Research Paper

Endocannabinoid 2-AG inhibits the release of dopamine from PC12 cells

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Abstract The endocannabinoid system (ECS) is known to be involved in the retrograde suppression of signal transduction at synapse. Although there are several reports investigating the effect of ECS on dopamine (DA) signaling, consistent results have not been reported. Therefore, in this study, PC12 cells differentiated with nerve growth factor were used as model cells for dopaminergic neuron, and the true effects of endocannabinoid on DA release in these cells were accurately evaluated using mass spectrometry. The induction of DA release by hexanal was significantly suppressed by co-treatment with endocannabinoid 2-arachidonylglycerol (2-AG). In addition, it was suggested that the changes in intracellular calcium kinetics mediated by cannabinoid receptors were involved in the suppression of DA release by co-treatment with 2-AG. We have also shown in previous studies that DA enhances the function of the ECS in glial cells. These results suggest that glial ECS may act as a suppression system for DA signaling in situations where DA signaling is abnormally enhanced in the central nervous system.

Key words: endocannabinoid system, 2-arachidonylglycerol, dopamine, PC12 cells, calcium kinetic

Introduction

After the 1990s, cannabinoid receptors were identified as receptors for tetrahydrocannabinol, the major psychotropic component of *cannabis sativa*. Currently, cannabinoid receptor type 1 and type 2 (CB1 and CB2) are known as the major cannabinoid receptors[1,2]. Thereafter, arachidonylethanolamide and 2-arachidonylglycerol (2-AG) have been identified as the endogenous ligand of these receptors[3−5]. In addition to these cannabinoid receptors and endocannabinoids, the system composed of enzymes involved in the synthesis and degradation of endocannabinoids (*N*-acyl phosphoatidylethanolamide phospholipase D, phospholipase C beta 4, diacylglycerol lipase alpha, fatty acid amide hydrolase and monoglyceride lipase) is known as the endocannabinoid system (ECS)[6]. This system has been shown to be involved in the regulation of various physiological functions[7−13].

The central nervous system (CNS) is composed of neurons and glial cells. Neurons play a central role in signal transduction in the CNS. When a stimulus above the threshold is applied to a neuron, depolarization occurs and an action potential is generated. The action potential conducts on the axon. At the axon terminal, synapse is formed with other neuron, and signal transduction between neurons is carried out by converting electrical signals into chemical neurotransmitters. The neurotransmitters released from presynaptic terminals induce depolarization or hyperpolarization of postsyn-

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aptic neuron. These conflicting signal transductions interact with each other to regulate neuronal activity. Glial cells, the other cells that constitute the CNS, are classified into oligodendrocytes, microglia, and astrocytes. These glial cells provide environmental maintenance and metabolic support in the CNS for neuronal survival and function. Oligodendrocytes are involved in axon signaling and microglia are involved in the immune response in the CNS. Astrocytes are involved in the regulation of signal transduction in the CNS by forming tripartite synapses with neurons[14].

The ECS is involved in the retrograde suppression of signal transduction at synapses. This mechanism begins with the release of endocannabinoids from postsynaptic neuron into the synaptic cleft. The endocannabinoids suppress the elevation of intracellular calcium ions in presynaptic neurons through binding to cannabinoid receptors. As a result, the release of neurotransmitters from presynaptic neuron is suppressed. Among such inhibition of synaptic transduction with ECS, the suppression of gamma-aminobutyric acid (GABA) release at inhibitory synapses is known as depolarization-induced suppression of inhibition (DSI)[15,16], and the suppression of glutamic acid (Glu) release at excitatory synapses is known as depolarization-induced suppression of excitation (DSE)[17]. Such DSI and DSE have been observed in many brain regions[18]. In addition to GABA or Glu signaling, it is known that various other neurotransmitter signaling are regulated by ECS[19–23].

In our previous study, we investigated the relationship between dopamine (DA) signaling and the ECS and found that long-term high concentrations of DA induced the 2-AG-productivity in glial cells[24]. From these facts, we speculate that glial ECS acts as an inhibitory mechanism of DA signaling when synaptic DA signaling is abnormally enhanced. Although there are several reports investigating the effect of ECS on DA signaling, consistent results have not been reported[25–29]. It seems that the reason why consistent results have not been reported is the experimental models and methods are not suitable. First, slice culture of the brain was used in these previous reports and it is possible that they detected the results of the interaction of multiple types of cells present there. Second, these results may differ from the real behavior of endogenous DA release, because they were investigating the effects of endocannabinoids on the release of radiolabeled-DA. In addition, other reports have detected the effect of endocannabinoid on the release of DA by electrophysiological techniques using voltammetry, which is only an indirect evidence. In order to improve these problems, we used PC12 cells as the model of dopaminergic neuron in this study. In addition, mass spectrometry was used to directly evaluate the effect of 2-AG on the release of endogenous DA in these cells. Furthermore, we also investigated the effect of 2-AG on intracellular calcium ion behavior because it is known that intracellular calcium dynamics are deeply involved in not only the release of neurotransmitters but the suppression of synaptic signal transduction by ECS. We considered that our experimental model could reveal the true effect of ECS on DA signaling.

