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A chemical genetic approach identifies piperazine antipsychotics as promoters of CNS neurite growth on inhibitory substrates

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Abstract

Injury to the central nervous system (CNS) can result in lifelong loss of function due in part to the regenerative failure of CNS neurons. Inhibitory proteins derived from myelin and the astroglial scar are major barriers for the successful regeneration of injured CNS neurons. Previously, we described the identification of a novel compound, F05, which promotes neurite growth from neurons challenged with inhibitory substrates *in vitro*, and promotes axonal regeneration *in vivo* (Usher et al., 2010). To identify additional regeneration-promoting compounds, we used F05-induced gene expression profiles to query the Broad Institute Connectivity Map, a gene expression database of cells treated with >1,300 compounds. Despite no shared chemical similarity, F05-induced changes in gene expression were remarkably similar to those seen with a group of piperazine phenothiazine antipsychotics (PhAPs). In contrast to antipsychotics of other structural classes, PhAPs promoted neurite growth of CNS neurons challenged with two different glial derived inhibitory substrates. Our pharmacological studies suggest a mechanism whereby PhAPs

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promote growth through antagonism of calmodulin signaling, independent of dopamine receptor antagonism. These findings shed light on mechanisms underlying neurite-inhibitory signaling, and suggest that clinically approved antipsychotic compounds may be repurposed for use in CNS injured patients.

Keywords

piperazine; phenothiazine; antipsychotics; chondroitin sulfate proteoglycans; myelin; regeneration; trifluoperazine; prochlorperazine

Introduction

A striking feature of the adult mammalian central nervous system (CNS) is that severed axons often fail to regrow and reform lost connections after injury. As a consequence, spinal cord injury (SCI) typically results in a lifelong loss of sensory and motor function below the level of the lesion. Unfortunately, there are currently no proven clinical therapies that can stimulate long distance regeneration and improve functional recovery for SCI patients.

CNS regenerative failure results from the poor intrinsic growth capacity of mature CNS neurons (Goldberg et al., 2002), together with the inhibitory environment of CNS lesions (Yiu and He, 2003). Genetic perturbations that stimulate intrinsic growth programs can improve regeneration (Liu et al., 2010; Moore et al., 2009; Park et al., 2008); however, environmental obstacles, such as those found in myelin and the glial scar, still limit axon growth through the lesion site (Yiu and He, 2003). Inhibitory proteins in myelin include Nogo (Huber and Schwab, 2000), myelin-associated glycoprotein (MAG; McKerracher et al., 1994), and oligodendrocyte myelin glycoprotein (Wang et al., 2002). Manipulations designed to overcome these myelin-derived molecules have resulted in limited improvements in regeneration (Atwal et al., 2008; Cafferty et al., 2010; Kubo et al., 2008; Lee et al., 2010; Walmsley and Mir, 2007). Similarly, glial scar proteins such as chondroitin sulfate proteoglycans (CSPGs) inhibit growth *in vitro* (Monnier et al., 2003; Snow, et al., 1990; Ughrin et al., 2003), and their expression correlates with regenerative failure *in vivo* (Davies et al., 1999; McKeon et al., 1991). Reducing expression of these CSPGs (Bradbury et al., 2002) or targeting their downstream signaling pathways (Koprivica et al., 2005; Shen et al., 2009; Sivasankaran et al., 2004) has led to a modest degree of regeneration and functional recovery after injury. Despite these successes, the signaling pathways responsible for growth inhibition by myelin proteins and CSPGs are not fully understood and regeneration-promoting therapies have yet to reach clinical practice.

The translation of novel therapies from the lab to the clinic is rife with obstacles. Failure rates for new drugs can be as high as 95%, and Food and Drug Administration (FDA)-approved new drugs spend an average of 13 years in testing at a cost of around \$1 billion (Collins, 2011). An increasingly attractive strategy to circumvent these problems is to discover new applications for drugs that are already clinically approved. One approach to achieve this “drug repurposing” is to compare the gene expression profiles of clinically approved compounds to profiles of perturbations that induce a phenotype of interest. Such strategies have been previously used to identify potentially novel therapies for hepatocellular carcinoma (Braconi et al., 2009; Chen et al., 2011), gastric cancer (Claerhout et al., 2011), ovarian cancer (Gullbo et al., 2011), lung cancer (Wang et al., 2011), colorectal cancer (Vilar et al., 2009), neuroblastoma (De Preter et al., 2009), and influenza (Josset et al., 2010). Here, we apply this comparative microarray approach to implicate novel strategies for improving regeneration after CNS injury.

Previously, we identified four novel compounds that promote CNS neurite outgrowth in the presence of an inhibitory myelin substrate *in vitro*. All four compounds also promoted growth on CSPG-derived substrates and on an *in vitro* model of the glial scar, but did not affect growth on permissive substrates (Usher et al., 2010). The signaling mechanisms through which these compounds act are not known, though they perturb growth cone microtubule dynamics. One compound, F05, promoted regeneration *in vivo* after acute transection of dorsal column sensory axons, as well as regrowth of retinal ganglion cell axons after optic nerve crush (Usher et al., 2010). These results suggest that the compounds can be exploited to identify convergent mechanisms of inhibitory environmental signaling and to develop treatment strategies for SCI.

