Cannabinoid type 1 receptor-containing axons innervate NPY/AgRP neurons in the mouse arcuate nucleus

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ABSTRACT

Objectives: Phyto- and endocannabinoids, such as THC and endocannabinoids, are well known to promote feeding behavior and to control energy metabolism through cannabinoid type 1 receptors (CB1R). However, the underlying mechanisms are not fully understood. Generally, cannabinoid-conducted retrograde dis-inhibition of hunger-promoting neurons has been suggested to promote food intake, but so far it has not been demonstrated due to technical limitations.

Methods: We applied immunohistochemical labeling of CB1R for light microscopy and electron microscopy combined with three-dimensional reconstruction from serial sections in CB1R-expressing and CB1R-null mice, which served as a negative control. Hunger-promoting neurons expressing Agouti-related protein and neuropeptide Y (AgRP/NPY) in the hypothalamic arcuate nucleus were identified in NPY-GFP and NPY-hrGFP mice.

Results: Using three-dimensional reconstruction from serial sections we demonstrated numerous discontinuous segments of anti-CB1R labeling in the synaptic boutons and axonal shafts in the arcuate nucleus. We observed CB1R in the symmetric, presumed GABAergic, synaptic boutons innervating AgRP/NPY neurons. We also detected CB1R-containing axons producing symmetric and asymmetric synapses onto AgRP/NPY-negative neurons. Furthermore, we identified CB1R in close apposition to the endocannabinoid (2-arachidonoylglycerol)-synthesizing enzyme diacylglycerol lipase-alpha at AgRP/NPY neurons.

Conclusions: Our immunohistochemical and ultrastructural study demonstrates the morphological substrate for cannabinoid-conducted feeding behavior via retrograde dis-inhibition of hunger-promoting AgRP/NPY neurons.

Keywords Hypothalamus; Arcuate nucleus; Agouti-related protein; Neuropeptide Y; Electron microscopy; 3D reconstruction

1. INTRODUCTION

Regulation of food intake and energy consumption is a complex system that includes multiple regulations of brain and peripheral organs [1–4]. As a group of lipid messengers, endocannabinoids (eCBs) have the capability to induce synaptic plasticity by retrograde modulation of glutamatergic and GABAergic neurotransmission in various brain areas as well as represent important metabolic signaling molecules in peripheral organs [1,5,6]. Indeed, cannabinoid type 1 receptors (CB1R) are well known to mediate the effects of cannabinoids on energy metabolism [7–10]. Novel data indicate that the anorexigenic proopiomelanocortin (POMC) neurons reverse their function in the presence of cannabinoids [11,12]. However, the cellular and molecular mechanisms behind hypothalamic CB1R activation in order to regulate energy expenditure and feeding behavior are still not fully understood [10,13]. POMC neurons affect whole body energy metabolism in tandem with neurons in the hypothalamic arcuate nucleus (ARC) that co-express Agouti-related protein (AgRP) and neuropeptide Y (NPY) [14]. At times of negative energy balance, AgRP/NPY neurons are directly activated by the appetite-inducing hormone ghrelin and by increased levels of free fatty acids that are utilized in these neurons via beta-oxidation [15]. Upon activation, AgRP/NPY neurons release neuropeptides as well as GABA to control behavior, in part by inhibiting POMC neurons in the ARC [16]. In parallel to the feeding-promoting hormone ghrelin, distinct eCBs such as 2-arachidonoylglycerol (2-AG) are up regulated in the hypothalamus during fasting, whereas satiety-promoting hormones such as leptin acutely reduce hypothalamic 2-AG levels [7,17,18]. This prandial state-dependent fluctuation of the retrograde messenger 2-AG indicates that eCBs might be involved in regulation of hypothalamic AgRP/NPY neurons. Previous
studies demonstrated moderate amounts of radioactive CB₁R ligand binding as well as CB₁R mRNA in hypothalamic nuclei [13,19–21]. However, AgRP/NPY neurons do not contain CB₁R mRNA and it is thus unlikely that cannabinoids directly affect the output synapses of AgRP/NPY neurons [19]. Nevertheless, retrograde eCB modification of presynaptic CB₁R on glutamatergic or GABAergic axon terminals innervating AgRP/NPY neurons was suggested as a putative mechanism of eCB-dependent control of AgRP/NPY activity, but CB₁R immunolabeling remained uncertain in the ARC [6,22–24]. This inspired our immunohistochemical investigation of eCB signaling in the ARC. Here, we used transgenic mouse models and methods of electron microscopy combined with three-dimensional (3D) reconstruction from serial sections to evaluate the morphological substrate of presynaptic CB₁R-dependent control of AgRP/NPY neurons in the ARC.