Materials and Methods

Reagents and chemicals

All reagents and chemicals were purchased from Merck (Darmstadt, Germany), and listed as follows: nerve growth factor (NGF), hexanal, 2-AG, isoproterenol, trifluoroacetic acid (TFA), acetonitrile (MeCN), *N*,*O*-bis(trimetylsilyl)trifluoroacetamide+trimetylchlorosilane (BSTFA+TMCS), calcium ionophore (A23187) and ethyleneglycol bis tetraacetic acid (EGTA). The primary antibodies (CB1 (ab23703) and CB2 (ab45942)) were purchased from Abcam plc (Cambridge, UK) and secondary antibody was purchased from Cell Signaling Technology (MA, USA).

Cell culture and treatment

The PC12 rat pheochromocytoma cell line was purchased from American Type Culture Collection (VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Merck, Darmstadt, Germany) supplemented with 10% v/v heat-inactivated horse serum (Merck, Darmstadt, Germany) and 5% v/v heat-inactivated fetal bovine serum (Merck, Darmstadt, Germany) in a humidified incubator at 37°C with an atmosphere of 5% CO_2. Cells were reseeded in the 6 well plates (for western blot assay and quantification of DA) or 96 well plates (for measurement of intracellular Ca^{2+}) at a density 4×10^4 cells/cm^2. To investigate the morphological change and expression change of cannabinoid receptors due to NGF treatment, PC12 cells were treated with 0 ng/mL NGF or 100 ng/mL NGF and designated as NGF(−)-PC12 or NGF(+)-PC12, respectively. The treatment with NGF was performed for 7 days, and cell morphological observation and sampling of total protein were performed every day from day 1 to day 7. To investigate the expression levels of CB1 and CB2 cannabinoid receptors, these cells were collected by using cell lysis

buffer (Cell Signaling Technology, MA, USA). For quantification of DA, reseeded cells were cultured with NGF for 4 days. The DA release from NGF(+)-PC12 was induced by treatment with 10 mM hexanal. In addition, to assess the effect of 2-AG on DA release from NGF(+)-PC12 with hexanal treatment, these cells were co-treated with hexanal and 2-AG. Five minutes after, culture supernatant and cells were collected for DA analysis and protein quantification, respectively. For measurement of change of intracellular Ca^{2+}, reseeded cells were cultured with NGF for 4 days and loaded with Fluo4-AM (DOJINDO, Kumamoto, Japan).

Western blot assay

The concentrations of total protein were determined by the Quant-iT Protein Assay kit and Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Twenty micrograms of total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (BIO RAD, CA, USA) and transferred to the polyvinylidene fluoride membrane (Merck, Darmstadt, Germany). The membrane was incubated with the primary antibody (1 : 1000 for CB1 and 1 : 500 for CB2) at room temperature for 1 h and with anti-rabbit secondary antibody (1 : 10000 for CB1 and 1 : 5000 for CB2) at room temperature for 1 h. The ECL select (Cytiva, Tokyo, Japan) was used to determine the expression levels of the proteins. Immunoreactive bands were analysed by densitometry using ImageJ 1.48 v (Weyne Rasband, national Institutes of Health).

Solid phase extraction and derivatization of DA

The DA in collected culture supernatants was extracted using Bond Elut PBA (Agilent, CA, USA). The cartridge was conditioned with MeCN, 1% TFA-MeCN and 50 mM phosphate buffer (pH10). After loading the sample (buffered to pH8.5), cartridge was washed with 10 mM phosphate buffer (pH8.5)+5% MeCN and 0.1% TFA-MeCN. The DA was eluted from the cartridge with 1% TFA-MeCN. Isoproterenol was added as an internal standard (I.S.) to eluted solution. The eluted solution was evaporated, and the residue was derivatized with BSTFA+TMCS for 1 h at 60°C. The trimethylsilyl (TMS) derivatized samples were analysed by gas chromatography/mass spectrometry (GC/MS).

