

# The FGF receptor uses the endocannabinoid signaling system to couple to an axonal growth response

Emma-Jane Williams,<sup>1</sup> Frank S. Walsh,<sup>2</sup> and Patrick Doherty<sup>1</sup>

<sup>1</sup>Molecular Neurobiology Group, Medical Research Council Centre for Developmental Biology, King's College London, London SE1 1UL, UK

<sup>2</sup>Discovery Research, Wyeth Research, Collegeville, PA 19426

A key role for DAG lipase activity in the control of axonal growth and guidance *in vitro* and *in vivo* has been established. For example, DAG lipase activity is required for FGF-stimulated calcium influx into neuronal growth cones, and this response is both necessary and sufficient for an axonal growth response. The mechanism that couples the hydrolysis of DAG to the calcium response is not known. The initial hydrolysis of DAG at the sn-1 position (by DAG lipase) will generate 2-arachidonylglycerol, and this molecule is well established as an endogenous cannabinoid receptor agonist in the brain. In the present

paper, we show that in rat cerebellar granule neurons, CB1 cannabinoid receptor antagonists inhibit axonal growth responses stimulated by N-cadherin and FGF2. Furthermore, three CB1 receptor agonists mimic the N-cadherin/FGF2 response at a step downstream from FGF receptor activation, but upstream from calcium influx into cells. In contrast, we could find no evidence for the CB1 receptor coupling the TrkB neurotrophin receptor to an axonal growth response in the same neurons. The observation that the CB1 receptor can couple the activated FGF receptor to an axonal growth response raises novel therapeutic opportunities.

## Introduction

Over the past 10 years or so, numerous molecules that can promote and/or inhibit axonal growth have been identified, and an emerging theme is that these molecules function by activating a limited number of signal transduction cascades in growth cones (Tessier-Lavigne and Goodman, 1996; Doherty et al., 2000). NCAM, N-cadherin, and L1 are cell adhesion molecules (CAMs)\* that promote axonal growth during development, and have a function in synaptic plasticity in the adult (Walsh and Doherty, 1997). Their ability to activate an FGF receptor–signaling cascade in growth cones is required for, and sufficient to explain, their positive effects on growth cone motility (Williams et al., 1994a; Saffell et al., 1997). The proximal steps in the FGF receptor signal transduction cascade involve activation of phospholipase C $\gamma$  to generate DAG (Hall et al., 1996), with the subsequent hydrolysis of DAG by an as yet uncharacterized DAG lipase coupling the pathway to an axonal growth response by stim-

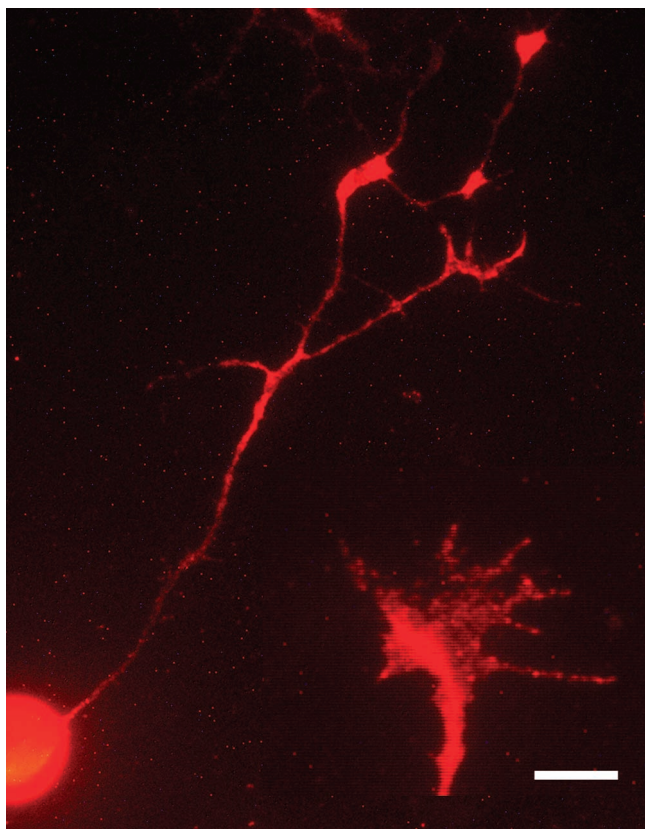
ulating calcium influx into the growth cone through N- and L-type calcium channels (Doherty et al., 1991a, 1995; Williams et al., 1994b, 1994c; Lom et al., 1998). Interestingly, under normal conditions, the calcium changes are not global, but instead are highly localized to the submembranous region of the growth cone (Archer et al., 1999; Chadborn et al., 2002). This is likely to be a very important feature of the pathway, as this type of highly localized change in calcium in growth cones is sufficient to induce the formation of new filopodia *in vivo* (Lau et al., 1999), and can also induce growth cone turning responses *in vitro* (Zheng, 2000). A key role for DAG lipase activity in the control of axonal growth and guidance *in vivo* has also been established (Brittis et al., 1996; Lom et al., 1998). Interestingly, the ability of N-cadherin to directly interact with the FGFR has also been implicated in tumor cell metastasis (Suyama et al., 2002), and N-cadherin–stimulated increases in tumor cell migration are also dependent on DAG lipase activity (Nieman et al., 1999).