Figure 1: Light and electron microscopy of the hypothalamus from CB₁R-expressing mice and CB₁R<sup>−/−</sup> littermates immunolabeled with anti-CB₁R serum. (A and B) Representative light micrographs of ARC from CB₁R<sup>+/−</sup> (A) and CB₁R<sup>−/−</sup> mice (B). Equal conditions of the tissue and micrograph preparation were applied. The micrographs show numerous immunopositive particles (arrows) in CB₁R<sup>+/−</sup> and similar but relatively rare staining in CB₁R<sup>−/−</sup>. (C and D) Electron microscopy analysis shows that, in the CB₁R<sup>+/−</sup>, numerous immunolabeling depositions are located in thin axon-like processes (arrows); atypical staining is also present. For example, it is unclear whether the DAB-Ni deposition between the axonal cell membrane and mitochondria (empty arrow) results from selective labeling of membranous CB₁R or cross reactivity with mitochondrial stomatin-like protein 2 [27–29]. Electron micrograph from CB₁R<sup>−/−</sup> shows non-selective DAB-Ni deposition in a dendrite (empty arrowhead) and mitochondrial labeling characteristic for stomatin-like protein 2 (double arrow). (E and F) 3D reconstructions from serial ultrathin sections of arbitrarily chosen axon-like processes (each depicted with different colors in semitransparent mode) that contain DAB-Ni depositions (depicted black; arrows). The processes in CB₁R<sup>+/−</sup> contain numerous immunopositive fragments, whereas axon-like processes contain single spots of DAB-Ni deposition in CB₁R<sup>−/−</sup>. Red line in E indicates positioning of the profiles shown in the electron micrograph in C where they are highlighted with the same semitransparent colors, respectively. (G and H) Electron microscopy quantifications of the anti-CB₁R immunolabeling in identified cell segments and organelles in ARC of the CB₁R-expressing (CB₁R<sup>+/−</sup> and CB₁R<sup>+/+</sup>, pooled) and CB₁R<sup>−/−</sup> mice. Notice that anti-CB₁R immunolabeling predominates in the axon-like processes in the CB₁R-expressing animals (G); this is not encountered in CB₁R<sup>−/−</sup> mice (H). This confirms presence of CB₁R in the axons while dendro-somatic and mitochondrial CB₁R locations are enigmatic. Averages of the measurements from 4 animals in each group ±SD are indicated. Abbreviations: d, dendrite; sb, synaptic bouton. (Scale bars in A and B = 10 μm; in C–F = 0.5 μm).
Figure 2: CB1R in synaptic boutons innervating AgRP/NPY-positive and -negative neurons in the ARC. (A–C) In 3D reconstruction, NPY-GFP-positive dendritic shaft (yellow) contacts with CB1R-positive axon (blue). Positions of profiles shown in electron micrographs (B and C) are indicated with red dotted lines b and c, respectively. (B) NPY-GFP-positive dendrite identified with diffuse DAB deposition (highlighted semitransparent yellow) produces symmetric synapse (arrowhead) with CB1R-positive synaptic bouton (semitransparent blue) that is identified with intense black DAB-Ni staining. (C) The NPY-GFP-positive dendrite is also innervated by two symmetric synapses (arrowheads) from CB1R-negative axons. Profile of axonal shaft of the reconstructed CB1R-positive axon is indicated with empty arrow. Profiles of other CB1R-positive axons not shown in the 3D.
2. METHODS

2.1. Animal maintenance
Mice were maintained with water and food freely available and housed on a 12 h light/12 h dark cycle. All mice were aged between 12 and 16 weeks at the time of killing. Procedures were approved by the Institutional Animal Care and Use Committee of Yale University.

