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Design Logic of a Cannabinoid Receptor Signaling Network That Triggers Neurite Outgrowth

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Abstract

Cannabinoid receptor 1 (CB1R) regulates neuronal differentiation. To understand the logic underlying decision-making in the signaling network controlling CB1R-induced neurite outgrowth, we profiled the activation of several hundred transcription factors after cell stimulation. We assembled an in silico signaling network by connecting CB1R to 23 activated transcription factors. Statistical analyses of this network predicted a role for the breast cancer 1 protein BRCA1 in neuronal differentiation and a new pathway from CB1R through phosphoinositol 3-kinase to the transcription factor paired box 6 (PAX6). Both predictions were experimentally confirmed. Results of transcription factor activation experiments that used pharmacological inhibitors of kinases revealed a network organization of partial OR gates regulating kinases stacked above AND gates that control transcription factors, which together allow for distributed decision-making in CB1R-induced neurite outgrowth.

Signaling through the cannabinoid receptor 1 (CB1R), which couples to the heterotrimeric guanine nucleotide-binding proteins (G proteins) G_i and G_o , regulates many physiological processes. In cultured mouse Neuro2A cells, CB1R stimulation induces neurite outgrowth through a signaling pathway from G_{α_o} that activates the protein kinase c-Src and the transcription factor signal transducer and activator of transcription 3 (Stat3) (1,2). CB1R signaling also has a key role during central nervous system development and in the adult brain (3,4). Furthermore, CB1R has been shown to modulate several neurological disorders (5). However, the organization of the CB1R signaling network involved in cellular state-change decisions has not been well defined. Delineation of the organization of signaling networks is useful in identifying emergent decision-making capabilities (6). To do so, we started with delineating individual pathways (1,2). However, simply verifying the presence and function of individual pathways will not advance our knowledge of the design of complex cellular regulatory networks and their decision-making capabilities. A key challenge in systems biology is to identify, experimentally verify, and understand the organizations of complex regulatory systems.

To broadly define the cellular network regulating CB1R-induced neurite outgrowth, we integrated transcription factor activity profiling, network biology, and cell biology. First, the CB1R-triggered activation of multiple transcription factors was profiled during neurite outgrowth. We then developed an in silico network in which the activated transcription factors were linked to known interactors and pathways that regulate them to identify new components and pathways involved in neuronal differentiation. Predictions were experimentally tested in

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cultured and primary neurons. We then used selective pharmacological inhibitors in transcription factor activation experiments to determine the hierarchy between three key kinases and transcription factors. These experiments allowed for construction of a map where partial OR gates at the level of G proteins regulating kinases are stacked on top of AND gates at the level of kinases regulating transcription factors, allowing for a distributed decision-making capability within the network.

CB1R-regulated transcription factors

We assayed transcription factor activation in response to the CB1R agonist HU-210 in Neuro2A cells by using a commercial array (7). Spotted on this array are 345 oligonucleotide transcription factor-binding sites (table S1), enabling the activation of a large number of transcription factors to be assayed simultaneously [see (7) for array details]. Studies have indicated that CB1R activation of $G\alpha_o$ can stimulate Stat3 (2), so we expected to observe activation of Stat3 on the array. Mouse Neuro2A cells were treated with HU-210 (2 μ M) for 20, 60, 120, and 360 min to assess transcription factor activation. Ongoing transcription was required for at least 360 min to induce neurite outgrowth in response to CB1R signaling (fig. S1). Nuclear extracts were obtained and processed for hybridization to the array. The Entrez Gene names of all the transcription factors activated over the 360-min time course are displayed in table S2. All of the transcription factor-activation arrays described in table S2 are shown in fig. S2A, and several transcription factors that were activated at 20 min are highlighted in Fig. 1A. Activated transcription factors fell into three main categories: those that were activated early and transiently, such as Stat3, Smad3, and Smad4; those that displayed sustained activation, including c-Myb and paired box 6 (PAX6); and those that were activated at later times, such as forkhead box I1 (FOXI1) and upstream transcription factor 1 (USF1). In all, 33 transcription factors were activated over the 6-hour time course of CB1R stimulation. Because the activations of homeobox D8 (HOXD8), HOXD9, and HOXD10 and Smad3 and Smad4 were each represented as single spots on the array, they were grouped together in table S2. For the computational analysis (see below), they were used individually. Stat3 was activated at 20 and 60 min, and this activation was confirmed by gel shift analysis (fig. S3A). cAMP response element-binding protein (CREB), a transcription factor known to be involved in neurite outgrowth (8), was also activated, and this result was verified when CREB was phosphorylated on Ser¹³³ in response to HU-210 (fig. S3B). It is likely that CREB is activated through $\beta\gamma$ subunit of G_o ($G\beta\gamma$)-mediated stimulation of p42 and p44 mitogen-activated protein kinase (MAPK) (9). MAPK was also activated in response to CB1R stimulation, and the treatment of cells with the upstream MAPK kinase (MEK)-1,2 kinase inhibitor PD 98059 (PD) attenuated phosphorylation of both MAPK and CREB (fig. S3E). Transfection of a dominant-negative (DN) CREB construct inhibited cannabinoid-induced neurite outgrowth, albeit to a lesser extent than did DN Stat3 (Fig. 1B). Retinoic acid receptor (RAR), another well-known regulator of neurite outgrowth (10), was also activated on the array, and this finding was confirmed by gel shift analysis (fig. S3C). We also examined several transcription factors, including c-Myb, activating protein 2 α (AP-2 α), and PAX6, that have not been shown to have a role in neurite outgrowth. Gel shift analysis confirmed the activation c-Myb (fig. S3D). These results validate several of the transcription factors that were activated on the arrays.

