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# Allele-specific Differences in Activity of a Novel Cannabinoid Receptor 1 (*CNR1*) Gene Intronic Enhancer in Hypothalamus, Dorsal Root Ganglia, and Hippocampus*

**Gemma Nicoll**[‡1], **Scott Davidson**[‡2], **Lynne Shanley**[‡], **Ben Hing**[‡], **Marissa Lear**[‡], **Peter McGuffin**[§], **Ruth Ross**[‡3], and **Alasdair MacKenzie**[‡4]

*From the* [‡]*School of Medical Sciences, Institute of Medical Sciences, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, Scotland and the* [§]*Medical Research Council Social, Genetic and Developmental Psychiatry Centre Centre, Institute of Psychiatry, King's College London, DeCrespigny Park, London SE5 8AF3, United Kingdom*

**Background:** Intron 2 of *CNR1* gene contains multiple disease-associated SNPs.
**Results:** Allelic variants of a novel enhancer in *CNR1* intron 2 affect its MAPK response in hypothalamus and hippocampus.
**Conclusion:** Alleles of enhancer may be functionally linked to obesity and addictive behavior.
**Significance:** Understanding the effects of *CNR1* polymorphisms on gene regulation will accelerate understanding of human disease.

Polymorphisms within intron 2 of the *CNR1* gene, which encodes cannabinoid receptor 1 (CB$_1$), have been associated with addiction, obesity, and brain volume deficits. We used comparative genomics to identify a polymorphic (rs9444584-C/T) sequence (ECR1) in intron 2 of the *CNR1* gene that had been conserved for 310 million years. The C-allele of ECR1 (ECR1(C)) acted as an enhancer in hypothalamic and dorsal root ganglia cells and responded to MAPK activation through the MEKK pathway but not in hippocampal cells. However, ECR1(T) was significantly more active in hypothalamic and dorsal root ganglia cells but, significantly, and in contrast to ECR1(C), was highly active in hippocampal cells where it also responded strongly to activation of MAPK. Intriguingly, rs9444584 is in strong linkage disequilibrium with two other SNPs (rs9450898 ($r^2 = 0.841$) and rs2023239 ($r^2 = 0.920$)) that have been associated with addiction, obesity (rs2023239), and reduced fronto-temporal white matter volumes in schizophrenia patients as a result of cannabis misuse (rs9450898). Considering their high linkage disequilibrium and the increased response of ECR1(T) to MAPK signaling when compared with ECR1(C), it is possible that the functional effects of the different alleles of rs9444584 may play a role in the conditions associated with rs9450898 and rs2023239. Further analysis of the different alleles of ECR1 may lead to a greater understanding of the role of *CNR1* gene misregulation in these conditions as well as chronic inflammatory pain.

The cannabinoid receptor 1 (CB$_1$)[5] is a G$_i$/G$_o$-protein-coupled receptor whose endogenous ligands include anandamide and 2-arachidonoyl glycerol (1, 2). CB$_1$ is strongly expressed in a number of different regions of the central nervous system such as the hypothalamus, hippocampus, and dorsal root ganglia (DRG) where it is known to play a role in modulating appetite (3–5), cognition and memory (6–8), and inflammatory pain, respectively (9).

Because of the role of CB$_1$ in these processes, there have been a number of genetic studies linking polymorphisms in and around the *CNR1* gene to conditions such as cognitive decline, drug addiction, schizophrenia, obesity, and inflammatory pain. For example, one polymorphism that occurs within intron 2 of the *CNR1* gene, rs2023239, has been associated with impulsivity (10), obesity (11), nicotine dependence (12), alcoholism (13), cannabis withdrawal and dependence (14, 15), substance addiction, and resistance to anti-depressive treatment (16). A second *CNR1* intron 2 polymorphism, rs9450898, was associated with smaller fronto-temporal white matter volumes and greater schizophrenia risk due to cannabis misuse (17). Intriguingly, rs9450898 and rs2023239 are separated by only 2 kb and are in strong linkage disequilibrium (LD; $r^2 = 0.915$). However, no mechanism has yet been discovered to explain the symptoms associated with rs9450898 or rs2023239.

