Induction of Krox-24 by Endogenous Cannabinoid Type 1 Receptors in Neuro2A Cells Is Mediated by the MEK-ERK MAPK Pathway and Is Suppressed by the Phosphatidylinositol 3-Kinase Pathway

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Neuro2a cells endogenously express cannabinoid type 1 (CB1) receptors. CB1 stimulation with HU210 activated ERK and induced the transcription factor Krox-24. A functional MEK-ERK pathway is an important requirement for CB1-mediated Krox-24 induction as blockade of MEK signaling by UO126 reduces both basal and CB1-mediated activation of Krox-24. CB1 receptor stimulation did not activate either JNK or p38 MAPK pathways or the pro-proliferation phosphatidylinositol 3-kinase (PI3K)-Akt pathway. However, serum removal or blockade of PI3K signaling by LY294002 transiently stimulated basal Krox-24 expression and increased CB1-mediated induction of Krox-24. This was consistent with a transient increase in pMEK, pERK, and pCREB levels following PI3K blockade. These data demonstrate that CB1-mediated activation of the Krox-24 transcription factor is negatively regulated through the PI3K-Akt pathway and reveals several points of signaling cross-talk between these two important kinase pathways.

In the brain the cannabinoid type 1 (CB1) receptor is largely responsible for mediating the effects of endocannabinoids and cannabinoid drugs like Δ9-tetrahydrocannabinol (THC). Detailed neuroanatomical and ultrastructural studies have demonstrated that CB1 receptors are localized pre-synaptically in populations of GABA-ergic and glutamatergic neurons in specific brain regions, including the basal ganglia, cerebellum, hippocampus, and cortex (1–8).

A major physiological function of the cannabinoid system at these synapses is to regulate the release of various neurotransmitters (8–10). Consequently, there has been much interest in understanding the signaling pathways that mediate both the short and long term effects of endocannabinoids in these brain regions. Several in vivo studies have shown a robust up-regulation of c-Fos and Krox-24 in specific neuronal populations within the striatum (11, 12) and hippocampus (10, 12, 13) following cannabinoid treatment. In an elegant study by Marsicano et al. (10), the CB1 receptors were knocked out specifically within the hippocampal glutamatergic principal neurons resulting in elevated glutamate toxicity leading to severe seizures and death. The CB1 activation of the ERK pathway, c-Fos, and Krox-24 were strongly implicated by the authors in the protection against glutamate toxicity (10).

As described above Krox-24 and c-Fos are physiologically regulated by CB1 in specific neuronal cells and are likely involved in the long term neuronal changes induced by cannabinoids. Krox-24 has been associated with important biological functions such as the stabilization of long lasting long-term potentiation (14, 15), cell differentiation (16–18), and as a cell survival or death signal in neuronal cells (18, 19). There is also evidence that Krox-24 is involved in the regulation of specific neurotransmitters and receptors (20–22).

Although a wealth of research has been conducted into CB1 receptor signaling, there is very little known about the precise molecular pathways involved in the coupling of CB1 to Krox-24 in neuronal cells. We are particularly interested in delineating the signaling systems recruited by the CB1 receptor in neurons as we hypothesize that these are major areas of dysfunction in various neurological diseases (23), such as Huntington disease where loss of CB1 receptors is one of the earliest known molecular markers of the human disease (24). Therefore, in order to prise apart the complex diversity of signaling pathways capable of coupling to CB1 in various cellular contexts (25, 26), we have used mouse Neuro2a neuronal cells. In these studies we investigated the involvement of the MAPK pathways in Krox-24 induction following CB1 receptor activation. We have demonstrated that CB1 can selectively activate the ERK pathway, which is crucially required for CB1-mediated Krox-24 induction. In addition, we have demonstrated that Krox-24 induction was not dependent upon PI3K/PKB signaling, and we discovered that this pathway can negatively regulate Krox-24 expression through intricate cross-talk signaling between the ERK and PI3K pathways.
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**EXPERIMENTAL PROCEDURES**

**Neuro2a Cell Culture**—Neuro2a cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen), buffered with 25 mM Hepes (referred to as complete media), and maintained at 37 °C in 5% CO₂. For harvesting, media were rinsed from cells with PBS, and cells were incubated with trypsin for 30–60 s. Cells were pelleted by centrifugation at 700 × g for 3 min and washed with complete media several times.