To test whether the activated transcription factors might regulate the induction of neurite outgrowth, we assessed 10 of the activated factors, representing all three categories (early, sustained, and late activation) in addition to CREB and Stat3. Depletion of AP-2 α , PAX6, and spleen focus forming virus proviral integration oncogene 1 (SPI1) with RNA interference (RNAi) inhibited cannabinoid-induced neurite outgrowth by ~60%, and RNAi of RAR α was also slightly inhibitory (Fig. 1B). In contrast, RNAi of Smad3, c-Myb, and nuclear transcription factor-Y α (NFYA) led to an enhancement of HU-210-stimulated neurite outgrowth. Ectopic expression of c-Myb reduced neurite outgrowth by ~30%. Off-target gene-silencing effects of

RNAi seemed unlikely because CB1R-induced neurite outgrowth was similar in cells treated with luciferase (Luc) small interfering RNA (siRNA) and a separate scrambled (SC) siRNA. These results suggest that transcription factor profiling is able to detect both positive and negative regulators of neurite outgrowth.

In silico network construction and predicting new components and pathways

Although the transcription factor arrays indicate that many transcription factors are activated during neurite outgrowth, they do not provide information about the cell signaling pathways and components that lead to their activation. We identified the upstream signaling pathways and components regulating the activated transcription factors by constructing a network in silico. For this we used available protein-protein interaction databases, graph-theory analysis, and statistical tests. We consolidated eight existing mammalian protein-protein interactions networks, the Biomolecular Interaction Network Database (BIND) (11), the Human Protein Reference Database (HPRD) (12), the Molecular Interaction database (MINT) (13), the Database of Interacting Proteins (DIP) (14), IntAct (15), BioGRID (16), Reactome (17), and the Protein-Protein Interaction Database (PPID) (18), with a neuronal signaling network we developed (19). To remove potentially low-confidence interactions, such as interactions reported from yeast two-hybrid screens, we filtered the nine consolidated data sets by removing all articles reporting more than three interactions. This method reduced the number of interactions in the consolidated database from 67,379 to 15,494 (Fig. 2A). Applying a shortest-path analysis, we first automatically found undirected paths of a limited path length (two steps, all direct- and second-neighbor interactions) between all the transcription factors, knowing the consensus-binding sequences on the transcription factor-activation array. Combining all the paths from this search resulted in a subnetwork made of 444 nodes and 1873 interactions from 1843 unique references. We merged this subnetwork with a large-scale curated signaling network we developed from the neuroscience literature (19). Again applying shortest-path analysis, we searched for directed paths with a limited threshold path length (seven steps) from the CB1R agonist HU-210 to the transcription factors associated with the consensus sequences on the array. We found paths from HU-210 to 104 transcription factors, including 17 of the 23 transcription factors that were activated within 20 min in table S2 (Fig. 2B) and 87 transcription factors that were not activated (fig. S4). Counting and comparing the number of times that components appeared in pathways to activated factors or non-activated factors enabled us to identify intermediate components in pathways predicted to participate in the regulation of the activated transcription factors (Fig. 2B and algorithm S1). BRCA1, the breast cancer susceptibility protein (20), was ranked highest as the most specific regulator of the activated transcription factors (Fisher exact test, $P = 0.05$; table S3, using equation S1; and Fig. 2B). One of the unanticipated pathways that emerged from using this method connected CB1R stimulation to PAX6 through phosphoinositol 3-kinase (PI3K) and the protein kinase Akt (table S3 and Fig. 2B). We applied a similar analysis by building a subnetwork that attempted to connect only the activated transcription factors (algorithm S2). Starting with a list of the 23 activated factors, we searched for paths of three links in length using the consolidated mammalian protein-protein interactions networks. This subnetwork contained 79 nodes and 328 links, significantly more links than those in subnetworks created from 20 randomly generated seed lists of the same size, created from factors that were not activated on the transcription factor-activation arrays (table S4; z test, $P < 0.001$). The clustering coefficients and characteristic path lengths (21) were similar (0.18 versus 0.21 ± 0.09 and 2.37 versus 2.46 ± 0.39) in the subnetwork of activated factors and the control subnetworks. We used a binomial proportions test to remove components that were found in the activated factors subnetwork but not specifically interacting with the activated factors, because these components interact with many proteins. Thus, their presence in the activated factors subnetwork might be by chance (22). Again, BRCA1 was identified as a specific interactor with the activated transcription

