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RECEPTOR REGULATION OF AXON GUIDANCE MOLECULE GENE EXPRESSION

Amy K. Jassen, Hong Yang, Gregory M. Miller, Elizabeth Calder, Bertha K. Madras

Department of Psychiatry, Harvard Medical School and the Division of Neurochemistry, New England Primate Research Center, 1 Pine Hill Drive, Southborough, MA 01772-9102

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Running title: GPCR Regulation of Axon guidance molecules

Corresponding author:

Bertha K. Madras

Department of Psychiatry, Harvard Medical School

Division of Neurochemistry, New England Regional Primate Research Center,

1 Pine Hill Drive, Southborough, MA 01772-9102,

Tel. 508-624-8073; Fax. 508-624-8166; E-mail: bertha_madras@hms.harvard.edu

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Non-standard Abbreviations: GPCR: G-protein Coupled Receptor; DCC: Deleted in colorectal cancer; CP: Crossing point, Ct: Crossing threshold; cAMP: 3'-5'-cyclic adenosine monophosphate; PBS: phosphate buffered saline

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Abstract

Axon guidance molecules, critical for neurodevelopment, are also implicated in morphological and other neurodaptative changes mediated by physiological or pharmacological events in adult brain. As an example, the psychostimulant cocaine markedly alters axon guidance molecules in adult brain of cocaine-treated rats. To decipher a potential link between drug-induced activation of G-protein coupled receptors (GPCRs) and modulation of axon guidance molecules, we investigated whether GPCR activity in a SK-N-MC human neuroepithelioma cell line (which expresses low levels of D1 dopamine receptors) affects gene expression of axon guidance molecules (semaphorins, ephrins, netrins and their receptors). Using real time PCR, we identified 17 of 26 axon guidance molecules in these cells, with varying levels of expression. Forskolin, which raised intracellular cAMP levels 340%, increased EphA5, EphB2 and Neuropilin1 expression, paralleling reported changes in the rat hippocampus after cocaine treatment. The dopamine receptor agonist dihydrexidine, which raised cAMP levels 22%, promoted regulatory changes in EphrinA1, EphrinA5, EphB1, DCC and Semaphorin3C, whereas SKF 81297 altered EphA5, EphrinA1 EphrinA5 and neuropilin1. cAMP and other signal transduction pathways may regulate axon guidance molecule gene expression, potentially linking monoamine receptor activation to signal transduction cascades, transcriptional regulation of axon guidance molecules and alterations in neural networks.

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Introduction

The pathfinding of axons is a tightly regulated process, guided by signaling of axon guidance molecules (ephrins, netrins, slits and semaphorins), a family of relatively high molecular weight receptors and associated ligands (Song and Poo, 2001). Neurodevelopment is the most extensively documented function of the axon guidance molecules, as their topographic guidance facilitates boundary and synapse formation, cell migration and axon guidance (Zhou, 1998; Klein, 2001; Knoll and Drescher, 2002). Axon guidance molecules are also expressed in adult brain and may contribute to synaptic function, neuroadaptation and response to brain injury (Hafner et al, 2004, Xiao et al, 2006, Gerlai, 2001; Yamaguchi and Pasquale, 2004). In the context of synaptic signaling, EphB receptor activity is essential for dendritic spine morphogenesis (Penzes et al., 2003; Murai et al., 2003) and plasticity in synaptic signaling (Gerlai 2001; Dalva et al., 2000; Contractor et al., 2002; Grunwald et al., 2004).

Axon guidance molecules are also implicated in adaptive responses to the psychostimulant drug cocaine in adult brain, as cocaine promotes significant changes in gene expression of axon guidance molecules, in a dosing regimen- and brain region–specific manner (Yue et al., 1999; Halladay et al., 2000; Bahi and Dreyer, 2005). Cocaine is an indirect agonist at dopamine and glutamate receptors, which are associated with promoting cocaine-induced behavioral effects and intracellular neuroadaptive cascades (Wolf et al., 2003; Kalivas, 2004). While these intriguing findings establish a potential causality between drug-induced receptor activity and changes in axon guidance molecule gene expression, there is no direct evidence linking changes in receptor activity or signaling with modulation of axon guidance molecule gene expression. We postulate that activation of GPCRs or augmentation of cAMP levels could

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alter axon guidance molecule gene expression. To decipher this potential link, we investigated whether forskolin-mediated elevation of cAMP or activation of GPCRs by monoamine receptor agonists (dihydrexidine or SKF 81297) changes expression of genes encoding axon guidance molecules. Our results support the underlying hypothesis, that modulation of receptor activity or cAMP levels, promotes adaptive responses in axon guidance molecule gene expression. Conceivably, our novel findings offer a molecular mechanism by which neurotransmitter or drug-receptor interaction may contribute to neuroadaptation relevant to synaptic strength, neuronal morphology and neural networks.