The mechanism that couples the hydrolysis of DAG to the calcium response in neurons is not known. The canonical pathway would involve the synthesis of two key second messengers in neurons. The initial hydrolysis of DAG at the sn-1 position (by DAG lipase) will generate 2-arachidonylglycerol (2-AG), with the subsequent hydrolysis of 2-AG generating arachidonic acid. At first sight, arachidonic acid

Address correspondence to Patrick Doherty, Molecular Neurobiology Group, Medical Research Council Centre for Developmental Biology, King's College London, New Hunt's House, London Bridge, London SE1 1UL, UK. Tel.: 44-207-848-6813. Fax: 44-207-848-6816. E-mail: patrick.doherty@kcl.ac.uk

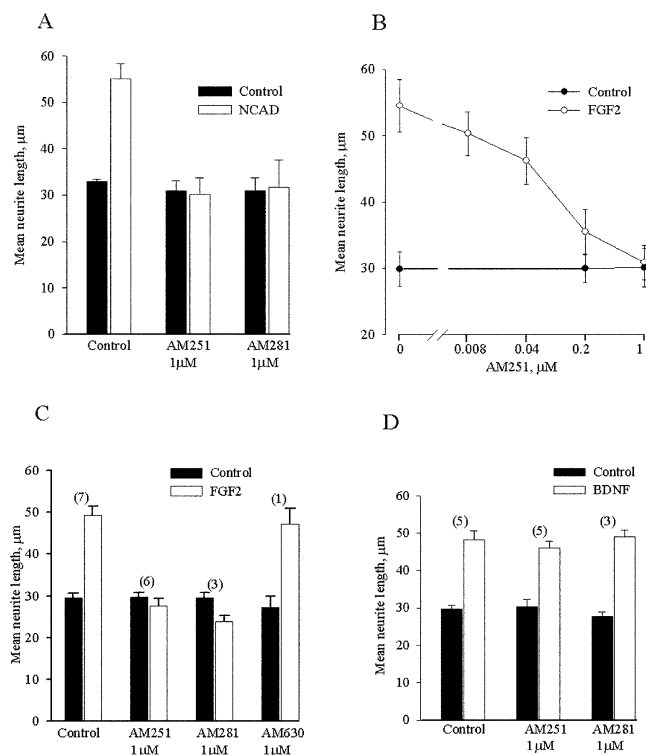
\*Abbreviations used in this paper: 2-AG, 2-arachidonylglycerol; CAM, cell adhesion molecule; NA, noladin ether.

