

The Endocannabinoid, 2-Arachidonoyl Glycerol, Induces Growth Cone Collapse and Neurite Retraction in Growing Peripheral Sensory Neurons

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Abstract

Cannabis has a detrimental impact on the developing nervous system. Therefore, regular consumption of cannabis by pregnant and lactating woman poses a potential risk to neuronal growth in fetuses and infants. Indeed, endogenous cannabis-like molecules called endocannabinoids regulate many physiological processes, including neurogenesis, axon guidance, and synaptic plasticity through CB1 receptors. To investigate the physiological role of CB1 receptors on peripheral sensory nerve growth, the endocannabinoid 2-arachidonoyl glycerol was added to cultured chick dorsal root ganglion neurons. This compound inhibited neurite elongation and induced growth cone collapse in a dose- and time-dependent manner. These data suggest that caution should be exercised regarding maternal cannabis use during pregnancy. Because ectopic sprouting and abnormal neuronal network connections are considered to be a cause of neuropathic pain, our current data imply an additional role of endocannabinoids as inhibitors of the formation of pain-maintenance networks.

Keywords

Endocannabinoid, CB1 Receptors, Dorsal Root Ganglion Neurons, Growth Cone

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1. Introduction

Cannabis is considered a relatively harmless recreational agent despite gathering evidence of its detrimental impact on both the adult and the developing central nervous system (CNS) [1]. This attitude may simply reflect people's recognition that the risk of cannabis is relatively low, particularly when compared to that of tobacco and alcohol [2].

Cannabis has a small molecular size and is generally lipophilic, enabling the compound to readily cross the blood-brain or other cellular (for example, placental) barriers. Thus, regular cannabis use during pregnancy may result in relatively high concentrations of active cannabinoids in the developing fetus [3]. Moreover, cannabis and its metabolites readily pass into breast milk. When cannabis is regularly consumed by breast-feeding mothers, human milk concentrations of delta-9-tetrahydrocannabinol (THC), the main component of cannabis with psychoactive properties, may be up to 8-fold higher than simultaneously measured maternal plasma concentrations [4]. The effects of cannabis on the developing fetus and infant remain uncertain, although some evidence suggests that perinatal cannabis exposure can negatively affect fetal growth [5].

CB1 receptors (CB1Rs) are widely distributed within the brain including cerebral cortex, hippocampus, basal ganglia, and amygdala, as major targets of exogenous cannabinoids such as THC. Moreover, the most abundant endocannabinoid (endogenous cannabinoid: eCB) in the CNS, 2-arachidonoyl glycerol (2-AG), also triggers a broad range of signaling events by acting on CB1Rs. The eCB system regulates many physiological processes including neurogenesis, axon guidance, and synaptic plasticity [6]-[8]. In addition to the CNS, CB1Rs are also expressed in the peripheral nervous system [9] [10]. However, few studies have demonstrated a physiological role for CB1Rs that are expressed in peripheral neurons [11]. Therefore, whether the activation of CB1Rs in developing peripheral sensory neurons affects neuronal growth and function should be determined.

Here we study dorsal root ganglion (DRG) neurons, which are peripheral sensory neurons. The afferents of DRG neurons relay sensory information that originates from the skin to the brain. Therefore, they play a critical role in nociception including inflammatory and neuropathic pain [12]. Cultured DRG neurons isolated from chick embryos can be utilized to evaluate the effects of drugs on neuronal growth during development [13]. We previously show that several local anesthetics have direct neurotoxic effects on cultured DRG neurons [14] [15]. The purpose of the present study is to elucidate the effects of cannabinoids on peripheral sensory nerve growth and regeneration by examining neurite extension and growth cone collapse in particular. For this purpose, cultured DRG neurons are exposed to 2-AG.

2. Materials and Methods

This study was approved by the Institutional Animal Care Committee. Chick neural tissues were isolated from day 7 embryos. To prepare peripheral neurons, DRGs were dissected from lumbar paravertebral sites. After removing the original neurites, the explants were plated on laminin-coated coverslips and cultured in F-12 medium supplemented according to Bottenstein's method [13], containing 100 µg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 ng/ml mouse 7S nerve growth factor. Cultures were maintained at 37°C in 5% carbon dioxide. 2-arachidonoyl glycerol (2-AG) was purchased from Sigma Co. Ltd. (10 mM stock in ethanol).