Quantification of DA by GC/MS

GC/MS analysis was performed on GCMS-QP2010 (Shimadzu, Kyoto, Japan) equipped with an AOC-20i auto-injector (Shimadzu, Kyoto, Japan). Analysis was performed in positive electron ionization mode. The GC/MS conditions were as follows: column, DB-5MS (30 m×0.25 mm, i.d. 0.25 μm, Agilent, CA, USA). The column oven temperature was programed to be held at 120°C for 3 min, increased at 10°C/min to 180°C, at 5°C/min to 190°C, and at 15°C/min to 280°C, then held for 5 min. The injector, interface, and ion source temperature, 270°C, 260°C and 200°C, respectively; carrier gas and flow rate, helium at 1 mL/min[30]. MS detection was carried out in selected ion monitoring (SIM) mode using *m/z* values of 280 for DA-TMS, *m/z* values of 355 for I.S.-TMS. The limit of detection and limit of quantification for DA-TMS were 2 ng/mL and 10 ng/mL, respectively. Six-point calibration curves were constructed for DA-TMS in the concentration range 100−600 ng/mL (r^2=0.999). In addition, the final concentration of DA was standardized by total protein concentration of cells derived from the same well.

Measurement of intracellular Ca^{2+} with Fluo4

To detect the change in $[Ca^{2+}]_i$ treated with hexanal and 2-AG, the Fluo4-AM dye, a calcium indicator, was loaded into our dopaminergic model cells (NGF (+)-PC12). The fluorescence intensity of Fluo4-loaded cells was measured by using ARVO X5 multilabel reader (Parkin Elmer, MA, USA). After baseline was collected at an emission wavelength of 535 nm and excitation wavelength of 485 nm for 20 s, hexanal and 2-AG were added to culture supernatant. The change of fluorescence intensity of Fluo4 was detected for 200 s at the same condition. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated using equation : $[Ca^{2+}]_i = K_d \times \{(F-F_{min})/(F_{max}-F)\}$. K_d represents the dissociation constant for Ca^{2+}-bound Fluo4 and is 345 nM. F represents the fluorescence of experimental samples. F_{min} represents the minimum fluorescence of Ca^{2+}-bound Fluo4 by adding EGTA. F_{max} represents the maximum fluorescence of Ca^{2+}-bound Fluo4 by adding A23187.

Statistical analysis

All data were shown as the mean±standard error (SE). A one way analysis of variance followed by Tukey−Kramer method was used to compare the significance of difference among all groups. Results were considered significant at $^{*}p < 0.05$ and $^{**}p < 0.01$.

Results

Change of cellular morphology and expression state of cannabinoid receptors in PC12 cells by NGF treatment

In NGF(−)-PC12, cell proliferation was observed, but no significant change in its morphology was observed (Fig. 1A). On the other hand, in NGF(+)-PC12, extension of neurite began to be observed after day 2 in addition to cell proliferation, and these neurites became a network structure with each passing day (Fig. 1B). The expression states of the cannabinoid receptors (CB1 or CB2) in these NGF(−)-PC12 or NGF(+)-PC12 were confirmed by western blotting method. Although the expression states of CB1 in NGF(−)-PC12 were slightly up-regulated with the progress of culture, the expression states of CB1 in NGF(+)-PC12 were highest on day 4, and this expression level was significantly higher than that in NGF(−)-PC12 (Fig. 1C). On the other hand, the expression states of CB2 were not significantly changed in either NGF(−)-PC12 or NGF(+)-PC12 (Fig. 1D). We used the NGF(+)-PC12 on day 4 as the model of dopaminergic neuron in subsequent experiments because the neurites are sufficiently extended and CB1 is the most highly expressed.

Effect of 2-AG on DA release

In Fig. 2A, chromatograms from DA-TMS and I.S.-TMS in collected sample were shown. The concentrations of extracellular DA after the treatment were as shown below: 0.17±0.01 ng/mg protein for 0 mM hexanal+0 nM 2-AG, 155.29±31.28 ng/mg protein for 10 mM hexanal+0 nM 2-AG, 73.85±20.15 ng/mg protein for 10 mM hexanal+1 nM 2-AG, 41.66±13.94 ng/mg protein for 10 mM hexanal +10 nM 2-AG, and 48.66±7.29 ng/mg protein for 10 mM hexanal+100 nM 2-AG (Fig. 2B). The DA release from NGF(+)-PC12 were markedly increased by hexanal treatment, and these induction of DA release were significantly decreased in a dose-dependent manner of co-treated 2-AG. Similar results were reproduced by multiple independent experiments.

