, Burlingame, CA, USA) and sheep anti-α-melanocyte stimulating hormone (MSH) antibody (1:10000; AB5087, Millipore, Billerica, MA, USA). On the next day, sections were washed in PB. For immunofluorescence staining, sections were incubated with the following secondary antibodies for 3 h at room temperature: goat anti-mouse Alexa Fluor 488 (1:500; A11001, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit Alexa Fluor 488 (1:500; A11018, Invitrogen), goat anti-mouse Alexa Fluor 647 (1:500; A21235, Invitrogen) and donkey anti-sheep Alexa Fluor 594 (1:500; A11016, Invitrogen). Stained brain sections were imaged using an FV-1200 confocal laser-scanning microscope (Olympus America, Inc., Center Valley, PA, USA).

IHC image analyses

The microglia contact with AgRP neurons was measured by using the ColocSurfasce Render plug-in in the Imaris 3D software (Bitplane, Zurich, Switzerland). The intensity

of AgRP and α-MSH fiber intensity and particle number, and CD39 and A1R intensity were measured using ImageJ V 1.50 software (National Institutes of Health, Bethesda, MD) in the PVN and ARC. Region of interest (ROI) within an image was manually selected with the Mouse Brain Atlas for ARC (ARC: between -1.46 and -1.82 mm from bregma). The images were converted to 8-bit images and a threshold was applied. The images were binarized to separate the immuno-positive cells from the background. The number of immune-positive cells in the hypothalamus was counted by unbiased observers.

Statistical analyses

Statistical analyses were performed in GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± SEM. The statistical significance between two groups was analyzed by unpaired Student's t-test.

Result

Fasted state affects the contact of microglia with AgRP neurons in the hypothalamic ARC

My previous studies showed that the expression of the P2Y12 receptor in the hypothalamic ARC was closely related to the energy state. In addition, the P2Y12 receptor of microglia was reported that direct contact with neurons. Thus, to determine whether energy deprivation affects hypothalamic microglia contact with AgRP neurons, I investigated changes in microglia contact in the hypothalamic ARC of fed and fasted mice, using an antibody for P2Y12 (Fig 1A, C). The fasted mice had decreased microglia contact with AgRP neurons compared to normally fed mice (Fig 1B) and decreased microglia contact coverage with AgRP neurons (Fig 1D). These findings suggest that decreased P2Y12 receptor activation in fasted mice affects contact with AgRP neurons.

P2Y12 receptor of microglia affects the contact with the AgRP neurons.

To determine whether the P2Y12 receptor activation affects microglia contact with AgRP neurons, I administered 2MEsADP with fasted mice (Fig 2C) and PSB 0739 with normally fed mice (Fig 2G) and analyzed microglia contact with AgRP neurons compared to each control mice (Fig 2A, E). In the fasted mice, after administration of 2MEsADP, microglia contact with AgRP neurons and microglia coverage on AgRP neurons is increased compared to the fasted mice (Fig 2B, D). However, after administration of PSB 0739 to normally fed mice, microglia contact with AgRP neurons was decreased (Fig 2F), as was microglia coverage on AgRP neurons (Fig 2H). These

results showed that microglia contact with AgRP neurons is closely related to P2Y12 receptor activation of microglia by energy state.

AgRP neuronal activation changes contact with microglia during the fasted state in the hypothalamic ARC.

I found decreased contact in microglia with the AgRP neurons in the hypothalamic ARC during the fasted state (Fig 1). It is well known the hypothalamic AgRP neurons activate in the fasted state. Thus, I aimed to test whether the change of microglia contact with AgRP neurons by neuronal activation. I used the c-fos, a representative neuronal activation marker, and investigate the microglia contact with c-fos positive AgRP and c-fos negative AgRP during the fasted state (Fig 3A). Interestingly, the contact of microglia with AgRP neurons during the fasted state is dramatically decreased in c-fos positive AgRP neurons compared to c-fos negative AgRP neurons (Fig 3B), and also decrease in microglia coverage to c-fos positive AgRP neurons (Fig 3C). These results suggest that microglia contact with AgRP neurons was associated with inhibition of neuronal activity.

P2Y12 receptor activation of microglia is important for AgRP neuronal activation in the fasted state.

Next, I aimed to investigate whether the change in microglia contact with AgRP neurons through 2MEsADP during the fasted state affects the AgRP neuronal activation in the hypothalamic ARC. Thus, I examined the number of contacted AgRP neurons in the microglia after administration of 2MEsADP in the fasted state. The

number of contacted AgRP neurons was increased by increasing the P2Y12 receptor activation of microglia during the fasted state (Fig 4A-C). Interestingly, at this time, c-fos positive AgRP neurons were decreased and c-fos negative AgRP neurons were increased in the hypothalamic ARC (Fig 4D-G) but did not differ in the total number of the AgRP neurons compared to fasted mice (Fig 4H). Next, I investigated whether increased P2Y12 receptor activation during the fasted state really affects the melanocortin system of the paraventricular nucleus (PVN) and feeding behavior. After administration of the 2MEsADP in the 18 h fasted mice, showed a decreased feeding response compared to fasted mice during the 1 to 2 hours (Fig 4I). In addition, 2MEsADP induced a decrease in the number and intensity of AgRP fibers in the PVN (Fig 4J-M), and alpha-MSH fibers number and intensity (Fig 4N-Q), as well as neuronal activation in the PVN (Fig R-T), were increased. These observations suggest that P2Y12 receptor activation of microglia is associated with AgRP neuronal activation during the fasted state.

P2Y12 receptor did not affect AgRP neuronal activation in the fed state.

To examine the effect of the P2Y12 receptor on AgRP neuronal activation, I administered PSB 0739 to normally fed mice and analyzed the number of contacted AgRP neurons in the microglia (Fig 5A-C). The number of contacted AgRP neurons to the microglia was decreased in the normally fed mice by PSB 0739 (Fig 5C). However, the number of c-fos negative (Fig 5F), c-fos positive AgRP (Fig 5G), and total AgRP neurons (Fig 5H) were not different compared to control mice. Thus, the P2Y12 receptor activation of microglia affects contact with AgRP neurons but not AgRP neuronal activation.

The AgRP neuronal activation through the P2Y12 receptor of microglia is associated with purinergic signaling.