We explored the hypothesis that polymorphic variation within *CNR1* intron 2 might change the activity of unidentified cis-regulatory regions. We used comparative genomics to identify highly conserved functional elements within *CNR1* intron 2 that might represent a cis-regulatory region. We then used molecular biology, primary cell culture, and pharmacology to isolate these sequences and to assess the effects of different alleles on the activity, tissue specificity, and signal transduction response of these polymorphic cis-regulatory regions. The significances of these results are discussed in the context of the role of the CB$_1$ receptor in the hypothalamus, hippocampus,

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and dorsal root ganglia, and consequences for disease susceptibility are explored.

## EXPERIMENTAL PROCEDURES

*Bioinformatic Analysis*—17-species vertebrate genome comparisons and detection of linkage disequilibrium was carried out using the Human (*Homo sapiens*) Genome Browser Gateway through the UCSC Genome Browser (see Fig. 1). Detection of transcription factor binding consensus sequences was carried out using the web-based program MATCH through BIO-BASE (18). Prediction of the effects of rs9444584 on transcription factor-DNA binding was carried out using the newly developed RegSNP website. Linkage disequilibrium between SNPs was quantified using SNAP LD.

*Plasmid Constructs*—ECR1 was amplified from placental human DNA using the Expand high fidelity PCR kit and the following primers, ECR1F-TAAGCTAGGGCATGGGTGTG and ECR1R-TAGTGGAGAGGCAGGTTTGC (657 bp), as described in the manufacturer's instructions (Roche Applied Science), and cloned into pGEM-T Easy as described (Stratagene) to form pECR1(C)-GEMT-Easy. The correct amplification and orientation of ECR1(C) within the pGEM-T Easy vector was checked with restriction enzyme digestion and sequencing. The pLuc plasmid (renamed for clarity from pTAL-Luc, Clontech, see Fig. 2*D*) contains a herpes simplex virus-thymidine kinase minimal promoter that has a low transcriptional activity. pECR1(C) was produced by removing the ECR1(C) fragment from pECR1(C)-GEMT-Easy using NheI and SmaI and cloning into the ApaI (made blunt-ended using Klenow) and SpeI sites of the pLuc vector (see Fig. 2*D*). pECR1(T) was recreated by mutating the ECR1(C) sequence within pGEM-T Easy vector using QuikChange II site-directed mutagenesis kit (Stratagene) and the following primers, ECR1F-GAGAGTTCATTACTAATATGGCTTAGG and ECR1RCCTAAGCCATATTAGTAATGAACTCTC, to produce ECR1(T). ECR1(T) was then cloned into pLuc in the same way as pECR1(C) to produce pECR1(T) (see Fig. 2*D*).

*Primary Cell Culture*—1–3-day-old rat neonates were humanely euthanized in accordance with current United Kingdom Home Office schedule 1 guidelines, and the hippocampal or hypothalamic regions were immediately dissected out in into DMEM/F12 (Invitrogen) under sterile conditions. The tissue was chopped into fine pieces and placed in 2 ml of trypsin/0.5 M EDTA for 6 min, and tissues were dissected as described previously (19). The tissue was then dissociated by gentle trituration in DMEM/F12 medium with a fire-polished glass pipette (~1-mm diameter) and centrifuged at 5000 rpm for 2 min. This process was repeated, and cells were counted on a hemocytometer and plated out onto 24-well plates at a density of $5 \times 10^4$/cm$^2$. Cultures were allowed to recover for 3 days at 37 °C in medium consisting of 90% DMEM/F12 media, 10% dialyzed FCS with penicillin/streptomycin until the majority of cells had formed neurite outgrowths. DRG cells were recovered and cultured as described previously (19, 20).

*Cell Transfection*—Magnetic transfection of plasmid constructs into primary lines was performed according to the manufacturer's instructions (OZ Biosciences). In summary, Neuro-Mag reagent was added to the plasmid preparation in the ratio

of 3 $\mu$l/1 $\mu$g of plasmid DNA in the medium. The DNA-media-NeuroMag solution was mixed and incubated at room temperature for 15 min. The DNA-media-NeuroMag solution was then added to previously prepared single cell cultures in 24-well plates and incubated on a magnetofection magnetic plate for 15 min in a cell culture incubator at 37 °C and 5% CO$_2$. Following transfection, cells were treated with 10 $\mu$M angiotensin II (Tocris) or angiotensin II plus a MEK kinase inhibitor (U0216, 1 $\mu$M, Tocris) a JNK II inhibitor (SP600125, 50 nM, Tocris), or a p38 kinase inhibitor (SB202190, 10 $\mu$M, Tocris) for 24 h.