factors (binomial test, z score 58.9; table S5 and Fig. 2C). We experimentally tested whether BRCA1 regulated neuronal differentiation and for the existence of a CB1R-to-PAX6 pathway.

Regulation of neuronal differentiation by BRCA1

Although the molecular mechanisms by which BRCA1 functions have remained enigmatic, BRCA1 is thought to participate in the response to DNA damage, centrosome amplification regulation, and mitotic spindle assembly (20,23,24). BRCA1 may also function in neural development, because mice with homozygous deletion of BRCA1 die as embryos because of neural defects (25). However, no cell-biological function for BRCA1 during neurogenesis has been reported. Several clinical case studies have linked BRCA1 mutant alleles found in breast cancer to epilepsy (26,27). Thus, BRCA1 may influence the pathology of neurological conditions.

To examine whether BRCA1 regulates neurite outgrowth, we inhibited BRCA1 expression by use of RNAi in Neuro2A cells (fig. S12D). Ablation of BRCA1 expression enhanced cannabinoid-induced neurite outgrowth by 70% (Fig. 3A). In addition, 80% of the neurite outgrowth normally observed in response to cannabinoid signaling was seen upon loss of BRCA1 expression in the absence of HU-210. This result raises the possibility that BRCA1 may also affect neuronal differentiation in the absence of cannabinoid signaling. Indeed, several of the transcription factors that interact with BRCA1 (28–30) and were activated through CB1R stimulation participate in neuronal differentiation in multiple contexts (8, 10, 31).

Network analysis indicated that BRCA1 interacts with several of the transcription factors, including Stat1, Stat3, and Smad3, within 20 min of cannabinoid treatment. We tested whether BRCA1 regulated Stat3 and Smad3 during CB1R stimulation. BRCA1 siRNA treatment resulted in an increase in the nuclear localization of Stat3 in response to HU-210 at 20, 60, and 120 min (Fig. 3B and fig. S5B) and in nuclear localization of Smad3 at 60 and 120 min (fig. S6). Stat3 also accumulated in the nucleus within 60 to 120 min after HU-210 treatment of Luc siRNA-treated cells (fig. S5A), but to a lesser extent than in cells treated with BRCA1 siRNA (fig. S5B). These results indicate that BRCA1 influences cannabinoid-regulated nuclear localization of Stat3 and Smad3. Consistent with these findings, stimulation of Neuro2A cells with HU-210 caused an ~40% decrease in amounts of BRCA1 mRNA by 60 min that was sustained until 120 min (Fig. 3C), which correlates with the time that Stat3 accumulates in the nucleus after HU-210 stimulation (fig. S5A). By 6 hours after treatment of cells with HU-210, amounts of BRCA1 mRNA were similar to those in unstimulated cells.