For the experiments, Neuro2a cells were prepared in complete media and seeded at 5–10,000 cells/well for 96-well plates or 0.5 × 10⁶ cells in 30-mm Petri dishes. Cells were allowed to recover overnight in complete media before undergoing treatments. All experimental treatments were prepared in complete media unless otherwise stated. HU210 (Tocris) and SR141716A (Tocris) stocks were prepared in ethanol and diluted in media to the appropriate concentration. The vehicle contained the equivalent concentration of ethanol (maximum of 0.1% ethanol). For the MEK inhibitor studies (UO126, Cell Signaling Technology), Neuro2a cells were pretreated with 10 μM UO126 or 0.1% Me₂SO vehicle in complete media for 45 min. UO126 was present for the entire stimulation period for Krox-24 assays in order to prevent recovery of MEK activity. Neuro2a cells were differentiated by serum reduction (2% FBS/DMEM) or 0.2% Triton X-100. Nuclei were stained using 0.8 g/ml merthiolate and incubated for 16 h at 4 °C. For intracellular targets, the immunobuffer was supplemented with 0.2% Triton X-100. Nuclei were stained using 0.8 μg/ml Hoechst-33258 in PBS for 15 min.

**PCR and Northern Analysis**—Total RNA was extracted from each sample using the Trizol (Invitrogen) extraction method and, following DNase I (Invitrogen) treatment, reverse-transcribed into cDNA using Superscript II (Invitrogen) each according to manufacturer’s instructions. PCR primers were as follows: CB1 forward, 5'-atgagctgactctagctgcagcttgcaga-3', and CB1 reverse, 5'-tcagagctcggcagcgagctg-3’ (27). CB1 primers amplify the 1.5-kb coding region of the CB1 receptor. *krox-24* PCR primer sequences were as follows: forward, 5'-tggagagggctgccacctggt-3', and reverse, 5’-ccagctctgacctctttg-3’. These primers were designed to amplify a 467-bp ampiclon. The PCR cycling parameters for CB1 and *krox-24* were initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s, with final extension at 72 °C for 7 min.

PCR amplicons were gel-purified (HighPure extraction kit; Roche Applied Science) and cloned into pGEM-T easy vector (Promega) using routine methods and sequenced (Centre for Genomics and Proteomics, University of Auckland). DNA probes were prepared using Ready-to-Go labeling beads (Amersham Biosciences) following the manufacturer’s instructions. Northern blots were prepared as described previously using 20 μg of total RNA per sample (28). Membranes were pre-hybridized with Rapid-Hyb buffer (RPN 1636; Amersham Biosciences) for 20 min at 65 °C and then hybridized with denatured labeled probe at 65 °C for 1 h. Nonspecific binding was removed by serial washes in 2× SSC, 0.1% SDS with the final stringent wash conducted at 60–65 °C using 0.1× SSC, 0.1% SDS for 30 min. Blots were placed against radiographic Hyperfilm (Amersham Biosciences) for an appropriate period of time (6 h to 5 days) to obtain sharp signals. Films were developed manually using Kodak D19 developer and Hypam fixer (Ilford Imaging UK Ltd.).

**Immunocytochemistry for CB1, Krox-24, Phospho-MEK (pMEK), and pERK**—Immunocytochemistry was conducted on cells grown in 96-well plates or on glass coverslips for confocal fluorescent microscopy. The C-terminal anti-CB1 primary antibody (L14) was generously gifted by Dr. Ken Mackie and has been characterized previously (5). In this study L14 was used at 1:1000. The N-terminal anti-CB1 antibody (CB1-NT catalog number 44-310; Biosource International) was used at 1:400. Both CB1 receptor antibodies were validated by Western analysis with Neuro2a cell lysates, which detected a prominent product of ~60 kDa (data not shown). Anti-Krox-24 (catalog number SC 189; Santa Cruz Biotechnology) was used at 1:2000. The pMEK antibody (catalog number 9121; Cell Signaling Technology,) was used at 1:500 and pERK (catalog number 9106; Cell Signaling Technology) at 1:1000. Anti-rabbit Alexa-fluor 488 and 594 (Molecular Probes) secondary antibodies were used for immunofluorescence at 1:400 dilution. For quantified immunocytochemistry experiments, biotinylated anti-rabbit IgG (B7389; Sigma) secondary antibody was used in conjunction with extravidin peroxidase (Sigma) followed by dianinobenzidine (Sigma) as substrate. Each antibody was diluted in immunobuffer consisting of PBS with 1% goat serum and 0.4 mg/ml merthiolate and incubated for 16 h at 4 °C. For intracellular targets, the immunobuffer was supplemented with 0.2% Triton X-100. Nuclei were stained using 0.8 μg/ml Hoechst-33258 in PBS for 15 min.