Previous studies showed that purinergic signaling in the microglia was important for various cell functions in the CNS. In addition, microglia's response to extracellular ATP was related to contact with neurons. Next, I investigated whether the AgRP neuronal activation through microglial contact with AgRP neurons was regulated by purinergic signaling. Thus, the purinergic signaling mediators such as vNUT (vesicular nucleotide transporter) and adenosine 1 receptor in AgRP neurons and CD39 in microglia were observed according to energy state. First, I observed the CD39 of microglia that is involved in hydrolysis of ADP and AMP through extracellular ATP. The CD39 intensity of microglia was decreased by the fasted state in the hypothalamic ARC. Next, I measured the number of vNUT positive AgRP neurons that are involved in ATP release to extracellular. The fasted state also reduced the number of vNUT positive AgRP neurons in the hypothalamic ARC. Additionally, I measured the A1R intensity in the AgRP neurons with normally fed and fasted mice. The A1R intensity of AgRP neurons was decreased by the fasted state in the hypothalamic ARC. These observations suggest that purinergic signaling is associated with an energy state such as the fasted state.

P2Y12 receptor activation of microglia is related to purinergic signaling

I found increased contact in microglia with the AgRP neurons after administration of 2MEsADP in fasted mice (Fig 2C, D), and decreased AgRP neuronal activation (Fig 4D-G). In addition, the purinergic signaling of microglia and AgRP neurons is affected by the energy state (Fig 6). Thus, I administered 2MEsADP with fasted mice and

analyzed purinergic signaling mediators (CD39, vNUT and A1R). After administration of 2MEsADP, CD39 intensity of microglia (Fig 7A-C), the number of the vNUT positive AgRP neurons (Fig 7D-F) and A1R intensity of AgRP neurons (Fig 7G-I) was decreased compared to fasted mice in the hypothalamic ARC. Collectively, these observations suggest that AgRP neuronal activation is regulated by microglia contact through the P2Y12 receptor activation and purinergic signaling.

Figures

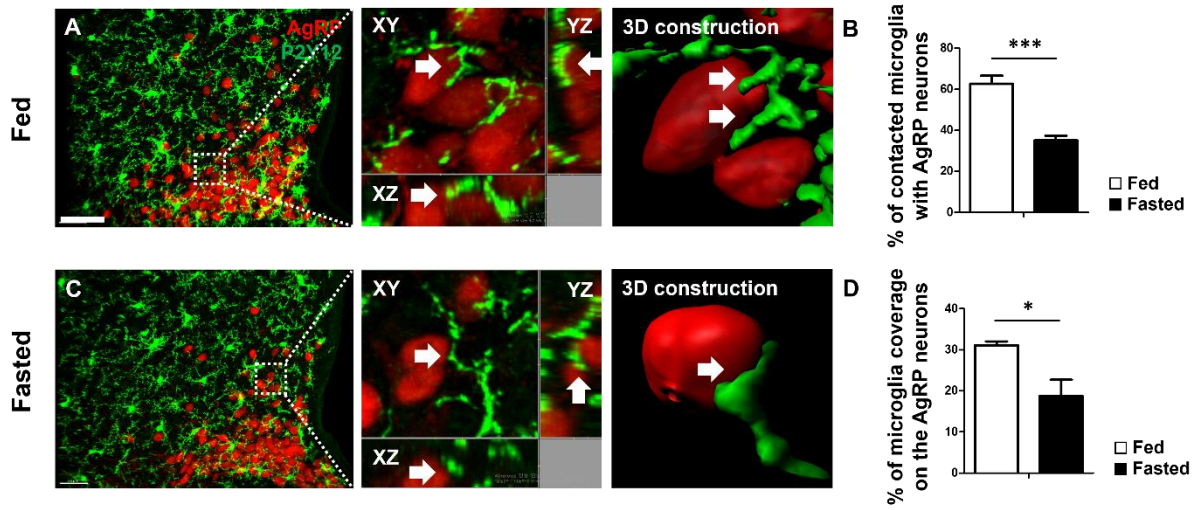

Figure 1. Change of microglia contact with AgRP neuron by the fasted state

IHC uses antibodies against P2Y12, a marker of homeostatic microglia, was performed the analysis of microglia contact with AgRP neurons (tomato signals) in the hypothalamic ARC using 3D reconstruction. Representative immunohistochemical images (A, C) and calculated graphs show that the ratio of contacted microglia to the AgRP neurons (B) and microglia coverage on AgRP neurons (C) was decreased in 18 h fasted mice compared to normally fed mice. Data are presented as mean ± SEM. (n=3 sections of 4 mice/group). *p<0.05 and ***p<0.001. Scale bar = 100 μm.