After culturing for 20 hr, DRGs were exposed to the agents. The neurite length and percent collapse were determined after exposure for 2, 6, or 24 hr. 2-AG was prepared in pre-warmed fresh culture medium and was gently added to the culture medium. The volume of the added solution was 1/100 of the total volume of the culture medium. Cell viability was determined by exposing the cells to vehicle solution for identical durations.

The tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4) containing 10% sucrose as described previously [14] [15] and viewed with a 40× phase objective using a phase-contrast microscope (Axiovert; Zeiss, Germany). Growth cones at the periphery of explants were scored for collapse if they were not in contact or close proximity to other growth cones or neurites. Fifty growth cones were viewed and scored per coverslip. Growth cones without filopodia and lamellipodia were counted as collapsed [14] [15]. The counting was performed by a trained examiner who was blinded to the experimental protocol.

The data are expressed as the mean ± SD of six independent measurements. Each data point for the neurite length and growth cone collapse assays was statistically analyzed with two-way analysis of variance with Bonferroni's method using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). P values less than 0.05 were considered significant.

3. Results

Significant neurite elongation of cultured DRG neurons was observed over time under control condition (*i.e.* without 2-AG). However, the endogenous CB1R agonist 2-AG induced significant inhibition of neurite elongation with growth cone collapse as shown in **Figure 1**. These effects were dose- and time-dependent (two-way ANOVA, **Figure 2**). 2-AG (10 μM) blocked axonal elongation at 6 and 24 hr after exposure ($P < 0.0001$, **Figure 2(a)**). The total neurite length 24 hr after exposure of 2-AG (10 μM) was even shorter than that before exposure suggesting severe neurotoxicity. 2-AG (10 μM) significantly increased the rate of growth cone collapse 2, 6, and 24 hr after exposure ($P < 0.0001$, **Figure 2(b)**). Even low concentrations of 2-AG (1 and 0.1 μM) increased the rate of collapse in a dose-dependent manner (**Figure 2(b)**). The effects of 2-AG on neurite length and rate of collapse 24 hr after exposure are summarized in **Figure 3(a)** and **Figure 3(b)**, respectively.

4. Discussion

In the present study, we showed that 2-AG induced growth cone collapse and inhibited axonal elongation in cultured peripheral sensory neurons. These actions were dose dependent and seemed to be pharmacological. However, we cannot completely exclude the possibility that the effect of 2-AG was not through CB1Rs, because we did not test whether the action of 2-AG was reversed by a CB1R antagonist such as AM251. Whether CB1R antagonists alone have any effect on neuronal growth of developing DRG neurons is also unclear. Testing with CB1R antagonists with neither independent neurotoxic nor neuro-pharmaceutical actions may be useful for