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Methods

Cell Culture and Drug Treatment

SK-N-MC neuroepithelioma cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured according to the recommended conditions. Cells were seeded at $5.5x10^5$ per well of a 6 well Costar tissue culture plate (Corning, NY). Two days later, the media was changed to serum free overnight followed by a 6 hour treatment with 10 μM forskolin (Sigma-Aldrich, St. Louis, MO), 10 μM dihydrexidine (gift from NIDA-NIH), 10 μM dihydrexidine plus 50 μM SCH23390 (Sigma-Aldrich), 10 μM dihydrexidine plus 50 μM eticlopride (Sigma-Aldrich) or vehicle (0.1% DMSO), 10 μM SKF 81297 (Sigma-Aldrich), 10 μM SCH 39166 (gift from Schering-Plough). The drugs were removed and cells were lysed by the addition of 800 μL Trizol Reagent (Invitrogen, Carlsbad, CA) per well. RNA isolation and reverse transcription were carried out using Superscript III reverse transcriptase and OligodTs (Invitrogen).

Axon Guidance Molecule Analysis: Real time RT-PCR and Western blot

Real time PCR was carried out using a Roche Light Cycler 2.0 system (Roche Diagnostics, Indianapolis, IN). Synthesized cDNA was diluted to 20ng/uL and used at 50 ng per reaction. The Taqman Master kit in combination with the Universal Probe Library (Human) was used to assess gene expression (generously supplied by Roche Diagnostics, Indianapolis, IN and Penzberg, Germany). PCR primers for Taqman/Probe Library assays were designed with the Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Two reference genes were used to normalize cDNA across

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samples; beta-actin (ACTB, NM_001101) and Hydroxymethylbilane synthase (HMBS, NM_000190). Analysis of real time PCR data was done using the ΔΔCt method: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{HOUSEKEEPING GENE gene}}$, $\Delta\Delta Ct = \Delta Ct_{\text{treated}} - \Delta Ct_{\text{vehicle}}$, where the change in gene expression as a result of drug treatment is given by: $2^{-\Delta\Delta Ct}$ (2 is the assumed efficiency). Without an efficiency correction, the ΔΔCt method gives an estimated quantification of changes in gene expression. However, the use of two distinct housekeeping genes and extensive replication of experiments provides greater validity to the ΔΔCt method.

Immunoprecipitation followed by western blot analysis was carried out for DCC, according to standard procedures. We probed for changes in DCC protein expression in SK-N-MC cell lysate or positive control lysate (Santa Cruz, Santa Cruz, CA). The mouse anti- human DCC antibody, G97-449, (BD Biosciences, San Jose, CA) was used for immunoprecipitation and western blot analysis was performed using the a rabbit anti- human DCC antibody from Santa Cruz (H-205, Santa Cruz, CA).

D1 dopamine receptor Profiling: Saturation and Competition Binding

SK-N-MC cells were washed once with ice cold PBS then lysed in 10 mM Hepes, pH 7.4 at 4°C for 15 minutes. SK-N-MC lysate was homogenized 15x and spun at 28,000xg then resuspended via homogenization in storage buffer (20 mM Hepes, 250 mM sucrose, pH 7.4). Membrane aliquots were snap frozen and stored at -80°C until used. The D1 dopamine receptor density (B_{max}) endogenously expressed in SK-N-MC cells was determined with [^{3}H]-SCH23390 in saturation experiments. SK-N-MC cell membranes were incubated with 8 concentrations of [^{3}H]-SCH23390 (Amersham Biosciences, Piscataway, NJ) in binding buffer (50 mM Hepes, 4 mM $MgCl_2$, pH 7.4 with KOH). Non-specific binding was determined using 1 μM SCH39166.

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Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Competition binding experiments were consistent with previously reported binding data for the D1 dopamine receptor. The SK-N-MC cell membranes were incubated with a single concentration of [^{3}H]-SCH23390 (1-2 nM) and 8 concentrations of the competing test ligand in buffer (50 mM Hepes, 4 mM $MgCl_2$, 0.01% Ascorbic acid, pH 7.4 with KOH). The K_i value for each test compound was determined using a Cheng-Prusoff equation (Cheng and Prusoff, 1973).

cAMP Accumulation

cAMP accumulation assays were carried out using the same procedure for drug treatment of SK-N-MC cells in a 6 well plate (as described above) with a 30 minute drug incubation. The assay was stopped with lysis buffer (250 μL) supplied with the HitHunter cAMP XS Kit (DiscoveRx, Fremont, CA) and a 2 μL lysate aliquot was added in triplicate to a 96 well plate to determine intracellular cAMP in response to 10 μM forskolin, 10 μM dihydrexidine, 10 μM forskolin plus 10 μM dihydrexidine, 50 μM SCH23390 (Sigma-Aldrich) plus 10 μM dihydrexidine or 50 μM SCH23390 alone.

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Results

SK-N-MC Cells Express D1 Dopamine Receptors and Axon Guidance Molecules

To investigate the hypothesis that SK-N-MC cells (Sidhu and Fishman, 1990), acting via cAMP, dopamine receptors or other GPCRs may modulate axon guidance molecule gene expression *in vitro*, we initially determined whether subtypes of dopamine (DA) receptor genes, which encode indirect and key receptor targets of cocaine in the brain, were expressed. With real time PCR, we confirmed the expression of dopamine D_1 receptors, discovered low expression of D_{2S} transcripts, but no evidence that genes encoding D_{2L}, D_3, D_4 or D_5 dopamine receptors were expressed in the SK-N-MC cell line. D1 dopamine receptor density (B_{max}) was approximately 45 fmol/mg protein and affinity (K_D) for [^{3}H]SCH23390 was 0.48 nM. [^{3}H]SCH23390 binding sites displayed properties consistent with D1 dopamine receptors, as Ki values were typical for D_1 receptor ligands: SCH39166: 4.6 nM; Butaclamol: 0.96 nM; SKF81297: 30 nM; Dihydrexidine: 18.1 nM.

