

Neuropeptide orphanin FQ inhibits dendritic morphogenesis through activation of RhoA

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Neuropeptide Orphanin FQ Inhibits Dendritic Morphogenesis through Activation of RhoA

Running title: OFQ inhibits dendrite growth via RhoA

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) plays a facilitatory role in neuronal development and promotion of differentiation. Mechanisms that oppose BDNF's stimulatory effects create balance and regulate dendritic growth. However, these mechanisms have not been studied. We have focused our studies on the BDNF-induced neuropeptide OrphaninFQ/Nociceptin (OFQ); while BDNF is known to enhance synaptic activity, OFQ has opposite effects on activity, learning and memory. We have now examined whether OFQ provides a balance to the stimulatory effects of BDNF on neuronal differentiation in the hippocampus. Golgi staining in OFQ knockout (KO) mice revealed an increase in primary dendrite length as well as spine density, suggesting that endogenous OFQ inhibits dendritic morphology. We have also used cultured hippocampal neurons to demonstrate that exogenous OFQ has an inhibitory effect on dendritic growth and that the neuropeptide alters the response to BDNF when pre-administered. To determine if BDNF and OFQ act in a feedback loop, we inhibited the actions of the BDNF and OFQ receptors, TrkB and NOP using ANA-12 and NOP KO mice respectively but our data suggests that the two factors do not act in a negative feedback loop. We found that the inhibition of dendritic morphology induced by OFQ is via enhanced RhoA activity. Finally, we have evidence that RhoA activation is required for the inhibitory effects of OFQ on dendritic morphology. Our results reveal basic mechanisms by which neurons not only regulate the formation of proper dendritic growth during development but also control plasticity in the mature nervous system.

Key words: brain-derived neurotrophic factor, orphanin FQ/nociceptin, neuropeptide, dendrite, RhoA

INTRODUCTION

Synaptic homeostasis results from a fine balance of stimulatory and inhibitory mechanisms that allow neurons to maintain stable function during periods of perturbation such as developmental and activity-dependent alterations in synapse number or strength (Turrigiano, 2012). These changes in synaptic function are associated with morphological modifications. Dendritic outgrowth and branching can be elaborated during development as well as by activity and learning (Horch, 2004). The ultimate morphology of a neuron is determined by transcriptional codes, which define the competence of cells to respond to extrinsic cues. Extrinsic factors implicated in dendritic branching include membrane bound as well as soluble compounds such as neurotrophins (Landgraf and Evers, 2005).

BDNF is a neurotrophin that is highly expressed in the hippocampus in an activity dependent manner (Isackson et al., 1991; Lindholm et al., 1994; Berninger et al., 1995; West et al., 2001). BDNF has been implicated in promoting synaptic plasticity and long-term potentiation (LTP) as well as the processes of dendritic branching, spine development, and functional maturation of synapses of

hippocampal neurons (Park and Poo, 2013); (Cohen-Cory, 2002). BDNF affects the dendritic branching of several different cell types (McAllister et al., 1995; Patel and McNamara, 1995; Vicario-Abejon et al., 1998; Labelle and Leclerc, 2000; Danzer et al., 2002; Jin et al., 2003; Wirth et al., 2003; Alonso et al., 2004; Ji et al., 2005; Woo et al., 2005). However, BDNF increases the number of primary dendrites in an activity dependent manner through receptor TrkB activation only in pyramidal and not non-pyramidal cells (McAllister et al., 1995; McAllister et al., 1996; Horch et al., 1999). Several signaling molecules downstream of TrkB have been implicated in the regulation of dendritic size and complexity by BDNF including, MAPK, Ras-PI3K-Akt (Dijkhuizen and Ghosh, 2005; Kumar et al., 2005) and Cyclin-dependent kinase 5 (Cdk5) (Cheung et al., 2007). A few studies have examined factors, which counter the effects of BDNF on dendritic growth. In cortical pyramidal cells, endogenous BDNF and neurotrophin 3 (NT3) oppose each other's actions on regulating dendritic growth in a layer specific manner (McAllister et al., 1995). In addition, a recent report has shown an opposing role of proBDNF to mature BDNF in neurite outgrowth via activation of the Rho pathway in DRG and cortical neurons (Sun et al., 2012). However, soluble factors that oppose BDNF in the hippocampus have not been characterized. In our previous work, we identified genes induced by BDNF treatment of hippocampal neurons including, the neuropeptide PreproOrphaninFQ/Nociceptin (preproOFQ) (Ring et al., 2006), an inhibitory peptide with opposing actions of BDNF.

PreproOFQ is translated into a precursor protein that is processed into four smaller peptides (OFQ, Nocistatin, PNP-4 and PNP-5) (Okuda-Ashitaka et al., 1998). OFQ expression is activated by cAMP via CRE (Zaveri et al., 2006) which is one mechanism by which BDNF regulates transcription (Calella et al., 2007). Heptadecapeptide OFQ is an endogenous ligand for the opioid receptor-like 1 (NOP) receptor with no appreciable affinity for the classical opioid receptors (Mollereau and Mouledous, 2000; Heinricher, 2003). Immunohistochemical, in situ hybridization and autoradiography studies of brain tissue demonstrate that OFQ ligand and the NOP receptor are expressed throughout the hippocampus (Manabe et al., 1998; Neal et al., 1999; Houtani et al., 2000). Specifically, in adult tissue, NOP receptor protein antibody revealed expression in the CA1-CA4 fields and the dentate gyrus, primarily in the fiber processes, and some cell bodies in the hilus of the dentate (Anton et al., 1996). Two groups have used NOP knockout mice with a β -galactoside reporter to confirm that NOP is in the pyramidal cells of the CA1-3 regions of the hippocampus and in the granule cells in a septotemporal gradient (Houtani et al., 2000; Manabe et al., 1998). Prepro-OFQ mRNA and protein are highest in the granule cell layer of the dentate gyrus, with lower levels in the molecular layer, and the polymorph layer. The mRNA and protein for the ligand are also in the stratum pyramidal of CA1 and CA3 with lower levels in CA2 (Neal et al., 1999). Finally, the mRNA for both NOP and prepro-OFQ are expressed in the mantle zone embryonically (Ikeda et al., 1998). These reports suggest that OFQ and NOP play roles in both the developing and

mature hippocampus, particularly in the CA1, CA3 pyramidal cells and dentate gyrus granule cells.

OFQ has been implicated in a variety of CNS-mediated behaviors including pain modulation, synaptic activity and learning, anxiety, locomotion, food intake and drug addiction (Zaveri, 2003). Many studies indicate that OFQ is inhibitory to LTP in the CA1, CA3 and dentate gyrus as well as learning and memory paradigms as shown by infusion of OFQ antagonists and KO mice (Sandin et al., 1997; Yu et al., 1997; Manabe et al., 1998; Yu and Xie, 1998; Wei and Xie, 1999; Noda et al., 2000). One study suggests that OFQ is a synaptically released endogenous inhibitor of LTP (Bongsebandhu-phubhakdi and Manabe, 2007). It is intriguing that a neuropeptide highly induced by BDNF has contrary effects to the neurotrophin on synaptic activity. Although the effect of OFQ on synaptic activity has been well studied, the role the neuropeptide and its receptor play in morphological plasticity has not been examined.

In this study we explore the effect of the BDNF-induced neuropeptide OFQ on dendritic morphology and test the hypothesis that the neuropeptide has opposing effects of BDNF. We find that mice lacking OFQ have exuberant dendrites and spines, suggesting that OFQ is inhibitory to dendritic morphogenesis. Furthermore, OFQ is able to attenuate the actions of BDNF on dendritic outgrowth *in vitro*. We demonstrate that the two soluble factors do not work through a negative feedback loop but rather act upon dendrites independently.

OFQ appears to activate the small GTPase Ras homologous member A (RhoA), which is known to inhibit neurite outgrowth (Koh, 2006), and our data indicate that RhoA is necessary for the effects of OFQ on neuronal morphology. Thus the inhibitory actions of OFQ on electrophysiological parameters in hippocampal neurons may be accompanied by structural alterations that render the effects of OFQ long-lasting.

METHODS

Mouse strains and sources

Nociceptin/Orphanin FQ peptide (OFQ) knockout (Kest et al., 2001), Nociceptin/OFQ receptor (NOP) knockout (Clarke et al., 2001) and wildtype (WT) mice, all 129S, were a gift from Dr. John Pintar. Adult (8-12 week) males were used for Golgi studies and fetuses obtained from timed mated females were used for hippocampal cell cultures.