*Dual-Luciferase Assay*—24 h after transfection and agonist/antagonist treatments, Dual-Luciferase assays were performed on lysates made from primary cell cultures according to the manufacturer's instructions (Promega). The Dual-Luciferase assay analysis was carried out on a GloMax 96 microplate luminometer (Promega) using 20 $\mu$l of cell lysate per well of a white 96-well plate.

*Statistical Analysis*—All experiments were repeated a minimum of three times on separate dates using separate groups of animals ($n \geq 3$). Two-tailed Student's $t$ tests or analysis of variance were used, where appropriate, to test the significance of data derived from primary cell cultures. Statistical analysis was done using Microsoft Excel.

## RESULTS

*A Highly Conserved Region within Intron 2 Contains an SNP in Strong LD with rs9450898 and rs2023239*—Many novel cis-regulatory elements display high levels of evolutionary conservation because of their critical role in modulating the tissue-specific expression of genes (19–20, 22–30). Because intron 2 of the *CNR1* gene contained a number of disease-associated SNPs (10–17), we asked whether intron 2 contained functional sequences such as cis-regulatory elements that could direct tissue-specific gene expression. Using comparative genomics, we identified a 402-bp region of high conservation (chr6: 88919055–88919457) that had been conserved from the common ancestor of birds and humans (310 million years; Figs. 1*A* and 2*A*). Intriguingly, we found that this sequence contained a polymorphism (rs9444584; C/T) that was in strong LD with rs9450898 and rs2023239 ($r^2 = 0.815$ and 0.920, respectively), which have been associated with different conditions in multiple association analyses (10–17). The two alleles of this 402-bp region of DNA were called ECR1(C) and ECR1(T).