Using cocaine-induced gene changes in rodent brain as a guide (Bahi and Dreyer, 2005), we then determined whether the SK-N-MC human neuroepithelioma cell line expressed 26 axon guidance molecules(Axon guidance molecules) of interest. We found 17 of the 26 axon guidance molecule target genes were expressed in this cell line (Table 1, Figure 1). We focused on 13 of the 17 expressed axon guidance molecules, which were consistent and of high yield, for further assay and analysis.

Forskolin and Dihydrexidine Elevate cAMP in SK-N-MC Cells

Based on our postulate that changes in cAMP or GPCR receptor activity could alter axon guidance molecule expression, we first determined the feasibility of increasing cAMP levels by

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forskolin or by activation of endogenous dopamine receptors. SK-N-MC cells treated for 30 min with 10 μM forskolin increased cAMP approximately 340% above vehicle. The D1 dopamine receptor agonist, dihydrexidine (10 μM dihydrexidine, 30 min) resulted in much lower stimulation of cAMP, 22% above vehicle controls, which was reduced to 10% following pre-treatment with the D1 dopamine receptor antagonist SCH23390 for 20 min. Longer incubation periods did not further enhance cAMP production over vehicle. Accordingly, dihydrexidine-induced cAMP increase was partially mediated by D_1 receptors or the antagonist concentration was insufficient to fully attenuate cAMP activation. The combined effects of dihydrexidine and forskolin were potentiated and not additive, as cAMP accumulation increased to ~507% over vehicle (Figure 2).

Forskolin, Dihydrexidine and SKF 81297 Modulate Axon Guidance Molecule Gene Expression

We then determined whether increased cAMP was associated with changes in Axon Guidance Molecule gene expression. Forskolin treatment (10 μM, 6 hours) consistently increased expression of three of the 13 genes tested (Figure 3A). All results are expressed as change relative to vehicle and normalized to β-Actin and HMBS. Forskolin increased EphA5, EphB2 and Neuropilin1 (NRP1) expression. Cells exposed to dihydrexidine, a dopamine receptor agonist (Figure 3B) responded to dihydrexidine (10 μM, 6 hr) with upregulation of EphrinA1 and down-regulation of EphrinA5, the ephrin receptor, EphB1, the netrin receptor, DCC and Semaphorin3C. To confirm that changes in protein expression paralleled changes in gene expression, we performed western blot analysis of DCC protein, using the same conditions as before. Dihydrexidine treatment decreased DCC protein levels in agreement with changes in

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DCC gene expression (Figure 3C). To extend these findings to another D1 dopamine receptor agonist, we conducted parallel studies with SKF 81927, using data from 3 of 4 experiments. SFK 81297 promoted an increase in EphA5, EphrinA1, EphrinA5, and NRP1 genes, paralleling forskolin-mediated increases in expression of EphA5 and NRP1 genes and dihydrexidine increases in Ephrin A1 (Figure 4). SKF also reduced expression of EphrinB3.

We attempted to block dopamine receptor agonist-mediated effects with dopamine receptor antagonists, but the D_1-like receptor antagonist SCH23390 alone (50 μM, 6 hr; n=8) regulated the two housekeeping genes and another D1 dopamine receptor antagonist SCH39166 (10 μM, n = 3) altered expression patterns of axon guidance molecules. Treatment of SK-N-MC cells with the D2 receptor antagonist eticlopride (50 μM, 6 hr; n=4) had no effect on the gene changes mediated by 10 μM dihydrexidine but increased the magnitude of the EphrinA1 increase mediated by dihydrexidine (data not shown).

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Discussion

To our knowledge, the present study is the first to link changes in gene expression of axon guidance moleculeswith alterations in cAMP production or drug-induced GPCR activation. In a neuroepithelioma cell line, forskolin and dihydrexidine altered expression of axon guidance molecules, following a steep (forskolin) or modest (dihydrexidine) rise of cAMP levels, and possibly other signaling cascades. Forskolin, dihydrexidine, and SKF 81297 induced a unique pattern of gene expression, with SKF 81297 effects overlapping results of the other treatments. Our findings provide the first association between drug-induced GPCR receptor activation, signal transduction, and transcriptional regulation of axon guidance molecules. The only precedent for this association are reports that a change in cAMP concentration modulates axonal attraction-repulsion via axon guidance molecules (Lohof et al., 1992; Song et al., 1997).

cAMP and GPCR Stimulation: Association with Axong Guidance Molecule Expression

Cells exposed to forskolin consistently increased expression of EphA5, EphB2 and NRP1. The discrete biochemical sequelae mediating gene induction are unknown, but conceivably, a surge of cAMP production can trigger transcriptional activity to augment expression of EphA5 and EphB2 receptor genes. EphA5 and EphB2 are implicated in synaptic plasticity and synaptogenesis in the adult rodent hippocampus (Gao et al., 1998; Martinez et al., 2005). EphA5 may also contribute to drug-induced modulation of long-term potentiation in the CA1 region and decreased neurogenesis in adult rat hippocampus (Thompson et al., 2005; Yamaguchi et al., 2005). EphB2 receptors subserve a different spectrum of functions by modulating LTP, NMDA receptor function and normal spine formation in the hippocampus (Grunwald et al., 2001; Henderson et al., 2001). The semaphorin receptor neuropilin1 binds

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semaphorin class 3 molecules, but neuropilin1 function in adult brain is poorly understood. Notwithstanding the caveats associated with a cell culture model system, cAMP regulation of axon guidance molecules in the SK-N-MC cell line may be relevant to cAMP modulation of axon guidance molecules in neurons.