2.2. Transgenic animals
CB2R-null (CB1R<sup>−/−</sup>) mice were on a C57/BL6 background [25]. NPY-GFP (B6.Cg-Tg(Npy-MAPT/Sapphire)1Rck/J, stock no 008321, The Jackson Laboratories, Bar Harbor, ME USA) and NPY-hrGFP (B6.FVB-Tg(Npy-hrGFP)1LowW/J, stock no 006417, The Jackson Laboratories) mice were used for identification of AgRP/NPY neurons in the ARC. Both lines were maintained on a C57/BL6 background.

2.3. Immunohistochemistry for electron and light microscopy
For correlative light/electron microscopy, CB1R<sup>+/+</sup>, CB1R<sup>+/−</sup>, CB1R<sup>−/−</sup> and NPY-GFP mice were perfused transcardially with a fixative containing 4% paraformaldehyde, 0.2% picric acid, and 0.2% glutaraldehyde in 0.1 M PB. The brains were removed and immersed overnight in the same fixative. Coronal brain sections (of 50 μm thickness) were cut with a vibratome. About half of the brain sections were immersed in 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min to block tissue peroxidase, whereas the remaining specimens were used for immunohistochemistry omitting this step. No difference in the immunolabeling was observed between these sections. For single immunolabeling, the sections were incubated with polyclonal sera against CB<sub>2</sub>R raised in guinea pig (1:1000; CB1-GP-AF530; Frontier Institute, Ishikari, Hokkaido, Japan), then, with biotinylated anti-guinea pig IgGs (1:300; Jackson ImmunoResearch, West Grove, PA, USA) and the Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with Ni-intensified 3,3′-diaminobenzidine-4HCl (DAB-Ni) as a chromogen. For CB1R/GFAP and CB2R/NPY-GFP double labeling, the sections were first immunolabeled for CB2R as above with DAB-Ni as a chromogen that produces intensive black staining; then, the sections were incubated with made-in-rat anti-GFAP (1:6000; Invitrogen, Eugene, OR, USA) or made-in-chicken anti-GFP (1:2000; Thermo Fisher Scientific, Rockford, IL, USA) sera. Thereafter, corresponding biotinylated anti-rat or anti-chicken IgGs (1:300; both from Jackson ImmunoResearch) and the Elite ABC kit were applied as above. 3,3′-diaminobenzidine-4HCl (DAB) producing diffuse electron-dense staining was used as a chromogen. The sections were post-fixed with 1% OsO<sub>4</sub>, dehydrated, embedded in durcupan (Fluka, Buchs, Switzerland) on microscope slides, and coveredslipped. Selected fragments of tissue were analyzed and photographed with an Axioplan 2 microscope (Zeiss, Jena, Germany) and re-embedded into durcupan blocks for electron microscopic investigation. The samples were cut with a Reichert ultramicrotome into 70-nm-thick sections. The sections were then stained with lead citrate and evaluated and photographed in a JEM 1010 electron microscope (JEOL, Japan) equipped with a Multiscan 792 digital camera (Gatan, Pleasanton, CA, USA).