**Quantification of Expression Using the Discovery 1 System**—The high content Discovery 1 system was used to quantify the expression of Krox-24. Images of four adjacent sites within the center of each well were acquired at ×100 magnification, using 4,6-diamidino-2-phenylindole UV light (405/465) and bright field on the Discovery-1 automated fluorescence microscope and image analysis system (Molecular Devices).

Images were analyzed using Metamorph (version 6.2.6) image analysis software (Molecular Devices). 4,6-Diamidino-2-phenylindole UV images (representing Hoechst-positive cell nuclei) were used to quantify total cell number within each image, whereas bright field images were used to quantify Krox-24 positive cells. The algorithm Find Spots identified positive cells based on region size (number of adjacent pixels within a specified radius) and staining intensity (contrast within immediate vicinity of stained objects).

**Time-lapsed Imaging**—Time-lapsed microscopy was conducted using a Zeiss Axiosvert 200 M microscope housed in a CO₂ environmental chamber to maintain samples at 37 °C with 5% CO₂. Images were captured using an AxioCam digital camera.

**Western Analysis**—For Western analysis cells were stimulated for the appropriate time then dishes were placed on ice and cells washed two times with ice-cold HBS (10 mM Hepes buffer, 150 mM NaCl, pH 7.8). Cells were harvested in 150 μl of HBS and 150 μl of SDS loading buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.01% bromphenol blue) and then denatured at 95 °C for 5 min. Samples were stored at −85 °C until required. 10 μl of each sample was separated on 12% SDS-polyacrylamide gel at 100 V for 2–3 h using the Bio-Rad mini Protean II
system (Bio-Rad). Proteins were transferred to polyvinylidene difluoride membrane (Millipore Corp), pre-wetted with methanol, using the Bio-Rad transfer system. Blots were probed with pAkt (1:2000; catalog number 9271, Cell Signaling Technology), pCREB (1:2000; catalog number 06-519 Upstate Biotechnology, Inc.), pELK-1 (1:1000; catalog number SC-8406; Santa Cruz Biotechnology), pERK (1:1000; catalog number 9101; Cell Signaling Technology), phospho-JNK (1:1000; catalog number 9251; Cell Signaling Technology), pMEK (1:1000; catalog number 9218; Cell Signaling Technology), phospho-p38 (1:1000; catalog number 9211; Cell Signaling Technology), pSEK-1 (1:1000; catalog number 9151; Cell Signaling Technology), and β-actin (1:30,000; catalog number AB6276; Abcam). Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were used in conjunction with the ECL kit (Amersham Biosciences) following the manufacturer’s recommendations. Blots were probed with β-actin to assess loading and transfer efficacy.

RESULTS

Expression of CB1 Receptors by Neuro2a Cells—cDNA from a number of neuronal cell lines was screened by PCR for the expression of the CB1 receptor gene. CB1-specific PCR primers amplified strongly from mouse Neuro2a cells and weakly from mouse P19 cells and human teratocarcinoma NT2 cells (data not shown). We did not detect endogenous CB1 receptors in human SK-N-SH cells or PC12 cells (negative control). These results were confirmed by Northern analysis, which demonstrated the existence of three abundant CB1 mRNA transcripts of 9.0, 6.0, and 1.5 kb. Fig. 1a shows the mRNA transcripts detected in two different preparations of untreated Neuro2a cultures.

The location and relative abundance of CB1 in Neuro2a cells was studied by immunocytochemistry. Cell surface or intracellular CB1 localization was determined using the CB1-NT antibody under nonpermeabilized or permeabilized conditions (Fig. 1b). This revealed that most Neuro2a cells expressed moderate levels of receptor; however, based on fluorescence intensity, the majority of receptors was localized to the cytoplasm rather than the cell membrane. We and others have observed a similar pattern of localization in cell lines transfected with the CB1 receptor (29) regardless of the expression level. The L14 antibody was used to validate total receptor localization as observed with the CB1-NT antibody. Confocal microscopy confirmed that the CB1 receptor was stored throughout the cytoplasm and also along the entire length of neuritic processes in Neuro2a cells (Fig. 1c).