In contrast to forskolin, dihydrexidine up- or down-regulated a different and larger repertoire of axon guidance molecules. The agonist-induced changes in gene expression were not fully blocked by D1 or D2 receptor antagonists, as the D1 dopamine receptor antagonists SCH 23390 and SCH 39166 had direct effects either on housekeeping genes (SCH 23390) or on axon guidance molecule expression (SCH 39166). Conceivably, endogenous receptors with constitutive activity can, by themselves, modulate axon guidance molecules, with implications for therapeutic receptor antagonist drugs. Despite the comparatively modest dihydrexidine-induced increase in cAMP, changes elicited by dihydrexidine were of higher magnitude than those of forskolin. Dihydrexidine induced up-regulation of EphrinA1, a ligand implicated in neurogenesis of neural stem cells via Rap1 and the MAPK pathway (Aoki et al., 2004) and associated with delineating the striatal matrix (Janis et al., 1999). Whether it functions in adult brain, or contributes to enhanced striosome predominance of early gene expression in brain of monkeys treated repeatedly with cocaine, remains unknown (Saka et al., 2004).

The dihydrexidine-mediated decrease in EphB1 gene expression in cell culture paralleled EphB1 down-regulation in the nucleus accumbens shell of rats self-administering cocaine (Kumaresan et al., SFN abstract#561.21, 2005). Dihydrexidine also reduced the gene encoding the netrin receptor DCC, required for both repulsive and attractive responses to netrin1 (Hong et al., 1999). In adult rodent brain, DCC protein is expressed in dopaminergic neurons and their terminal fields (Osborne et al., 2005).

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Why do Forskolin and Dihydrexidine Modulate Axon Guidance Molecules Differently?

GPCR activation or cAMP production was associated with axon guidance molecule modulation, but not via a simple stoichiometric relationship. Non-parallel changes in axon guidance molecule gene expression elicited by forskolin or receptor agonists were anticipated, in view of the significant differences in forskolin- and D_1 receptor-mediated signaling events, Forskolin-stimulated cAMP production was > 10-fold higher than dihydrexidine, possibly triggering a different cascade of concentration-dependent signaling actions. Furthermore, at 10 μM, dihydrexidine is likely to activate both D_1 and D_2 dopamine receptors, and possibly alpha 2 adrenergic receptors which are expressed in SK-N-MC cells (Schaak et al., 1997). It is noteworthy to mention that dihydrexidine potentiated forskolin induction of cAMP, indicating that dihydrexidine did not stimulate G_i/G_o coupled GPCRs. Furthermore, the D1 dopamine receptor mediates other signaling cascades, in addition to cAMP and can heterodimerize with other receptors (Gines et al., 2000; O'Dowd et al., 2005). Axon guidance molecule gene expression levels engendered by SKF 81297 overlapped with, but were not identical to changes elicited by forskolin or dihydrexidine. This finding was also anticipated as, at the concentrations we used, SKF 81297 conceivably would activate a range of receptors, including 5-HT2C, 5-HT2A, α2-adrenergic receptors, if expressed in this cell line (NIMH Psychoactive Drug Screening Program (http://pdsp.cwru.edu/pdsp.htm). Drug-induced modulation of axon guidance molecule expression is likely to reflect an array of signal transduction sequelae triggered by activating various GPRCs receptors, as a function of agonist concentration.

Axon Guidance Molecules and Neuroadaptation

In cultured neuroepithelioma cells, exogenous compounds increased cAMP levels, and enhanced cAMP levels were associated with altered axon guidance molecule gene and protein

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expression. We recognize the perils of extrapolating observations and mechanisms based on cultured cells to organized brain tissue, as cultured cells do not model complex neuronal interactions, time course, and neuron-specific responses. Notwithstanding these caveats, the relevance of our findings to drug-induced modulation of axon guidance molecules in brain merit exploration. Axon guidance molecules are expressed in adult human and nonhuman primate brain (Hafner et al., 2004; Xiao et al., 2006) and are increasingly implicated in synaptic function and neuroadaptation (Yamaguchi and Pasquale, 2004; Pasquale, 2005) as well as response to the psychomotor stimulant cocaine. Various dosing regimens of cocaine modulate axon guidance molecules in a region-specific manner (Bahi and Dreyer, 2005), suggestive of causality between psychostimulant induced receptor activation, axon guidance molecule changes and neuroadaptation. The mechanisms by which cocaine might modulate axon guidance molecules are unknown, but cocaine-induced neurotransmitter activation of the G_s-coupled D1 dopamine receptors (or other receptors) and consequent elevation of cAMP may be linked to axon guidance molecule modulation (Yue et al., 1999; Halladay et al., 2000; Bahi and Dreyer, 2005, Xiao et al 2006). Cocaine-mediated neuroadaptation extends beyond the cellular level, to changes in synaptic strength, neuronal morphology and neural networks (Dong et al., 2004; Robinson and Kolb, 2004; Saka et al., 2004), processes that conceivably involve axon guidance molecule function. It is noteworthy that several axon guidance molecule genes altered by forskolin or dihydrexidine, were regulated in the same direction by cocaine administration in rat brain (Bahi and Dreyer, 2005). Enhanced cAMP levels, via AMG transcriptional regulation may also contribute to hippocampal plasticity. Axon guidance moleculesin hippocampus are implicated in modulating LTP and NMDA receptor function (Henderson et al., 2001; Contractor et al., 2002). Of relevance to the present study, forskolin is frequently used to induce LTP (Otmakhov et al.,

2004), via enhanced cAMP that modulates axon guidance molecule gene expression. Although highly speculative, axon guidance molecules may also contribute to antidepressant drug-induced hippocampal neurogenesis, by guiding integration of newly formed neurons (Santarelli et al., 2003).