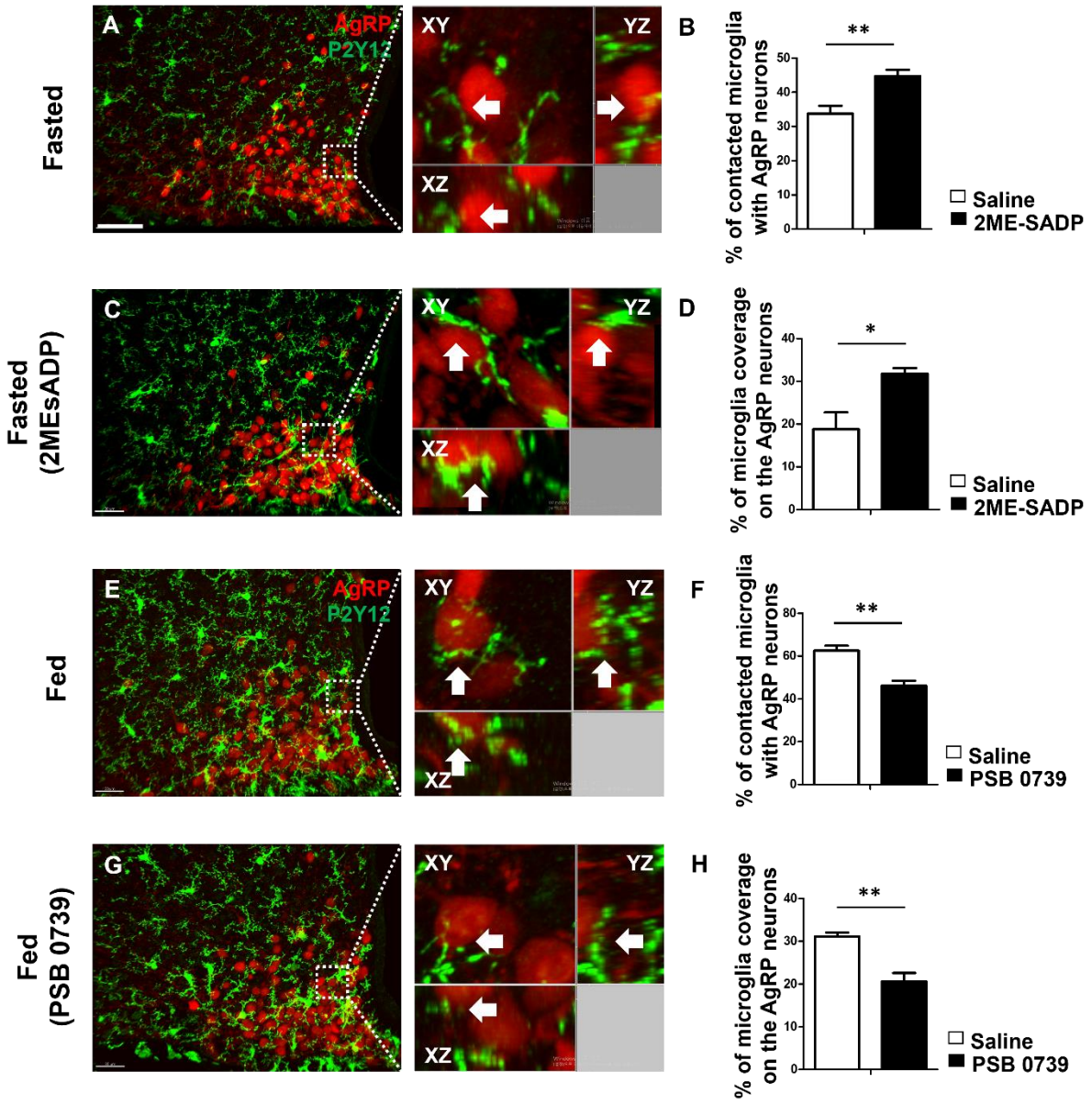

Figure 2. Effects of the P2Y12 receptor activation on the contact with AgRP neurons

P2Y12 receptor agonist induces the increase of microglia contact with AgRP neurons after 18 h fasting in the hypothalamic ARC. In addition, the P2Y12 receptor antagonist induces the decrease of microglia contact with AgRP neurons in normally fed mice. To identify the contact effect of the P2Y12 receptor agonist and antagonist, overnight

fasted mice were icv injection of P2Y12 receptor agonist 2MEsADP (100 μmol/2 μl) and normally fed mice were icv injection of P2Y12 receptor antagonist PSB 0739 (3ug/ul). Representative images (A, C) showing P2Y12 of microglia and AgRP-specific tomato signals in the hypothalamic ARC of AgRPcre; Ai14 mice and calculated graphs (B, D) show an increase of microglia contact with AgRP neurons after 18 h fasting by the 2MEsADP compared to control mice. Representative images (E, G) showing P2Y12 of microglia and AgRP-specific tomato signals in the hypothalamic ARC of AgRPcre; Ai14 mice and calculated graphs (B, D) show a decrease of microglia contact with AgRP neurons normally fed mice by the PSB 0739 compared to control mice. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). *$p<0.05$ and **$p<0.01$. Scale bar = 100 μm.

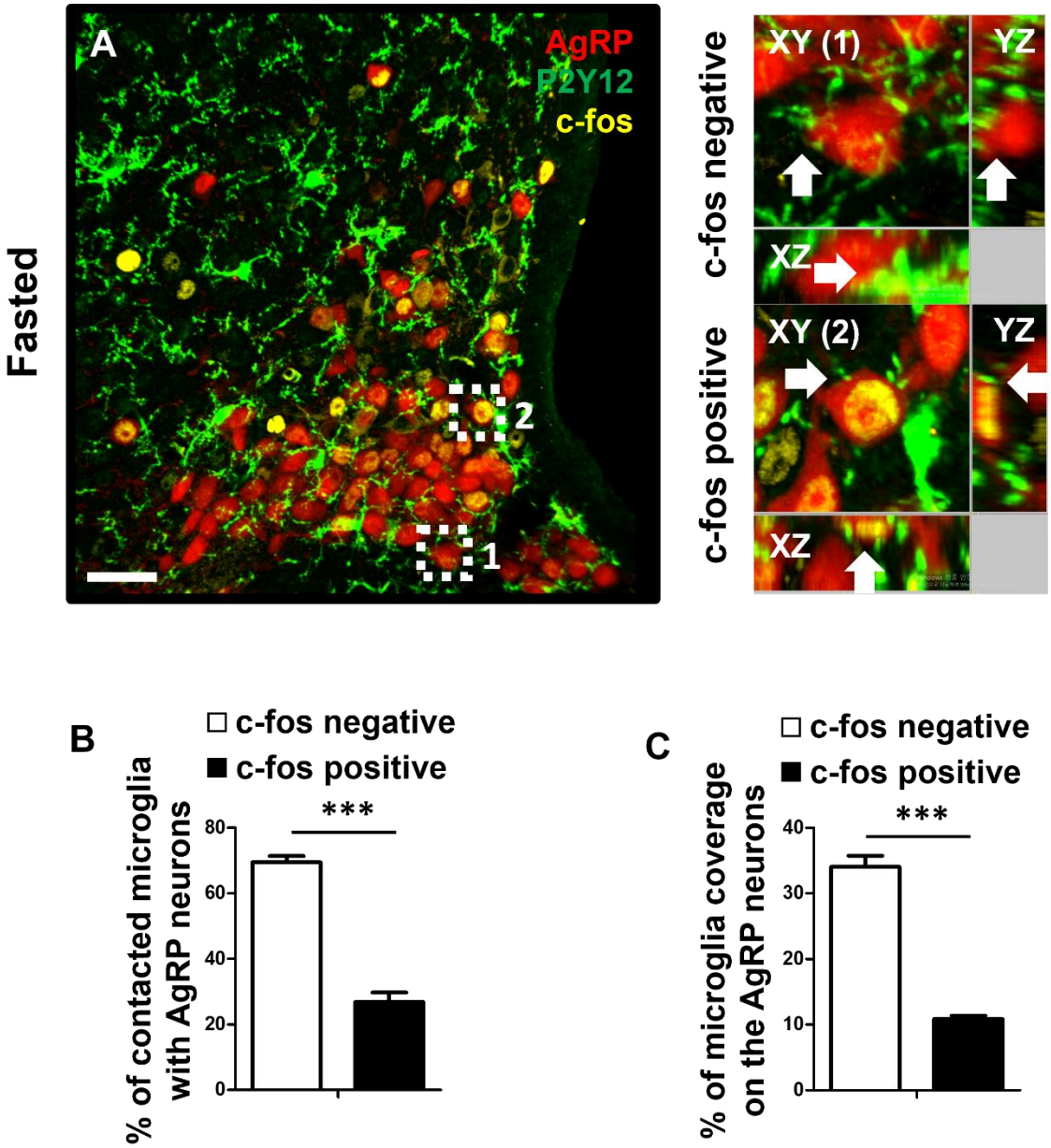

Figure 3. Change of contact with AgRP neuron of microglia by neuronal activation in fasted state