Examination of the population diversity of these alleles demonstrated that in Eurasian populations, the T allele was comparatively rare and was only found in 13–20% of the alleles within the population (Fig. 2*B*). However, in sub-Saharan Africa, the frequency between C and T alleles was nearly equal (47 and 53%, respectively, Fig. 2*B*). Predictive software programs (Match and Transfac) were used to show the presence of androgen receptor (AR), CCAAT enhancer-binding protein (C/EBP), transcription factor II A (TFII-A), and activator protein-1 (AP1) binding sites within ECR1 (Fig. 2*A*). Using our newly developed RegSNP program, we were able to predict that ECR1(C) would have relatively low affinity for the AP-1 transcription factor ($p$-core binding = 0.448), whereas the ECR1(T) allele would have a significantly increased affinity ($p$-core binding = 0.736).
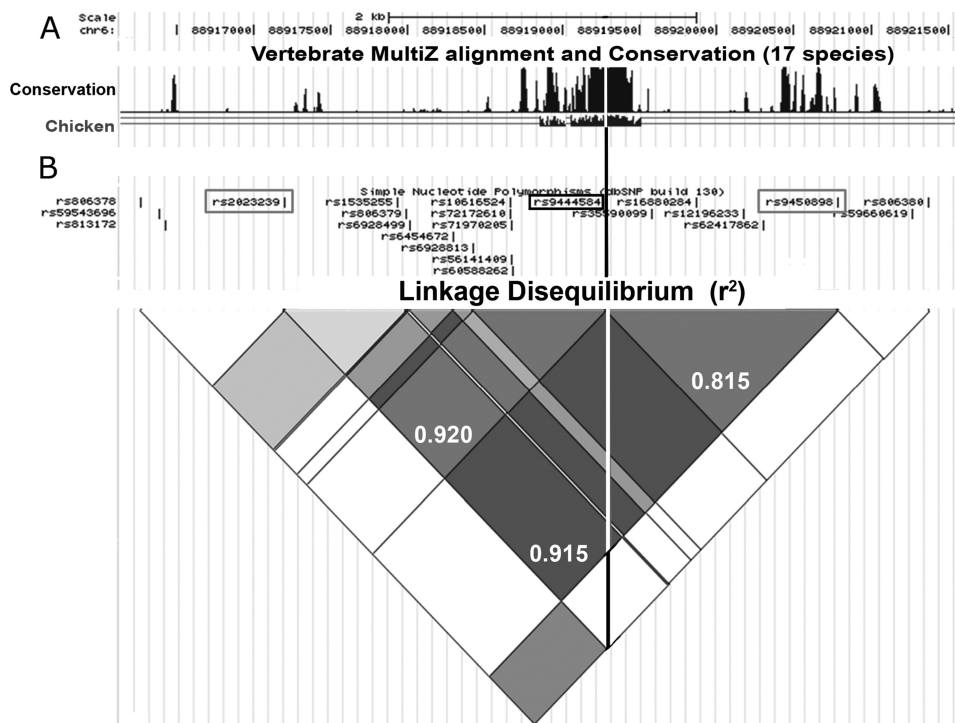
FIGURE 1. **rs9444584 is in strong LD with rs92023230 and rs9450896 and occurs within a region of *CNR1* intron 2 that has been conserved for 310 million years.** *A*, a 17-species vertebrate alignment plot from the UCSC Genome Browser highlights the presence of a putative enhancer region conserved between birds and humans. The *white line* represents the location of rs944458 within this sequence. The *top scale bar* represents linear distance (2 kb), and the *second scale bar* represents coordinates in base pairs along the length of human chromosome 6. *B*, an LD heat map analysis of SNPs within *CNR1* intron 2 derived from the UCSC browser demonstrating levels of LD ($r^2$) between the different SNPs. This map is in linear register with the graph in *A*. Exact measurements of LD (as calculated by SNAP LD) are displayed as decimal fractions in the *grey diamonds* linking the three SNPs of interest (rs9444584, rs92023230, and rs9450896). The location of rs9444584 is highlighted using a *black box* and *line*. The locations of rs92023230 and rs9450896 are highlighted using *gray boxes*.
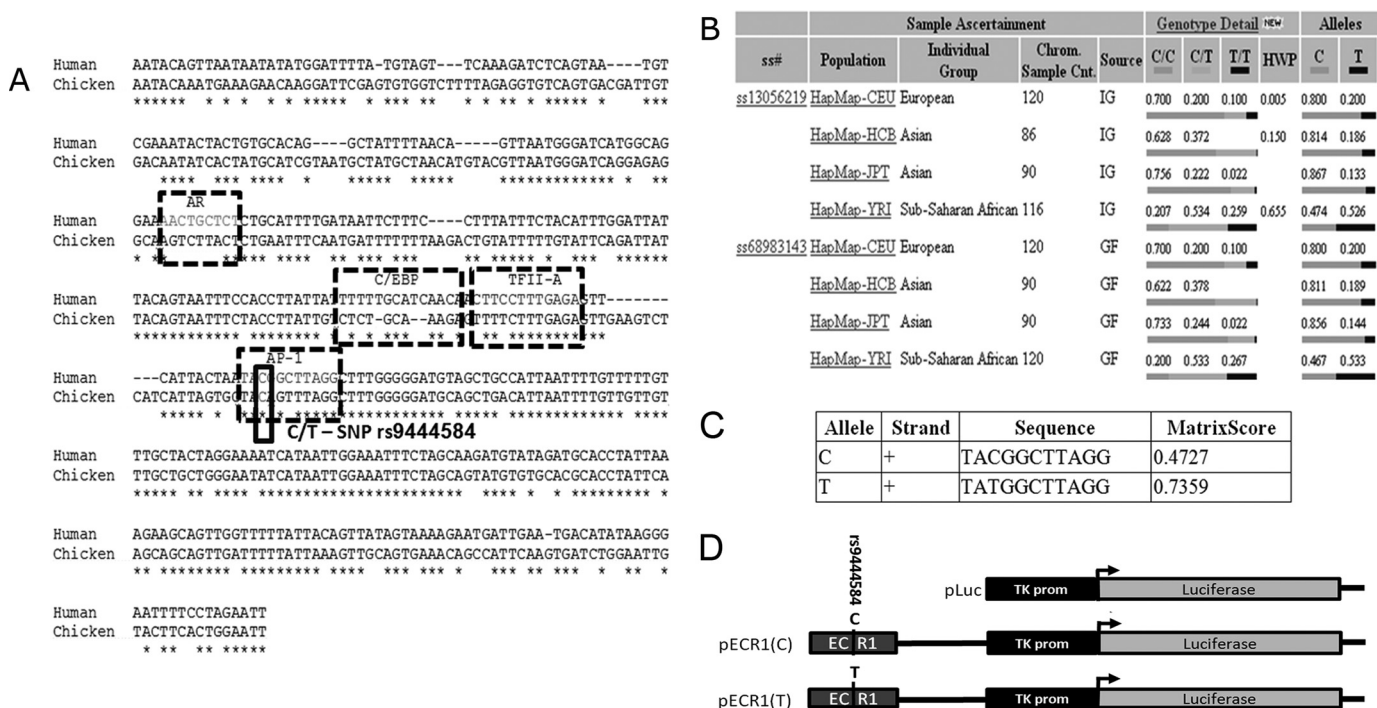


