Nerve growth factor scales endocannabinoid signaling by regulating monoacylglycerol lipase turnover in developing cholinergic neurons

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Endocannabinoid, particularly 2-arachidonoyl glycerol (2-AG), signaling has recently emerged as a molecular determinant of neuronal migration and synapse formation during cortical development. However, the cell type specificity and molecular regulation of spatially and temporally confined morphogenetic 2-AG signals remain unexplored. Here, we demonstrate that genetic and pharmacological manipulation of CB1 cannabinoid receptors permanently alters cholinergic projection neuron identity and hippocampal innervation. We show that nerve growth factor (NGF), implicated in the morphogenesis and survival of cholinergic projection neurons, dose-dependently and coordinately regulates the molecular machinery for 2-AG signaling via tropomyosine kinase A receptors in vitro. In doing so, NGF limits the sorting of monoacylglycerol lipase (MGL), rate limiting 2-AG bioavailability, to proximal neurites, allowing cell-autonomous 2-AG signaling at CB1 cannabinoid receptors to persist at atypical locations to induce superphrenic neurite extension. We find that NGF controls MGL degradation in vitro and in vivo and identify the E3 ubiquitin ligase activity of breast cancer type 1 susceptibility protein (BRCA1) as a candidate facilitating MGL’s elimination from motile neurite segments, including growth cones. BRCA1 inactivation by cisplatin or genetically can rescue and reposition MGL, arresting NGF-induced growth responses. These data indicate that NGF can orchestrate endocannabinoid signaling to promote cholinergic differentiation and implicate BRCA1 in determining neuronal morphology.

Results and Discussion

CB1 Requirement of Cholinergic Projection Neuron Development. We have recently shown that endocannabinoid-mediated axonal growth and guidance requires the precisely ordered molecular assembly of 2-AG signaling networks during corticogenesis (2, 16). Here, we defined, by in situ hybridization, that CB1 mRNA was present in cholinergic territories, particularly the medial septum (MS) (Fig. 1 A–A′ and Fig. S1 A–A′) from embryonic day (E) 14.5 until birth in mice. We found CB1Rs perisomatically in and distributed along the processes of bipolar genetically tagged choline acetyltransferase (ChAT) neurons (Fig. 1 B–B′), reminiscent of migrating CB1;R γ-aminobutyric acid-containing interneurons (17). In addition, CB1Rs were localized to cholinergic


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axons and their putative growth cones traversing the hippocampus (Fig. 1C–D) and the corpus callosum (Fig. S1E). 

If endocannabinoid signaling at CB1Rs impacts cholinergic cell identity and differentiation, then their pharmacological or genetic disruption might impair the developmental organization of the cholinergic basal forebrain. In CB1R−/− fetuses, we observed ectopic localization of cholinergic neurons with a projection-like neurochemical makeup, coexpressing ChAT, the low-affinity neurotrophin receptor p75 (p75NTR), and the vesicular acetylcholine transporter (VACHT) (18), in the fetal dorsolateral striatum, which otherwise lacked p75NTR+ cells in wild-type littermates (5) (Fig. 1B–E and Fig. S2A–B). CB1R antagonism by AM 251 during pregnancy recapitulated genetic loss of function (Fig. 1E and Fig. S2B and C). Ectopic p75NTR+/ChAT+ neuron density is likely an underestimate because our analysis coincided with the expression onset of both markers. 

Loss of CB1R function disrupts neuronal morphogenesis (2, 16). Therefore, we asked whether neurite complexity of cholinergic neurons routing normally to the fetal basal forebrain is impaired upon manipulating CB1R. Sholl analysis of septal ChAT+ neurons (E18.5) demonstrated significantly increased numbers of neurites emanating from cholinergic somata in disrupted CB1R function (G and G′) Quantitative densitometry of ChAT+ cholinergic neurons at select mediolateral coordinates in the striatum of adult CB1R−/− and wild-type mice. Surplus cholinergic neurons accumulated in the dorsal striatum. (H–I) The density of ChAT+ profiles in stratum pyramidale of the CA1 subfield was significantly reduced in CB1R−/− animals relative to wild-type littermates. Pyr, stratum pyramidale; Rad, stratum radiatum. (J) Reduced density of MGL+ profiles in the stratum pyramidale of hippocampal CA1 in CB1R−/− animals relative to wild-type controls. Data were expressed as means ± SEM. *P < 0.01; **P < 0.05. (Scale bars: E, 200 μm; A1 and A2, 100 μm; B, D, and F, 10 μm; H, 5 μm; B2, B3, C, C′, and D2, 3 μm.)
subfield (CA1) (Fig. 1 F–H2 and Fig. S2E) coincident with significantly reduced MGL immunoreactivity in putative pre-synapses of the same layer in CB1R−/− mice (Fig. 1F and Fig. S2F–G). Thus, our data imply that 2-AG signaling at CB1Rs contributes to defining the neurochemical specificity, final positions, morphology, and connectivity of basal forebrain cholinergic neurons.