CB1 Receptor Activation of MAPK Pathways—We investigated the activation of the ERK, p38, and JNK MAPK pathways following activation of CB1 in Neuro2a cells by immunocytochemistry and Western analysis. The levels of pERK1/2 were increased by stimulation with the CB1 receptor agonist HU210 in a concentration-dependent manner (Fig. 2a). Both MEK and ERK were phosphorylated rapidly, and this pathway remained in an activated state throughout the time course. In some experiments there was a moderate increase in pERK levels in control cells following media replenishment, which contains fresh serum; however, there were no detectable changes in the levels of pMEK following media change (Fig. 2b). Antibodies specific to phospho-p38, phospho-JNK (data not shown), and phospho-SEK-1 (pSEK-1) failed to detect activation of these pathways in the Neuro2a cells by immunocytochemistry (data not shown) or Western blotting over 5–40 min.

Time course of CB1-mediated Krox-24 Induction in Neuro2a Cells—Krox-24 is a member of the inducible transcription factor family. The CB1 agonist HU210 was tested across a range of concentrations from 10 pM to 10 μM inclusive with maximal increases in Krox-24 mRNA/protein observed at 100 nM.

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![FIGURE 1. Endogenous expression of CB1 by Neuro2a cells. a, Northern analysis revealed the presence of three distinct CB1 mRNA species of ~9, 6, and 1.5 kb. b, localization of CB1 using CB1-NT under nonpermeabilized (panel i, cell surface) and permeabilized (panel ii, total) conditions. c, confocal microscopy with L14 confirmed the presence of CB1 in the cytoplasm (×60 objective). Scale bars represent 20 μm.](image)

![FIGURE 2. Concentration- and time-dependent activation of MEK-ERK MAPK pathway by CB1 in Neuro2a cells. a, Western analysis of phospho-ERK1/2 levels following stimulation of cells with HU210 (10 nM to 10 μM) show a concentration-dependent stimulation. b, analysis of the temporal activation of MAPK pathways by CB1 in Neuro2a cells. Results are representative of 2–3 experiments.](image)
HU210 (data not shown). We found krox-24 mRNA levels to be elevated in Neuro2a cells 40–60 min after CB1 stimulation with 100 nM HU210 by Northern analysis (Fig. 3a).

We subsequently investigated the nuclear accumulation of the Krox-24 protein following cannabinoid stimulation over a similar time course. Basal Krox-24 staining was observed in ~3–5% of cells under these culture conditions. In HU210-treated cells, the number of Krox-24 positive cells was increased from 60 min onward with expression peaking around 90–120 min and remaining elevated for the remainder of the 24-h study (Fig. 3b). The Krox-24 response showed considerable heterogeneity with typically 15–25% of cells strongly stained 90–120 min after initiation of cannabinoid agonist treatment (Fig. 3c). Following 24 h of agonist stimulation, total cell number was not significantly different from control wells. Nor did we observe any significant changes in cell morphology such as neurite sprouting when compared with controls.

The expression levels of c-Fos and activation of CREB and ELK-1 were also measured as they are known downstream effectors of the ERK pathway. Surprisingly, there were no cannabinoid-mediated changes in fos mRNA or protein levels, although both were increased by forskolin stimulation of Neuro2a cells (data not shown). Weak stimulation of both pCREB and pELK-1 was observed (Fig. 3d).

CB1 Receptor-mediated Krox-24 Activation Is pERK MAPK-dependent—We then investigated how CB1 signaling pathways were coupled to the up-regulation of Krox-24 levels in Neuro2a cells. We used the MEK inhibitor UO126 to block...
the phosphorylation of ERK1/2 by pMEK, and we measured pERK levels and Krox-24 induction following treatment with 100 nM HU210. UO126 drastically reduced basal pERK levels and prevented the increase in pERK levels stimulated by HU210 (Fig. 4a). The blockade of pMEK activity with UO126 resulted in a reduction of basal Krox-24 nuclear staining and complete suppression of the HU210-mediated Krox-24 response (Fig. 4b). The effect of UO126 on basal and CB1-mediated Krox-24 induction was consistent with the MAPK-ERK pathway playing an important role in the activation of this inducible transcription factor.

To confirm that the observed effects of HU210 on Krox-24 were CB1-mediated, we treated the cells with the CB1 inverse agonist SR141716A. As expected, the HU210-induced increase in Krox-24 levels was blocked completely in the presence of 1 μM SR141716A (Fig. 4c). Furthermore, we observed a significant reduction (p < 0.01) in basal Krox-24 levels in the cells that received SR141617A only (Fig. 4c), indicating that some of the basal Krox-24 activity in the Neuro2a cells may be through constitutive activation of CB1. Alternatively, SR141716A may have blocked cannabinoids present in the serum or produced by the Neuro2a cells, in either case demonstrating the involvement of CB1 in the Krox-24 response.