Because the loss of BRCA1 expression induced neurite outgrowth in the absence of CB1R stimulation, we investigated whether BRCA1 played a general role in neuronal differentiation. In primary cultures of rat hippocampal neurons, the neurons initially form neurites that further develop into a single axon and multiple dendrites (32). To assess the role of BRCA1 in differentiation, primary hippocampal cultures were transfected with BRCA1 siRNA after the cells had adhered to the culture plates (fig. S12E). After 30 hours, cells were fixed and stained with β -tubulin to mark neurites, and neurite outgrowth was analyzed morphometrically. In three of four experiments, RNAi of BRCA1 appeared to decrease the number of processes per cell by 10 to 15% (Fig. 4A and fig. S7A), suggesting BRCA1 is a positive general regulator of neuronal differentiation. Because several case studies have linked BRCA1 mutations to epileptic seizures (26,27), we also examined whether BRCA1 regulated synapse formation in primary hippocampal cultures. Synapses are proposed to form between neurons through protrusions by dendritic filopodia, which extend toward axon terminals and form stable contacts during differentiation (33). Rat primary hippocampal cultures were transfected with Luc siRNA or rat BRCA1 siRNA on the third day that cells were cultured (fig. S12F). On day

7, cultures were fixed and stained with antibodies to the synaptic vesicle marker synaptophysin and β -tubulin to mark dendrites (Fig. 4B and fig. S7B). The loss of BRCA1 expression in primary cultures resulted in an increase in the punctuate synaptophysin staining in hippocampal cultures, indicating that BRCA1 may function in regulating locations where synapses may be forming during differentiation of hippocampal neurons. This was not due to an effect of BRCA1 on cell viability, as the number of live and dead cells was similar in neurons treated with Luc or BRCA1 siRNA (fig. S8). Overall, these findings indicate that BRCA1 is a regulator of cannabinoid-mediated and general neuronal differentiation and raise the possibility that loss or dysregulation of BRCA1 may also contribute to abnormal neuronal morphology and neurological disorders.

PI3K signaling to PAX6 during CB1R-stimulated neuronal differentiation

Network analysis also predicted that a CB1R-PI3K-Akt pathway regulates neurite outgrowth and linked this pathway to PAX6. To assess this possibility, we first examined whether the PI3K pathway was activated during neurite outgrowth in Neuro2A cells. Stimulation of CB1R resulted in Akt activation, as demonstrated by phosphorylation of Ser⁴⁷³, which is required for activation of Akt (Fig. 5A) (34). This activation was blocked by the selective PI3K inhibitor LY 294002 (LY), suggesting that Akt activation was occurring through PI3K. Treatment of Neuro2A cells with LY also inhibited neurite outgrowth by ~50%, similar to the effects of the MAPK pathway inhibitor PD and DN CREB (Fig. 1B and Fig. 5B). Blockade of both MAPK and PI3K pathways led to further inhibition of neurite outgrowth (Fig. 5B). This inhibition is similar to that observed with DN Stat3 (Fig. 1B) or the upstream kinase Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (Fig. 5B). These results suggest that PI3K regulates cannabinoid-induced neurite outgrowth and that PI3K and MAPK may act independently to induce neurite outgrowth.

We assessed with gel shift analysis whether PI3K signaling activated PAX6 in Neuro2A cells. Treatment of Neuro2A cells with LY before HU-210 blocked the shift of a PAX6 consensus site after 20 min of stimulation but not at later time points (Fig. 5C), suggesting that PI3K acts in the early activation of PAX6 during cannabinoid-induced neurite outgrowth. The role of PAX6 in cannabinoid signaling in primary hippocampal neurons was also examined. PAX6 is activated by phosphorylation on Ser and Thr residues (35,36). To assess whether PI3K is involved in PAX6 activation in primary hippocampal cultures, we cultured neurons for 3 days, treated them with LY or dimethyl sulfoxide (DMSO), and then stimulated them with HU-210 (1 μ M) for 30 or 60 min. PAX6 was immunoprecipitated and then immunoblotted with a phosphothreonine antibody to examine PAX6 phosphorylation. Stimulation of CB1R with HU-210 led to the phosphorylation of PAX6, and blockade of PI3K inhibited this effect (Fig. 5D and fig. S9), indicating that PI3K may influence PAX6 activation in response to CB1R signaling.