Conceivably, exogenous compounds that activate neuron-specific membrane receptors and ion channels converge to trigger unique intracellular signaling cascades that govern the repertoire of Axon guidance molecules. In this way, axon guidance molecule gene expression may modulate neuronal activity and connectivity at many levels to provide a rich array of rapid or gradual neuroadaptive responses. The bidirectional signaling of membrane-associated Axon guidance molecules renders them well suited to mediate anterograde and retrograde directed information at synapses. This initial study provides a initial view and potential link between cAMP modulation by physiological or pharmacological compounds and changes in axon guidance molecules. The novel underlying hypothesis and implications of this work may be relevant, not only to neuroadaptive responses elicited by addictive and therapeutic drugs (e.g cocaine, antipsychotics, antidepressants), but to other receptor-mediated mechanisms in neurons, such as learning and memory.

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Footnotes.

a) Supported by DA06303 (BKM), DA11558 (BKM), DA15305 (BKM), RR00168. The research was presented in abstract form at the annual meeting of the Society for Neuroscience: Jassen A.K., Yang H, Miller G.M. and Madras B.K. Axonal Guidance Molecule Gene Expression is Regulated by cAMP stimulants: Implications for Drug-induced Neuroadaptation in Brain. Program No. 1032.14, 2005, Abstract viewer/Itinerary planner, Washington D.C., Society for Neuroscience.

b) Reprint requests: Bertha K. Madras

Department of Psychiatry, Harvard Medical School

Division of Neurochemistry, New England Regional Primate Research Center,

1 Pine Hill Drive, Southborough, MA 01772-9102,

Tel. 508-624-8073; Fax. 508-624-8166; E-mail: bertha_madras@hms.harvard.edu

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Legends for Figures

Figure 1. Example of Real time PCR curves for targets tested compared to no template control (NTC) curves. Representative amplification curves obtained using primer sets for 14 axon guidance molecules and two housekeeping genes in combination with the Universal Probe Library. Note: the flat NTC lines show that there is no contribution of contamination or primer secondary structure to the specific amplification curves.

Figure 2. Left: cAMP accumulation in response to 30 minute drug treatment of SK-N-MC cells. 10 μM dihydrexidine (DHX), 10 μM forskolin (FSK), forskolin+dihydrexidine, the drug combination all increased cAMP, whereas the D1 dopamine receptor antagonist SCH 23390 (SCH) decreased cAMP in SK-N-MC cells. Right: Forskolin data are removed and data expanded to reveal cAMP enhancement with dihydrexidine, attenuation of dihydrexidine effects by SCH 23390 and SCH 23390 reduction of cAMP. Data are the mean ± SEM from 3-7 independent experiments each performed in triplicate. Statistical significance of cAMP accumulation in response to drug as compared to vehicle treated was evaluated by the two-tailed Student's t test (*P=0.01; **P=0.007; ***P=0.0001).

Figure 3. Changes in axon guidance molecule gene expression in response to (**A**) 10 μM forskolin (FSK), (**B**) 10 μM dihydrexidine (DHX) treatment for 6 hours. Data are expressed as the means of ratios (drug treatment versus vehicle treatment) relative to two different housekeeping genes (Beta-Actin and HMBS) using the ΔΔCt method. Data are the average of 3-5 independent experiments ± SEM each performed in triplicate. (**C**) DCC protein expression is

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decreased after 6hr 10 μM dihydrexidine treatment as determined by western blot. Bands are duplicate lanes for each treatment and shown is a representative blot from 4 independent experiments. The control lane is a positive control lysate for DCC.

Figure 4. Changes in axon guidance molecule gene expression in response to 10 μM SKF 81297 treatment for 6 hours. Data re expressed as the means of ratios (drug treatment versus vehicle treatment) relative to two different housekeeping genes (Beta-actin and HMBS) using the ΔΔCt method.

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Table 1. Axon guidance molecule genes assayed for expression in SK-N-MC cells using the Universal Probe Library (Human). For those targets which we could not detect transcript at least two different primer sets were tested. Expression values are defined as follows: "++++"=CP<25.5; "+++"=CP25.5-28.5; "++"=CP28.5-31.5; "+"=CP31.5-34.5; "+/-" =CP34.5-40; "-" = CP>40 or negative. CP is the crossing point at which the real time PCR signal is first detectable above background (also referred to as the Ct or crossing threshold). Drug treatment effects were not assessed on axon guidance molecules with an expression level of "+/-" or "-". For amplification curves see Figure 1.