For 3D reconstruction, 20–30 serial images were made with 15,000× magnification of electron microscope. Neuronal fragments were chosen for the 3D reconstruction of axon-like processes in a random manner while avoiding cell bodies and blood vessels when possible. The micrographs were aligned using the computer program Reconstruct [26], publicly available at http://www.bu.edu/neural/Reconstruct.html. For fluorescent microscopy, NPY-hrGFP mice were perfused with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB. The brains were removed and immersed overnight in the same fixative. Coronal brain sections (of 50 μm thickness) were cut with a vibratome. For double immunofluorescence staining, the sections were incubated in blocking solution (5% normal goat serum and 0.2% Triton X-100 in 0.1 M PB) for 60 min. The primary polyclonal antibodies (guinea pig anti-CB<sub>2</sub>R IgG (CB1-GP-AF530); Frontier Institute, Ishikari, Hokkaido, Japan) and rabbit anti-diacylglycerol lipase-alpha (DAGL) IgG (DGLa-Rb-Af380; Frontier Institute) were both diluted at 1:300 and concomitantly applied overnight at room temperature. The next day, sections were washed 3 times in 0.1 M PB and were concomitantly incubated with the secondary antibodies (goat anti-guinea pig IgG (H+L) Alexa Fluor<sup>®</sup> 568 conjugate, A-11075; and, goat anti-rabbit IgG (H+L) Alexa Fluor<sup>®</sup> 633 conjugate; both from Thermo Fischer Scientific, Waltham, MA, USA) both at a dilution of 1:500 for 1 h at room temperature. Finally, the sections were coveredslipped in DAKO mounting medium and confocal laser scanning microscopy was performed using a Zeiss LSM Meta 510.

In previous studies, we extensively analyzed specificity of the anti-CB<sub>2</sub>R labeling using mass-spectrometry, Western Blots, and immunohistochemistry at light and electron microscopy levels; we demonstrated that anti-CB<sub>2</sub>R IgG (CB1-GP-AF530; Frontier Institute) in parallel to CB<sub>2</sub>R also recognize a conformational epitope in mitochondrial stomatin-like protein 2 [27–29]. Specific anti-DAGL labeling (neuronal somato-dendritic surface expression) was confirmed for immunohistochemistry at levels of light and electron microscopy using wild type and DAGL-null mice [30–32]. Specific anti-GFP and anti-GFAP labeling was confirmed in numerous studies [e.g., [33,34]].

2.4. Statistical analysis
Quantifications of the anti-CB<sub>2</sub>R immunolabeling in identified cell segments and organelles in ARC of the CB<sub>2</sub>R-expressing (CB1R<sup>+/−</sup>and CB1R<sup>−/−</sup> pooled) and CB2R<sup>−/−</sup> mice were performed at 15,000× magnification of electron microscope. Percentages of the anti-CB<sub>2</sub>R immuno-precipitation locations in axon-like, dendro-somatic, and mitochondrial profiles were calculated for every measurement. Then, averages of 4 measurements from every one of 4 animals in each group ±SD were calculated using Excel 2013 (Microsoft) software.

3. RESULTS

3.1. CB<sub>2</sub>R concentrates in axon-like processes in the ARC
Raised in guinea pig anti-CB<sub>2</sub>R serum provides intensive staining of CB<sub>2</sub>R-expressing cell bodies and axons with a commercial CB<sub>2</sub>R antibody (Jackson, CA, USA). Anti-CB<sub>2</sub>R labeling is specific in a dramatic difference in anti-CB<sub>2</sub>R labeling

reconstructions are also seen (arrows in C), (D and E) 3D reconstruction from serial ultrathin sections demonstrates three axons (each depicted with different colors in semitransparent mode) that contain plural anti-CB<sub>2</sub>R DAB-Ni depositions (depicted black; small arrows) and innervate NPY-GFP-negative cells. Red dotted lines indicate positioning of the profiles shown in the electron micrograph in E where they are highlighted with the same semitransparent colors, respectively. The yellow synaptic bouton produces symmetric synapses (arrowhead) with the NPY-GFP-negative cell body (semitransparent orange). The green synaptic bouton produces asymmetric synapses (empty arrowheads) with the NPY-GFP-negative cell body and a dendrite. Several CB2R-positive axon-like profiles (not shown in 3D) are highlighted semitransparent red. (Scale bars = 1 μm.).
between CB1R-expressing and CB1R−/− animals. We found that each CB1R-immunopositive process contains several discontinued depositions of anti-CB1R DAB-Ni immuno-precipitation in the CB1R wild type (CB1R+/+) and heterozygous (CB1R+/-) mice. In contrast, only a single spot of staining was detected in every 3D-reconstructed process in CB1R−/− mice, designating occasional binding of the antibodies (Figure 1E, F).