P2Y12 receptor of contact with c-fos positive AgRP neurons was decreased during the 18 h fasting state compared to c-fos negative AgRP neurons. To analyse the difference in microglia contact of activated AgRP neurons compared to non-activated AgRP neurons using 3D reconstruction. Representative images (A) and calculated graphs

(B, C) show a decrease in the number of microglia contact (B) and coverage (C) with c-fos positive AgRP neurons in the fasting state compared to c-fos negative AgRP neurons. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). ***p<0.001. Scale bar = 100 μm.

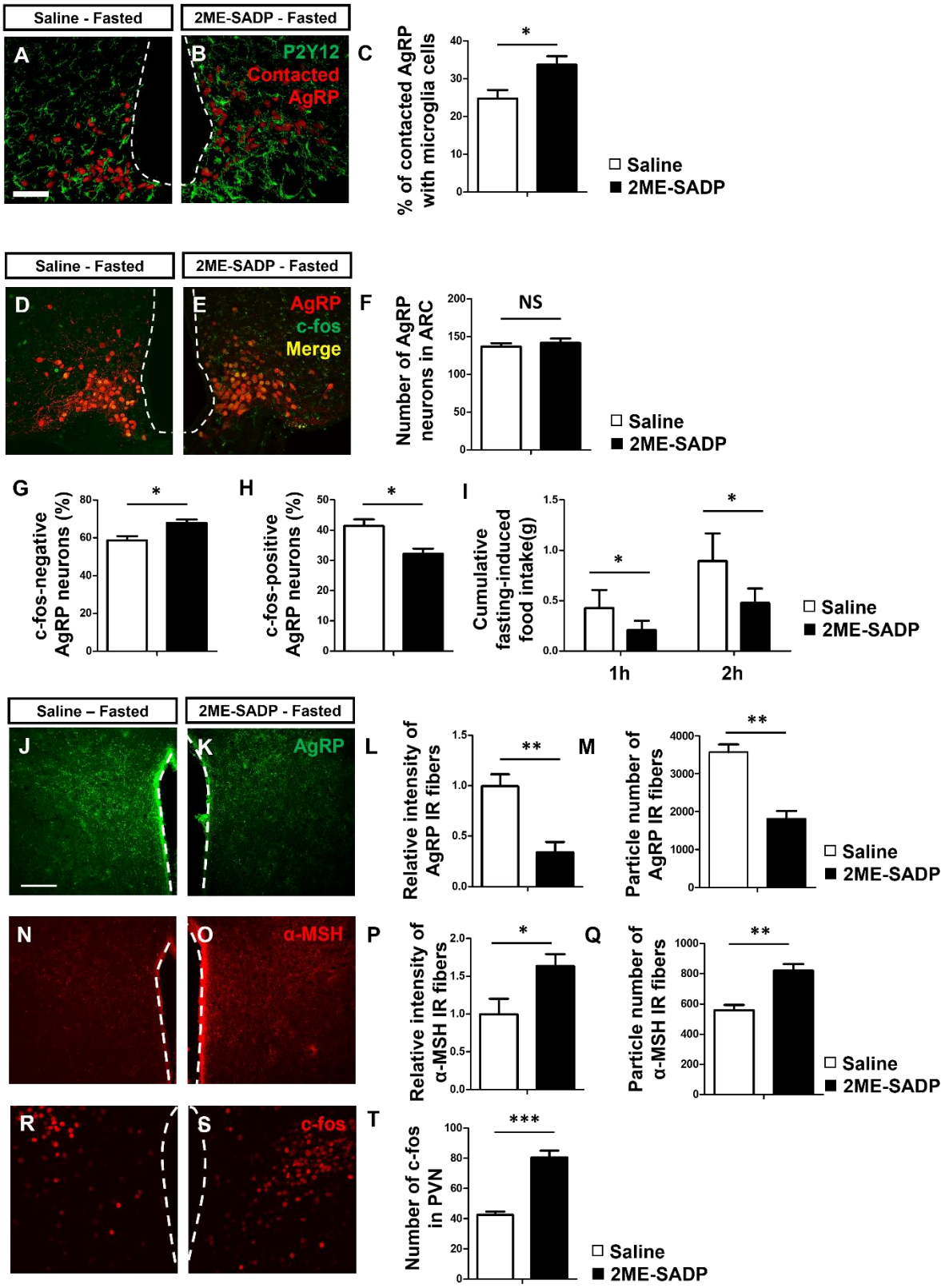
Saline - Fasted
2ME-SADP - Fasted
A
B
P2Y12
Contacted
AgRP
C
% of contacted AgRP with microglia cells
*
Saline
2ME-SADP
D
E
AgRP
c-fos
Merge
F
Number of AgRP neurons in ARC
NS
G
c-fos-negative AgRP neurons (%)
H
c-fos-positive AgRP neurons (%)
I
Cumulative fasting-induced food intake(g)
1h
2h
Saline – Fasted
J
K
AgRP
L
Relative intensity of AgRP IR fibers
**
M
Particle number of AgRP IR fibers
N
O
α-MSH
P
Relative intensity of α-MSH IR fibers
Q
Particle number of α-MSH IR fibers
R
S
c-fos
T
Number of c-fos in PVN
***

Figure 4. The regulation of P2Y12 receptor activation affects hypothalamic AgRP neuronal activation in the fasted state.