Signal processing for neuronal differentiation

Many of the transcription factors that we identified by the profiling approaches are involved in neurite outgrowth. This, taken together with the validation of the network predictions that BRCA1 is an important regulator and that PI3K-to-PAX6 is a signaling pathway regulating neuronal differentiation, suggests the validity of the network that we are identifying by using this combination of experiments and bioinformatics. However, these experiments do not shed light on the design logic of this network. We sought to determine the relationship between the upstream kinases and downstream transcription factors as an approach to understand how the different logic gates might be organized within the network. We used the transcription factor activation arrays to assess how Src, MAPK, and PI3K signals influence the 23 transcription factors that are activated after 20 min of stimulation of CB1R (table S2) in the presence of their

pharmacological inhibitors LY, PD, and PP2, respectively (fig. S2). Each inhibitor affected the activation of a group of transcription factors and activation of some transcription factors was inhibited by several inhibitors (Fig. 6A). As expected, PD inhibited CREB activation and PP2 inhibited Stat3. Both LY and PP2 inhibited PAX6 activation (Fig. 6A), suggesting that in addition to PI3K, Src signaling may also influence PAX6 activation. Blockade of either PI3K or Src inhibited Akt activation (fig. S10). However, other molecules in addition to Src may signal to Akt because the inhibition of Src did not completely abrogate the activation of Akt. The inhibition of MAPK enhanced Akt activation, suggesting that the PI3K pathway may compensate for the loss of MAPK signaling during neurite outgrowth. A simplified schematic of the signal flow during cannabinoid-induced neurite outgrowth is shown in Fig. 5E.

Network organization and cell state–change decisions

This study provides the framework to explore the mechanistic details of individual interactions during neuronal differentiation. These relationships are likely to be cell type–specific as highlighted by BRCA1 inhibition of neurite outgrowth in Neuro2A cells and stimulation of outgrowth in hippocampal neurons. This study has enabled us to develop a systems-level logic diagram for cell state–change decisions in Neuro2A cells (Fig. 6B). For this we used the results of this study and the experimental literature on the G_i and G_o signaling pathways (fig. S13 and table S6). The picture that emerges has a set of partial OR (pOR) gates that connect the $G\alpha_i$, $G\alpha_o$, and $G\beta\gamma$ subunits to the kinases PI3K, Src, and MAPK. Src itself may stimulate both PI3K and MAPK. This redundancy of pathways indicates that the upstream region of the network is abundant in positive feed-forward motifs that function as pOR gates, a topology reminiscent of what we have observed in our literature-based signaling network of the hippocampal neuron (19). The three pOR gates are stacked on top of three AND gates that connect the kinases to many transcription factors. Akt also appears to participate in a single-input module–type motif (37) connecting to a number of transcription factors, but the role of most of these transcription factors, except RAR, in neurite outgrowth in Neuro2A cells is not clear. In contrast, AND gates connect kinases to c-Myb, Stat3, PAX6, NFYA, and CREB, all of which function in CB1R neurite outgrowth as shown by functional ablation experiments. This organization of pOR gates stacked on top of AND gates suggests a distributed decision-making process. This provides a balance between redundancy of response pathways at the upper level and a balanced funneling of signals at the lower level, in which the AND gates can serve as filters. Such filtering would ensure that only signals of sufficient intensity and duration turn on the transcription factors to trigger state change. Thus, this overall organization allows for reliable state-change responses to appropriate signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and Notes