Key words: CAM; CB1; 2-AG; cannabinoid; N-cadherin



**Figure 1. CB1 receptor expression on cerebellar neurons.** Cerebellar neurons were cultured overnight in media containing 5 ng/ml FGF2 before being fixed and stained for the CB1 receptor (see Materials and methods). The above picture shows a representative example of a stained cerebellar neuron. The inset shows an individual growth cone at higher magnification. The bar is 10  $\mu\text{m}$  relative to the larger image, and 5  $\mu\text{m}$  relative to the inset. No staining was seen when the primary antibody was omitted.

appeared to be the best candidate for the “instructive” signal for axonal growth in the CAM/FGF receptor pathway, as the direct application of arachidonic acid to primary neurons fully mimics the neurite outgrowth response stimulated by FGF2 and the aforementioned CAMs (Williams et al., 1994a, 1994c). However, arachidonic acid can stimulate the accumulation of 2-AG in cells (Ueda et al., 2000), and this raises the possibility that it might be 2-AG that normally couples the FGF receptor signaling cascade to the calcium response. Interestingly, 2-AG is a ligand for the CB1 and CB2 cannabinoid receptors (Di Marzo et al., 1998), and in some instances cannabinoid receptors have been shown to positively couple with calcium channels (Okada et al., 1992; Sugiura et al., 1996; Rubovitch et al., 2002). Based on these observations, we tested for “cross-talk” between the FGF receptor and endocannabinoid signaling systems. Now, we provide compelling evidence that signaling via the CB1 receptor is not only required for, but can also mediate, the neurite outgrowth response stimulated by N-cadherin and FGF2, and that it does so by coupling DAG hydrolysis to a signaling cascade that depends upon calcium influx into neurons via both N- and L-type calcium channels.



**Figure 2. CB1 antagonists inhibit N-cadherin and FGF2 responses.**

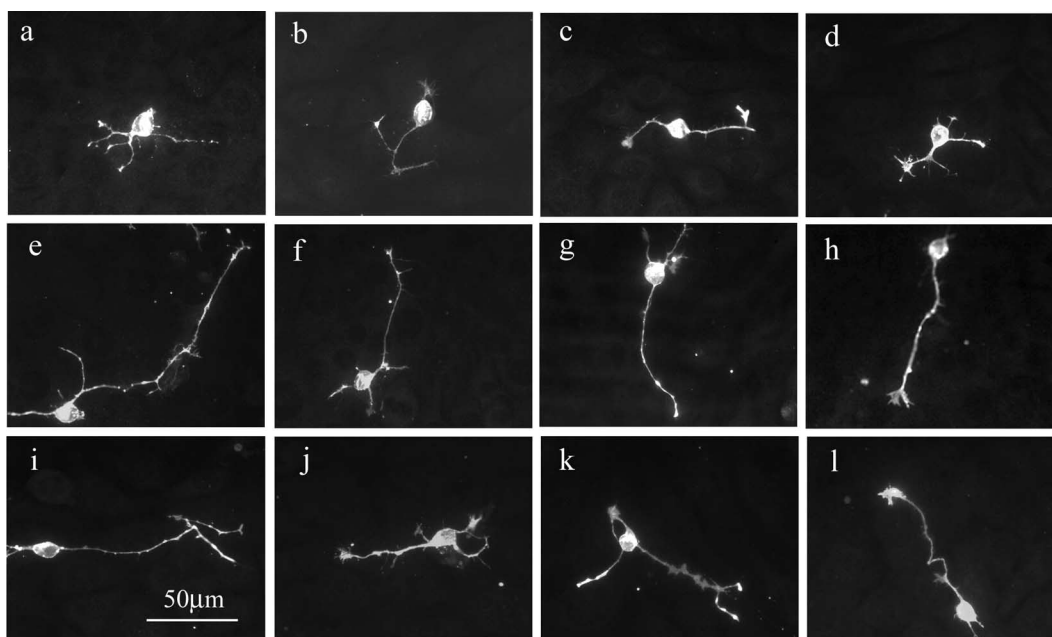
(A) Cerebellar neurons were cultured on 3T3 monolayers or N-cadherin-expressing monolayers in control media or media supplemented with 1  $\mu\text{M}$  AM 251 or AM 281 as indicated. (B) Cerebellar neurons were cultured over 3T3 cell monolayers in control media or media supplemented with 5 ng/ml FGF2 in the presence of increasing concentrations of AM 251 as indicated. (C and D) Cerebellar neurons were cultured over 3T3 monolayers in control media or media supplemented with 5 ng/ml FGF2 or 5 ng/ml BDNF in the presence of 1  $\mu\text{M}$  AM 251, AM 281, or AM 630 as indicated. In each instance, the cultures were fixed after 18 h, and the length of the longest neurite per cell was determined from  $\sim 120$  neurons. The results show absolute neurite length. In A and B, results are shown for an individual representative experiment. In C and D, results were pooled from the given number of independent experiments. Bars show the SEM.