FIGURE 2. **rs9444584 allele frequency varies greatly in different human populations and changes the predicted affinity of AP1 transcription factor.** *A*, pairwise alignment of the human and chicken ECR1 sequences showing predicted transcription factor binding sites and allelic variants of rs944458. AR, androgen receptor; *C/EBP*, CCAAT enhancer-binding protein; *TFII-A*, transcription factor II A. *B*, table from the National Center for Biotechnology Information (NCBI) SNP web site showing population diversity of rs944458 in European, Asian, and sub-Saharan African populations. *C*, table generated by the RegSNP algorithm showing predicted relative binding affinities of each allele of rs944458 to the AP1 transcription factor. Matrix score and core score values represent the probability that AP1 will bind to each allelic binding site. *D*, diagrammatic representation of each of the plasmid constructs used in the current study (not to scale). *Bent arrows* represent the transcriptional start site of the luciferase gene. *ss#*, assigned submitter SNP ID, *Chrm. Sample. Cnt*, chromosome sample count, *IC*, individual Genotype, and *GF*, genotype frequency.
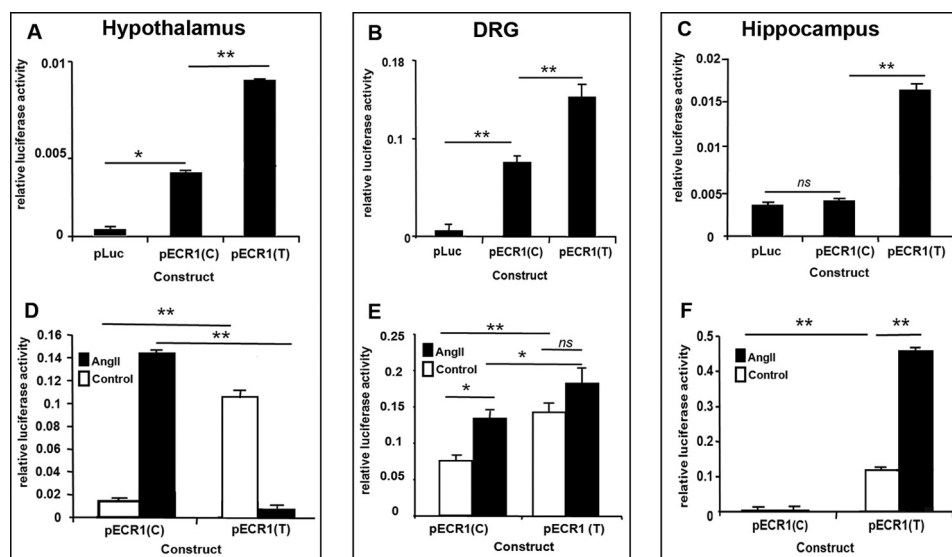
FIGURE 3. **Allele-specific properties of ECR1 in different primary cell types and following stimulation of MAP kinase pathways.** *A–F*, graphs showing the results of Dual-Luciferase analyses of primary cells derived from neonate rat hypothalamus (*A* and *D*), DRG (*B* and *E*), and hippocampus (*C* and *F*) following transfection with the different constructs shown in Fig. 2*D* and treatment with angiotensin II for 24 h (*D–F*; 10 $\mu M$). Cells were lysed, and relative luciferase activity was determined and normalized to an internal *Renilla* luciferase control (pRL-CMV). *ns* = not significant, *, $p = \leq 0.05$, **, $p = \leq 0.01$.