Analysis of Cannabinoid-responsive Neuro2a Cells—From the Krox-24 time course (Fig. 3), it was evident that the majority of cells did not produce a Krox-24 response. Analysis of pMEK and pERK immunocytochemistry revealed that there were few strongly positive cells in the presence of serum following vehicle treatment (Fig. 5a). Upon stimulation with HU210 (5 min), pMEK staining was observed in cells indicated with the long arrows in Fig. 5a. These cells were generally large, typically with a diameter of 20–30 μm, and some clearly had the morphology of dividing cells, with evidence of telomeric separation. These cells were not abundant and only accounted for 6–10% of all cells.

Consistent with our Western analysis, media replenishment in the absence of agonist increased the number of cells with low levels of pERK. Following HU210 stimulation strong pERK staining was observed in large cells that were morphologically similar to pMEK positive cells. In addition, cytoplasmic staining was also present in a number of the smaller Neuro2a cells, albeit at a lower level (Fig. 5a, short arrows). Approximately 50–60% showed specific pERK staining 5 min after HU210 administration. This pattern of staining was verified by pretreatment with UO126, which reduced pERK to below detectable levels in both the vehicle and HU210 conditions (Fig. 5a). In the absence of primary antibodies, the secondary antibody binding was negligible (data not shown).

Changes in cellular morphology were studied using time-lapsed microscopy. As the Neuro2a cells began dividing, neuritic projections were withdrawn and cells began to appear spherical. During division, the cells maintained this spherical morphology for ~50–60 min before membrane pinching occurred and two small daughter cells were born (Fig. 5b). The process from expansion to separation took ~60–90 min, consistent with the time delay between pMEK and Krox-24 induction.

Krox-24 positive cells were predominantly small in size and often present as pairs of positive cells (Fig. 3c and Fig. 5). Our observation that the pMEK/pERK positive cells appeared morphologically to be mitotic suggested that the Krox-24 positive cells had recently divided. To test this hypothesis, we reduced the rate of cell proliferation by serum reduction (2% FBS) and differentiation with retinoic acid (20 μM RA in 2% FBS). The RA induces pronounced morphological changes and appears to further reduce
the rate of proliferation imposed by the serum reduction (Fig. 5c).
Following 3 days of differentiation, the basal level of Krox-24 is very low in these cells (<2% positive cells). Following HU210 stimulation, Krox-24 induction was observed in ~9% of cells cultured in 2% FBS and less than 5% of cells differentiated with RA. The reduction in Krox-24 induction under these differentiating conditions suggests that Krox-24-responsive cells may be derived primarily from mitotic Neuro2a cells.
Presence of Serum Abrogates CB1 Receptor Activation of Krox-24—MAPK and Krox-24 assays revealed that greater numbers of cells responded to cannabinoid stimulation at the MAPK level than the Krox-24 level. This suggested suppression of the CB1 pathway downstream of MAPK. One possibility is that factors present in serum may drive activity through a distinct pathway, such as the PI3K-Akt pathway, which suppresses signaling via the MEK-ERK cascade. We therefore investigated whether removal of serum had any impact on the expression of Krox-24 and CB1 activation of Krox-24. Complete removal of serum resulted in the transient stimulation of Krox-24 with a peak around 2 h, supporting the idea of an inhibitory input conferred by serum factors. Stimulation of Krox-24 levels with 100 nM HU210 was also enhanced in the absence of serum, more so than the additive effects of either treatment alone (Fig. 6a).

We then conducted a time course study of pMEK and pERK levels following serum withdrawal to determine the level in the signaling cascade at which the suppression was occurring. To our surprise there was little difference in pMEK or pERK levels within the first 20 min of serum removal, although a small increase was observed in pMEK levels at 40–60 min after serum removal (data not shown). These data would suggest that the serum-mediated suppression of the MEK-ERK cascade is primarily occurring downstream of ERK.