## Results and discussion

### CB1 receptor antagonists inhibit the neurite outgrowth response stimulated by N-cadherin and FGF2, but not BDNF

In the adult brain, cannabinoid receptor agonists released from postsynaptic neurons act as retrograde messengers to suppress neurotransmitter release from the presynaptic axon. The effect is largely mediated by the CB1 receptor, and involves the coupling of a pertussis toxin-sensitive G protein to inhibition of calcium influx through N-type calcium channels (for review see Wilson and Nicoll, 2002). However, the CB1 receptor is also expressed in the embryonic nervous system (Buckley et al., 1998; Berrendero et al., 1999), and this suggests additional functions for the CB1 receptor during development.

When postnatal cerebellar neurons are cultured over monolayers of transfected 3T3 cells that express physiological levels of N-cadherin, N-cadherin promotes neurite



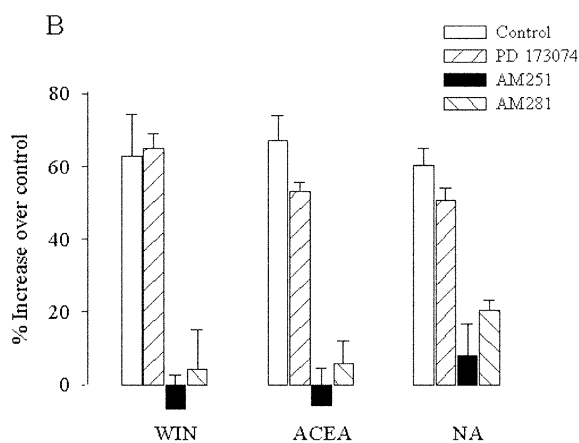
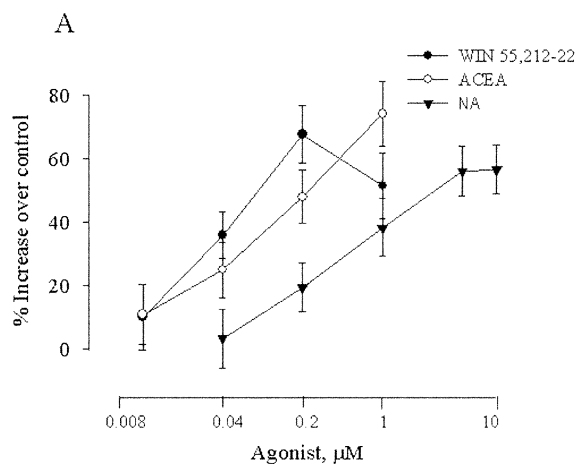
**Figure 3. The effect of FGF2 and CB1 agonists on neuronal morphology.** Cerebellar neurons were cultured overnight on 3T3 cell monolayers in control media (a–d) containing 5 ng/ml FGF2 (e–h) or media containing 0.2  $\mu$ M WIN 55,212–2 (i–l) before being fixed and stained for GAP-43. Representative examples of neurite-bearing cells are shown.