Additional evidence of selective labeling in the CB1R-expressing animals was obtained with quantification of the sites of DAB-Ni immuno-precipitations in electron microscopy. Analysis of occasional single ultrathin sections shows that 82.2±4.2% of the immuno-precipitations in the CB1R-expressing mice are located in axon-like processes, whereas the remaining number of immuno-precipitations is distributed between dendrites, cell bodies, and mitochondria (Figure 1G). Predomination of a certain location of anti-CB1R labeling was not encountered in CB1R−/− mice. In those, non-CB1R binding of the CB1R antibodies (presumed background or labeling of other molecules) was detected in nearly equal proportions in axon-like processes, dendro-somatic cell segments, and mitochondria (Figure 1H).

Mind that similar mitochondrial labeling is evident in the CB1R-expressing and CB1R−/− mice, confirming previously demonstrated binding of the anti-CB1R serum with mitochondrial stomatin-like protein 2 rather than revealing mitochondrial location of CB1R [27–29]. In three random segments of ARC neuropil from CB1R+/+ mice that were subject of electron microscopy analysis and 3D reconstruction (each analyzed volume ~100 μm³), we identified in total 32, 26, and 13 CB1R-positive axon-like processes per a reconstructed segment (Supplementary Figure 2). No dendrite-like processes containing CB1R accumulation were observed in these neuropil segments. Detected CB1R-positive axon-like processes are separated by immunonegative tissue and do not form CB1R-enriched bundles that would be easily identifiable with light microscopy. Thus, our quantitative electron microscopy analysis and 3D reconstruction from serial sections demonstrate numerous CB1R-positive axon-like processes in the hypothalamus.

3.2. CB1R-expressing axons innervate AgRP/NPY-positive and -negative neurons

To analyze whether the hypothalamic CB1R-expressing axons could produce synapses innervating AgRP/NPY neurons in the ARC, we performed double immunolabeling for CB1R (DAB-Ni) and GFAP (DAB) combined with electron microscopy 3D reconstruction. CB1R-containing synaptic bouton (depicted semitransparent yellow in 3D and serial electron micrographs) innervates an immunonegative dendritic shaft (depicted semitransparent indigo). The synaptic contact is shown in red in 3D and indicated with red arrowheads in the electron micrographs. Anti-CB1R intensive black DAB-Ni depositions (arrows, depicted black in 3D) are seen in both, the synaptic bouton and axonal shafts. Another CB1R-containing axon-like process (semitransparent yellow; empty arrows) is also seen. Notice that several of the anti-CB1R DAB-Ni depositions (arrows) contact the GFAP-positive astroglial cell (identified with diffuse DAB staining; highlighted semitransparent light blue), but they are not inside of it. (Scale bars = 0.5 μm).

Figure 3: A three-somatic synapse in ARC from CB1R+/+ mouse identified with double immunolabeling for CB1R (DAB-Ni) and GFAP (DAB) combined with electron microscopy 3D reconstruction. (A and B) 3D reconstruction images rotated 90° relative to each other. (C and D) Representative serial micrographs used for the 3D reconstruction. CB1R-containing synaptic bouton (depicted semitransparent yellow in 3D and serial electron micrographs) innervates an immunonegative dendritic shaft (depicted semitransparent indigo). The synaptic contact is shown in red in 3D and indicated with red arrowheads in the electron micrographs. Anti-CB1R intensive black DAB-Ni depositions (arrows, depicted black in 3D) are seen in both, the synaptic bouton and axonal shafts. Another CB1R-containing axon-like process (semitransparent yellow; empty arrows) is also seen. Notice that several of the anti-CB1R DAB-Ni depositions (arrows) contact the GFAP-positive astroglial cell (identified with diffuse DAB staining; highlighted semitransparent light blue), but they are not inside of it. (Scale bars = 0.5 μm).
neurons, supporting the hypothesis for retrograde eCB signaling in
the ACR and predominance of eCB-conducted dis-inhibition of the AgRP/
NPY hunger promoting neurons. Further morphological study (for
example, immuno-gold labeling that provides more precise location of
the antigen) of CB1R location in the hypothalamic nuclei is warranted.