Direct Blockade of PI3K-Akt Pathway Stimulates Krox-24 Expression—Factors present in serum are known to activate the PI3K pathway (30), and in the presence of serum Neuro2a cells have abundant levels of Akt, a key substrate of the PI3K pathway. We therefore investigated the consequence of inhibition of the PI3K pathway on Krox-24 induction. The PI3K inhibitor LY294002 (Sigma) stimulated Krox-24 expression in the presence of serum in a concentration-dependent manner (data not shown). 10 μM LY294002 produced optimal stimulation of Krox-24 without inducing cell death, whereas 50 μM LY294002 induced death in some cells (data not shown). LY294002 induction of Krox-24 occurred in ~60% of the cells within 2 h (Fig. 6b). Furthermore, when the PI3K pathway was blocked, stimulation of CB1 by HU210 induced Krox-24 induction in close to 100% of the cells.

A second PI3K inhibitor, wortmannin (Sigma), also stimulated Krox-24 expression in a concentration-dependent manner with peak expression occurring around 2–3 h (data not shown). These data indicated that in the presence of serum the active PI3K pathway is capable of suppressing stimulatory inputs that would otherwise result in increased Krox-24 expression.

We sought to investigate the relationship between the serum-activated PI3K pathway and suppression of CB1 signaling. LY294002 rapidly inhibits PI3K and significantly reduces the level of phospho-Akt (pAkt) within 5 min of treatment. This inhibition is sustained for at least 60 min (Fig. 6c). Following blockade of PI3K signaling, the levels of pMEK, pERK, pCREB, and pATF-1 are each elevated transiently (Fig. 6c). Time course studies revealed that pMEK levels were increased at 10–20 min with pERK levels increased at 20 min and pCREB levels increased at 20–40 min (Fig. 6c). These temporal changes are consistent with signal transduction from PI3K through pMEK, then pERK, to pCREB. Interestingly, the levels of pELK-1 were moderately reduced following suppression of PI3K, indicating that some pELK-1 activity was mediated through PI3K.

Effect of MEK Blockade on pELK-1 and pCREB—The PI3K inhibitor studies demonstrated cross-talk between the PI3K pathway and the MEK-ERK pathway. We next investigated whether this cross-talk was reciprocal by inhibiting MEK activity with UO126 (Fig. 7). Western analysis revealed that within 10 min, UO126 effectively prevented phosphorylation of ERK that was sustained for ~4 h. During this period there was a pronounced increase in pAkt levels that became detectable at 20 min after MEK inhibition. This demonstrated that the level of pAkt was influenced by the activity of the MEK-ERK pathway.
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FIGURE 7. The effects of suppressing the MEK-ERK pathway on the phosphorylation status of Akt, CREB, and ELK-1. Western analysis was used to investigate the consequence of blocking MEK phosphorylation of ERK using UO126 over a 4-h time course. Representative of two experiments.

Next we measured the phosphorylation status of the ELK-1 and CREB transcription factors following UO126 treatment (Fig. 7). Phospho-CREB levels were reduced; however, there was little difference in the phosphorylation status of ELK-1. The decreases in pCREB levels correlated with decreases in pERK and increases in pAkt, indicating that pCREB levels were positively correlated with ERK and negatively with pAkt.

DISCUSSION

There has been much interest in elucidating the complexity of the neuronal cannabinoid system as follows: from understanding the precise localization of the receptors (5, 7, 8, 31), regulation of neurotransmitter release (9, 32–35), identifying endogenous ligands (8, 36–38), and signaling pathways evoked following CB1 receptor activation (10–13). We are particularly interested in the latter aspect as there are many unanswered questions regarding the signaling pathways employed by CB1 receptors in different brain regions that mediate both short and long term cannabinoid effects on neurons. Several in vivo studies have shown that ERK is activated in specific neuronal populations following cannabinoid administration (10, 12, 13, 39, 40). CB1-mediated ERK signaling has been shown to play a role in hippocampal neurogenesis and synaptic integrity (39). The ERK pathway has also been implicated in the induction of Krox-24 in the striatum (12) and hippocampus (13) following THC treatment. However, in the studies by Caboche and coworkers (12, 13), the observed ERK and Krox-24 responses were thought to occur via indirect effects of THC on the striatum and hippocampus as they were abrogated by antagonists of dopamine and N-methyl-D-aspartic acid receptors. To determine the direct role of the MAPK pathways in the CB1-mediated induction of Krox-24, we have utilized the mouse neuronal Neuro2a cell line that endogenously expresses CB1 receptors (41). We first measured the phosphorylation of important kinases within each of the MAPK cascades following CB1 stimulation was concentration- and time-dependent following CB1 stimulation and remained elevated for at least 20 min. As expected, pMEK levels followed a similar profile. We also measured the phosphorylation of p38 and JNK. The phosphorylated kinases were present at very low levels under basal conditions and interestingly did not change following HU210 treatment. pSEK-1, the kinase that phosphorylates JNK, also showed no detectable response to CB1 activation. These findings demonstrated that in the Neuro2a cells, CB1 was preferentially coupled to the activation of the MEK-ERK pathway. These data are consistent with the CB1-mediated induction of the ERK pathway in various brain regions (10, 12, 13, 40) and the specific activation of the ERK pathway reported in other cell types (42).