*ECR1(C) Is Active within Primary Hypothalamic Cell Cultures and DRG but Not in Primary Hippocampal Cells*—Because *CNR1* is expressed in many different parts of the brain and peripheral nervous system such as the hypothalamus, hippocampus, and DRG, we explored the hypothesis that the most common allele, ECR1(C), acted as an enhancer of promoter activity within cells derived from specific regions of the brain. ECR1(C) was amplified using high fidelity PCR from human placental DNA and cloned into the pTAL-Luc vector (Clontech) that contains a TATA-like promoter (*PTAL*) region from the herpes simplex virus-thymidine kinase promoter linked to a luciferase reporter (Fig. 2*D*). This commercially available plasmid has been widely used to assay gene regulatory sequences in the past and will subsequently be referred to as pLuc. Because of the known function of $CB_1$ in the hypothalamus, DRG, and hippocampus in appetite, cognition, and pain, respectively, we examined whether the ECR1(C) acted as an enhancer of promoter activity in primary cells derived from these different tissues. We also chose to use primary lines as the activity of many enhancer regions has been shown to be highly context-dependent (19–20, 22, 23, 25, 28–30). pLuc and pECR1(C) were magnetofected into primary cell cultures derived from the hypothalamus, hippocampus, or DRG, and after 24 h, Dual-Luciferase assays were performed. It was observed that ECR1(C) was able to act as an enhancer of promoter activity in hypothalamic and DRG cells (Fig. 3, *A* and *B*) but not in hippocampal cells (Fig. 3*C*).

*ECR1(C) Enhancer Activity Is Responsive to MAP Kinase Activation in Primary Hypothalamus- and DRG-derived Cells but Not in Hippocampal Cells*—Angiotensin II (angiotensin) is a widely recognized inducer of many different MAP kinase pathways at concentrations ranging from 500 nM to 10 $\mu M$ (31–33). We cultured different primary cells transfected with pECR1(C) in the presence of angiotensin II and demonstrated that ECR1(C) could respond to angiotensin in both primary DRG and hypothalamic cells (Fig. 3, *A* and *B*). However, we

were unable to detect any significant up-regulation of ECR1(C) in hippocampus-derived cells.

*Response of ECR1(C) to Angiotensin in Hypothalamic Cells Is Modulated by MEK Kinase and p38 Kinase Pathways*—We explored which of the three major MAP kinase pathways were involved in mediating the effects of angiotensin on ECR1(C) by culturing pECR1(C)-transfected hypothalamic cells in the presence of angiotensin and an antagonist specific for the MEK kinase (U0216), JNK (SP6000125), or p38MAP (SB202190) kinase pathways in hypothalamic cells. The JNK kinase inhibitor had no significant effect on the ability of angiotensin to induce the activity of ECR1(C) (Fig. 4*B*). However, incubation with the p38 agonist halved the induction of ECR1(C) induced by angiotensin, and treatment with the MEK kinase inhibitor completely abolished the action of angiotensin (Fig. 4, *A* and *C*).

*ECR1(T) Allele Demonstrates Significant Differences from ECR1(C) as an Enhancer in Hippocampal Cell Cultures*—Using bioinformatics, we predicted that ECR1(T) would bind the AP1 protein with a higher affinity (Fig. 2*C*). This is an interesting prediction in the context of MAP kinase signaling as AP1 activity is known to be modulated by MAP kinase pathways including JNK and MEK kinase (34). We used site-directed mutagenesis of the pECR1(C) plasmid to produce pECR1(T) (Fig. 2*D*). We compared pECR1(T) activity with that of pECR1(C) in hypothalamic, DRG, or hippocampal cell cultures. ECR1(T) demonstrated 50 and 40% higher enhancer activity in primary cells derived from hypothalamus and DRGs, respectively, than the ECR1(C) allele (Fig. 3, *A* and *B*). Significantly, and in strong contrast to ECR1(C), it was observed that ECR1(T) demonstrated potent enhancer activity in hippocampal neurons where ECR1(C) was inactive (Fig. 3*C*).