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Gene of interest	Accession #	Expression
Housekeeping genes (4)		
Beta-Actin	NM_001101	++++
HMBS	NM_000190	++
GUSB	NM_000181	++++
GAPDH	NM_002046	++++
Axon guidance molecule Genes (26)		
EphA1	NM_005232	-
EphA3	NM_005233	-
EphA4	NM_004438	+++
EphA5	NM_182472	++
EphA8	NM_020526	-
EphB1	NM_004441	+++
EphB2	NM_004442	++
EphrinA1	NM_004428	+++
EphrinA3	NM_004952	-
EphrinA5	NM_001962	++
EphrinB1	NM_004429	+/-
EphrinB2	NM_004093	+
EphrinB3	NP_001397	+
Neuropilin1 (NRP1)	NM_003873	++
Sema3A	NM_006080	+/-
Sema5A	NM_003966	-
Sema6A	NM_020796	++
Sema7A	NM_003612	-
Sema3C	NM_006379	+++
Sema3E	NM_012431	-
Sema4G	NM_017893	+/-
DCC	NM_005215	++
Neogenin	NM_002499	+++
UNC5A	XM_030300	+/-
Netrin1	NM_004822	-
Reelin	NM_173054	-
Dopamine Receptor Genes (6)		
D_1dopamine receptor	NM_000794	++
D_{2S}dopamine receptor	NM_016574	+
D_{2L}dopamine receptor	NM_000795	-
D_3dopamine receptor	NM_033660	-
D_4dopamine receptor	NM_000797	-
D_5dopamine receptor	NM_000798	-

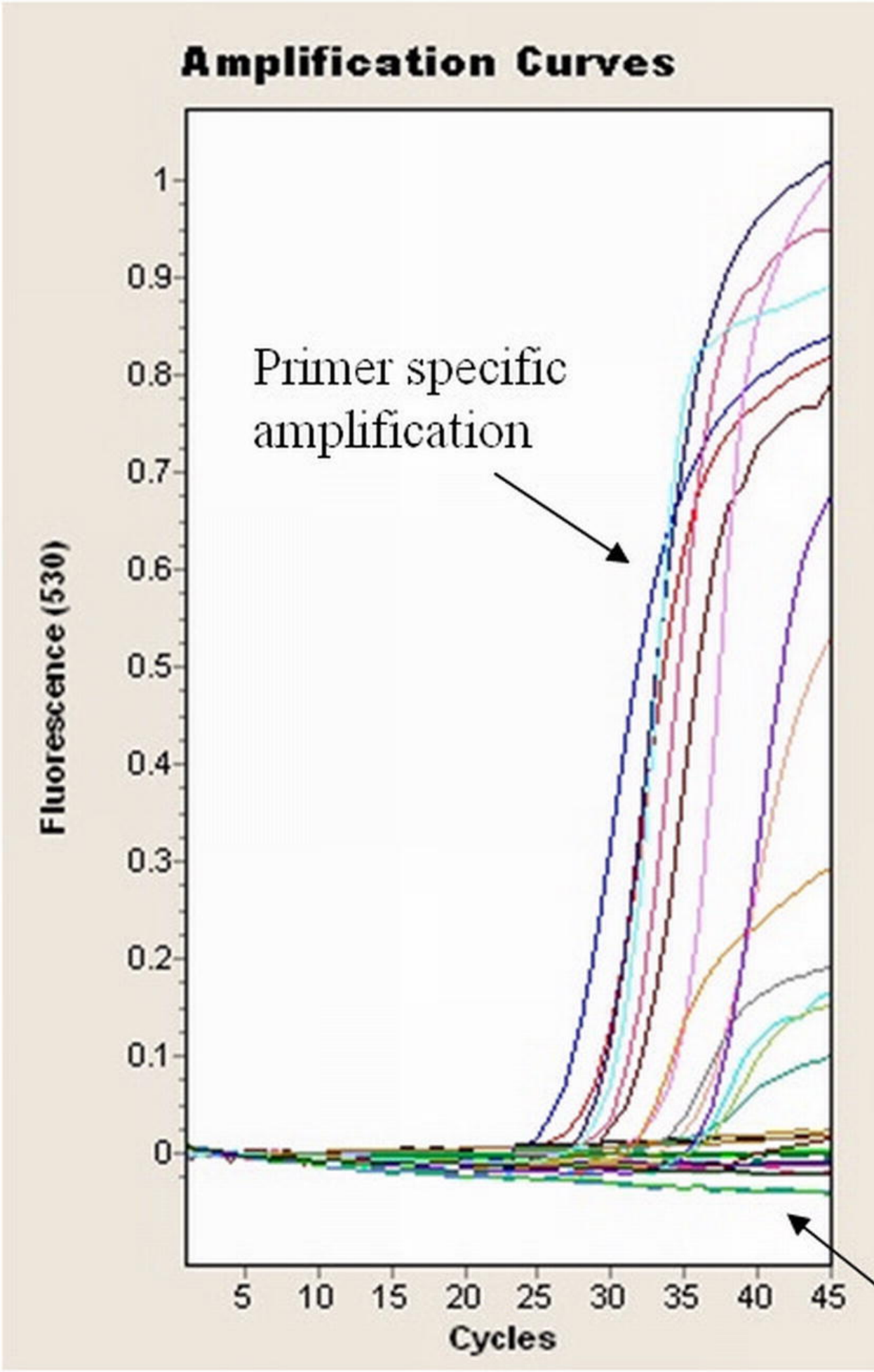

Amplification Curves
Fluorescence (530)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
35
40
45
Cycles
Primer specific amplification
NTC curves