Neuropeptides and Inhibitors

OFQ was obtained from Phoenix Pharmaceuticals (Burlingame, CA) and BDNF from Peprtech (Rocky Hill, NJ). BDNF was used at 25 or 50 ng/ml based on our previous studies (Thakker-Varia et al., 2001). Both concentrations of BDNF have been shown to have similar effects on dendrite growth (Sokolowski, 1997). OFQ was used at 3 μ M (Ring et al., 2006). Naloxone benzoylhydrazone was used at 10 μ M (Sigma, St. Louis, MO) to inhibit NOP receptors. ANA12 was used at 10 μ M (Sigma, St. Louis, MO) to inhibit TrkB receptors (Cazorla et al., 2011) with a 30

min pretreatment prior to addition of OFQ from 7-10 days *in vitro* (div). **KCl was used at 20mM to induce depolarization (Wayman et al., 2006) prior to addition of OFQ.** C3 Transferase (Cytoskeleton, Inc., Denver, CO) was used at 0.3 μ g/ μ l to inhibit RhoA activation. Cells were pretreated with C3 Transferase for 4hr prior to treatment with OFQ (3 μ M) from 7-10 div.

Golgi staining procedure

Protocol for Golgi staining followed manufacturers recommendations (FD Rapid GolgiStain kit, FD NeuroTechnologies, Ellicott City, MD). Briefly, tissue samples were exposed to impregnation solution (A + B) for 3 weeks in the dark at room temperature. They were then transferred to solution C for 48 hours at 4°C. Brains were rapidly frozen in isopentane and coronal sections 80 μ m thick through the entire hippocampus were cut on a cryostat and mounted onto gelatin-coated slides. The slides were developed by rinsing in distilled water followed by incubation in 1:1:2 mixture of Solutions D and E and water. The sections were then dehydrated in a series of graded ethanol and HistoClear (National Diagnostics, Charlotte, NC) before coverslipping. Quantification of dendritic morphology was performed using a camera lucida drawing tube and BioQuant software. All analyses were done blind to the observer. Study inclusion criteria for individual brains were well-impregnated neurons with no evidence of incomplete or artificial staining and nondescript precipitate did not obscure neuron or branches. Neurons were selected without regard to the number of tertiary and quaternary branches with cut-ends to prevent bias toward

neurons with smaller dendritic arbors. Selected dendrites of CA1 pyramidal neurons that were analyzed met the following criteria: 1) labeled cells were located in the sections with hippocampal formation containing CA1 and dentate gyrus; 2) the dendritic segments and spines were completely filled; 3) to capture the entire dendritic arbor, images were taken at multiple focal planes. **Protrusions were always analyzed on secondary dendrites which are variable distances from the soma but consistent among the fact that they are the same branch order. Approximately three secondary branches per neuron were used for analysis and 10 μ M segments were scored using Camera Lucida drawing and averaged.**

Hippocampal cell culture

Hippocampi were obtained from time-mated embryonic day 16 (E16) C57BL/6 mice (Hilltop Laboratories, Scottsdale, PA) or E18 Sprague Dawley rats (Charles River, Wilmington, MA) and killed by CO₂ asphyxiation in accordance with institutional guidelines for care and use of animals. Rat embryos were used to obtain neurons for the RhoA GLISA assays due to the large number of embryos required to generate enough cells. Pooled tissue from each litter was mechanically triturated in Eagle's Minimum Essential Medium (MEM) with glucose and 7.5% fetal bovine serum and plated on poly-D-lysine-coated petri dishes at 250,000 cells/35mm dish. Cultures were maintained in serum-free Neurobasal medium containing B27 (Invitrogen, Grand Island, NY) and glutamine (SFM) at 37 °C in a 95% air/5% CO₂ humidified incubator as previously described (Thakker-Varia et al., 2001) and contained virtually pure neurons with

5.05 ± 0.64% astrocyte contamination as assessed by immunocytochemistry for Glial Fibrillary Acidic Protein (GFAP).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and blocked with goat serum prior to incubation in anti Nociceptin/OFQ (1:150 Santa Cruz FL-176, Santa Cruz, CA), anti NOP/KOR-3 (1:50, Santa Cruz H-85, Santa Cruz, CA), PSD-95 (1:200, MA1-045, Pierce, Rockford, IL) or mouse anti-Microtubule-Associated Protein 2 (MAP2) antibodies (1:2000; clone HM-2, Sigma, St. Louis, MO) overnight at 4°C. Controls with no primary antibody added were used to test for specificity of the staining. Cultures were incubated in goat anti-rabbit secondary antibodies or goat anti-mouse secondary antibodies conjugated to AlexaFluor 594 (1:1000, Invitrogen, Grand Island, NY) at RT for 1h. 4',6-diamidino-2-phenylindole (DAPI) (1:1000, Invitrogen, Grand Island, NY) was used to label nuclei. The cells were coverslipped with Fluoromount-G antifade reagent (Southern Biotech, Birmingham, AL) and the images captured on a Zeiss Apotome microscope at either 20X or 40X using Axiovision software.

Dendritic Analysis

Upon completion of imaging, the pictures of neurons were analyzed using either Axiovision 4.5 by tracing the **primary and secondary** dendrites and quantifying with the Measure function or the Bonfire v1.0 program (Kutzing et al., 2010; Langhammer et al., 2010). Briefly for the Bonfire program, ImageJ with the

NeuronJ plugin was used to trace the neurons and define the positions of all dendrites and then MATLAB was used to convert the file into a format that could be read in NeuronStudio where dendritic traces were confirmed. This allowed us to determine the structure of each cell's dendritic arbor in two-dimensional form and convert it in a digital format. MATLAB was then used to export the digitized traces into numerical data for each condition, e.g. number of primary dendrites, number of secondary dendrites etc. The center-out also known as inside-out labeling was chosen to analyze the neurons which allows measurements of dendrites starting at the cell body due to the fact that we were **quantifying only** primary and secondary rather than higher order dendrites.

RhoA Assay

RhoA activation was measured using the G-LISA assay kit (Cytoskeleton, Inc, Denver, CO). Briefly, hippocampal cultures (6 div) cells were incubated in Neurobasal without B27 overnight prior to assay. Cells were then treated with 3 μ M OFQ for 3 hours, followed by harvesting in cell lysis buffer with protease inhibitor cocktail and samples were flash frozen. Protein assay was performed using the Precision Red Advanced Protein Assay reagent and protein levels were normalized. Following treatment with antigen presenting buffer, Anti-RhoA primary antibody (1:250) and secondary antibody horse radish peroxidase (HRP) conjugated (1:62.5) were each applied for 45 min at room temperature. HRP detection was performed for 15 min at 37°C and the reaction was read at 490nm

on a Bio-Tek plate reader. Buffer was used as a blank and samples were analyzed in triplicate.

Statistical Analysis

Power Analysis was performed when necessary to determine the appropriate number of observations. A Power of 0.80 was used as a standard for adequacy.

Statview software was used for statistical analysis of all data. Data were analyzed using two-tailed Student t test or ANOVA followed by Fishers PLSD post hoc test for multiple comparisons. $p < 0.05$ is considered significant.

RESULTS

OFQ Deletion Enhances Dendritic Outgrowth

The effects of deletion of OFQ on dendritic growth were analyzed *in vivo* by performing analysis of adult OFQ knockout (KO) mice using the Golgi-impregnation technique. Increased dendritic arborization in excitatory cells of the granule cells in the hippocampus of OFQ KO mice was observed compared to WT mice (Fig 1A-D,G-H). Specifically, we observed significant increases in the number of primary dendrites (WT 3.00 ± 0.33 , OFQ KO 4.1 ± 0.23 , $n = 10$, $p = 0.015$) and total length of primary dendrites which is the sum of the length of all primary dendrites for a given cell and then averaged for all the cells quantified (WT $90.85 \pm 18.82\mu\text{m}$, OFQ KO $154.41 \pm 23.10\mu\text{m}$, $n = 10$, $p = 0.047$). There was a trend to increased dendritic number (WT 3.60 ± 0.43 , OFQ KO 5.20 ± 0.68 , $n = 10$, $p = 0.062$) and length in secondary dendrites as well but without

significance (WT $195.95 \pm 80.21\mu\text{m}$, OFQ KO $399.04 \pm 80.21\mu\text{m}$, $n = 10$, $p = 0.196$). The average **primary and secondary** dendritic length, which is the sum of dendritic lengths per cell divided by the number of dendrites and then averaged for all cells, showed a trend but no significance for both primary (WT $28.85 \pm 2.95\mu\text{m}$, OFQ KO $36.45 \pm 4.21\mu\text{m}$, $n = 10$, $p = 0.156$) and secondary (WT $44.72 \pm 12.75\mu\text{m}$, OFQ KO $62.43 \pm 6.64\mu\text{m}$, $n = 10$, $p = 0.234$) dendrites. **Therefore, while OFQ KO mice have significantly more primary dendrites than the WT mice and total primary dendritic length is increased, the average primary dendritic length per primary neuron is not significantly altered. These findings indicate, that there are more dendrites rather than longer ones and suggest that OFQ may have a role in inhibiting dendrite initiation rather than in preventing elongation. Therefore for the subsequent experiments, we focused on the number and total length of primary dendrites for all analyses.** The density of protrusions representing spines was also evaluated on the secondary dendritic branches of CA1 pyramidal cells from OFQ KO mice in three $10\mu\text{m}$ -long segments per cell. We observed a significant increase in the number of protrusions in the OFQ KO mice relative to controls (WT 6.15 ± 0.82 , OFQ KO 9.89 ± 0.74 , $n = 11,12$ cells, $p = 0.003$) (Fig 1E,F,I). Together these data suggest that endogenous OFQ is inhibitory to dendritic morphogenesis as well as spine formation.