Due to the complexity of neurotransmission in vivo, it is difficult to differentiate signaling events occurring as a consequence of altered neurotransmission or direct ligand-mediated events (12, 13). Therefore, we wished to investigate whether Krox-24 was regulated by ERK in Neuro2a cells. CB1-stimulated Krox-24 expression occurred rapidly, and the elevated mRNA and subsequent protein levels were transient, consistent with the characterization of Krox-24 induction previously (43). We then employed UO126, a potent MEK inhibitor, to determine the role of the ERK pathway in CB1-mediated Krox-24 induction. UO126 effectively blocked both CB1-mediated and basal expression of Krox-24. These results confirm that the MEK-ERK pathway is an essential requisite for CB1-mediated Krox-24 induction. We also measured c-Fos induction following cannabinoid treatment and found that both mRNA levels and fos protein were unaffected by CB1 stimulation. This is in contrast to the THC-mediated induction of c-Fos (12, 13) but in agreement with observations in human astrocytes (44) demonstrating differential regulation of c-Fos by cannabinoids in a cell type-dependent manner.

During the immunocytochemical analysis of the CB1-mediated Krox-24 induction, several important observations were made. The majority of Krox-24 positive cells had a distinct morphology being small in size and often present in pairs. Krox-24 induction was typically observed in only 15–25% of cells in the presence of serum. This suggested either heterogeneity in responsiveness or the activation of differential signal transduction pathways, some of which may have inhibited the Krox-24 response. Analysis of the responsive Neuro2a cells by immunocytochemistry for pERK revealed that ~50–60% of cells had inducible staining, preventable by pretreatment with UO126. This clearly demonstrated that the percentage of cannabinoid-responsive cells was considerably greater than the resultant Krox-24 positive cells. We observed a small population of pERK positive cells (~6–10%) that were considerably larger in size and showed very strong pERK staining. Similar observations were made with the pMEK antibody. These cells appeared to be mitotic, based on a nuclear morphology consistent with telomeric separation. We conducted time-lapse microscopy to study the morphological changes as Neuro2a cells divided. The morphology and size of the strongly stained pMEK cells matched the morphology of the mitotic Neuro2a cells observed during the time-lapse experiments. Cells that were Krox-24 positive showed morphology consistent with daughter cells
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born during the time lapse. These data suggested that the ERK signal only gave rise to a Krox-24 response in cells that were mitotic at the time of stimulation when cultured in the presence of serum. This suggested strongly that the CB1-ERK signaling in nondividing cells was abrogated by a suppressive pathway preventing induction, transcription, or translation of the krox-24 mRNA.

The PI3K pathway has been shown to inhibit ERK signaling in Neuro2a cells following insulin stimulation (30). Insulin can independently activate the ERK and PI3K pathways in Neuro2a cells; however, PI3K-Akt signaling was shown to dominate and abrogate the ERK pathway at the level of Raf-1 (30). FBS contains insulin and IGF-I, which are both capable of potently activating the PI3K pathway (45). We therefore investigated the effects of serum withdrawal and blockade of the PI3K-Akt pathway on CB1-mediated Krox-24 induction. Both of these treatments resulted in the stimulation of Krox-24 expression in the majority of cells. Following blockade of the PI3K-Akt pathway and stimulation of CB1 with HU210, Krox-24 expression was observed in close to 100% of the cells within 2 h. Together these data indicate that the suppression of the CB1-ERK signaling achieved by the action of serum factors is transduced through the PI3K pathway and that by directly blocking PI3K more cells are able to activate the krox-24 gene in response to cannabinoid activation. It has been suggested that activation of PI3K-Akt can induce ERK activation in certain cell types (46, 47); however, several studies to date have shown that PI3K does not stimulate ERK in the brain (40, 48). Our data are consistent with and extend the latter findings to show that PI3K can inhibit CB1-mediated ERK signaling.