outgrowth via a mechanism that requires activation of a neuronal FGF receptor signal transduction cascade (Williams et al., 2001). Given the requirement of DAG lipase activity for the axonal growth response, and considering that the hydrolysis of DAG will generate the CB1 agonist 2-AG, we decided to test whether CB1 function was required for the N-cadherin response. In this context, there is ample evidence that cultured cerebellar neurons express the CB1 receptor on cell bodies and neurites (for review see Nogueron et al., 2001), and we have extended this observation to cerebellar growth cones (Fig. 1). Our results show that two independent CB1 antagonists (AM 251 and AM 281) completely inhibit the N-cadherin component of the neurite outgrowth response (Fig. 2 A). If the CB1 antagonists are acting at a step downstream from the FGF receptor, they should also inhibit the neurite outgrowth response stimulated by FGF2. Fig. 2 B shows a representative example of an experiment where various doses of AM 251 were tested for their ability to inhibit the response stimulated by 5 ng/ml FGF2. A substantial inhibition ( $\sim$ 80%) of the response can be seen at an AM 251 concentration of 0.2  $\mu$ M, with a complete inhibition found at 1  $\mu$ M. A series of pooled experiments show that at 1  $\mu$ M, both AM 251 and AM 281 completely inhibit the FGF2 response (Fig. 2 C). In contrast, a specific CB2 receptor antagonist (AM 630) had no effect on the FGF2 response (Fig. 2 C). BDNF stimulates axonal growth by activating the TrkB receptor tyrosine kinase, and as this response does not depend on the hydrolysis of DAG (Lom et al., 1998), there is no obvious basis for postulating a role for the endocannabinoid signaling in the response. In agreement, AM 251 and AM 281 did not inhibit the neurite outgrowth response stimulated by BDNF (Fig. 2 D).

#### Activation of the CB1 receptor stimulates neurite outgrowth

Next, we determined whether activation of the CB1 receptor could mimic the FGF2 response, and whether this requires FGF receptor function. When neurons were treated with 5 ng/ml FGF2 or 0.2  $\mu$ M of the CB1 agonist WIN 55,212–2, there was a clear increase in neurite outgrowth with no obvious difference in the nature of the neuronal response stimulated by these agents (Fig. 3). In total, we found that three CB1 receptor agonists (WIN 55,212–2, ACEA, and noladin ether [NA]) stimulated neurite outgrowth in a dose-dependent manner with maximal responses found in the low  $\mu$ M range (Fig. 4 A). Again, neurons treated with the CB1 agonists were not obviously different from those treated with FGF2 or N-cadherin. The responses to all three CB1 agonists were fully inhibited by AM 251 and AM 281 (Fig. 4 B), confirming that they reflect activation of the CB1 receptor. A specific FGF receptor inhibitor (PD 173074) that fully inhibits the FGF2 and N-cadherin responses (Skaper et al., 2000; Williams et al., 2001) had no effect on the responses stimulated by all three CB1 agonists (Fig. 4 B), and this demonstrates that CB1 function is downstream from FGF receptor activation. Direct application of arachidonic acid to primary neurons can also fully mimic the neurite outgrowth response stimulated by CAMs and FGF2 at a site downstream from FGF receptor activation, but upstream from calcium influx into growth cones (Williams et al., 1994a, 1994c). The arachidonic acid response is also fully inhibited by the two CB1 receptor antagonists, demonstrating that arachidonic acid also acts upstream of the CB1 receptor, possibly by stimulating 2-AG synthesis in cells (unpublished data).