Effect of 2-AG on change of $[Ca^{2+}]_i$

In the control sample (cont.), no significant change was observed in $[Ca^{2+}]_i$ (Fig. 3). When the cells were treated with 10 mM hexanal alone, $[Ca^{2+}]_i$ reached the highest value (16505±2287 nM) at 10 s after the addition of the agent (Fig. 3). The $[Ca^{2+}]_i$ when co-treated with hexanal and 2-AG also reached the highest value at 10 s after the addition of the agent, and these values were shown below: 4176±1086 nM for hexanal+1 nM 2-AG, 2977±288 nM for hexanal+10 nM 2-AG, and 1725±183 nM for hexanal+100 nM 2-AG, (Fig. 3). The $[Ca^{2+}]_i$ were markidly increased by hexanal treatment ($P<0.01$, compared with cont.), and this increase was significantly suppressed in dose-dependent manner of co-treated 2-AG ($P<0.01$, compared with 10 mM hexanal+0 nM 2-AG).

Discussion

PC12 cells were established by Greene and Tischler in 1976 from rat adrenal pheochromocytoma[31]. These cells have the ability to release some neurotransmitters (noradrenaline, catecholamines, and DA) by vesicles. In addition, since this cell differentiates into a sympathetic nerve in response to NGF, it is often used as a model cell for exocytosis studies of such neurotransmitters[32]. CB1 and CB2 are known as major cannabinoid receptors. The distribution of these receptors is distinctly different: CB1 is expressed mainly in the nervous system, whereas CB2 is expressed mainly in the peripheral immune system tissue[33]. It was considered that the sensitivity to endocannabinoid may change by differentiation with NGF. Therefore, to use PC12 cells as a model of dopaminergic neuron in this study, we first confirmed the expression state of cannabinoid receptors (CB1 and CB2) by NGF-treated PC12. As a result, the expression level of CB1 increased due to the differentiation of PC12 cells by NGF treatment, although no significant change was observed in the expression level of CB2. These increase in expression level of CB1 associated with the differentiation of PC12 cells into neuron-like cell by NGF treatment suggest not only morphological change but change of cell properties into neuron-like cell. Based on these facts, PC12 cells differentiated by NGF (NGF(+)-PC12) were used as the model of dopaminergic neuron in the subsequent our studies.

Generally, the release of neurotransmitters from presynaptic terminals begins with the influx of calcium ions into the cell through voltage-gated calcium channels after the action potential reaches the presynaptic terminal. The elevation of intracellular calcium ions causes synaptic vesicles containing neurotransmitters to fuse with the presynaptic membrane and release neurotransmitters into the synaptic cleft. In other words, the progress of signal transduction at synapses is triggered by an increase in $[Ca^{2+}]_i$. In this study,