*ECR1(T) Demonstrates Significant Allele-specific Differences from ECR1(C) in Its Response to Activation of MAP Kinases*—We have demonstrated that ECR1(C) responded to MAP kinase activation in both hypothalamus-derived and DRG-derived primary cultures but not in hippocampal cultures (Fig. 3,
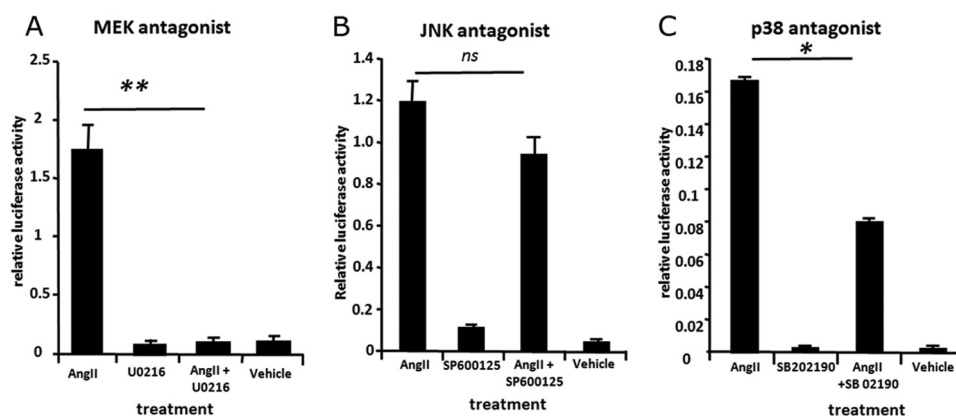
FIGURE 4. **ECR1(C) activity is controlled by MEK/p38 kinase signaling in hypothalamic neurons.** Primary rat hypothalamic neurons were cultured for 3 days and then transfected with a pECR1(C) construct and treated with angiotensin II (*AngII*, 10 $\mu$M) in the presence/absence of MEK kinase inhibitor (U0216, 1 $\mu$M) (*A*), JNK II inhibitor (SP600125, 50 nM) (*B*), or p38 kinase inhibitor (SB 202190, 10 $\mu$M) (*C*). The control is the pECR1(C) construct treated with a respective vehicle. Cells were incubated with treatment for 24 h and then assayed for luciferase activity. Cells were lysed, and a relative luciferase measurement was determined and normalized to an internal *Renilla* luciferase control (pRL-CMV). *ns* = not significant, *, $p = \leq 0.05$, **, $p = \leq 0.01$.

*D–F*). However, in hippocampal cells treated with angiotensin, and in direct contrast to ECR1(C), activity of ECR1(T) was increased by over 300% when compared with the untreated sample (Fig. 3*F*). Surprisingly, although activation of MAP kinases in these hypothalamic cells increased the activity of ECR1(C) by nearly 10-fold, application of angiotensin to hypothalamic cultures transfected with pECR1(T) virtually inactivated ECR1(T) activity in these cells (Fig. 3*D*). Similarly, although treatment of DRG neurons with angiotensin increased activity of ECR1(C), no significant increase in ECR1(T) activity was observed (Fig. 3*E*).

## DISCUSSION

Using a unique combination of *in silico* genetics, comparative genomics, molecular biology, and primary cell culture, we present evidence of a novel cis-regulatory region, ECR1(C), that is active in hypothalamus- and DRG-derived cells where it is responsive to MAP kinase signaling, but inactive in the hippocampus. However, the most significant finding from the current study is that, in contrast to ECR1(C) a second allele of ECR1 present in the population, ECR1(T), acts as a potent MAP kinase-inducible enhancer in hippocampus cells. These intriguing observations may have important implications for understanding disease because rs9444584 is in strong LD with two other polymorphisms that have been previously linked to diseases such as obesity, addiction, and reduced frontal brain volumes in schizophrenia as a result of cannabis misuse.

We explored the possibility that MAP kinase pathways were involved in controlling ECR1 as MAP kinase pathways in the hypothalamus and DRG have been shown to play a role in the modulation of appetite (35) and pain (36), processes in which the $CB_1$ receptor is also involved (3–5). Furthermore, $CB_1$ activation is known to trigger MAP kinase pathways (37). Thus, it is possible that one role for the ECR1(C) enhancer in the hypothalamus would be to allow a response to MAP kinase pathways, triggered by $CB_1$ activation, to modulate $CB_1$ levels in response to food availability. For example, MEK/ERK kinase pathways are induced by activation of $CB_1$ in N1E-115 neuroblastoma cells, supporting the existence of a $CB_1$-MAP kinase autocrine loop in certain groups of neurons (38). In keeping with these studies, we were able to demonstrate that the MAP kinase pathways responsible for the activity of ECR1(C) in hypothalamic cells were primarily the MEK kinase pathways with input from p38MAP kinases. These are interesting observations as the T allele of rs2023239, which is in strong LD with rs9444584, has been associated with higher body mass index in both Swiss obese subjects and Danish individuals (11). Considering the role of the $CB_1$ gene in appetite in the hypothalamus and the triggering of MAP kinase signaling by $CB_1$ activation, it can be hypothesized that increased sensitivity of ECR1(T) to MAP kinase signaling might play a role in perturbing $CB_1$ expression in the hypothalamus, leading to changes in appetite and increased body mass index.