Hypothalamic AgRP neuronal activation was decreased in the hypothalamic ARC by the 2MEsADP after 18 h fasting. To determine whether increased microglia contact with AgRP neurons by the 2MEsADP affects AgRP neuronal activation, fasted mice were icv injected with 2MEsADP and analyzed the number of activated AgRP neurons, melanocortin system of the PVN and feeding behavior. (A-C) Representative images (A, B) and calculated graphs show an increase in the number of contacted AgRP neurons to microglia compared to fasted mice. (D-H) Representative images (D, E) and calculated graphs show did not differ the number of total AgRP neurons (F). c-fos negative AgRP neurons were increased (F) and c-fos positive AgRP neurons were decreased (G) in the fasted state by the 2MEsADP. (n=3 sections of 5 mice/group). The administered mice with 2MEsADP were decreased food intake compared to fasted mice (I). (J-M) Representative images (J, K) and calculated graphs show a decrease AgRP immuno-positive signals such as intensity (L) and particle number of AgRP (M) in the PVN by the 2MEsADP. (N-Q) Representative images (N, O) and calculated graphs (P, Q) show an increase of α-MSH immuno-positive signals such as intensity (P) and particle number of α-MSH (Q) in the PVN by the 2MEsADP. (R-T) Representative images (R, S) and the calculated graph show an increase in the number of c-fos (T) in the PVN by the 2MEsADP. Data are presented as mean ± SEM (n=3 sections of 4 mice/group). *$p<0.05$, **$p<0.01$ and ***$p<0.001$. ns, not significant. Scale bar = 100 μm.

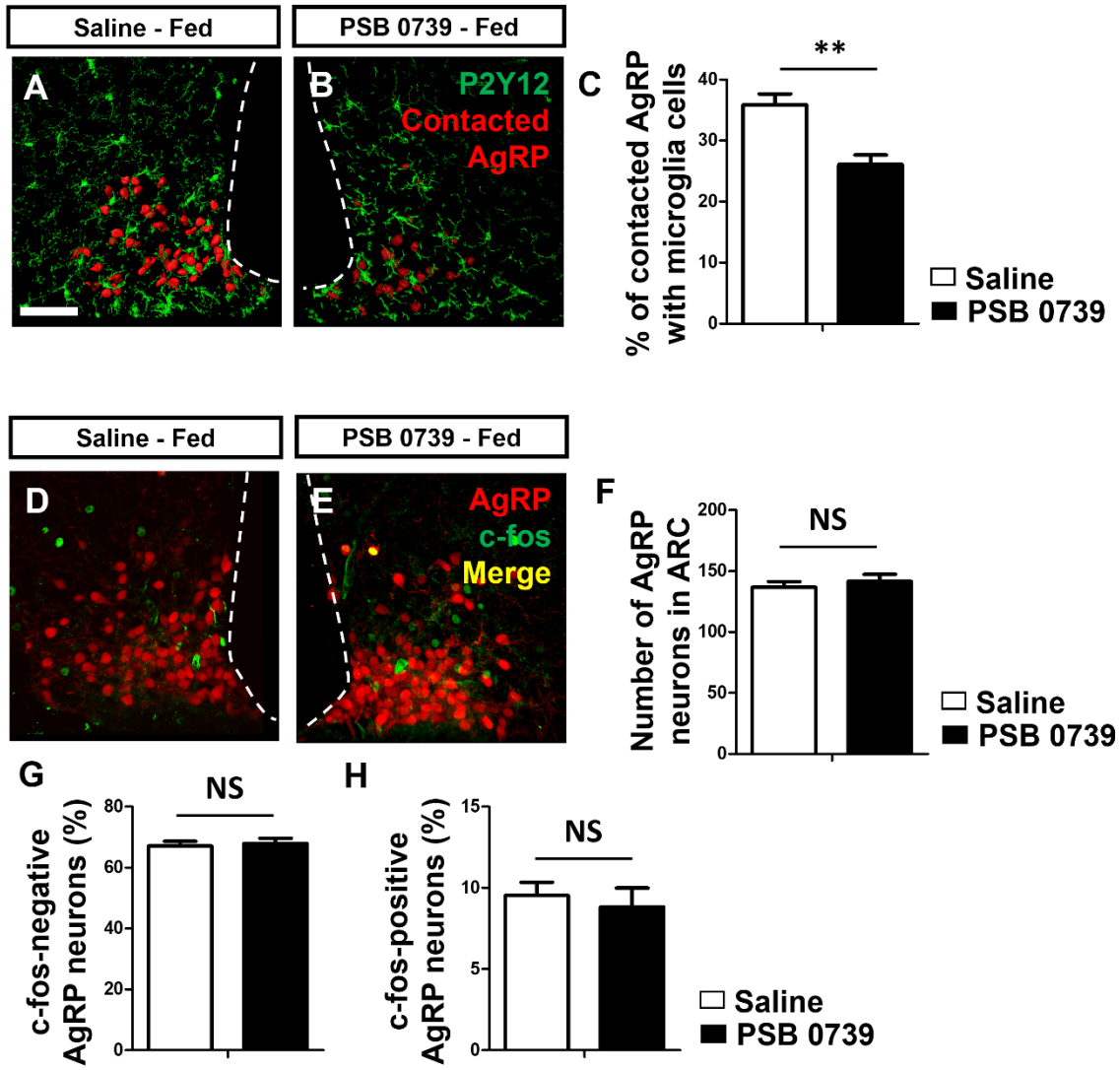

Figure 5. The regulation of P2Y12 receptor activation did not affect AgRP neuronal activation in the normally fed mice.

Hypothalamic AgRP neuronal activation was not changed in the normally fed mice by the PSB 0739. To determine whether decreased P2Y12 receptor activation in the normally fed mice affects AgRP neruonal activation, normally fed mice were icv injected with PSB 0739 and analyzed the number of activated AgRP neurons. (A-C) Representative images (A, B) and calculated graph show a decreased contacted AgRP neuron (C) to microglia by the PSB 0739. (D-H) Representative images (D, E)

and calculated graphs show not differences was observed the number of total AgRP neurons (F), c-fos negative AgRP neurons (G) and c-fos positive AgRP neurons (H) after administration of PSB0739 in the normally fed mice. Data are presented as mean ± SEM (n=3 sections of 5 mice/group). **p<0.01. ns, not significant. Scale bar = 100 μm.