1. Jordan JD, et al. *J Biol Chem* 2005;280:11413. [PubMed: 15657046]
2. He JC, et al. *J Biol Chem* 2005;280:33426. [PubMed: 16046413]
3. Harkany T, et al. *Trends Pharmacol Sci* 2007;28:83. [PubMed: 17222464]
4. Chevaleyre V, Takahashi KA, Castillo PE. *Annu Rev Neurosci* 2006;29:37. [PubMed: 16776579]
5. Centonze D, Finazzi-Agro A, Bernardi G, Maccarrone M. *Trends Pharmacol Sci* 2007;28:180. [PubMed: 17350694]
6. Bhalla US, Ram PT, Iyengar R. *Science* 2002;297:1018. [PubMed: 12169734]
7. Materials and methods are available as supporting material on *Science* Online.
8. Segal RA. *Annu Rev Neurosci* 2003;26:299. [PubMed: 12598680]
9. Gutkind JS. *Sci STKE* 2000;2000:re1. [PubMed: 11752597]
10. Clagett-Dame M, McNeill EM, Muley PD. *J Neurobiol* 2006;66:739. [PubMed: 16688769]
11. Bader GD, Betel D, Hogue CWV. *Nucleic Acids Res* 2003;31:248. [PubMed: 12519993]
12. Mishra GR, et al. *Nucleic Acids Res* 2006;34:D411. [PubMed: 16381900]
13. Chatr-aryamontri A, et al. *Nucleic Acids Res* 2007;35:D572. [PubMed: 17135203]
14. Xenarios I, et al. *Nucleic Acids Res* 2001;29:239. [PubMed: 11125102]
15. Kerrien S, et al. *Nucleic Acids Res* 2007;35:D561. [PubMed: 17145710]
16. Stark C, et al. *Nucleic Acids Res* 2006;34:D535. [PubMed: 16381927]
17. Joshi-Tope G, et al. *Nucleic Acids Res* 2005;33:D428. [PubMed: 15608231]
18. Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG. *Nat Neurosci* 2000;3:661. [PubMed: 10862698]
19. Ma'ayan A, et al. *Science* 2005;309:1078. [PubMed: 16099987]
20. Venkitaraman AR. *Cell* 2002;108:171. [PubMed: 11832208]
21. Watts DJ, Strogatz SH. *Nature* 1998;393:440. [PubMed: 9623998]
22. Berger SI, Posner JM, Ma'ayan A. *BMC Bioinform* 2007;8:372.
23. Gudmundsdottir A, Ashworth A. *Oncogene* 2006;25:5864. [PubMed: 16998501]
24. Joukov V, et al. *Cell* 2006;127:539. [PubMed: 17081976]
25. Gowen LC, Johnson BL, Latour AM, Sulik KK, Koller BH. *Nat Genet* 1996;12:191. [PubMed: 8563759]
26. Eccles DM, Barker S, Pilz DT, Kennedy C. *J Med Genet* 2003;40:24e.
27. Eccles D, Bunyan D, Barker S, Castle B. *J Med Genet* 2005;42:e42. [PubMed: 15994867]
28. Houvras Y, et al. *J Biol Chem* 2000;275:36230. [PubMed: 10945975]
29. Gao B, et al. *FEBS Lett* 2001;488:179. [PubMed: 11163768]
30. Dubrovskaya A, et al. *Oncogene* 2005;24:2289. [PubMed: 15735739]
31. Garcia-Campmany L, Marti E. *Development* 2007;134:65. [PubMed: 17138664]
32. Govek EE, Newey SE, Van Aelst L. *Genes Dev* 2005;19:1. [PubMed: 15630019]
33. Ziv NE, Smith SJ. *Neuron* 1996;17:91. [PubMed: 8755481]
34. Alessi DR, et al. *EMBO J* 1996;15:6541. [PubMed: 8978681]
35. Lang D, Powell SK, Plummer RS, Young KP, Ruggeri BA. *Biochem Pharmacol* 2007;73:1. [PubMed: 16904651]
36. Yan Q, et al. *J Biol Chem* 2007;282:13954. [PubMed: 17374606]
37. Shen-Orr SS, Milo R, Mangan S, Alon U. *Nat Genet* 2002;31:64. [PubMed: 11967538]
38. Miralem T, Avraham HK. *Mol Cell Biol* 2003;23:579. [PubMed: 12509456]

Fig. 1.