Because our algorithm predicted that AP1 binding affinity would be increased in ECR1(T), we were not surprised to observe that it became more responsive to MAP kinase activation in hypothalamic cells. However, we were surprised to observe such a clear difference in the ability of the ECR1(C) and ECR1(T) alleles to activate promoter activity in hippocampal cells. In addition, the ability of ECR1(T) to respond to MAP kinase activation in hippocampal cells, in clear contrast to the response of ECR1(C) in these cells, was also unexpected. These may be important observations as MAP kinase signaling, in the form of ERK-MEK kinase, in the hippocampus is involved in $CB_1$-mediated neurogenesis and the synaptic integrity required for stable long term memory formation and cognition (39–42).

In addition, changes in the structure of the hippocampus and in plasticity of hippocampal neurons have been linked to cannabis-related psychosis (43–45). Intriguingly, rs94580898 is in strong LD with rs9444584 and has been associated with reductions in white matter in schizophrenia patients following cannabis overuse (17). The heightened sensitivity of ECR1(T) to MAP kinase signaling in the hippocampus in comparison with ECR1(C) may represent a possible mechanisms to explain the cognitive deficits and reduced white matter experienced by many schizophrenia sufferers and heavy marijuana users.

Consistent with the results of the current study, previous research has demonstrated that that $CB_1$ receptor coupling to G-proteins differs between different brain regions and there-

fore depends on the cellular environment, *i.e.* it is context-dependent. One possible mechanism governing this context dependence is receptor coupling. For example, Breivogel *et al.* (46) demonstrated that receptor coupling efficacy is relatively higher in the hypothalamus than the hippocampus. From autoradiographic analysis, the relatively sparse cannabinoid receptors in the hypothalamus appeared to activate nearly as many G-proteins as in the receptor-dense hippocampus. These data imply that the $CB_1$ receptor-mediated initiation of downstream signaling in the hypothalamus and hippocampus differ significantly and may have consequences for the initiation of MEK-mediated regulation of *CNR1* expression in the hippocampus. This is in line with our results demonstrating regional difference in control of *CNR1* expression via MAPK signaling. The findings have far reaching implications for the effects of cannabis in the brain as studies have demonstrated that the MEK pathway is implicated in regional differences in the development of tolerance to $\Delta^9$THC, the major psychoactive constitute of cannabis (47). Furthermore, cannabis smoking in adolescence is associated with an increased risk of psychosis, particularly in individuals with a family history of schizophrenia (48). Studies reveal that $CB_1$ receptors in adolescent hippocampus have lower functional coupling to G-proteins and desensitize more slowly in response to THC treatment than those of adults (49). It could be speculated that polymorphisms in ECR1 may increase susceptibility of adults and adolescents to develop psychosis when exposed to high potency cannabis.

Further evidence for the possible harmful effects of ECR1(T) comes from the observation that following the migration of Eurasian ancestors out of Africa and into the Middle East 40,000 years ago, ECR1(T) was clearly selected against within these populations where the allelic frequency of ECR1(T) has been reduced from 50% in African populations to as little as 13% of the population in Europe and Asia. It may not be entirely coincidental that this reduction in the frequency of ECR1(T) occurred subsequent to the arrival of Eurasian ancestral populations in a region of the world where *Cannabis sativa* was endemic and in which it had been used for religious, medicinal, and recreational purposes for many thousands of years (50). Further support for this hypothesis comes from a number of studies that have reported a higher incidence of psychosis in individuals of African and Caribbean origin (21, 51–53), although the role of cannabis use in these studies was not fully explored.

A possible mechanism can be inferred if we consider that $CB_1$ activation is known to trigger MAP kinase pathways (37) in neuronal cells and that the ECR1(T) allele responds more strongly to MAP kinase activation in hippocampal cells, where the ECR1(C) allele does not. Thus the current study raises the possibility of the presence of a potentially harmful $CB_1$-MAP kinase-ECR1(T) autoregulatory loop in hippocampal neurons in a proportion of the human population that contributes to susceptibility to many of the conditions associated with rs9450898 and rs2023239 or the negative effects of high potency cannabis use.

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