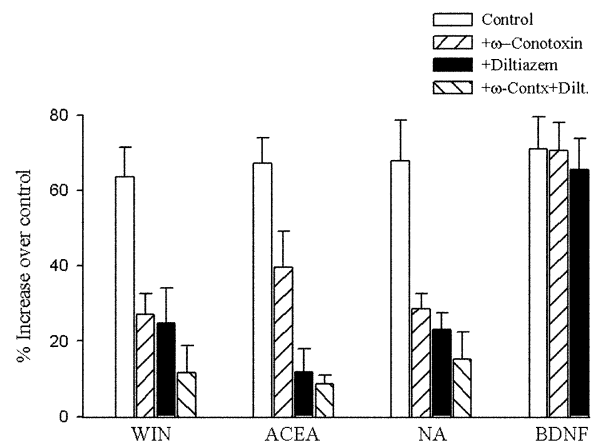




**Figure 4. CB1 receptor agonists stimulate neurite outgrowth.** (A) Cerebellar neurons were cultured on 3T3 monolayers in control media or media supplemented with the given concentrations of three CB1 receptor agonists (WIN 55,212-2, ACEA, and NA) as indicated. In each instance, the cultures were fixed after 18 h, and the length of the longest neurite per cell was determined from ~120 neurons. The results are from a single representative experiment, and each value shows the percent increase in mean neurite length relative to control media. Bars show the SEM. (B) Cerebellar neurons were cultured on 3T3 monolayers in control media or media supplemented with 0.2 μM WIN 55,212-2 (WIN), 1 μM ACEA, or 1 μM NA, as indicated. The experiments were done in control media and media supplemented with 500 nM PD 173074, 1 μM AM 251, or 1 μM AM 281. In each instance, the cultures were fixed after 18 h, and the length of the longest neurite per cell was determined from ~120 neurons. The results are pooled from three independent experiments and show the percent increase in mean neurite length relative to control media. Bars show the SEM.

### N- and L-type calcium channel antagonists inhibit the neurite outgrowth response stimulated by CB1 agonists

DAG hydrolysis couples the FGF receptor pathway to an axonal growth response by stimulating calcium influx into the growth cone through N- and L-type calcium channels (Williams et al., 1994b, 1994c; Archer et al., 1999; for review see Doherty et al., 2000). If endocannabinoid signaling mediates this response, then L- and N-type calcium channel antagonists should inhibit the response to the CB1 agonists, as



**Figure 5. The response to the CB1 agonists requires calcium influx into neurons.** Cerebellar neurons were cultured on 3T3 monolayers in control media or media supplemented with 0.2 μM WIN 55,212-2 (WIN), 1 μM ACEA, 1 μM NA, or 5 ng/ml BDNF as indicated. The experiments were done in control media (SATO supplemented with 2% FCS) or media further supplemented with 250 nM ω-conotoxin, and/or 10 μM diltiazem as indicated. In each instance, the cultures were fixed after 18 h, and the length of the longest neurite per cell was determined from ~120 neurons. The results are pooled from three independent experiments and show the percent increase in mean neurite length relative to control media. Bars show the SEM.

these agents inhibit the FGF2 response from all tested neurons, including rat cerebellar granule cells (Williams et al., 1994b). To test this, we cultured neurons with the various CB1 receptor agonists in media further supplemented with an L-type calcium channel antagonist (diltiazem at 10 μM), an N-type calcium channel antagonist (ω-conotoxin, 250 nM), or a combination of both. The CB1 agonists failed to elicit a neurite outgrowth response when N- and L-type calcium channels were simultaneously inhibited, and they only elicited small responses when individual channels were blocked (Fig. 5). Thus, we can conclude that the CB1 agonists promote neurite outgrowth via a mechanism that requires calcium influx into neurons through N- and L-type calcium channels. Interestingly, the neurite outgrowth response stimulated by BDNF was completely unaffected by the calcium channel antagonists (Fig. 5), and this further demonstrates that two receptor tyrosine kinases (the FGF receptor and TrkB receptor) use different signal transduction cascades to couple to an axonal growth response in cerebellar neurons.

At first sight, calcium influx into neurons might seem an unlikely mechanism for the CB1 agonist driven neurite outgrowth response, given the paucity of data in the literature reporting positive effects of cannabinoid agonists on calcium levels in cells. However, it should be noted that under normal conditions, the calcium changes induced by CAMs and FGF2 are not detectable by conventional imaging, as they are highly localized to the submembranous region of the growth cone (Archer et al., 1999; Chadborn et al., 2002). Furthermore, the notion that cannabinoid receptor activation can have positive effects on calcium influx is not without precedence, as it has been shown that the CB1 receptor can positively modulate L-type calcium channels in a neu-

ronal cell line (Sugiura et al., 1996; Rubovitch et al., 2002). In addition, low concentrations of the natural cannabinoid receptor agonist  $\Delta^9$ -THC enhance  $K^+$  induced increases in calcium levels in cells (Okada et al., 1992). The stimulation of calcium influx into cells by cannabinoid receptor agonists is insensitive to pertussis toxin (Sugiura et al., 1996; Rubovitch et al., 2002), with the available evidence suggesting that it might be mediated by the now well-established stimulatory effects of cannabinoids on adenylate cyclase (Glass and Felder, 1997; Felder et al., 1998; Calandra et al., 1999; Ishii and Chun, 2002). Interestingly, we have found that pertussis toxin does not inhibit the cannabinoid agonist responses reported in this study (unpublished data).