OFQ Reduces Dendrite Outgrowth *In Vitro*

To determine the cell types in our cultures expressing OFQ and NOP, immunocytochemistry was performed. Pyramidal cells at 14 days *in vitro* (div)

exhibit the typical morphology of excitatory neurons in the hippocampus. Non-pyramidal cells have the bipolar morphology of inhibitory interneurons. We detected OFQ and its receptor NOP in both pyramidal and non-pyramidal neurons of the hippocampus *in vitro* shown (Fig 2) as has been reported extensively for pyramidal cells of the CA region and the dentate gyrus *in vivo* (Anton et al., 1996; Ikeda et al., 1998; Manabe et al., 1998; Neal et al., 1999; Houtani et al., 2000; Higgins et al., 2002). Quantification of the immunocytochemistry revealed that 85.61 ± 2.34 cells were positive for OFQ and 87.89 ± 1.59 ($n = 6$) were positive for NOP, indicating that the majority of the cultured cells expressed both the neuropeptide and its receptor. The specificity of the antibodies was determined by the lack of background in a no primary control.

The effect of exogenously applied OFQ on the morphological characteristics of pyramidal cells was explored since the effect of OFQ deletion was apparent in CA1 pyramidal neurons *in vivo*. To examine the effect of exogenous OFQ on primary dendrites in pyramidal cells *in vitro*, embryonic hippocampal cells in culture were treated with OFQ peptide and MAP2 positive dendrites were analyzed. The morphological events of hippocampal neuronal differentiation in culture are characterized by protrusion of primary dendrites from the cell body at 1 div until 10 div followed by extension of dendritic branches from the primary branches between approximately 6-12 div. Finally, spine formation and maturation occurs from 12-21 div resulting in synaptic contacts (Dotti et al., 1988;

Craig and Banker, 1994). Dendritic initiation was assayed by quantifying the number of primary dendrites at an early time in development. OFQ treatment of dissociated hippocampal neurons from 2-5 div resulted in significantly fewer primary dendrites compared to control (Control 5.72 ± 0.18 , OFQ 5.06 ± 0.16 , $n = 50$, $p = 0.014$) (Fig 3). Dendritic maturation was assayed by measuring dendrite outgrowth and the formation of secondary dendrites at a slightly later time in development. There was a reduction in the total primary dendritic length (Control $178.18 \pm 12.34\mu\text{m}$, OFQ $127.39 \pm 14.80\mu\text{m}$, $n = 11$, $p = 0.033$) and the number of branch points (Control 5.92 ± 0.31 , OFQ 4.88 ± 4.88 , $n = 25$, $p = 0.050$) when cells were treated from 5-8 div. These data indicate that OFQ reduces dendritic initiation as well as dendritic complexity. No significant effect was observed in the length of secondary dendrites. Spines are not developed at this stage *in vitro* so no analysis of dendritic protrusions was performed. In support of the inhibitory effect of OFQ on dendritic outgrowth, there was an increase in the number of primary dendrites in cultures treated with a competitive NOP receptor antagonist (Naloxone benzoylhydrazone $10\mu\text{M}$) compared to vehicle controls (Controls 5.72 ± 0.18 , Naloxone 6.30 ± 0.15 , $n = 50$, $p = 0.014$) (Supplemental Fig 1), indicating the role of endogenous OFQ on dendrite growth.

The interaction between BDNF and OFQ on dendritic growth was examined. BDNF increased the average number of primary dendrites (Control 5.72 ± 0.18 , BDNF 6.58 ± 0.22 , $n = 50$, $p = 0.002$) and branch points (Control 5.92 ± 0.31 , 7.04 ± 0.50 , $n = 25$, $p = 0.036$) relative to control as has been reported (Cheung

et al., 2007) and there was a trend to increased total **primary** dendritic length by BDNF (Control $178.18 \pm 12.34\mu\text{m}$, BDNF $213.28 \pm 20.72\mu\text{m}$, $n = 11$, $p = 0.175$) (Fig 3). Pre-treatment of cells with OFQ for 30 min significantly attenuated the BDNF-induced increase in primary dendrite number (BDNF 6.5 ± 0.22 , BDNF + OFQ 5.42 ± 0.19 , $n = 50$, $p = 0.001$) and number of branch points (BDNF 7.04 ± 0.50 , BDNF + OFQ 5.52 ± 0.35 , $n = 25$, $p = 0.005$) such that cells treated with OFQ followed by BDNF resembled control cultures (Fig 3). We have also observed that OFQ significantly attenuates the number of PSD95+ puncta induced by BDNF, *in vitro* (Control 22.59 ± 1.04 , OFQ 21.83 ± 1.15 , BDNF 27.94 ± 1.47 , BDNF + OFQ 24.28 ± 1.15 , $n = 17,18$, BDNF vs BDNF + OFQ $p = 0.036$) (Supplemental Fig 2). Thus it appears that OFQ and BDNF have opposite effects and that their actions on dendritic growth can counteract each other.

OFQ and BDNF Do Not Act in a Feedback Loop to Regulate Dendrite Outgrowth

To determine if OFQ and BDNF act in a negative feedback loop, we assayed dendritic morphology in cells treated with ANA-12 to inhibit TrkB receptors and hippocampal cells derived from NOP KO mice to eliminate NOP activation. If a feedback loop exists then cells lacking NOP should have an even more robust increase in dendritic morphological parameters in response to BDNF relative to wildtype cells. Similarly, cells treated with ANA-12 should exhibit a more dramatic decrease in dendritic growth when treated with OFQ relative to cells treated with OFQ alone. We chose to focus on primary dendrite number and total **primary** dendritic length for these experiments, thus the cells were treated

from 7-10 div. Consistent with the previous experiment, there was a significant decrease in total **primary** dendritic length (Control $1131.26 \pm 100.00\mu\text{m}$, OFQ $686.75 \pm 51.28\mu\text{m}$, $n = 25,23$ $p = 0.0002$) and number of primary dendrites (Control 7.84 ± 0.57 , OFQ 6.39 ± 0.39 , $n = 25,23$ $p = 0.032$) in wildtype cells treated with OFQ (Fig 4A and B), confirming that OFQ is inhibitory to dendritic outgrowth. There was also a trend to a decrease in dendrite length (Control $1131.26 \pm 100.00\mu\text{m}$, ANA-12 $958.36 \pm 75.81\mu\text{m}$, $n = 25,33$, $p = 0.100$) and dendrite number (Control 7.84 ± 0.57 , ANA-12 7.69 ± 0.37 $n = 25,33$, $p = 0.816$) in cells treated with ANA-12 alone, suggesting that endogenous BDNF contributes to dendritic morphology. However, we did not detect a more inhibitory effect of OFQ in the ANA-12 pre-treated cells relative to those exposed to OFQ alone either for total **primary** dendritic length (OFQ $686.75 \pm 51.28\mu\text{m}$, OFQ + ANA-12 $760.14 \pm 59.36\mu\text{m}$, $n = 23,32$, $p = 0.497$) or primary dendrite number (OFQ, 6.39 ± 0.39 , OFQ + ANA-12 6.03 ± 0.41 , $n = 23,32$, $p = 0.570$).

With regard to the WT and NOP KO mice, there was a significant increase in total **primary** dendritic **length** (Control WT $642.41 \pm 67.97\mu\text{m}$, BDNF WT $846.18 \pm 98.40\mu\text{m}$, $n = 30$, $p = 0.033$) and number of primary dendrites (Control WT 4.40 ± 0.35 , BDNF WT 6.20 ± 0.446 , $n = 30$, $p = 0.001$) when cells derived from wildtype mice were treated with BDNF alone (Fig 4C and D). There was also a trend toward increased number of primary dendrites in the NOP KO cells relative to control (Control WT 4.40 ± 0.35 , Control NOP KO 5.23 ± 0.233 , $n = 30$, $p = 0.081$) as would be expected if OFQ is inhibitory to dendrites. However, there

was not an amplified effect of BDNF in the NOP KO cells for either dendrite number (BDNF WT 6.20 ± 0.446 , BDNF NOP KO 5.70 ± 0.263 , $n = 30$, $p = 0.293$) or dendrite length (BDNF WT $846.18 \pm 98.40\mu\text{m}$, BDNF NOP KO $708.98 \pm 47.23\mu\text{m}$, $n = 30$, $p = 0.149$). Together these data suggest that there is no feedback loop between the BDNF and OFQ pathways and that they may use independent mechanisms to enhance and inhibit dendritic growth, respectively.