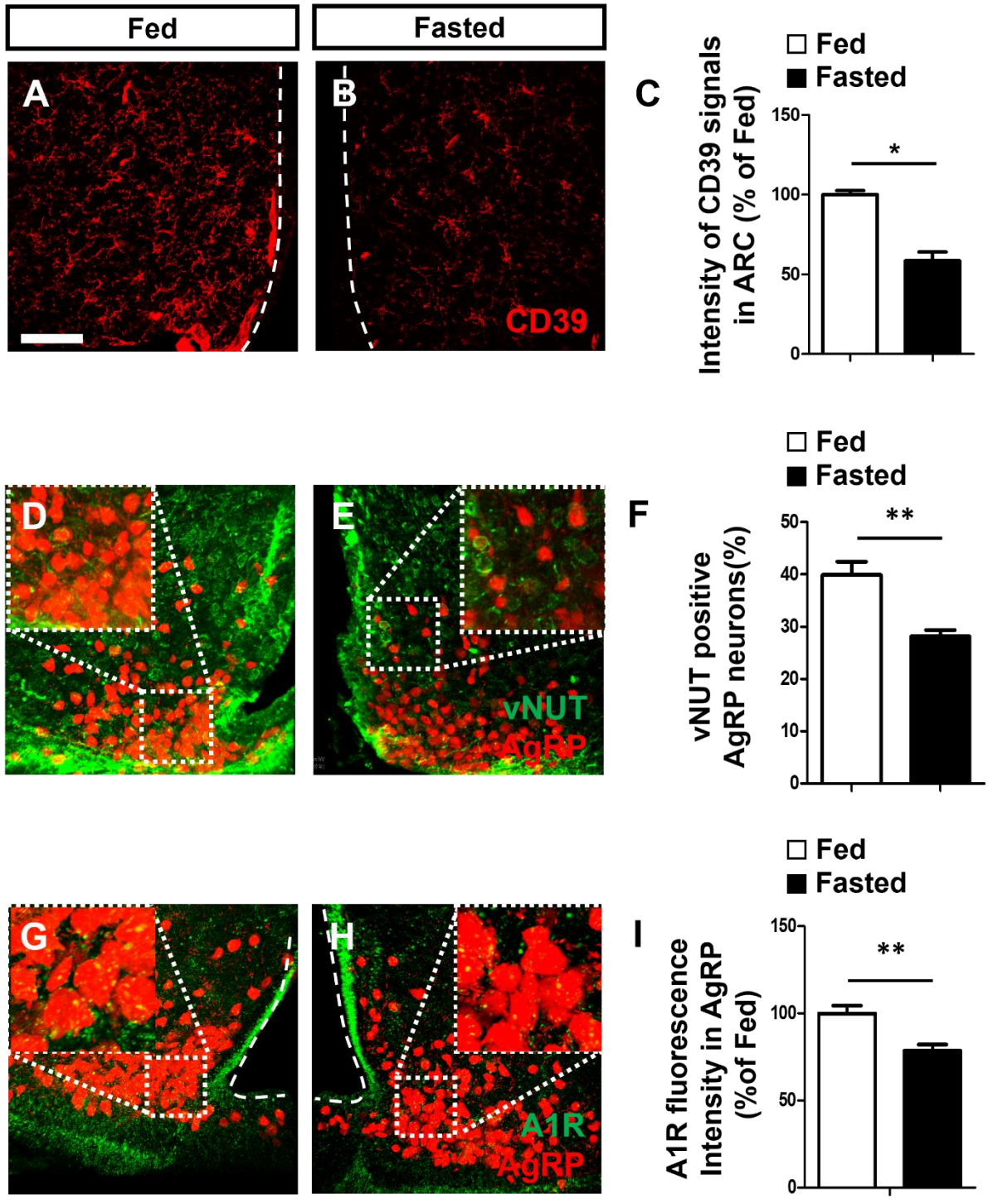
Fed
Fasted
A
B
CD39
C
Fed
Fasted
Intensity of CD39 signals in ARC (% of Fed)
150
100
50
0
*
D
E
vNUT
AgRP
F
Fed
Fasted
vNUT positive AgRP neurons(%)
50
40
30
20
10
0
**
G
H
A1R
AgRP
I
Fed
Fasted
A1R fluorescence Intensity in AgRP (%of Fed)
150
100
50
0
**

Figure 6. Purinergic signaling in the hypothalamic ARC is affected by the energy state

To identify the effects of change in activation of AgRP neurons by the energy state, immunohistochemical analyses were performed to normally fed mice and fasted mice. (A-C) Representative images (A, B) and calculated graph show a decrease of CD39 intensity in the microglia during 18 h fasting state compared to normally fed mice. (D-F) Representative images (D, E) and calculated graph show a decrease of vNUT positive AgRP neurons (F) in the hypothalamic ARC during 18 h fasting state compared to normally fed mice. (G-I) Representative images (G, H) and calculated graph show a decrease of A1R intensity in the AgRP neurons during 18 h fasting state compared to normally fed mice. Data are presented as mean ± SEM (n=3 sections of 4~5 mice/group). *$p<0.05$ and **$p<0.01$. Scale bar = 100 μm.