There are a number of potential molecular interaction points at which the PI3K pathway could suppress ERK signaling. The main route described in the literature to date is Raf1-Akt cross-talk, whereby activated Akt can inhibit the activity of Raf-1 directly (49, 50). We therefore measured the phosphorylation status of MEK and ERK following blockade of PI3K signaling. There was a small transient increase in pMEK and pERK levels following PI3K inhibition, consistent with cross-talk at or above MEK possibly due to inhibition of Raf-1. An additional point of cross-talk at or above MEK may involve the transcription factors controlling Krox-24 expression. The krox-24 promoter region contains multiple serum-response elements (SRE), a single cAMP element, an NFkB-binding site, and several SP1 elements in both rodents and humans (20). There is also an EBS-binding site that allows binding by Krox-24 to suppress its own transcriptional activity (20). The SREs are thought to play a major role in krox-24 regulation (20). Two distinct transcription factors, the serum-response factor and the ternary complex factor, dimerize following phosphorylation and interact with the SRE to initiate transcription. ELK-1 has been demonstrated to be the ternary complex factor involved in the activation of the SRE for the krox-24 gene in endothelial cells (51) and dorsal striatum (12). In our studies, however, we did not detect robust changes in the phosphorylation status of ELK-1 following either CB1 stimulation or inhibition of the ERK or PI3K pathways. The phosphorylation level of these transcription factors is generally considered a good indicator of their activation by the ERK pathway. However, parameters other than phosphorylation may play a role in the regulation of activation, such as dimerization with serum-response factor or nuclear localization. In contrast to ELK-1, there was a robust increase in pCREB levels following PI3K inhibition, concordant with a decrease in pAkt levels. This suggested a point of cross-talk downstream of ERK at which activated Akt suppresses CREB. This is supported by two observations. First, the greater proportion of pERK positive than Krox-24 positive cells implies that an abrogation of signaling occurs downstream of ERK. Second, temporally, the increase in pCREB levels preceded the detectable change in pERK but occurred shortly after the loss of pAkt activity. We also investigated the reciprocal regulation of Akt by the MEK-ERK pathway. The transient elevation of pAkt levels for 20–60 min following suppression of the ERK pathway suggested that Akt activity is also finely tuned by ERK activity. Conversely, pCREB levels were decreased following ERK suppression. This further supports our hypothesis that the potential cross-talk may involve pAkt suppression of CREB activation.

Akt is the major known target for activated PI3K (reviewed in Ref. 52), and it has been demonstrated that Akt can directly phosphorylate Raf-1 on Ser-259 (50). This promotes binding of the 14-3-3 protein, which then reduces Raf-1 activation by other kinases (50). It has been postulated that phosphorylation of Ser-259 by Akt, or indeed cAMP-dependent protein kinase (reviewed in Ref. 53), may suppress subsequent signaling through the MEK/ERK pathway (49, 52). To date several studies have shown that CB1 signaling can directly activate PI3K through the G-protein βγ-subunit and that PI3K was a prerequisite for MAPK-ERK activation (46, 47). However, our data support the hypothesis that CB1 activation of ERK in vivo is not necessarily mediated by PI3K activation and also demonstrates for the first time that the PI3K pathway can actually suppress CB1 signaling in neuronal cells. The PI3K ERK cross-talk that we have observed in the Neuro2a cells may subserve an inherent feedback or feed-forward regulatory mechanism allowing opposing pathways to control the magnitude of signaling of the other. CB1 receptors are primarily localized presynaptically in GABergic and glutamatergic synapses where complex neurocommunication occurs involving signaling from multiple GPCRs, ion channels, and growth factor receptors. Therefore, it is highly probable that these signaling events are involved with CB1 signaling in vivo.

This study has investigated the signaling pathways involved in the CB1-mediated activation of the Krox-24 transcription factor and has revealed the absolute requirement of a functional MEK-ERK transduction pathway. We have also identified reg- ulatory cross-talk between the two major signaling networks involved in growth, proliferation, and survival, the MEK-ERK and PI3K-Akt pathways. Fig. 8 provides a schematic summarizing these findings. It is not surprising that these major signaling systems have the capacity to control or influence the signaling of other pathways to either enhance or suppress their output. This is exemplified by our data that show that the CB1-mediated activation of Krox-24 in nondividing cells not only requires
the positive input from MEK-ERK but also requires cessation of suppression from the PI3K pathway. The signaling cross-talk observed here for the CB1 receptor may be representative of the complex integration of signals received by neuronal cells at the synapse.

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