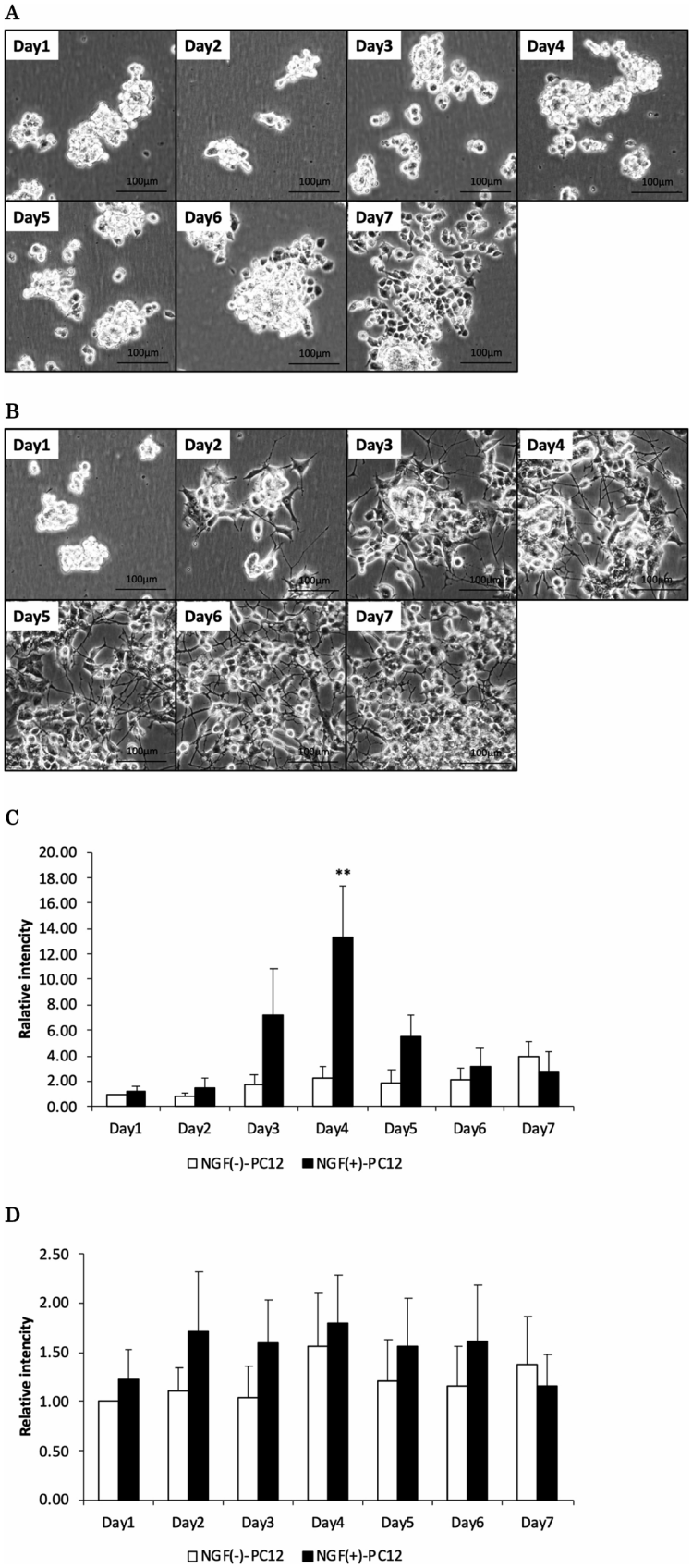

Fig. 1. Change of cellular morphology and expression state of cannabinoid receptors in PC12 cells by NGF treatment.
(A) and (B) show the cellular morphology of PC12 cells treated with 0 ng/mL NGF (NGF(−)-PC12) and 100 ng/mL NGF (NGF(+)-PC12), respectively. (C) and (D) show the result of western blot of CB1 and CB2, respectively. White bars refer to NGF(−)-PC12 and black bars refer to NGF(+)-PC12. $^{**}P<0.01$, compared with NGF(−)-PC12.

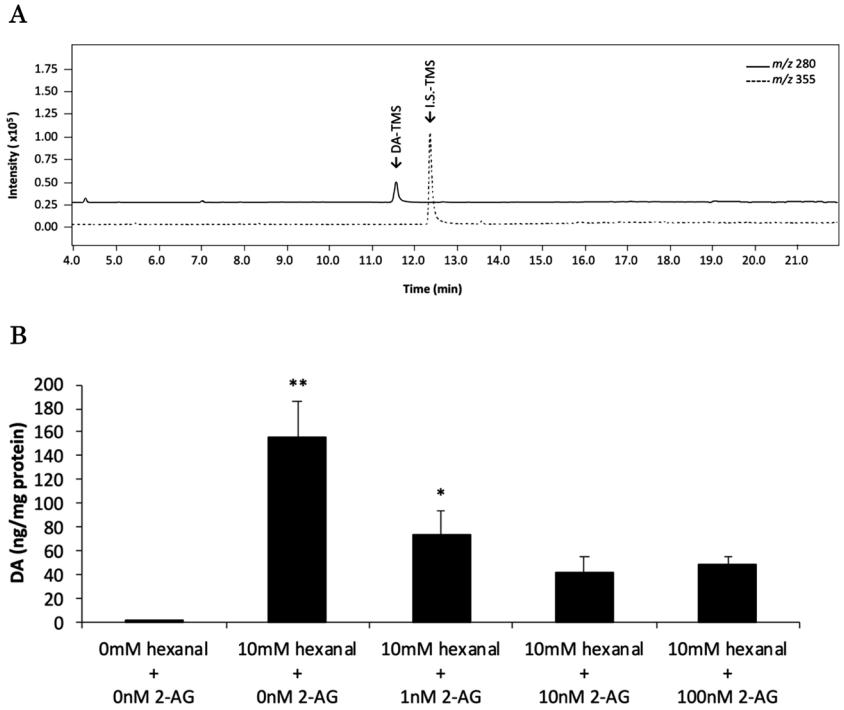

Fig. 2. Effect of 2-AG on DA release of NGF(+)-PC12 cells.
(A) The SIM chromatograms of DA-TMS and I.S.-TMS in collected sample. (B) DA released into culture supernatant by co-treated with hexanal and 2-AG. All results were showed as mean±SE (n=3). $^{*}P<0.05$ and $^{**}P<0.01$, compared with 0 mM hexanal+0 nM 2-AG.