To explore the role of synaptic activity in the effect of OFQ on dendrite outgrowth, cells were exposed to high KCl (20mM) to depolarize the cells (Wayman et al., 2006) and the cells were treated with OFQ. If OFQ needs to inhibit synaptic activity in order to inhibit dendritic growth than the addition of KCl should prevent the effect of OFQ on dendrite growth. Relative to control, OFQ inhibited dendritic total primary length (Control $1131.26 \pm 100.00\mu\text{m}$, OFQ $686.75 \pm 51.28\mu\text{m}$, $n = 25,23$ $p = 0.0002$) and number of primary dendrites (Control 7.84 ± 0.57 , OFQ 6.39 ± 0.39 , $n = 25,23$ $p = 0.032$) as we have previously observed (Fig 4D and E). Moreover, KCl showed increased total primary dendritic length (Control $1131.26 \pm 100.00\mu\text{m}$, KCl $1424.52\mu\text{m}$, $n = 25,30$, $p = 0.013$) as would be expected from enhanced synaptic activity although not much change in primary dendritic number (Control 7.84 ± 0.57 , KCl 8.36 ± 0.42 , $n = 25,30$, $p = 0.422$). However, treatment of the cells with KCl did not reverse the effect of OFQ on dendrite growth to control levels, but rather the KCl + OFQ group resembles the OFQ data for length (OFQ $686.75 \pm 51.28\mu\text{m}$, KCl + OFQ $646.26 \pm 35.63\mu\text{m}$, $n = 23,26$, $p = 0.731$) and number (OFQ 6.39 ± 0.39 , KCl + OFQ 5.31 ± 0.25 , $n =$

23,26, $p = 0.100$). These data suggest that increasing activity does not rescue the effect of OFQ and that synaptic activity is not an intermediate to the effect of OFQ on dendrite growth. Therefore, OFQ likely acts upon dendrite growth mechanisms directly.

OFQ Activates RhoA and RhoA Is Required for the Effects of OFQ on Dendrite Growth

We then focused our studies on signaling cascades that may be activated or inhibited by OFQ that are independent of the BDNF pathway. The RhoA/Rac/Cdc42 pathway is a good candidate since RhoA is activated by G-protein coupled receptors such as NOP and RhoA activation inhibits neurite outgrowth in several cell types (Kozma et al., 1997; Gehler et al., 2004; Kubo et al., 2007; Gu et al., 2013). We employed a sensitive assay that detects active, GTP-bound Rho followed by a colorimetric G-LISA method to determine if OFQ activates RhoA. We detected an increase in RhoA activation following 3 hours of exposure of hippocampal neurons to OFQ relative to cells treated with vehicle (Control 1.00, OFQ 1.75 ± 0.27 , $n = 3$, $p = 0.008$) (Fig 5). Longer treatments of hippocampal neurons with OFQ for 3 days did not maintain the elevated RhoA levels (Control 1.00, OFQ 1.04 ± 0.12 , $n = 3$, $p = 0.717$) (Supplemental Fig 3). These findings indicate that the RhoA pathway is robustly but transiently induced by OFQ treatment. To determine if OFQ induces RhoA directly or through another pathway, we used the NOP inhibitor, Naloxone. Pretreatment of the cultures with Naloxone prevented the OFQ induced RhoA activation relative to

control (OFQ 1.75 ± 0.27 , OFQ + Naloxone 1.16 ± 0.21 , $n = 3$, $p = 0.049$) (Fig 5). Moreover, to confirm the specificity of the RhoA activation assay, we used the RhoA inhibitor C3 Transferase. The cell permeable form of C3 Transferase was used which inactivates RhoA but not related GTPases such as Cdc42 or Rac1. C3 Transferase inhibits Rho proteins by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase (Cytoskeleton, Inc.). Pretreatment of the cultures with C3 Transferase significantly inhibited the RhoA activation induced by OFQ (OFQ 1.75 ± 0.27 , OFQ + C3 0.872 ± 0.31 , $n = 3$, $p = 0.008$). Naloxone and C3 Transferase had no significant effects on RhoA activation relative to vehicle (Control 1.00, Naloxone 1.29 ± 0.23 , C3 Transferase 1.05 ± 0.12 , $n = 3$, $p = 0.329$ and 0.872 , respectively). Therefore, the activation of RhoA in hippocampal neurons in vitro appears to be a direct and specific effect of treatment with the neuropeptide OFQ.

To explore if the OFQ induced RhoA activation is required for the inhibitory effects of the neuropeptide on dendritic morphology, the RhoA inhibitor C3 Transferase was applied 4 hr prior to OFQ treatment. OFQ significantly inhibited total **primary** dendritic length (Control $1129.36 \pm 90.49\mu\text{m}$, OFQ $792.49 \pm 60.67\mu\text{m}$, $n = 41,45$, $p = 0.015$) and the number of primary dendrites (Control 9.34 ± 0.46 , OFQ 7.89 ± 0.41 , $n = 41,45$, $p = 0.049$) relative to vehicle as expected (Fig 6). C3 Transferase alone had no effect on dendritic length (Control $1129.36 \pm 90.49\mu\text{m}$, C3 $1112.22 \pm 60.67\mu\text{m}$, $n = 41,45$, $p = 0.900$) or number (Control 9.34 ± 0.46 , C3 8.67 ± 0.66 , $n = 41,45$, $p = 0.358$). C3

Transferase attenuated the inhibitory effect of OFQ such that cells treated with C3 Transferase plus OFQ were not significantly different relative to vehicle treated cells for dendrite length (Control $1129.36 \pm 90.49\mu\text{m}$, C3 + OFQ 1205.37 ± 90.33 , $n = 41,44$, $p = 0.581$) or number (Control 9.34 ± 0.46 , C3 + OFQ 9.02 ± 0.48 , $n = 41,44$, $p = 0.666$). Thus RhoA activity is necessary for the inhibitory effect of OFQ on dendritic outgrowth, indicating the signaling pathway used by the neuropeptide.

DISCUSSION

This study demonstrates that the neuropeptide OFQ inhibits dendritic outgrowth of excitatory neurons in the hippocampus. Although BDNF has been shown to upregulate OFQ expression (Ring et al., 2006) and OFQ exhibits opposite effects to BDNF on dendritic growth, we find no evidence for a feedback loop between the two pathways. This data suggest that BDNF and OFQ act in parallel pathways to exert their actions on dendritic outgrowth.

Our findings are consistent with the opposing effects of BDNF and OFQ on synaptic activity, LTP and learning and memory. BDNF enhances LTP and promotes learning and memory while OFQ inhibits these processes (Park and Poo, 2013); (Cohen-Cory, 2002); (Zaveri, 2003). Endogenous molecules are therefore important to maintain order in the central nervous system. Interestingly, although neurotrophins are thought to be growth-promoting factors, not all of

them have the same effects. NT-3 has been shown to have opposite actions on dendritic growth when compared to BDNF in the same population of neurons. In layer 4 of the cortex, BDNF stimulates dendritic growth, while NT-3 inhibits dendritic growth but in layer 6 the actions of these molecules are reversed, indicating that the cues from the local environment may have an effect on neuronal morphology (McAllister et al., 1997). Similarly for neuropeptide OFQ, despite the predominate function being described as an inhibitory neuropeptide, there are reports showing opposite effects which can be attributed to either mode and location of administration and the dose (Grisel et al., 1996; Mogil et al., 1996; Xu et al., 1996; Tian et al., 1997; Okuda-Ashitaka et al., 1998; Hawes et al., 2000; Sandin et al., 2004).

In our previous study we found a role for OFQ in promoting neurite outgrowth. This can be attributed to several differences in experimental design between that study and the current one. For example, in the prior experiment, hippocampal neurons were treated at time of plating with OFQ and in this study the cells were allowed to establish themselves for several days up to a week prior to treatment with OFQ. These more established neurons may express NOP at a higher level and therefore the response to OFQ may be more specific. The concentration of OFQ that elicited the greatest and, for some parameters, the only significant effect in the previous study was 10 μ M while in this report we used 3 μ M as a more physiological concentration. Indeed there are reports that the effects of OFQ can vary dramatically depending on the concentration used (Heinricher,

2003). Nocistatin which has been claimed to have differing effects to OFQ (Okuda-Ashitaka et al., 1998) also increased several parameters related to dendritic outgrowth in our previous study, suggesting that some of the effects may be non-specific at the highest concentration. Finally, the earlier report only examined the effect of OFQ after a 24 hr exposure. The current study explores the long-term effect of OFQ on structural changes that may occur following prolonged exposure to OFQ, after the electrophysiological effects of OFQ on LTP, learning and memory. For example, the fast changes in synaptic efficacy by BDNF may be translated into structural alterations when the synapses are exposed to BDNF for a longer period of time (Martinez et al., 1998; Pozzo-Miller et al., 1999). Finally, although Zaveri et al (Zaveri et al., 2006) have shown that agents that increase preproOFQ mRNA such as cAMP, retinoic acid, serum and HDAC inhibitor induce dendrite outgrowth in both neuroblastoma and progenitor cell lines, these observations are correlative; there is no evidence of the direct effect on process outgrowth and the results could be explained by the effect of factors such as BDNF that are also induced by those treatments.