Cell-Autonomous 2-AG Signaling in Fetal Cholinergic Neurons. CB1R expression is spatially and temporally coordinated with 2-AG synthesis and degradation in the developing cerebrum (2). DAGLa, producing 2-AG (12) (Fig. 2A and A1 and Fig. S1J), and MGL (Fig. 2 B and B1) were localized to the perikarya and processes of cholinergic projection neurons by E18.5. Cholinergic neurons isolated from the fetal basal forebrain retained the expression of VACHT, CB1Rs, and 2-AG metabolizing enzymes in vitro (Fig. 2C–C1). DAGLaβ and MGL were differentially targeted along the developing VACHT+ primary neurite (Fig. 2C2), the prospective axon (18). MGL was restricted to the proximal neurite stem. In contrast, CB1R and DAGLaβ partitioned to the distal neurite, suggesting the spatial confinement of 2-AG signaling to the motile neurite segment including the growth cone (Fig. 2C3). Accordingly, agonist-induced CB1R activity facilitated neurite outgrowth and inhibited growth cone differentiation (16) (Fig. 2D). These data suggest the dominance of cell-autonomous 2-AG signaling during cholinergic development.

NGF Regulates 2-AG Signaling. Endocannabinoids are unlikely to function as a solitary signaling system to define cholinergic morphology and connectivity, particularly because CB1R deletion does not arrest cholinergic differentiation or survival. In contrast, NGF is required for cholinergic projection neurons to survive, reach morphological and neurochemical maturity, and establish and maintain axonal projections (9). We hypothesized that NGF signaling might use endocannabinoids and that the coordinated action of neurotrophin and 2-AG signals could determine cholinergic morphology, particularly axonal complexity. Coincident targeting of TrkA and CB1Rs to cholinergic growth cones identified a subcellular platform for molecular interactions (Fig. 3 A and A1). NGF [2–4 d in vitro (DIV)] (10) increased neurite outgrowth and enhanced the formation of multiple VACHT/Tau-2/collapsin response mediator protein 2 (CRMP-2)+ processes (Fig. 3 B1 and C and Fig. S3 A and B), suggesting that NGF alters neuronal polarity (and probably induces a multi-axonal phenotype) on the expense of the elongation of the primary VACHT+ neurite (Fig. 3B2) but not cholinergic commitment or survival (Fig. S3C).

If CB1Rs escape desensitization and 2-AG signaling remains nonsaturated and dynamic upon NGF treatment, then pharmacological manipulation of CB1Rs might modify the ensuing cholinergic phenotype. Accordingly, WIN55,212-2, a CB1R agonist (16), induced neurite outgrowth (Fig. 3B), reinstated VACHT+ neurite identity (Fig. 3B1), and extended the VACHT+ neurite (the quiescent axon) in NGF-treated cholinergic neurons (Fig. 3B1 and Fig. S3E) while maintaining cholinergic growth cones in undifferentiated, motile states (Fig. S3D). Next, we tested whether NGF-induced neurite outgrowth requires CB1R activation. O-2050, a silent CB1R antagonist (2), occluded NGF-induced morphogenesis, being particularly potent to reduce the outgrowth and number of VACHT+ neurites (Fig. 3 B and C and Fig. S3E). Similarly, DAGL inhibition by O-3841 (SI Text) arrested cholinergic neuritogenesis (Fig. S3 F and G).