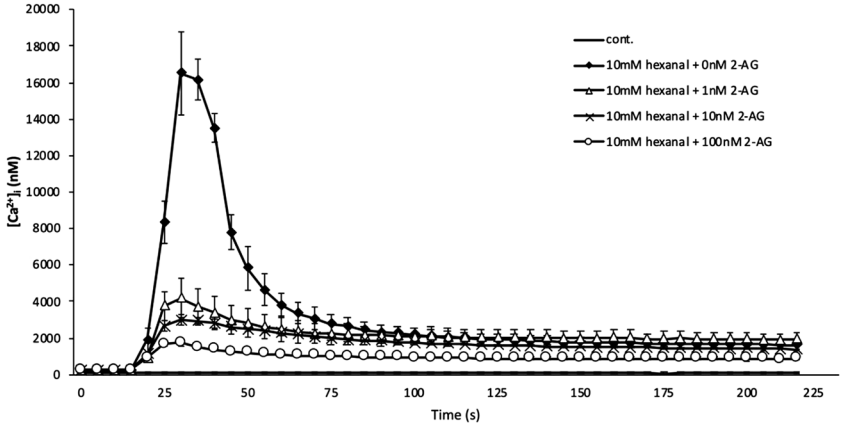

Fig. 3. Effect of 2-AG on change of $[Ca^{2+}]_i$.
Intracellular Ca^{2+}kinetics in NGF(+)-PC12 cells co-treated with hexanal and 2-AG were measured using Fluo4. All results were expressed as the mean±SE from three independent preparations.

we used PC12 cells as the model of dopaminergic neuron to investigate the effect of 2-AG on the release of DA inherent in these cells. Hexanal was used to induce the release of DA from these cells. In addition, the ECS is involved in retrograde suppression of signal transduction at synapses. Therefore, we examined whether endocannabinoid 2-AG acts suppressively on DA release from the model of dopaminergic neuron (NGF(+)-PC12). As a result, it was clarified that the extracellular DA level was markedly increased by the treatment of hexanal, and these induction of release of DA by hexanal was significantly suppressed in a dose-dependent manner of 2-AG. Hexanal is straight-chain aliphatic aldehyde having 6 carbon atoms, and is known to induce the release of DA from PC12 cells by increasing the $[Ca^{2+}]_i$[34]. It seems that the induction of release of DA by hexanal is a good experimental model of the physiological process of release of neurotransmitters at synapses. From these facts, we speculate that 2-AG also suppresses the

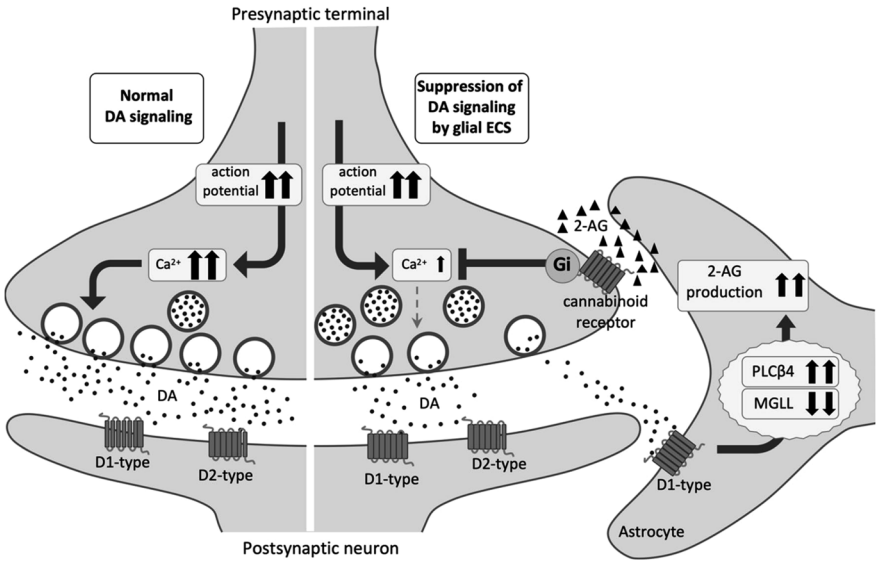

Fig. 4. Schema of inhibitory mechanism of DA release by glial endocannabinoid 2-AG.
Generally, DA release from synaptic terminal of dopaminergic neuron is promoted in a $[Ca^{2+}]_i$-depending manner. DA release from DA neurons is also abnormally enhanced by the action of addictive drugs such as stimulants. These enhancements in DA signaling are detected by astrocytes coexisting at synapses. The productivity of 2-AG in astrocytes are induced due to the changes in the expression of factors involved in the turnover of 2-AG in these cells. The 2-AG released from astrocytes into the synaptic cleft binds to cannabinoid receptors exist at presynaptic terminals. The action of $G_{i/o}$ protein coupled to these receptors suppresses the increase in $[Ca^{2+}]_{i,}$ resulting in the suppression of DA release from presynaptic terminals. In other words, astrocytic ECS acts as the inhibitory mechanism for DA signaling.

release of DA from dopaminergic neuron in vivo.