We have focused this study on the excitatory neurons of the hippocampus. NOP and OFQ are highly expressed in the dentate gyrus granule cells, in CA1 and CA3 pyramidal cells and interneurons in tissue sections (Anton et al., 1996; Ikeda et al., 1998; Manabe et al., 1998; Houtani et al., 2000; Higgins et al., 2002). We confirmed this broad expression pattern for OFQ and NOP in cultured hippocampal neurons. BDNF affects the dendritic branching of several cell types

including excitatory pyramidal neurons (McAllister et al., 1995; Labelle and Leclerc, 2000; Alonso et al., 2004), inhibitory interneurons (Vicario-Abejon et al., 1998; Jin et al., 2003; Wirth et al., 2003; Woo et al., 2005) and dentate granule cells (Patel and McNamara, 1995; Danzer et al., 2002). However, BDNF increases the number of primary dendrites in an activity dependent manner only in pyramidal neurons and not in nonpyramidal cells (McAllister et al., 1995; McAllister et al., 1996; Horch et al., 1999), suggesting that the effect of BDNF on excitatory neurons is related to changes in synaptic activity. Thus our findings are consistent with the model that BDNF enhances the activity and dendrite outgrowth of excitatory neurons and upregulates OFQ which in turn inhibits the activity and morphological changes of the excitatory neurons. Our previous study was performed using an automated cell reader so it could not distinguish between excitatory cells and interneurons (Ring et al., 2006) and may also explain some of the differences in findings between the studies. Future studies examining the effect of OFQ on inhibitory interneurons may reveal alternative effects on dendritic outgrowth. This study examined the effect of OFQ on BDNF-induced dendritic growth and we observed that OFQ attenuates the BDNF response. We pre-treated the hippocampal neurons with OFQ prior to BDNF addition because in preliminary biochemical studies looking at the effect of OFQ on BDNF-induced activation of downstream signaling molecules, we observed that simultaneous treatment of OFQ and BDNF did not have an effect on BDNF-mediated phosphorylation. In contrast, pre-treatment of OFQ prior to BDNF inhibited the effects of BDNF. It is likely that BDNF is more potent than other

factors and that its signaling pathway will preempt others if not given a chance to become activated beforehand.

Our results reveal a more dramatic effect of OFQ on primary dendrites as opposed to secondary branches. The OFQ KO mice have significantly more **primary** dendrites than the WT mice and while the total **primary** dendritic length is increased, the average dendritic length for **primary** neurons is not significantly altered, suggesting that there are more **primary** dendrites rather than longer ones. These findings indicate a role of OFQ in inhibiting dendrite initiation rather than in preventing elongation. Treatment of hippocampal cultures with OFQ also supports the conclusion that OFQ inhibits dendrite initiation, as there are fewer primary dendrites and less branching in conjunction with a reduction in total dendritic length. There is also an increased density of spines in the OFQ KO implying that OFQ may be involved in synapse formation as also suggested by the attenuation of BDNF-induced PSD95 expression by OFQ, although our current study examines the effects of OFQ on dendrite formation and growth, prior to synapse formation.

Our findings suggest that BDNF and OFQ do not act in a negative feedback loop. We initially attempted to inhibit the BDNF and OFQ pathways through shRNA methods but were unable to completely eliminate TrkB or NOP expression in individual cells. Therefore we employed alternative methods to test the presence of the feedback loop. The involvement of NOP signaling on the actions of BDNF

was examined in NOP KO mice and since TrkB KO mice are not viable, we selected ANA-12 a new TrkB antagonist, to pharmacologically inactivate TrkB receptors in a non-competitive and selective manner (Cazorla et al., 2011) and test the effects of OFQ. Since the NOP and OFQ receptors were non-functional in all cells in the culture, this KO experimental approach was more reliable than shRNA and definitively demonstrated the absence of a feedback loop between the two factors.

There are a number of examples of positive feedback between BDNF and synaptic factors such as neuregulins (Loeb et al., 2002; Ma et al., 2011), GABA release (Obrietan et al., 2002), nitric oxide (Cheng et al., 2003), MeCP2 (Klein et al., 2007), Norepinephrine (Chen et al., 2007) as well as BDNF itself (Vaynman et al., 2003). Fewer examples of negative feedback exist but do include the upregulation of the Sprouty (Spry1) by BDNF. Spry proteins are ligand-inducible inhibitors of TrkB-dependent signaling pathway so they inhibit differentiation and survival (Gross et al., 2007). Rather than using a direct feedback loop, it is possible that the interaction between BDNF and OFQ represent a version of synaptic scaling which is one mechanism by which neurons maintain homeostasis. BDNF mediates postsynaptic forms of synaptic scaling (Turrigiano, 2007). Chronic activity deprivation can be reversed by BDNF and inhibiting TrkB activation mimics the effects of activity blockade (Rutherford et al., 1998; Desai et al., 1999). A recent study demonstrates that BDNF and MAPK along with MSK1 are involved in homeostatic synaptic scaling, which increases GluA1 on

cell surface via the immediate early gene Arc (Correa et al., 2012). It is possible that prolonged exposure to BDNF, which causes chronic activity elevation, results in upregulation of OFQ to reverse the hyperactivity and the morphological consequences. The two factors need not act in contrast upon the same pathway to exert their opposing actions on the neurons and maintain morphological homeostasis.

The role of activity in OFQ mediated inhibition of dendritic growth was examined using KCl to depolarize the cells. Our data suggest that increasing activity does not reverse the effect of OFQ and that synaptic activity is not an intermediate to the effect of OFQ on dendrite growth. Therefore, OFQ likely acts upon dendrite growth mechanisms directly, perhaps through the induction of RhoA, rather than the dendritic growth inhibition being a result of the reduced synaptic activity caused by OFQ. Similar experiments have been performed on neurotrophin-induced dendritic growth (McAllister et al., 1996). Those previous studies demonstrated that dendritic growth by BDNF requires activity since inhibitors such as sodium and calcium channel blockers or glutamate receptor antagonists prevented the BDNF-induced dendritic outgrowth. Thus the effects of OFQ directly on the mechanism of dendritic growth are in contrast to the manner by which BDNF induces dendritic growth via activity. The importance of endogenous OFQ on dendritic growth was demonstrated by the fact that Naloxone treatment causes a small but significant increase in the number of

primary dendrites, again suggesting that the NOP receptor is directly involved in controlling dendritic growth.

We demonstrate that RhoA is upregulated by OFQ and is required for the inhibitory effects of the neuropeptide on dendritic outgrowth. The Rho family members of GTPases play an important role in dendrite growth by linking growth and guidance molecules to effectors that influence the cytoskeleton. Specifically, in neurons RhoA, Rac1, and Cdc42 have been shown to affect neurite outgrowth (Bradke and Dotti, 1999; Ahmed et al., 2006) by regulating actin cytoskeletal dynamics (Kaibuchi et al., 1999; Burridge and Wennerberg, 2004; Jaffe and Hall, 2005). In general, Rac1 and Cdc42 are positive modulators, whereas RhoA is an inhibitory molecule that causes growth cone collapse and axon retraction (Lindsley et al., 2011) and also has an adverse affect on dendrite formation. We also showed that by inhibiting RhoA with pretreatment with C3 Transferase resulted in attenuation of the activation effect of OFQ validating the role of RhoA in this process and the specificity of the assay. When the cells were pretreated with Naloxone, a potent antagonist of classic opioid and NOP receptors, the RhoA activation was again reduced after OFQ treatment. These finding indicate that OFQ activates RhoA directly via its own NOP receptor rather than it being an indirect effect through another receptor or pathway. The activation of RhoA by OFQ is transient, which is consistent with other studies showing transient activation of RhoA following treatments with molecules such as tumor necrosis factor (Hunter et al., 2003), thrombin (Narayan et al., 2010), vascular endothelial

growth factor (Sun et al., 2006; Yokomori et al., 2009), and carbachol (Liu et al., 2006). Acute activation of RhoA may induce downstream signaling pathways that then have effects on dendrite outgrowth. For example, neurotrophin signaling activates protein kinase Akt through the PI3 kinase pathway, which in turn influences the activity of Rac1 and Cdc42 (Dickson, 2001). Rac1 and Cdc42, affect actin polymerization and myosin activity within the growth cone (Ruchhoeft et al., 1999; Yuan et al., 2003; Chen et al., 2006) to mediate the promotion of neurite outgrowth by BDNF (Cohen-Cory et al., 2010; Numakawa et al., 2010).

In addition to the studies on the positive effects of Rac1 and Cdc42 on neurites, there are reports demonstrating that RhoA is inhibitory to dendritic growth. While RhoA activation enhances the outgrowth of axons, it reduces the morphology of dendrites (Ahnert-Hilger et al., 2004). RhoA inhibits cypin expression and subsequent microtubule assembly thus decreasing dendrite number in cultured hippocampal neurons (Chen and Firestein, 2007). Upregulation of RhoA expression by knocking-down an mRNA binding protein hnRNP-Q1 inhibits dendritic outgrowth (Xing et al., 2012). Finally, a recent study indicates that proBDNF which, like OFQ, has opposing actions to BDNF and causes neurite collapse, also requires RhoA for its effects (Sun et al., 2012). Thus, the positive effects of BDNF on neuronal morphology may be mediated by Rac1 and Cdc42 GTPases, whereas the inhibitory effects of the BDNF-induced neuropeptide OFQ on dendritic growth may be regulated by the GTPase RhoA. These opposing

actions may be critical for maintaining neuronal stability and homeostasis during development and times of altered synaptic activity in the adult brain.