3.3. CB1R and DAGL are co-localized at AgRP/NPY neurons in ARC
To further elucidate potential CB1R signaling in ARC, we performed
immunofluorescence assay for the enzyme DAGL, which is known to
be located at postsynaptic sites catalyzing biosynthesis of the eCB
retrograde messenger 2-AG [38,39]. We observed dotted DAGL
immunolabeling as in GFP-expressing AgRP/NPY neurons so in GFP-
negative cells. Multiple immunofluorescence exemplified a close
spatial relationship between CB1R and DAGL in the ARC, particularly at
NPY-GFP neurons (Figure 4). The latter finding supports a retrograde
mode of action of 2-AG at AgRP/NPY neurons, as has been demon-
strated in other brain segments [5,29].

4. DISCUSSION
Relatively low anti-CB1R immuno-reactivity in the hypothalamus
makes identification of CB1R-containing axons with light microscopy
and in single electron micrographs difficult. Here, we addressed this
problem using electron microscopy with 3D reconstruction from serial
sections. We show that (1) numerous axons in ARC contain several
discontinuous CB1R-positive segments; (2) CB1R-positive axons
innervate AgRP/NPY neurons with mostly symmetric, presumed
inhibitory, synapses; (3) unlabeled neurons receive both symmetric
and asymmetric synaptic contacts with CB1R-positive axons; (4) DAGL
— the enzyme catalyzing biosynthesis of the eCB retrograde
messenger 2-AG — is in close apposition to CB1R at AgRP/NPY neu-
rons. Taken together, the data demonstrate the morphologic substrate
for eCB/CB1R-conducted retrograde dis-inhibition of the orexigenic
neurons in ARC.
CB1R and eCBs were shown to be involved in the regulation of food
intake and energy consumption in several hypothalamic nuclei,
including the lateral and dorsomedial hypothalamus as well as ARC
and paraventricular nucleus [1,2,11,13,19,40,41]. Moreover, eCB
prandial regulation apparently includes synaptic and non-synaptic
mechanisms [10,12,42]. A hypothalamic role of CB1R-containing
synapses in eCB regulation of food intake was suggested [19,22], but it
was not experimentally demonstrated. Our findings unravel a yet un-
known synaptic mechanism of eCB control of food intake that may act
in parallel with recently demonstrated intracellular eCB control of
mitochondrial respiration [12].
Thus, our results show that eCBs and cannabis drugs may conduct
prandial mechanisms through retrograde synaptic dis-inhibition and
dis-excitation in the hypothalamus. Further investigations are war-
ranted to determine the location and neurochemical properties of
CB1R-expressing inhibitory neurons innervating AgRP/NPY neurons in
ARC; and, to determine what neurons provide the CB1R-containing
inputs to other neurons of the hypothalamus.

Figure 4: Immunolabeling for CB1R (red) and DAGL (blue) in ARC from the NPY-hrGFP transgenic mouse. CB1R/DAGL-double positive spots [some of those are at NPY-hrGFP expressing cells (green)] are indicated with arrows. CB1R-positive DAGL-negative spots are indicated with arrowheads. (Scale bar = 10 μm).
AUTHOR CONTRIBUTION

YMM and MK designed and executed experiments, analyzed the data, and wrote the manuscript. TLH and PR analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

All authors declare no conflict of interests.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.01.004.

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