The retrograde suppression of signal transduction at synapses by ECS is initiated by the binding of endocannabinoids to cannabinoid receptors present at presynaptic terminals. These cannabinoid receptors are classified as G-protein-coupled receptors, and CB1 is coupled with $G_{i/o}$. The action of $G_{i/o}$ proteins through activation of these receptors by endocannabinoids induce not only the inhibition of voltage-gated calcium channel but the activation of voltage-gated and inward-rectifier potassium channels. As a result, the influx of calcium ions into the cell is suppressed, and finally the release of neurotransmitters from the presynapse is suppressed. The calcium ion dynamics are deeply involved in both the release of neurotransmitters at synapses into the synaptic cleft and the suppression of synaptic signal transduction by ECS. Therefore, we investigated whether calcium ion dynamics was involved in the suppression of DA release by 2-AG observed in our experimental model. As a result, the increase in $[Ca^{2+}]_i$ by hexanal was significantly suppressed in dose-dependent manner of 2-AG. These facts demonstrated that our experimental system sufficiently reproduced the mechanism of suppression of signal transduction at synapses by ESC at the cultured cell level.

We have been investigating the effects of glial endocannabinoid systems on DA signaling. In our previous study, we revealed that the long-term exposure of high-concentration DA induced the 2-AG-productivity in glial cells through changes in the expression state of factors involved in 2-AG turnover[24]. A common site affected by addictive drugs is the dopaminergic neuron, which projects from the ventral tegmental area to the nucleus accumbens[35–37]. Such dopaminergic neuron, also called the reward system, is deeply involved in the formation of drug dependence. It is thought that repeated use of addictive drugs causes abnormalities in CNS function due to long-term stimulation of the reward system, resulting in drug dependence. From the results of our previous studies, it is suggested that the function of ECS in glial cells present at synapses is enhanced under the condition that DA at synaptic cleft is abnormally high due to long-term repeated use of addictive drugs. Furthermore, in this study, we revealed that endocannabinoid 2-AG actually suppresses the release of DA from dopaminergic neuron. Thus, these results suggest that glial ECS may act as a suppression system for DA signaling in situations where DA signaling is abnormally enhanced in the central nervous system (Fig. 4).

Conclusion

The purpose of this study was to reveal the true effect of ECS on DA signaling by improving the problems of previous report that investigated the effect of ECS on the release of DA. Using PC12 cells as a model for dopaminergic neurons, we investigated the effect of 2-AG on the release of endogenous DA in these cells. As a result, it was clarified that the release of DA under physiological conditions was inhibited by suppressing the increase in $[Ca^{2+}]_i$ by 2-AG. Considering the results of our previous studies and this study, it is suggested that glial ECS coexisting at synapses may act as a inhibitory mechanism for abnormally enhanced DA signaling. In other words, ECS may have an antagonistic effect on addictive drugs, and it is suggested that extrinsic activation of this system may be applicable to the treatment of drug addiction.

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Conflict of Interest

There are no conflict of interest relevant to this article.

References

1) Matsuda LA, Lolaittou SJ, Brownstein MJ, Young AC, Bonner TI: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346: 561−564, 1990.
2) Munro S, Thomas KL, Abu-Shaar M: Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365: 61−65, 1993.
3) Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, et al: Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258: 1946−1949, 1992.
4) Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, et al: Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 50: 83−90, 1995.
5) Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, et al: 2-Arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215: 89−97, 1995.
6) Lu HC, Mackie K: An introduction to the endogenous cannabinoid system. *Biol Psychiatry* 79: 516−525, 2016.
7) Morena M, Campolongo P: The endocannabinoid system: An emotional buffer in the modulation of memory function. *Neurobiol Learn Mem* 112: 30−43, 2014.
8) Bellocchio L, Cervino C, Pasquali R, Pagotto U: The endocannabinoid system and energy metabolism. *J Neuroendocrinol* 20: 850−857, 2008.
9) Wang L, Yang T, Qian W, Hou X: The role of endocannabinoids in visceral hyposensitivity induced by rapid eye movement sleep deprivation in rats: Regional differences. *Int J Mol Med* 27: 119−126, 2011.
10) Basu S, Dittel BN: Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. *Immunol Res* 51: 26−38, 2011.
11) Kwok CH, Devonshire IM, Imraish A, Greenspon CM, Lockwood S, et al: Age-dependent plasticity in endocannabinoid modulation of pain processing through postnatal development. *Pain* 158: 2222−2232, 2017.
12) Fraga D, Zanoni CI, Rae GA, Parada CA, Souza GE: Endogenous cannabinoids induce fever through the activation of CB1 receptors. *Br J Pharmacol* 157: 1494−1501, 2009.
13) Zlebnik NE, Cheer JF: Drug-induced alterations of endocannabinoid-mediated plasticity in brain reward regions. *J Neurosci* 36: 10230−10238, 2016.
14) Perea G, Araque A: GLIA modulates synaptic transmission. *Brain Res Rev* 63: 93−102, 2010.
15) Ohno-Shosaku T, Maejima T, Kano M: Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. *Neuron* 29: 729−738, 2001.
16) Wilson RI, Nicoll RA: Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 410: 588−592, 2001.
17) Kreitzer AC, Regehr WG: Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* 29: 717−727, 2001.
18) Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M: Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev* 89: 309−380, 2009.
19) Ishac EJ, Jiang L, Lake KD, Varga K, Abood ME, et al: Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br J Pharmacol* 118: 2023−2028, 1996.
20) Gifford AN, Ashby CR, Jr: Electrically evoked acetylcho-