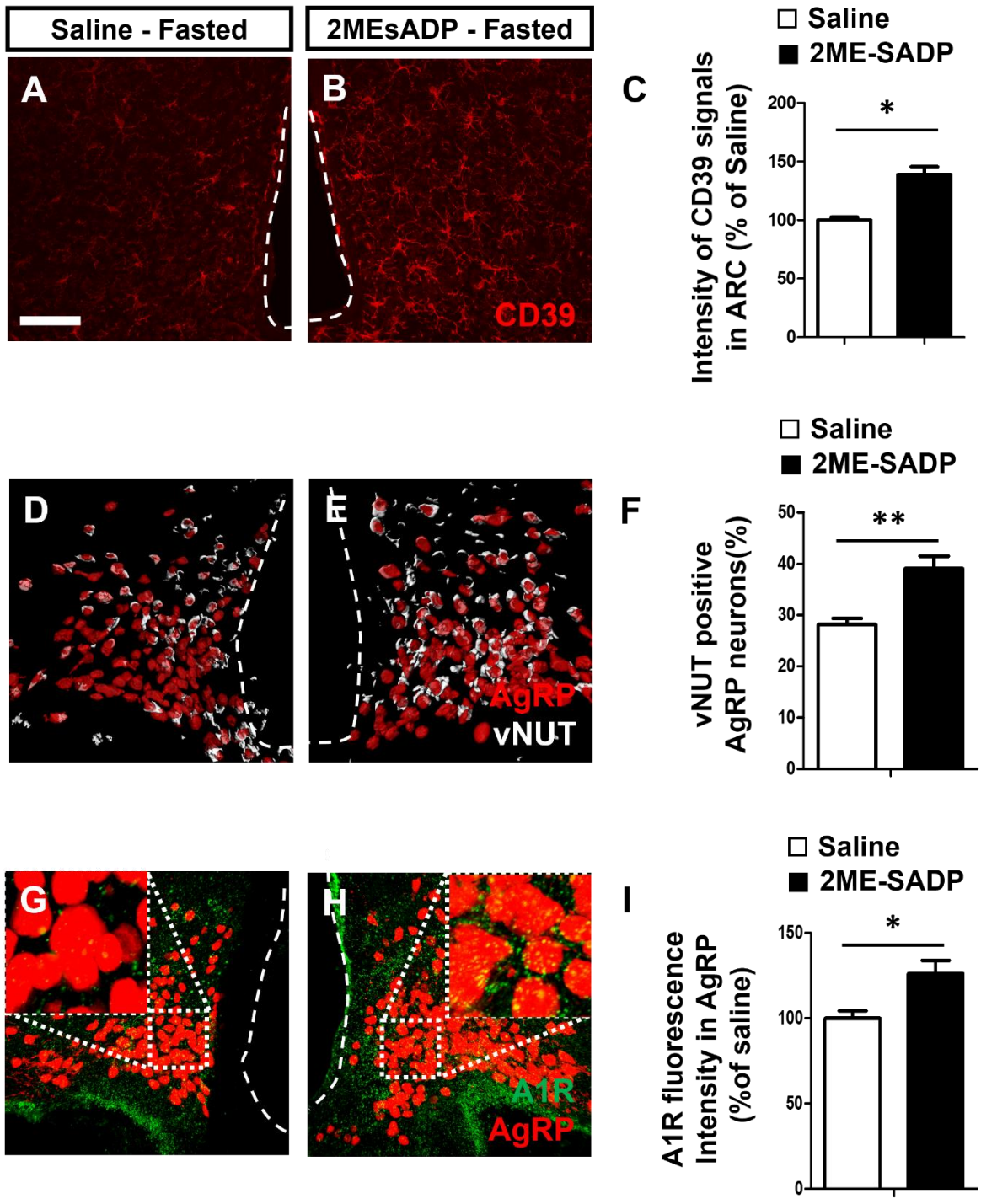

Figure 7. Purinergic signaling of microglia is affected by P2Y12 receptor activation

To investigated whether the after administration of 2MEsADP to the fasted mice the

increase of microglia contact with AgRP neurons and inhibition of AgRP neuronal activity were affected by the purinergic signaling, after administered 2MEsADP with the fasted mice were performed to immunohistochemistry analyses. (A-C) Representative images (A, B) and calculated graphs show an increase of CD39 intensity in microglia (C) by the 2MEsADP. (D-F) Representative images (D, E) and calculated graphs show an increase of vNUT positive AgRP neurons (F) in the hypothalamic ARC by the 2MEsADP. (G-I) Representative images (G, H) and the calculated graph show an increase of A1R intensity in the AgRP neurons (I) by the 2MEsADP. Data are presented as mean ± SEM (n=3 sections of 4~5 mice/group). *p<0.05 and **p<0.01. Scale bar = 100 μm.

Discussion

In this study, I found that microglia P2Y12 receptor activity is regulated by energy state and, is closely related to microglia contact with AgRP neurons. The contact is important in regulation of hypothalamic AgRP neuronal activation. These results show that hypothalamic microglia is a crucial mediator of AgRP neuronal activation according to the energy state.

The contact of microglia is induced by various soluble factors and synaptic plasticity-related genes in the neurons in the central nervous system (15). It has recently been reported that the P2Y12 receptor of microglia is important for contact with neuronal secreted ATP (6). In addition, the microglia contact affects neuronal activities such as neural survival and circuitry formation, which is essential for neuronal activation, in a healthy brain (1-3). Previous studies showed that the P2Y12 receptor of microglia is a homeostatic marker of microglia, which is involved in the microglia process extension or retraction (3, 16). Thus, the P2Y12 receptor of microglia is a crucial regulator of hypothalamic neuronal activation. My previous research found that microglia morphological alterations are related to P2Y12 receptor activation and that regulation of P2Y12 receptor activation affects hypothalamic neuronal activation. These findings suggested that the microglia P2Y12 receptor is an important regulator of microglia morphology and neuronal activation in the hypothalamic ARC. Therefore, I observed whether contact of the P2Y12 receptor in microglia on the AgRP neurons was changed during energy starvation. The contact of microglia was decreased for the AgRP neurons during overnight fasting in the hypothalamic ARC. In particular, when P2Y12 receptor activation was regulated through a P2Y12 agonist and an antagonist,

microglia contact was changed for the AgRP neurons by energy state. This change suggests that the P2Y12 receptor of microglia is an important regulator for the hypothalamic microglia process as well as contact with AgRP neurons

It is well known that activated hypothalamic AgRP neurons induce increased food intake and decreased energy expenditure during overnight fasting and then regulate energy homeostasis (17-19). At this time, c-fos that is a neuronal activity marker is increased in the AgRP neurons of the hypothalamic ARC. Thus, I examined whether the contact of microglia in activated AgRP and non-activated AgRP neurons is changed during overnight fasting. Interestingly, the contact of microglia with the c-fos positive AgRP neurons was dramatically decreased during overnight fasting compared to the c-fos negative AgRP neurons. Previous studies reported that microglia contact with the neurons is involved in the decrease in neuronal activation (6). On the basis of the aforementioned study, this observation suggests that contact of microglia with the AgRP neurons is associated with neuronal inactivation. Therefore, I focused on the number of non-activated AgRP neurons induced by the administration of 2MEsADP after overnight fasting. Interestingly, the number of c-fos negative AgRP neurons contacted with microglia is increased after the administration of 2MEsADP after overnight fasting, but the number of c-fos positive AgRP neurons contacted with microglia was decreased. In association with this, after administration of PSB 0739 to normally fed mice, I investigated the number of AgRP neurons contacting with the microglia. In normally fed mice, the number of AgRP neurons in contact with microglia decreased after administration of PSB 0739, but the number of c-fos negative and positive AgRP neurons did not change when compared to control mice. Thus, these observations suggest that the contact of microglia for the AgRP neurons in the fasting

status affects the hypothalamic AgRP neuronal activation.