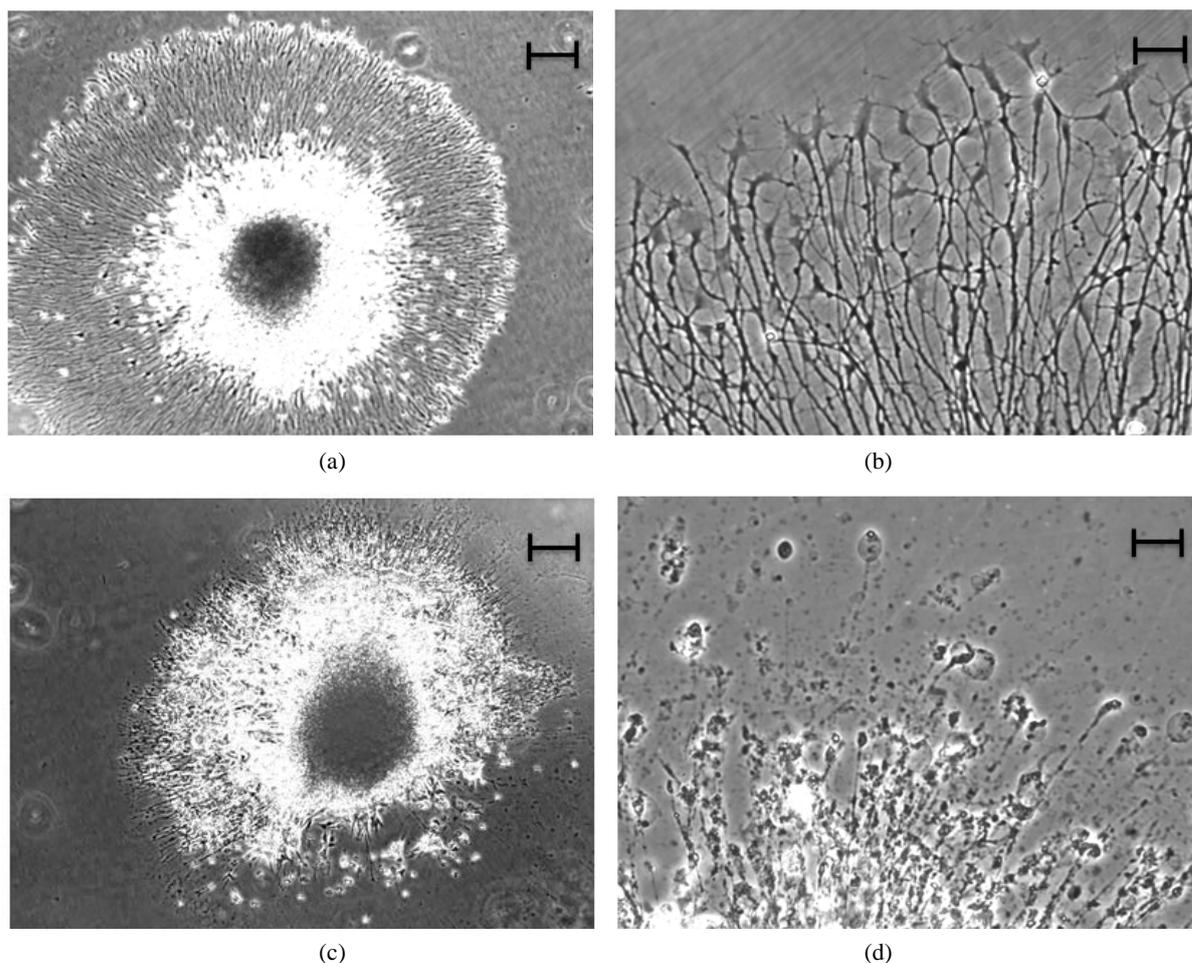


Figure 1. Typical neurite retraction and growth cone collapse induced by 2-AG in cultured DRG neurons. (a) (b) The neuron before the application of 2-AG (preexposure); (c) (d) The neuron after exposure to 10 μM 2-AG for 24 hr. Scale bars in (a) and (c) = 10 μm ; bars in (b) and (d) = 60 μm .

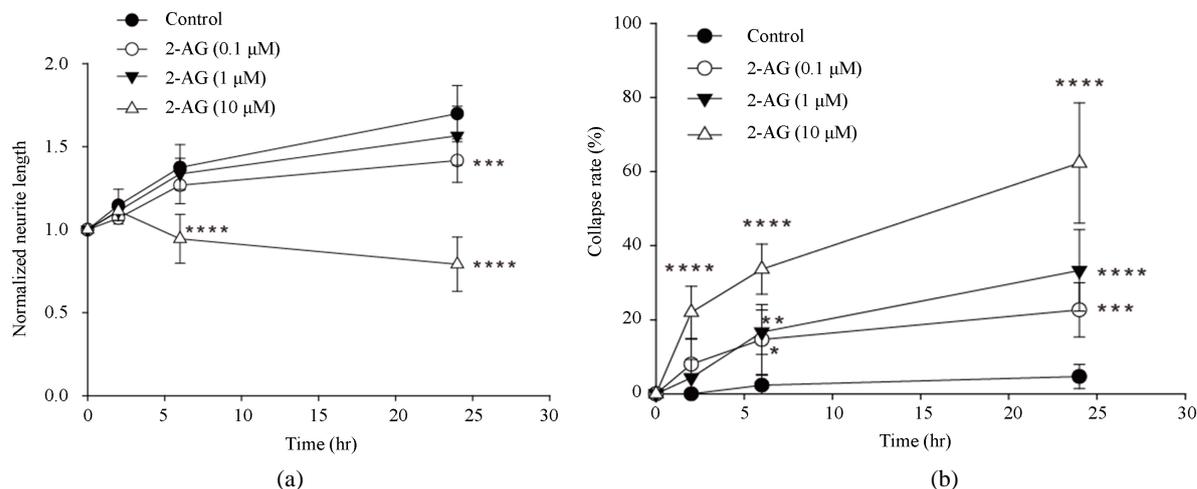


Figure 2. The effect of 2-AG on neurite length (a) and collapse rate of cultured DRG neurons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, two-way ANOVA with a post-hoc Bonferroni test.