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FIGURE LEGENDS

Figure 1 OFQ deletion enhances dendritic morphogenesis. A-D, Representative excitatory granule cell dendrites visualized from (A,C) WT and (B,D) OFQ KO adult male mice using the Golgi staining technique. Scale bar A,B = 100 μ m, C,D = 40 μ m. E and F, Representative images of dendritic protrusions from CA1 neurons in (E) WT and (F) OFQ KO mice. Scale bar = 10 μ m. G-I, Quantifying of primary and secondary dendrites including the (G) number of dendrites and (H) total **primary and secondary** dendritic length (n=10). (I) Quantifying of secondary dendrite average protrusion density \pm SEM per 10 μ m segment (n = 11,12). * indicates $p < 0.05$, t-test.

Figure 2 OFQ and NOP are expressed in both pyramidal and non-pyramidal cells *in vitro*. Hippocampal cultures (7 div) were immunolabeled with anti-OFQ (A) and anti-NOP antibodies (B) (red). Pyramidal cells (arrows) express OFQ as do the non-pyramidal bipolar neurons (arrowheads). (C) Representative image of immunostaining with no primary antibody as a negative control, demonstrating the lack of background. DAPI staining (blue) indicates presence of cell nuclei. Scale bar = 40 μ m. (D) Quantification of average number of OFQ and NOP positive cells per field \pm SEM (n = 6).

Figure 3 OFQ reduces dendrite initiation and branching *in vitro*. A-D, Representative images of hippocampal cells treated with (A) vehicle, (B) OFQ

(3 μ M), (C) BDNF (50ng/ml) or (D) OFQ plus BDNF from 2-5 div for dendrite initiation and 5-8 div for dendrite length and branching and visualized with MAP2. Scale bar = 30 μ m. E-G, Quantifying of dendritic morphology including (E) average primary dendrite number \pm SEM (n=50), (F) total **primary** dendritic length \pm SEM (n=11) and (G) average number of branch points \pm SEM (n=25). * indicates $p < 0.05$ relative to control or between indicated groups, ANOVA Fisher's PLSD.

Figure 4 OFQ and BDNF do not act in a feedback loop. A,B Wild type hippocampal cells 7 div were treated with ANA-12 (10 μ M) for 30 min followed by the addition of OFQ (3 μ M) for 3 days in the continued presence of ANA-12 (n = 23-33). C,D Alternatively NOP KO embryonic mice were used to prepare hippocampal cultures that were treated at 7 div with BDNF (25ng/ml) for 3 days (n = 30). **E,F Wild type hippocampal cells 7 div were treated with KCl (20mM) for 30 min followed by the addition of OFQ (3 μ M) for 3 days in the continued presence of KCl (n = 23-30).** Cells were fixed and stained with MAP2. Quantifying of dendritic morphology including (A,C,E) total **primary** dendritic length \pm SEM and (B,D,F) number of primary dendrites \pm SEM. * indicates $p < 0.05$ relative to control, ANOVA Fisher PLSD.

Figure 5 OFQ activates RhoA. Wild type hippocampal cells 7 div were treated with vehicle or OFQ (3 μ M) for 3 hours. In some dishes C3 Transferase (0.3 μ g/ml) or Naloxone (10 μ M) was added prior to OFQ. The dishes were then

subjected to G-LISA RhoA activation assay with a colorimetric reaction. Data were normalized to baseline RhoA activation by vehicle control. Nine dishes per condition were pooled (n=3). * indicates $p < 0.05$, ANOVA Fisher PLSD.

Figure 6 RhoA is required for the effects of OFQ on dendrites. A-D, Representative images of hippocampal cells (7 div) treated with (A) vehicle, (B) C3 Transferase, (C) OFQ (3 μ M) or (D) OFQ plus C3 Transferase for 3d and visualized with MAP2. Scale bar = 50 μ m. E-F, Quantifying of dendritic morphology including (E) total **primary** dendritic length \pm SEM (n=50) and (F) average primary dendrite number \pm SEM (n=41-45). * indicates $p < 0.05$ relative to control or between indicated groups, ANOVA Fisher's PLSD.

Supplemental Figure 1 Naloxone increases primary dendrite number in hippocampal neurons. Quantifying of average primary dendrite number \pm SEM (n=50). * indicates $p < 0.05$ relative to control, t-test.

Supplemental Figure 2 OFQ inhibits BDNF-induced PSD-95 puncta. Neurons (16 div) were treated with OFQ (3 μ M), BDNF (50ng/ml) or both for 3d. Average number of PSD95+ puncta/50 μ m segment \pm SE (n=17,18). *indicates $p < 0.05$ relative to control or between indicated groups, ANOVA Fisher's PLSD.

Supplemental Figure 3 RhoA is not activated by OFQ after a 3-day exposure. Wild type hippocampal cells 4 div were treated with vehicle or OFQ (3 μ M) for 3

days and then subjected to G-LISA RhoA activation assay with a colorimetric reaction. Data were normalized to baseline RhoA activation by vehicle control. Nine dishes per condition were pooled (n=3). $p > 0.05$, t-test.