Identification of positive and negative regulators of CB1R-induced neurite outgrowth. **(A)** Arrays of transcription factor activation in Neuro2A cells treated with DMSO as a control or 2 μ M HU-210 (CB1R agonist) for 20 min. The right panel highlights several of the activated transcription factors. The colors in the panel correspond to the circled spots in the arrays. TCF1, T cell factor 1. **(B)** Effects of transcription factor inhibition on neurite outgrowth. Neuro2A cells were transfected with the indicated siRNAs or transfected with DN Stat3 constructs [Y $\rightarrow$ F and DNA-binding domain (DBD)], DN CREB, wild-type c-Myb, or pcDNA3 (see fig. S11 for construct expression) and then stimulated with HU-210 to induce neurite outgrowth. Error bars, mean $\pm$ SEM ($n = 3$ independent experiments); *, $P < 0.05$ (statistically significant difference by Student's t test) versus the control Luc siRNA; **, $P < 0.05$ (Student's t test) versus control pcDNA3. The figure is a composite of multiple experiments. The siRNA transfections were performed as two experimental sets. Set 1: Luc, AP-2, PAX6, c-Myb, and USF1. Set 2: Luc, NR3C1, Smad3, RAR α , CEBP α , NFYA, and SPI1. Transfections of each DN construct were first done independently and then repeated as one experimental set. Depletion of transcription factor expression was confirmed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) or immunoblot (fig. S12).

Fig. 2. Construction of networks and identification of BRCA1 and a PI3K-AKT-PAX6 pathway as regulators of CB1R-induced neurite outgrowth. **(A)** Eight mammalian protein-protein interaction databases and one signaling network were consolidated into a single network made of 67,379 human protein-protein and protein-ligand interactions (I). This network was filtered by removing interactions from research articles that reported more than three interactions. The lists of activated and nonactivated transcription factors (TFs) at 20 min were used as input nodes to find direct and neighboring interactions and to identify paths from the CB1R receptor to the transcription factors (II), enabling us to identify and rank regulators within the network (III). **(B)** A subnetwork created by finding the shortest paths of a maximum of seven steps from

the HU-210 node (HU) to the 23 activated transcription factors (orange nodes) at 20 min. (I) Paths were found for 17 out of the 23 factors. BRCA1, PI3K, and AKT1 are highlighted (green nodes). HU and CB1R nodes are highlighted in blue. (II) Pathway connecting CB1R to PAX6 through PI3K and AKT1 (edited manually after literature review). (III) Table showing the ranking of components in pathways detected in a control subnetwork (fig. S4 and table S3) versus the activated subnetwork using the ranking method described in the supporting online material (SOM) (7). I, II, and III in (B) correspond to I, II, and III in (A), respectively. CREBBP, CREB-binding protein; PIK3CA, PIK3 catalytic, alpha; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate. (C) Subnetwork created by finding the shortest paths of a maximum of two steps between the 23 activated transcription factors. Nineteen of the factors were connected using this method (orange nodes). A binomial proportions test was used to prune out most of the less important intermediates. BRCA1 is highlighted in green.

Fig. 3.

Regulation of CB1R-induced neurite outgrowth by BRCA1. (A) Effect of BRCA1 siRNA on cannabinoid-induced neurite outgrowth. Neuro2A cells were transfected with Luc siRNA or BRCA1 siRNA and stimulated with 2 μ M HU-210 to induce neurite outgrowth or with DMSO as a control. Amounts of neurite outgrowth in cells exposed to Luc siRNA and HU-210 were normalized to 1, and baseline amounts of neurite outgrowth were normalized to 0 (7). Treatment of cells with an SC siRNA (SC siRNA HU) resulted in similar amounts of cannabinoid-induced neurite outgrowth as Luc siRNA (Luc siRNA HU). Error bars, mean $\pm$ SEM ($n = 3$ independent experiments), *, $P < 0.01$ (student's t test) versus Luc siRNA HU-210 control. (B) Regulation of Stat3 localization. Neuro2A cells were transfected with Luc siRNA or BRCA1 siRNA and

treated with DMSO or HU-210 for 20 min. Cells were fixed, permeablized, and stained with Stat3 antibodies. Purple and yellow arrows indicate cytosolic and nuclear Stat3 localization, respectively. Scale bars indicate distance in micrometers. Nuclei were visualized with Hoescht stain. (C) Decreased BRCA1 expression in response to CB1R stimulation. Neuro2A cells were stimulated with HU-210 and RNA was isolated at the indicated times. Quantitative real-time RT-PCR was performed as described in the SOM (7). Error bars, mean $\pm$ SEM ($n = 4$ independent experiments); *, $P < 0.01$ (Student's t test) versus 0 min control.

Fig. 4.