Cholinergic neurons responded to NGF by up-regulating CB1R, DAGLa, and MGL protein levels in a time-dependent (Fig. S4A and B) and dose-dependent (Fig. 3D) fashion, resulting in increased intracellular 2-AG concentrations (Fig. 3E). NGF induced MGL (Fig. 3F) and CB1R (Fig. S4C) accumulation at atypical locations in the proximal stem of multiple neurites. Tyrophostin (AG 879), an inhibitor of TrkA signaling (19), acutely reduced neurite outgrowth (Fig. 3G) and coincidently allowed MGL to venture into the actin-rich motile neurite tip (Fig. 3G1 and G2 and Fig. S4E). This suggests that TrkA activation can adjust the length of 2-AG–responsive neurite segments by regulating MGL availability. Cumulatively, we interpret these findings that NGF alters the morphological complexity of cholinergic neurons by
NGF Affects MGL Stability in Cholinergic Neurons. NGF affects the differentiation and connectivity of postnatal cholinergic neurons via TrkA/extracellular signal-regulated kinase (Erk) signaling (9). Here, we used a pharmacological approach to dissect NGF’s receptor requirements and downstream signaling inducing 2-AG signaling in fetal cholinergic neurons in vitro. Inhibition of TrkA phosphorylation by K252a eliminated the NGF-induced coordinated increase of DAG1α, CB1Rs, and MGL expression, verifying TrkA involvement (Fig. 4A). In contrast, p75NTR blockade limited cholinergic survival, confirming that p75NTR is a “dependence” or survival) receptor in this cell type (20). We found that Erk inhibition (PD98059) reduced NGF-induced DAG1α and CB1Rs but not MGL protein levels, confirming neurotrophin-induced Erk signaling in cholinergic neurons (9). However, inhibition of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B pathway associated with endocannabinoid-induced neurite outgrowth (21), but not the Src or phospholipase C pathways, eliminated NGF-dependent protein, particularly MGL expression (Fig. 4A). This finding is significant because MGL activity in growth domains can limit neurite outgrowth (2).

Next, we explored the molecular mechanism by which NGF regulates MGL protein levels. We excluded NGF-dependent induction of MGL transcription by quantitative PCR (Fig. 4B), using early growth response protein (Egr)1 as positive control (22) (Fig. 4B; see Fig. S5A–A2 for CB1Rs and DAG1α/β). These findings raise the possibility of an NGF-induced increase in the translation efficacy of MGL (Mgll) mRNAs. We tested this hypothesis in pheochromocytoma cell line 12 (PC12) cells, which respond to NGF by increased Egr1 mRNA (Fig. 4C) and MGL protein expression (Fig. S5B), recapitulating cholinergic responsiveness. By using absolute PCR combined with sucrose gradient fractionation of mRNAs bound to polysomes, we found that NGF affected neither the copy number nor the recruitment of MGL (Mgll) mRNAs to free ribosomes or polysomes (Fig. 4D–D2).

In vitro data suggest that MGL’s proteasomal degradation (2) may be a candidate mechanism to facilitate focal 2-AG signaling in CB1Rs cholinergic growth cones. Therefore, we sought to determine whether NGF posttranslationally controls MGL in vivo. Because available genetic tools favor the molecular dissection of NGF effects on cholinergic neurons during postnatal life (9), we used presymptomatic AD11 mice (≤3 mo of age) that express an NGF-neutralizing antibody (7) to assess NGF’s effects on MGL mRNA and protein when NGF withdrawal is yet to significantly disrupt cholinergic neurotransmission (Fig. 5C). By combining genome-wide microarray analysis and comparative mRNA profiling, we found that NGF deprivation significantly reduced CB1Rs and DAG1α but not MGL mRNA levels in the basal forebrain (Fig. 4E). In contrast, Western blotting demonstrated the robust loss of MGL protein (Fig. 5C). Quantitative morphometry revealed the loss of MGL+ presynapses in the cerebral cortex (Fig. 4F–F2) and basal forebrain (Fig. 5D1 and D2) in AD11 mice relative to wild-type controls, confirming that NGF regulates MGL protein but not mRNA levels in vivo.

BRCA1 Is Expressed in Cholinergic Neurons: Implications for MGL Turnover. Inhibiting the proteasome by lactacystin stabilized MGL in cholinergic neurons (Fig. 4D). Therefore, we hypothesized that NGF could alter MGL protein turnover by a mechanism operating focally in cholinergic growth cones (Fig. 5A). BRCA1 possesses E3 ubiquitin ligase activity (15), accumulates at leading edges in migrating cells (23), and is expressed during brain development (24), particularly in proliferative zones (Fig. 5F). Therefore, we tested whether BRCA1 is a candidate ubiquitin ligase to destine MGL toward proteasomal degradation in cholinergic neurites. We detected BRCA1 in the basal forebrain (Fig. 5C–D2), with BRCA1 being particularly noticeable in leading processes of cholinergic neurons (Fig. 5E–F2).

Next, we addressed whether NGF regulates BRCA1 expression and whether altered BRCA1 levels correlate with those of MGL.
NGF progressively induced BRCA1 mRNA expression in cultured cholinergic neurons (Fig. 5G) and PC12 cells (Fig. S6A). In AD11 mice, BRCA1 levels were slightly diminished (Fig. S5C), suggesting that reduced BRCA1 expression is sufficient to underpin physiological BRCA1 functions.