Fig 1

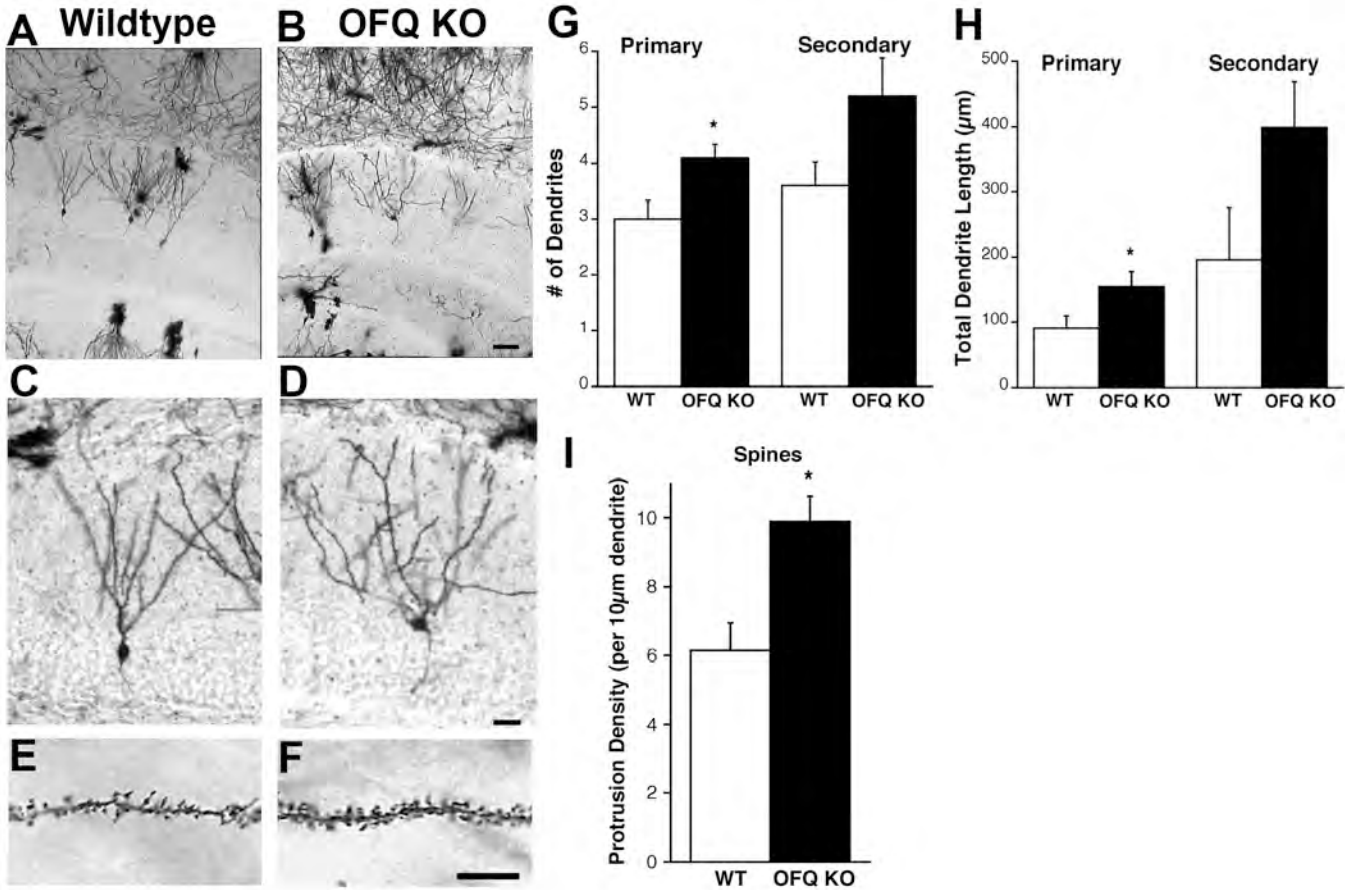


Fig 2

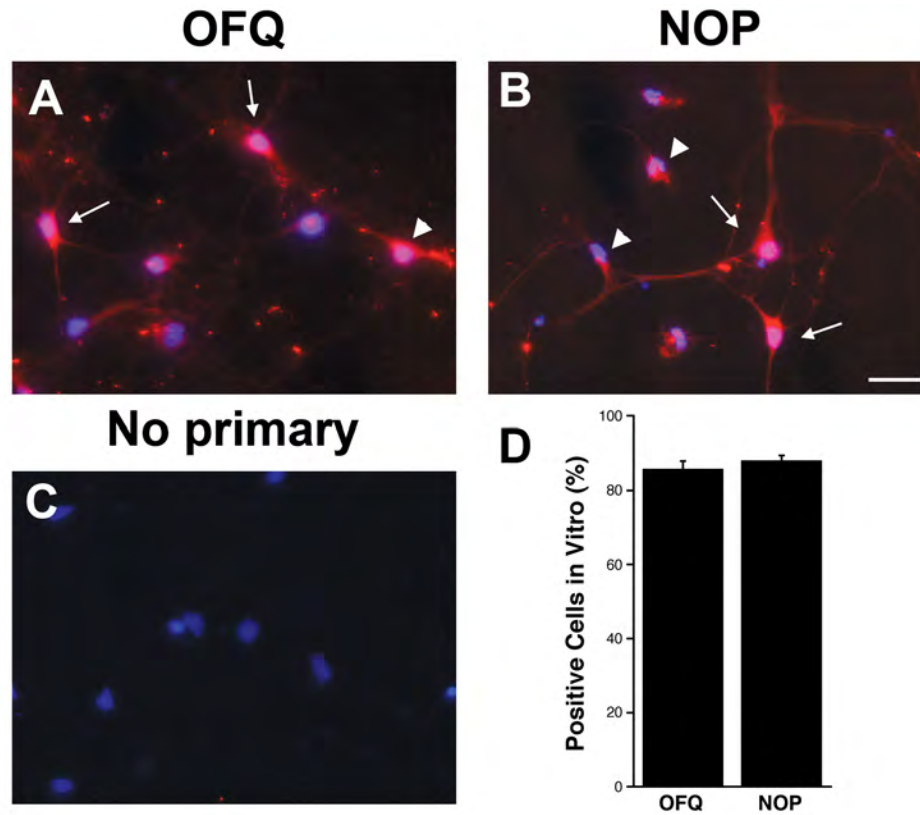


Fig 3

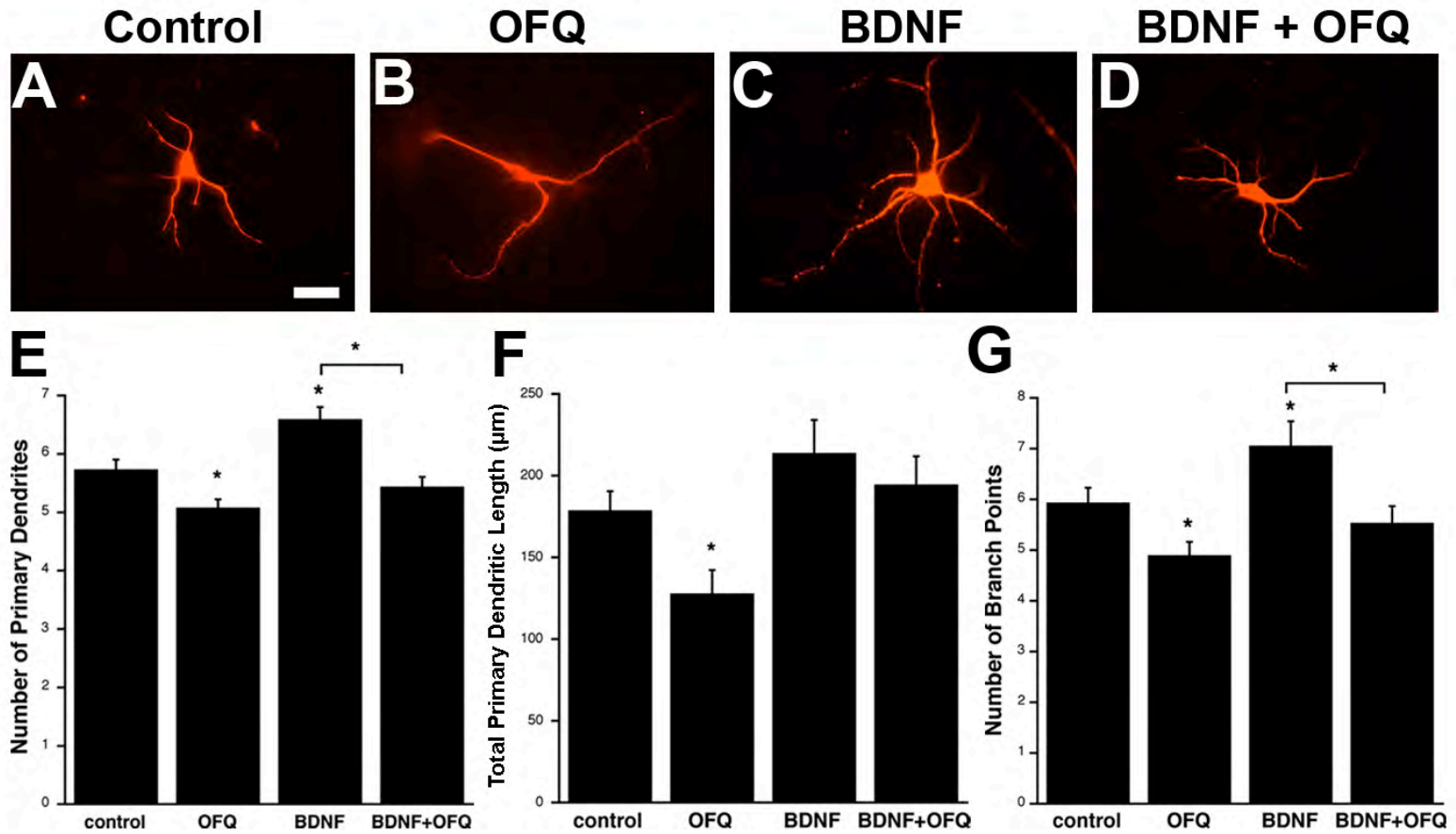


Fig 4

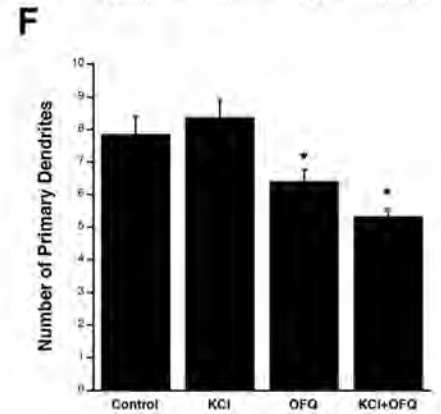
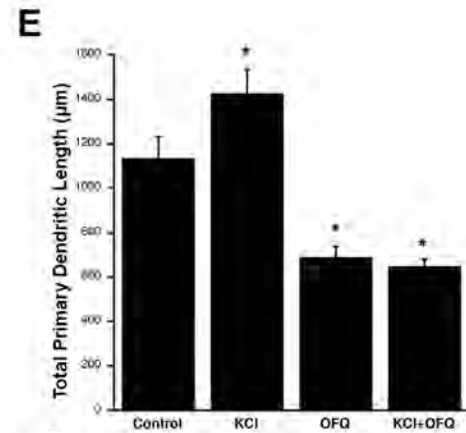
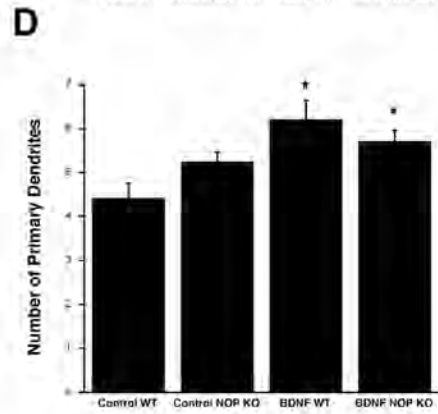
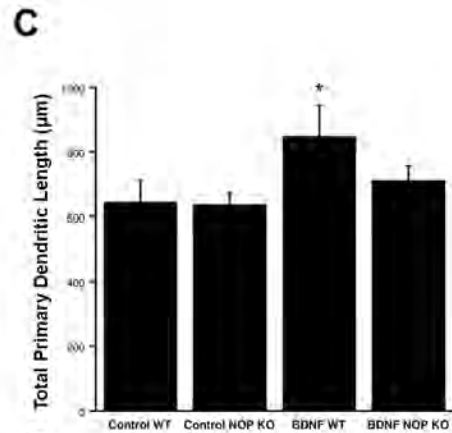
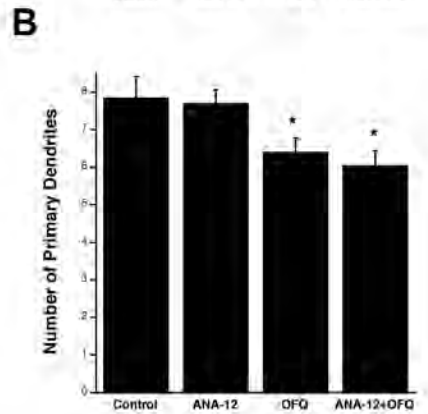
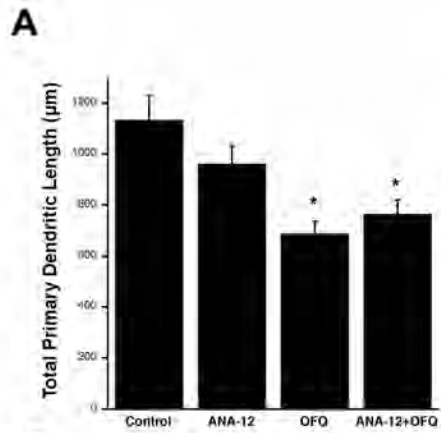