Regulation of neuronal differentiation by BRCA1. **(A)** BRCA1 regulates neurite outgrowth in rat primary hippocampal neuron cultures. Hippocampal cultures were transfected with Luc siRNA or BRCA1 siRNA after plating and adhesion. Cells were fixed 30 hours after transfection. The mean number of processes per cell in each field was analyzed morphometrically. The mean number of processes for neurons treated with Luc siRNA was normalized to 1. Error bars, mean $\pm$ SEM ($n = 10$ wells of four fields per well for each experiment set); *, $P < 0.05$ (Student's t test) versus Luc siRNA control. **(B)** BRCA1 regulates synaptic density in hippocampal neuron cultures. Neurons were transfected with Luc siRNA or BRCA1 siRNA using NeuroPORTER after 3 days in cultures. Four days later, cells were fixed and stained with synaptophysin (red) and β -tubulin (blue) antibodies. Yellow arrows denote synaptophysin puncta. Quantification is shown in fig. S7B.

Fig. 5.

Effects of PI3K-Akt signaling to PAX6 on CB1R-induced neurite outgrowth. **(A)** Phosphorylation of Akt. Neuro2A cells were stimulated with 2 μ M HU-210 for the indicated times in the absence (-LY) or presence of (+LY) the PI3K inhibitor LY. Cells were lysed and immunoblot analysis was performed with antibodies to phospho Akt (pAkt) or total Akt. pAkt levels were normalized to total Akt. Error bars, mean $\pm$ SEM ($n = 3$ independent experiments); *, $P < 0.05$ (Student's t test) versus 0 min control. **(B)** Effects of pharmacological inhibitors on neurite outgrowth. Neuro2A cells were either treated with the indicated inhibitors or transfected with DN CREB and then stimulated with HU-210 to induce neurite outgrowth. Error bars, mean $\pm$ SEM ($n = 4$ independent experiments for PD, $n = 3$ for all others); *, $P < 0.02$ (Student's t test) versus DMSO control; **, $P < 0.01$ (Student's t test) versus pcDNA3 control. The figure is a composite of multiple experiments. DN CREB + LY was performed with the other DNA transfections in Fig. 1B. **(C)** Gel shift assay of PAX6 binding. Neuro2A cells were stimulated with 2 μ M HU-210 for the indicated times in the absence (-LY) or presence (+LY) of LY. Nuclear extracts were prepared and gel shift assays were performed with oligonucleotides containing consensus-binding sites for PAX6. **(D)** PAX6 phosphorylation in rat primary hippocampal neuron cultures. Hippocampal cultures were stimulated with 1 μ M HU-210 for the indicated times in the absence (-LY) or presence (+LY) of 10 μ M LY 294002. Cells were lysed, and immunoprecipitations were performed with rabbit antibodies to PAX6 or rabbit immunoglobulin G as a control. Immunoblot analysis was performed with mouse antibodies directed against phospho-Thr. Phospho-Thr levels were normalized to PAX6 after stripping and re-probing the blots with PAX6 antibodies. Values were averaged from two independent experiments. **(E)** Simple schematic of signal flow through Src, MAPK, and PI3K during neurite outgrowth. CB1R stimulation by HU-210 (HU) activates the α subunits of G_i and G_o ($\alpha_{i/o}$) and leads to activation of Stat3 through the kinase Src. BRCA1 is depicted in blue. The putative interaction between BRCA1 and Akt is shaded gray (38).

Fig. 6.

Integration of activated transcription factors with the upstream signaling network during CB1R-induced neurite outgrowth. **(A)** Pharmacological inhibition of transcription factor activation during CB1R stimulation. The Venn diagram (data from fig. S2B) shows the inhibition of transcription factor activation by the PI3K inhibitor LY (blue), the MAPK pathway inhibitor PD (yellow), and the Src inhibitor PP2 (magenta). **(B)** Proposed decision logic for cell-state change during CB1R-stimulated neurite outgrowth. A set of three pOR gates connects the alpha subunits of G_i and G_o ($G\alpha_{i/o}$ for clarity) and the $G\beta\gamma$ subunit to the kinases PI3K, Src, and MAPK. This study's results and the experimental literature (table S6) suggest that these gates are not true OR gates and are thus represented as pOR gates (see fig. S13 for details). Stacked below are three AND gates that connect the kinases to the transcription factors. The components and connections are in black. The gray arrows and gate symbols are in gray to denote information flow and the abstract nature of the pOR and AND gates.