Recent studies show that regulation of neuronal activation through microglia is related to purinergic signaling by the contact of microglia (6). In a healthy brain, microglia are involved in the synaptic pruning and synaptic functions, which are partly regulated through purinergic signaling of microglia with neurons. In particular, crucial mediators in the purinergic signaling are extracellular ATP and CD39, adenosine and adenosine receptors (20, 21). The various evidences from the previous studies suggested that contact of microglia with AgRP neurons in the hypothalamic ARC is associated with purinergic signaling. First, the ATP generation of hypothalamic AgRP neurons is decreased by the fasting status. Second, the A1R of hypothalamic AgRP neurons, which is involved in inhibition of the neuronal activation, is decreased by the fasting status. Thus, I examined whether the purinergic signaling is involved in the hypothalamic AgRP neuronal activation through contact between microglia and AgRP neurons in the fasting status. In the hypothalamic ARC, vesicular nucleotide transporter (vNUT), which is involved in the ATP release to extracellular, and A1R of the AgRP neurons and CD39 of microglia were decreased during the fasting state. Interestingly, the contact of microglia with AgRP neurons was increased by 2MEsADP. 2MEsADP also increased purinergic signaling mediators CD39, vNUT and A1R in the fasting state. These results show that changes in contact of microglia with AgRP neurons caused by energy state affect the regulation of AgRP neuronal activation through purinergic signaling of CD39, ATP, and A1R in microglia and AgRP neurons.

Overall, the current data show that overnight fasting affects P2Y12 receptor activation in microglia, which affects not only the process length of microglia but also changes in

contact with neurons. In addition, this change in microglia contact is important in the regulation of AgRP neuronal activation in the hypothalamic ARC through purinergic signaling.

References

1. Li Q, Barres BA: Microglia and macrophages in brain homeostasis and disease. Nat Rev Immunol. 2018;18:225-242

2. Yin J, Valin KL, Dixon ML, Leavenworth JW: The Role of Microglia and Macrophages in CNS Homeostasis, Autoimmunity, and Cancer. J Immunol Res. 2017;2017:5150678

3. Woodburn SC, Bollinger JL, Wohleb ES: The semantics of microglia activation: neuroinflammation, homeostasis, and stress. J Neuroinflammation. 2021;18:258

4. Bernier LP, Bohlen CJ, York EM, Choi HB, Kamyabi A, Olesen LD, Hefendehl JK, Collins HY, Stevens B, Barres BA, MacVicar MA: Nanoscale Surveillance of the Brain by Microglia via cAMP-Regulated Filopodia. Cell Rep. 2019;10:2895-2908

5. Madry C, Kyrargyri V, Arancibia-Carcamo IL, Jolivet R, Kohsaka S, Bryan RM, Attwell D: Microglia Ramification, Surveillance, and Interleukin-1β Release Are Regulated by the Two-Pore Domain K^+. Neuron. 2018;97:299-312

6. Badimon A, Strasburger HJ, Ayata P, Chen X, Nair A, Ikegami A, Hwang P, Chan AT, Graves SM, Uweru JO, Ledderose C, Kutlu MG, Wheeler MA, Kahan A, Ishikawa M, Wang YC, Loh YHE, Jiang JX, Surmeier DJ, Robson SC, Junger WG, Sebra R, Calipari ES, Kenny PJ, Eyo UB, Colonna M, quintana FJ, Wake H, Grandinaru Vm Schaefer A: Negative feedback control

of neuronal activity by microglia. Nature. 2020;7829:417-423

7. Cserep C. Posfia B, Lenart N, Fekete R, Laszlo ZI, Lele Z, Orsolit B, Molnar G, Heindl S, Schwarcz AD, Ujvari K, Kornyei Z, Toth K, Szabadits E, Sperlagh B, Baranyi M, Csiba L, Hortobagyi T, Magloczky Z, Martinecz B, Szabo G, Erdeyi F, Szipocs R, Tamkun MM, Gesierich B, Duering M, Katona I, Liesz A, Tamas G, Denes A: Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science. 2020;6477:528-537
8. Haigh JL, New LE, Filippi BM: Mitochondrial Dynamics in the Brain Are Associated With Feeding, Glucose Homeostasis, and Whole-Body Metabolism. Front Endocrinol. 2020;11:580879
9. Schmitt K, Grimm A, Dallmann R, Oettinghaus B, Restelli LM, Witzig M, Ishihara N, Mihara K, Ripperger JA, Albrecht U, Frank S, Brown SA, Eckert A: Circadian control of DRP1 activity regulates Mitochondrial Dynamics and Bioenergetics. Cell Metab. 2018;27;657-666
10. H.Huang ML, Chiang S, Kalinowski DS, Bae DH, Sahni S, Richardson DR: The Role of the Antioxidant Response in Mitochondrial Dysfunction in Degenerative Diseases: Cross-Talk between Antioxidant Defense, Autophagy, and Apoptosis. Oxid Med Cell Longev. 2019;7:6392763
11. Moriyama Y, Hiasa M, Sakamoto S, Omote H, Nomura M: Vesicular nucleotide transporter (VNUT): appearance of an actress on the state of purinergic signaling. Purinergic Signal. 2017;13:387-404

12. Hasuzawa N, Moriyama S, Moriyama Y, Nomura M: Physiolopathological roles of vesicular nucleotide transporter (VNUT), an essential component for vesicular ATP release. Biochim Biophys Acta Biomembr. 2020;1862:183408

13. Taruno A: ATP Release Channels. Int J Mol Sci. 2018;19:808

14. Henry FE, Sugino K, Tozer A, Branco T, Sternson SM: Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight—loss. Elife. 2015;4:e09800

15. Szepesi Z, Manouchehrian O, Bachiller S, Deierborg T: Bidirectional Microglia-Neuron Communication in Health and Disease. Front Cell Neurosci. 2018;12:323

16. Koizumi S, Ohsawa K, Inoue K, Kohsaka S: Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. Glia. 2013;61:47-54

17. Varela L, Horvath TL: AgRP neurons: a switch between peripheral carbohydrate and lipid utilization. EMBO J. 2012;31:4252-4

18. Ramamoorthy TG, Begum G, Harno E, White A: Developmental programming of hypothalamic neuronal circuits: impact on energy balance control. Front Neurosci. 2015;9:126

19. Deem JD, Faber CL, Morton GJ: AgRP neurons: Regulators of feeding, energy expenditure, and behavior. FEBS J. 2022;289:2362-2381

20. Illes P, Rubini P, Ulrich H, Zhao Y, Tang Y: Regulation of Microglial Functions by Purinergic Mechanisms in the Healthy and Diseased CNS. Cells. 2020;9:1108

21. Burnstock G: Purinergic signalling and disorders of the central nervous system. Nat Rev Drug Discov. 2008;7:575-90