Figure 2

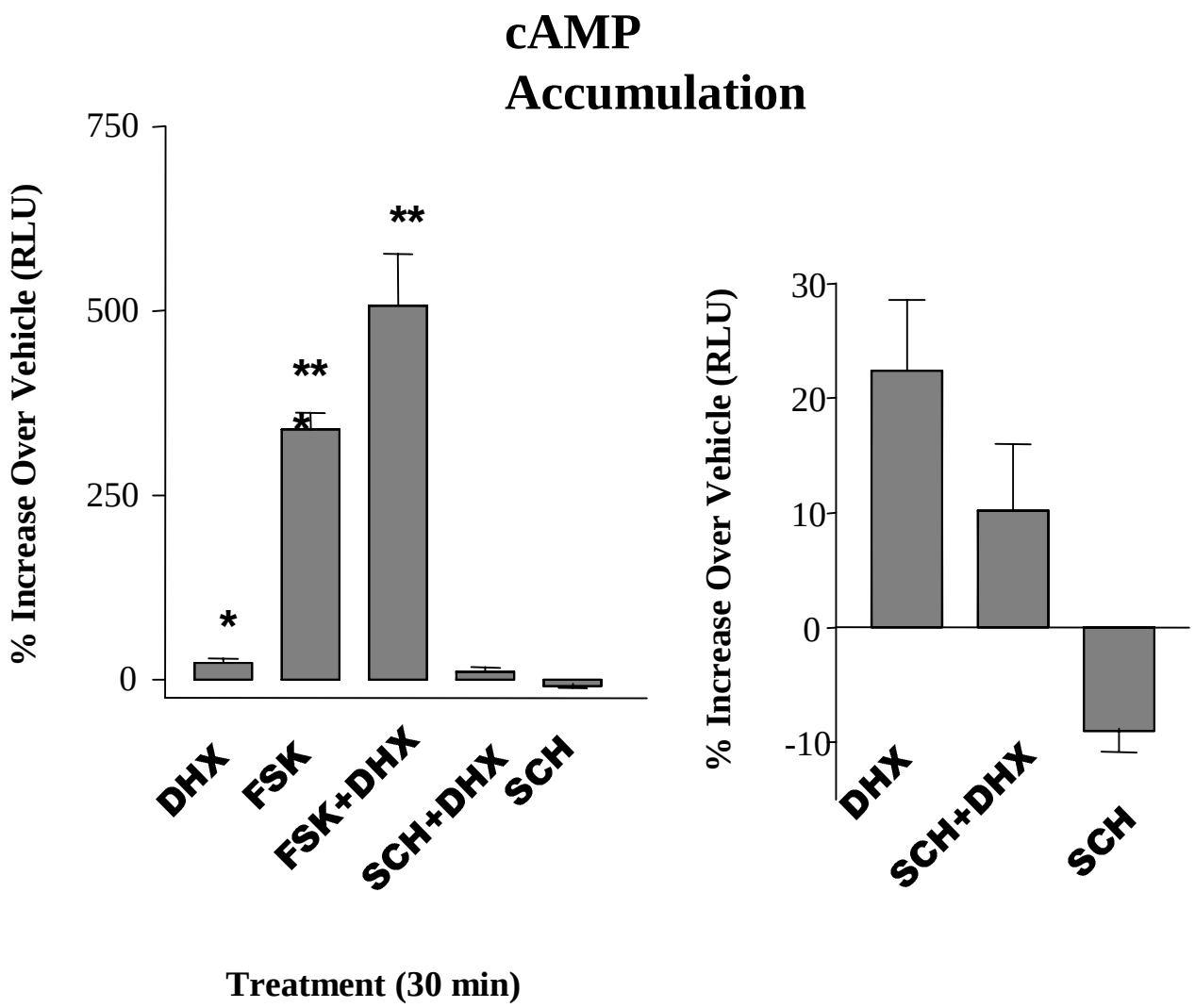


Figure 3 Part A

6 Hour 10 μM FSK Treatment

Gene	AVG ± SEM Beta- Actin	AVG ± SEM HMBS
EphA5	1.61 ± 0.06	1.57 ± 0.10
EphB2	1.53 ± 0.09	1.35 ± 0.08
NRP1	1.40 ± 0.08	1.26 ± 0.03