line release from hippocampal slices is inhibited by the cannabinoid receptor agonist, WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. *J Pharmacol Exp Ther* 277: 1431−1436, 1996.

21) Nakazi M, Bauer U, Nickel T, Kathmann M, Schlicker E: Inhibition of serotonin release in the mouse brain via presynaptic cannabinoid CB1 receptors. *Naunyn Schmiedebergs Arch Pharmacol* 361: 19−24, 2000.
22) Beinfeld MC, Connolly K: Activation of CB1 cannabinoid receptors in rat hippocampal slices inhibits potassium-evoked cholecystokinin release, a possible mechanism contributing to the spatial memory defects produced by cannabinoids. *Neurosci Lett* 301: 69−71, 2001.
23) Jennings EA, Vaughan CW, Christie MJ: Cannabinoid actions on rat superficial medullary dorsal horn neurons in vitro. *J Physiol* 534: 805−312, 2001.
24) Ochi H, Hirata Y, Hamajima M, Isobe I, Igarashi K: Dopamine enhances the glial endocannabinoid signaling. *Med Mass Spectrom* 5: 2−10, 2021.
25) Cadogan AK, Alexander SP, Boyd EA, Kendall DA: Influence of cannabinoids on electrically evoked dopamine release and cyclic AMP generation in the rat striatum. *J Neurochem* 69: 1131−1137, 1997.
26) Kathmann M, Bauer U, Schlicker E, Göthert M: Cannabinoid CB1 receptor-mediated inhibition of NMDA- and kainate-stimulated noradrenaline and dopamine release in the brain. *Naunyn Schmiedebergs Arch Pharmacol* 359: 466−470, 1999.
27) Szabo B, Müller T, Koch H: Effects of cannabinoids on dopamine release in the corpus striatum and the nucleus accumbens in vitro. *J Neurochem* 73: 1084−1089, 1999.
28) Steffens M, Engler C, Zentner J, Feuerstein TJ: Cannabinoid CB1 receptor-mediated modulation of evoked dopamine release and of adenylyl cyclase activity in the human neocortex. *Br J Pharmacol* 141: 1193−1203, 2004.
29) Köfalvi A, Rodrigues RJ, Ledent C, Mackie K, Vizi ES, et al: Involvement of cannabinoid receptors in the regulation of neurotransmitter release in the rodent striatum: A combined immunochemical and pharmacological analysis. *J Neurosci* 25: 2874−2884, 2005.
30) Park ST, Kang BX, Li Q, Kim HS, Lee J, et al: Chemical derivatization of catecholamines for gas chromatography-mass spectrometry. *Bull Krean Chem Soc* 30: 1497−1504, 2009.
31) Greene LA, Tischler AS: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* 73: 2424−2428, 1976.
32) Westerink RH, Ewing AG: The PC12 cell as model for neurosecretion. *Acta Physiol (Oxf)* 192: 273−285, 2008.
33) Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, et al: International union of pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 54: 161−202, 2002.
34) Kobayashi Y, Kako H, Yokogoshi H: Contribution of intracellular Ca^{2+}concentration and protein dephosphorylation to the induction of dopamine release from PC12 cells by the green odor compound hexanal. *Cell Mol Neurobiol* 30: 173−184, 2010.
35) Koob GF, Sanna PP, Bloom FE: Neuroscience of addiction. *Neuron* 21: 467−476, 1998.
36) Nestler EJ: Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* 2: 119−128, 2001.
37) Mizoguchi H, Yamada K, Mizuno M, Mizuno T, Nitta A, et al: Regulations of methamphetamine reward by extracellular signal-regulated kinase 1/2/ets-like gene-1 signaling pathway via the activation of dopamine receptors. *Mol Pharmacol* 65: 1293−1301, 2004.