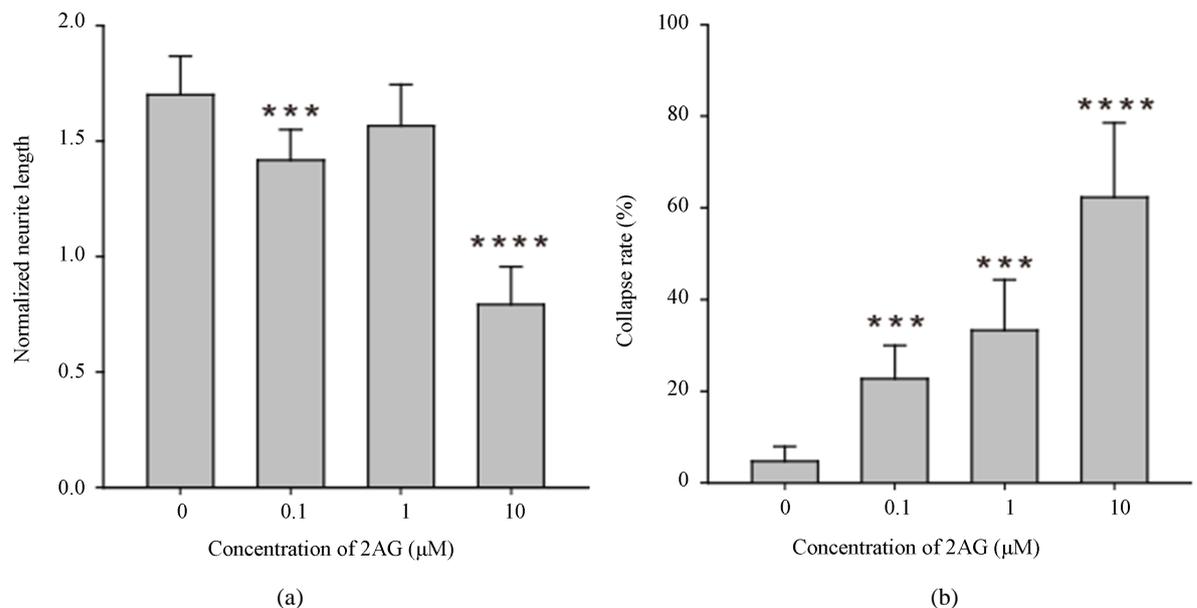


Figure 3. The effect of exposure to 2-AG for 24 hr on neurite length (a) and the rate of growth cone collapse (b) of cultured DRG neurons. *** $P < 0.001$, **** $P < 0.0001$, two-way ANOVA with post-hoc Bonferroni test.

elucidating the pharmacological action of cannabinoids on developing neurons. Further studies are needed to clarify the mechanisms underlying the inhibitory effect of 2-AG on DRG neurons.

We used chick explant cultures for analysis of the pharmacological actions of 2-AG because growth cones in chick explant cultures have wide fan-like shapes that are suitable for morphological analysis. The growth cone collapse assay is often used for biological analysis of endogenous factors and externally applied substances, both in developmental and regeneration studies. We previously reported that this system is applicable for pharmacological and toxicological studies of neurotropic factors, lipid mediators, and analgesic drugs [14]-[17]. However, the action of eCB may be species specific. Studies with human and other mammalian nervous tissues will be necessary for further analysis of the therapeutic use of cannabinoids.

CB1Rs are targets of exogenous cannabinoids such as cannabis. The current study suggested that activation of CB1Rs during development led to serious negative effects on neuronal growth in the peripheral nervous system. Therefore, high cannabis consumption by pregnant and lactating women may result in sensory system dysfunction in fetuses and infants, although the critical period(s) and doses of cannabis consumption in such situations

are unclear.

The expression of CB1Rs, as well as endocannabinoid levels in the spinal cord, increased in a rat model of neuropathic pain [18] [19]. The authors speculated that eCBs function as endogenous analgesics in neuropathic pain. Clinical studies showed that cannabinoids are applicable in both acute and chronic pain patients as analgesics [20]-[22]. Because ectopic sprouting and abnormal neuronal network connections are considered a cause of neuropathic pain, the actions of eCBs on neurite extension may be crucial for inhibiting painful conditions.

5. Conclusion

Although the precise physiological mechanism is still unknown, our current data imply an additional action of eCBs in inhibiting formation of pain-maintenance networks. Further studies should be performed to clarify the underlying signaling cascades and morphological regulatory roles of eCBs in pain.

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