A

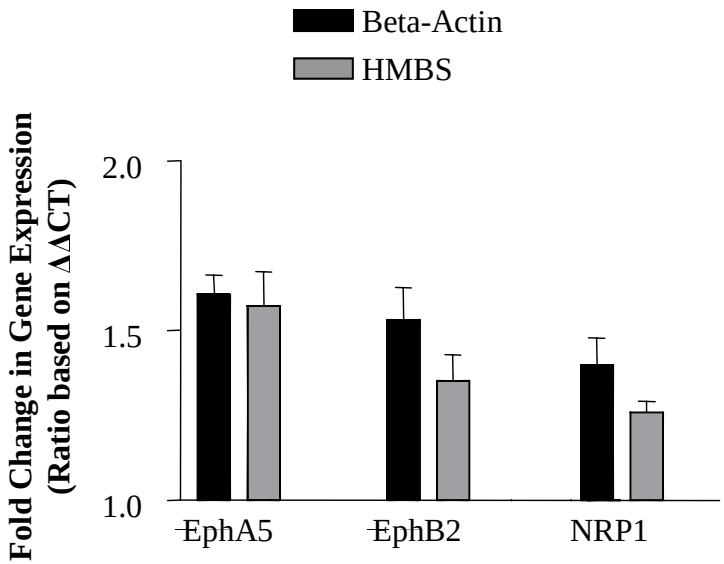
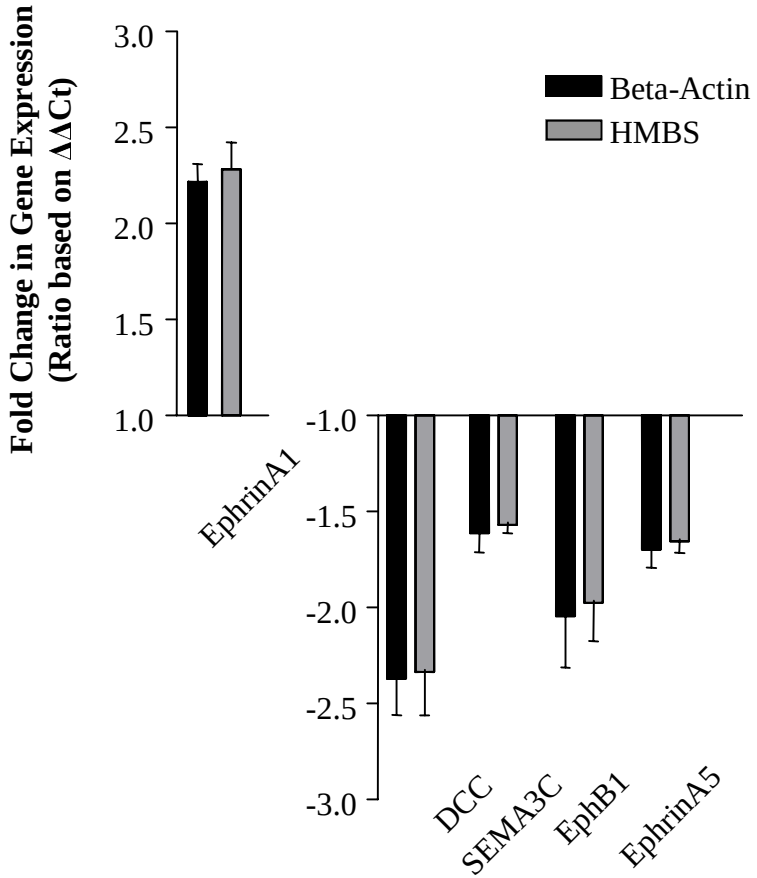


Figure 3B

6 Hour 10 µM DHX Treatment

Gene	AVG ± SEM Beta- Actin	AVG ± SEM HMBS
EphrinA1	2.22 ± 0.09	2.28 ± 0.14
DCC	-2.37 ± 0.19	-2.34 ± 0.23
Sema3C	-1.61 ± 0.10	-1.57 ± 0.05
EphB1	-2.05 ± 0.27	-1.97 ± 0.20
EphrinA5	-1.70 ± 0.01	-1.65 ± 0.06

B



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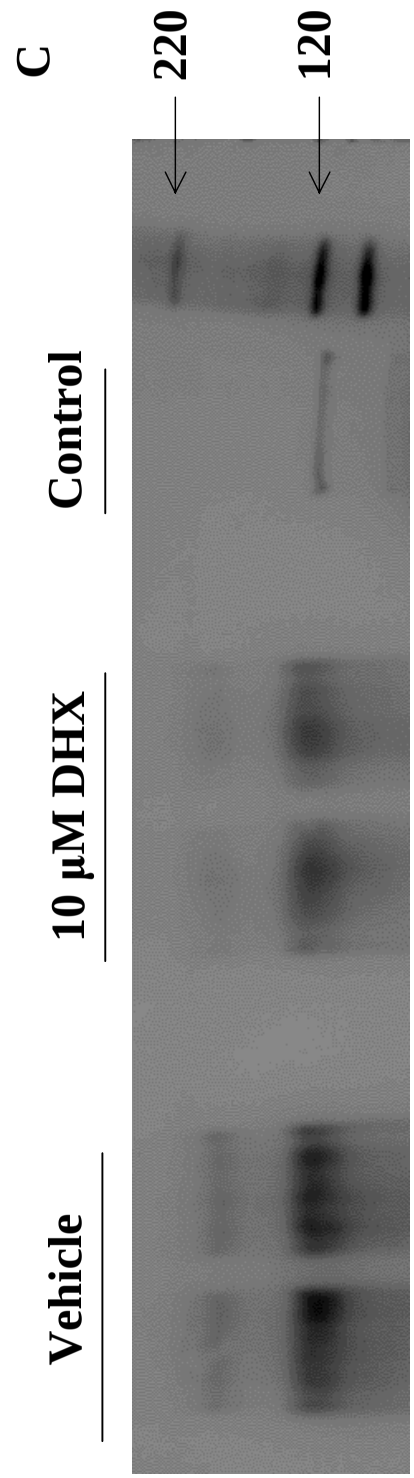

Figure 3C

Figure 4

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6 Hour 10 uM SKF 81297 Treatment