Biphasic responses to cannabinoids have been noted in biochemical, physiological, and behavioral studies (Okada et al., 1992; Glass and Felder, 1997; Sulcova et al., 1998). The data have been interpreted as evidence for the concomitant activation of two parallel pathways by the activated CB1 receptor, namely a stimulatory pertussis toxin-insensitive pathway, and an inhibitory pertussis toxin-sensitive pathway. An analogous situation might explain the biphasic nature of the neurite outgrowth response to FGF2 and arachidonic acid, and the fact that in some circumstances activation of the FGF receptor can inhibit axonal growth (Williams et al., 1994a, 1994c, 1995). In this scheme, the more classical CB1-mediated inhibition of calcium channels (Caulfield and Brown, 1992; Mackie and Hille, 1992) might account for the desensitization/inhibitory component of the response.

In summary, to our knowledge, this is the first explicit demonstration of cross-talk between a neuronal receptor tyrosine kinase and the endocannabinoid system. This work also suggests that the emerging roles for adhesion molecules such as N-cadherin in synaptic plasticity in the adult (for review see Goda, 2002; Togashi et al., 2002) might be considered alongside their ability to activate the endocannabinoid signaling system.

## Materials and methods

### Neurite outgrowth assays and immunocytochemistry

Cerebellar neurons were cultured over monolayers of parental 3T3 cells or an established transfected 3T3 cell line that expresses physiological levels of chick N-cadherin (the LK8 cell line; for review see Doherty et al., 1991b) as described previously (Williams et al., 1994a). For establishment of the cocultures, ~80,000 monolayer cells were plated into individual chambers of an eight-chamber tissue culture slide (precoated with poly-L-lysine and fibronectin) and maintained overnight in DME/10% FCS. The medium was removed, and ~6,000 dissociated cerebellar neurons (taken from postnatal day 2 rats) were plated into each well in SATO medium supplemented with 2% FCS. Test reagents were added as indicated in the text, and the cocultures were maintained for 18 h. The cocultures were then fixed and stained for GAP-43 expression, and the mean length of the longest neurite per cell measured for ~120–150 neurons as described previously (Williams et al., 1994a). For CB1 receptor expression, cerebellar neurons were cultured overnight in eight-chamber tissue culture slides precoated with poly-L-lysine (as above) and 10  $\mu$ g/ml laminin (in DME for 2 h at 37°C). The cultures were fixed for 1 h with 4% PFA, and after blocking nonspecific binding sites with PBS/0.5% gelatin for 30 min at RT, were incubated with 20  $\mu$ g/ml of affinity-purified rabbit antibodies against the CB1 receptor (CB11-A; Alpha Diagnostic International, Inc.) overnight at 4°C. All other steps in the staining procedure were as described previously for GAP-43 (see above).

### Materials

ACEA (arachidonyl-2 $\alpha$ -chloroethylamide N-[2-chloroethyl]-5Z,8Z,11Z,14Z-eicosatetraenamide), NA (2-arachidonylglycerol ether; 2-[(5Z,8Z,11Z,14Z-

eicosatetraenyl)-1,3-propanediol], WIN 55,212-2 mesylate ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate), AM 281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) and AM 251 (N-[piperidin-1-yl]-5-[4-iodophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide), and AM 630 (6-lodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone) were obtained from Tocris Cookson, Ltd., and were used at concentrations recommended by the supplier. BDNF and FGF2 were obtained from R&D Systems. Arachidonic acid was obtained from Affinity Bioreagents, Inc., and was used as described previously (Williams et al., 1994c). Diltiazem hydrochloride was obtained from Calbiochem, and  $\omega$ -conotoxin GVIA was obtained from Alomone Labs; both were used as described previously (Doherty et al., 1991a).

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