Fig 5

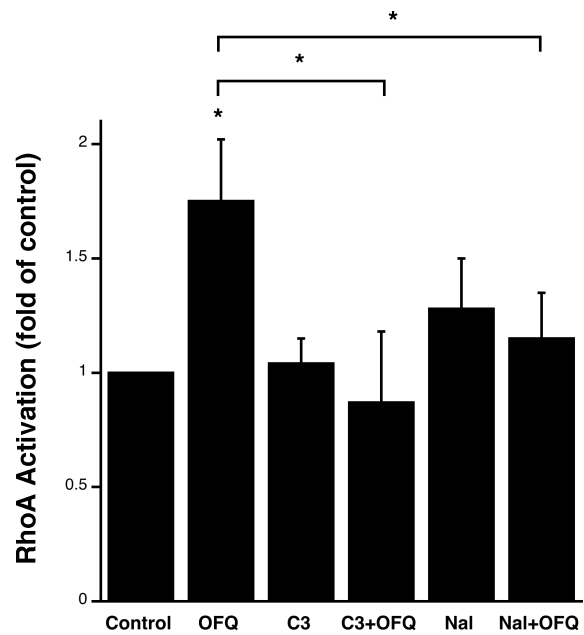
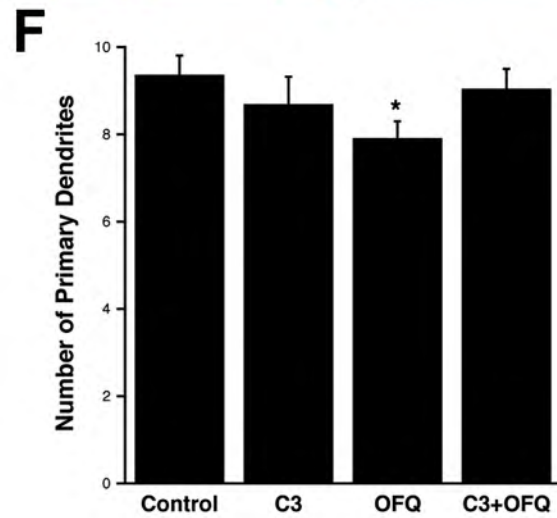
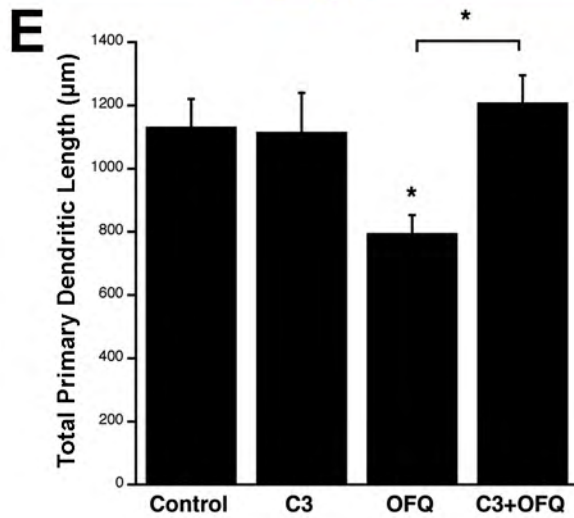
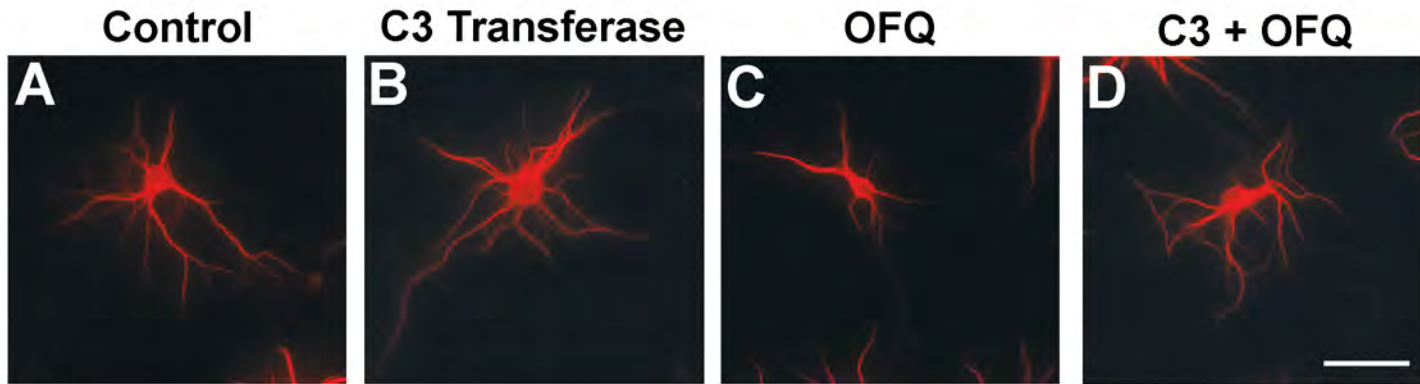
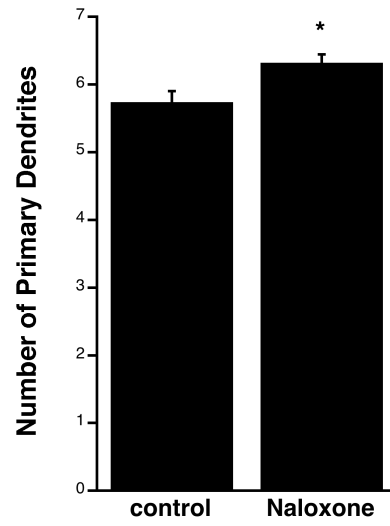


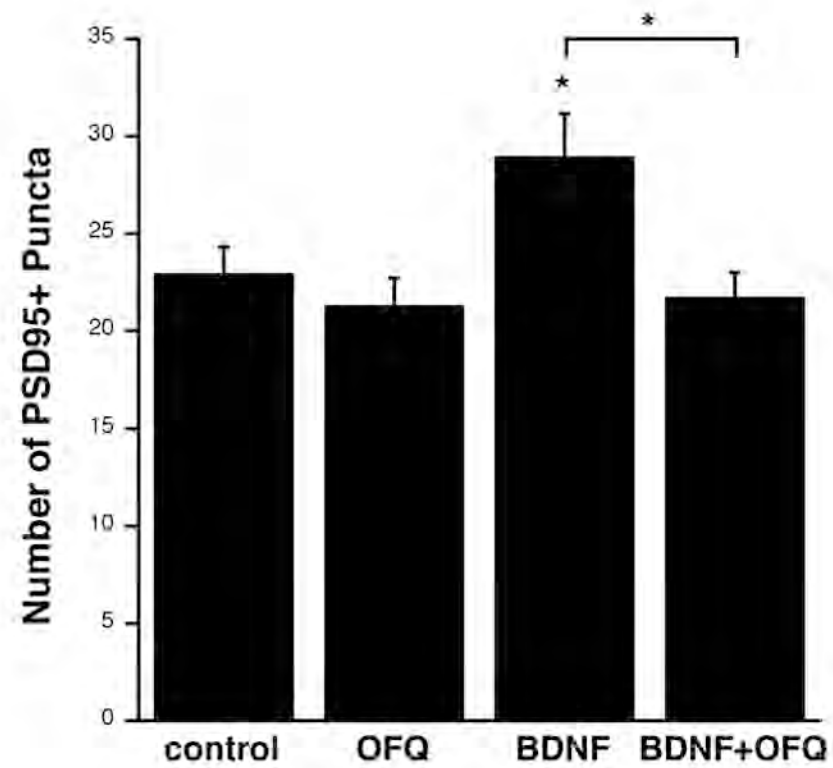
Fig 6



Supp Fig 1



Supp Fig 2



Supp Fig 3