BRCA1’s subcellular distribution in cholinergic neurites, particularly in motile filopodia (Fig. 5 H–I2 and Fig. S6 B and B1), is mutually exclusive with MGL, suggesting that BRCA1 may contribute to regulating MGL turnover. We tested this possibility by exposing basal forebrain neurons to cisplatin (15). We show that inhibition of BRCA1’s ubiquitin ligase activity by the platinum-based anticancer drug (15) stabilized MGL in growth cones under control conditions (Fig. 5 I and I1). Moreover, cisplatin limited neurite outgrowth from NGF-treated cholinergic neurons (Fig. 5 I and I1) and PC12 cells (Fig. S6 D and D1) by reinstating MGL’s subcellular distribution (Fig. 5J). We validated these observations by showing MGL stabilization upon siRNA-mediated BRCA1 silencing in growth cones of basal forebrain neurons (Fig. 5 J–J2 and Fig. S6 E and E1), as well as the SH-SYSY neuroblastoma cell line (Fig. S6F). Collectively, our findings highlight a mechanism coupling NGF signaling at TrkA receptors to neurite outgrowth via sequential regulation of BRCA1 and MGL in cholinergic neurons.
Conclusions
The contribution of endocannabinoid signaling to the formation of neuronal networks in the developing forebrain is increasingly appreciated (2, 4, 16). Available models implicate neurotrophin-induced Ca^{2+} increase as an upstream signal activating 2-AG generation (25) and emphasize DAGLx requirements of the initiation of neurite outgrowth (13). Our study provides an alternative to this “DAGL centric” view, favoring positional enzymatic ligand inactivation as a means of rate-limiting temporal and spatial endocannabinoid availability during developmental processes.

Although endocannabinoids are increasingly recognized for their impact on synapse structure and function (1, 16), the upstream regulation of endocannabinoid metabolism and signaling at CB_{1}Rs remains poorly understood. Our observations, together with the proposed regulation of BRCA1 by CB_{1}R activity (21, 26), highlight BRCA1 as a molecular “hinge” to integrate coexistent permissive signals and outline a bidirectional signaling loop that emerges as a key component of neuronal differentiation and synaptic coupling (21). Identifying a regulatory pathway involving TrkA and CB_{1}R coincidently modulating BRCA1 acting as a “feedback amplifier” is broadly relevant to the orchestration of neurotrophin/endocannabinoid interplay in cancer (19, 26), pain (27), or neurodegeneration (1, 7). Understanding developmental mechanisms that allow the integration of specific neuronal subtypes into large-scale neuronal networks may be rewarding to devise translational restorative strategies aimed to prevent disease-related modifications of synaptic connectivity. Our evidence that CB_{1}R-mediated 2-AG signals convert NGF-induced axonal sprouting into a regulated growth process in cholinergic neurons reconciles the decade-long dilemma on NGF’s ineffectiveness in promoting the growth of cholinergic grafts, even when exogenous sources of NGF are present. Recognizing the role of NGF on secondary signaling systems to exert coordinated and directional growth presents possibilities for maintaining effective cholinergic neurotransmission under disease conditions.

Materials and Methods
Mice and Drug Treatment. Tissues from wild-type and transgenic mice were characterized and processed as described (2). AM 251 was administered at a dose of 3 mg/kg, with male embryos harvested on E18.5 (SI Materials and Methods).

Histo- and Cytochemistry. Multiple immunofluorescence labeling of fetal mouse brains, cultured neurons, and PC12 cells was performed by applying select mixtures of affinity-purified antibodies (Fig. S1 and Table S1). In situ hybridization was carried out by using digoxigenin-labeled riboprobes (16). Images were acquired on a Zeiss 710LSM confocal laser-scanning microscope (2) (SI Materials and Methods).

mRNA Detection and Protein Biochemistry. Gene expression profiling was done using the two-color protocol by Agilent with reference experimental design. Quantitative PCRs were performed on a Bio-Rad MyQ thermal cycler (2) using primer sets listed in Table S2. Polyribosyme profiling in PC12 cells was on linear sucrose gradients (15–50%). Basal forebrain neurons or SH-SY5Y human neuroblastoma cells were transfected with either non-targeting (scrambled) siRNA or a pool of BRCA1-specific siRNAs (21). Protein samples from basal forebrain, cultured neurons after treatment (Table S3), and immortalized cells were analyzed under denaturing conditions. Antibodies used for Western blotting are listed in Table S1.

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