In this study, we sought to uncover the signaling pathways affected by F05 and to identify clinically relevant regeneration-promoting compounds. Our approach makes use of the Broad Institute “Connectivity Map”, a repository of gene expression signatures for over 1,300 small molecules (Lamb et al., 2006). Importantly, the database derives signatures from both nondrug bioactive compounds as well as a variety of FDA-approved compounds, thereby allowing the opportunity to suggest novel uses for currently prescribed drugs. Our analysis identified a subclass of antipsychotics (piperazine phenothiazines) that induce remarkably similar changes in gene expression to those seen with F05. We found that this structural class of antipsychotics promotes neurite growth in different types of cultured CNS neurons challenged with either myelin proteins or CSPGs through a mechanism dependent on antagonism of calmodulin signaling.

Materials & Methods

Microarray

Microarray data from F05 and vehicle treated samples were obtained using methods similar to those employed by the Broad Institute to generate gene expression signatures for the Connectivity Map (Lamb et al., 2006). Human MCF7 breast adenocarcinoma cells (American Type Culture Collection) were grown to ~70% confluency over a period of 24 hours in DMEM media containing 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine (Gibco). The cells were then treated for 6 hr with F05 (5 μ M) or vehicle (DMSO, 0.05%) in the same media. Following treatment, total RNA was isolated using a standard TRIzol isolation procedure (Invitrogen), and purified using the RNeasy Kit (Qiagen, Valencia, CA). RNA quantity and quality (absorbance ratios 260/280>2; 260/230>1.8) were assessed using a NanoDrop[®] Spectrophotometer (ND-1000, Thermo Scientific). Three biological replicates were analyzed for each of the two conditions.

Total RNA was submitted to the Duke University Microarray Facility through the NINDS/NIMH Microarray Consortium. Samples were processed using Ambion MessageAmp Premier Target Labeling Service and hybridized to Affymetrix Human U133 2.0 Plus Arrays. Average probe set signal intensities (background subtracted, perfect match - mismatch) and detection calls for each array were calculated using MAS 5.0 software (Affymetrix). The data are publically available (GEO accession #GSE34331). Genes affected by F05 were identified using the following criteria: $p < 0.05$ using a t-test between the average signal intensities across the chips for DMSO vs. F05 treated; fold change ratios of F05/DMSO > 1.5 or < -1.5 ; and detection calls of “present” for all 6 chips (3 DMSO, 3 F05 treated). Probes meeting these criteria were used as “up tags” or “down tags” to query the Connectivity Map. The database automatically calculates a “Connectivity Score” using a non-parametric rank based pattern matching strategy (Gene Set Enrichment Analysis; Subramanian et al., 2005) that yields a value between +1 and -1 for each of the reference compounds. A value close to +1 indicates a high degree of similarity between the query and

reference compound, whereas a value close to -1 essentially indicates a reversal of expression patterns.

Cell culture

Tissue culture dishes were prepared by coating with 100 $\mu\text{g/mL}$ poly-D-lysine overnight followed by another overnight incubation with 5 $\mu\text{g/mL}$ laminin (Cultrex[®], Trevigen[®]) either alone or in combination with 1 $\mu\text{g/mL}$ CSPG (Millipore CC117; Ernst et al., 1995; Monnier et al., 2003). For the MAG experiments, hippocampal neurons were grown on a confluent monolayer of MAG or control-transfected Chinese hamster ovarian (CHO) cells (generously donated by Roman Giger, University of Michigan) using methods previously described (Mukhopadhyay et al., 1994).

Hippocampal neurons were prepared from E18 rats as described (Banker and Cowan, 1977; Bradke and Dotti, 1997) and cultured for 48 hours at 37°C, 5% CO₂ in a Neurobasal medium containing 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 2 mM glutamax, 10 mM Hepes, and 1X Neurocult[®] SM1 Neuronal Supplement (Stemcell). Retinal ganglion cells (RGCs) were harvested from P6 Sprague Dawley rats and purified by sequential immunopanning (Barres et al., 1988; Meyer-Franke et al., 1995). RGCs were grown for 48 hours at 37°C, 10% CO₂ in a defined serum-free medium (Meyer-Franke et al., 1995; Wang et al., 2007) containing 50 ng/mL BDNF, 10 ng/mL CNTF, 5 $\mu\text{g/mL}$ insulin, and 5 μM forskolin.

Neurons were plated and allowed to adhere to the dish for 1 hour in 2/3 the final volume of media, prior to addition of compounds (Table 1) or vehicle controls at 3X concentration to make up the final volume of media. Unless otherwise indicated, all tissue culture reagents were from Invitrogen (Gibco). Antipsychotic compounds were obtained from Spectrum Chemicals (Ellisville, MO), Calp1 was from Tocris Bioscience (New Brunswick, NJ), and all other compounds were from Sigma (St. Louis, MO). Supplemental Table I shows all the compounds used, their abbreviations, sources, and concentrations used.

Immunocytochemistry

After two days *in vitro* (DIV), neurons were fixed with 4% paraformaldehyde/4% sucrose in PBS. The blocking and antibody buffers for the hippocampal neurons contained 0.2% fish gelatin and 0.03% Triton X-100 in PBS. For the RGC and MAG-CHO experiments, cells were blocked in 20% normal goat serum, 0.02% Triton X-100 in antibody buffer (150 mM NaCl, 50 mM Tris base, 1% bovine serum albumin, 100 mM L-Lysine, 0.04% sodium azide, pH 7.4). After blocking for 1 hour at RT, cells were incubated in primary antibody overnight at 4°C. Primary antibodies included mouse anti- β -tubulin (E7, Developmental Studies Hybridoma Bank, University of Iowa) to assess neurite outgrowth and an antibody against the N-terminal extracellular domain of the D2 receptor (Chemicon #AB1558). For the RGC and MAG-CHO experiments a neuronal specific rabbit anti- β -III tubulin antibody was used (Sigma #SAB4500088). Secondary antibodies (goat anti-mouse or rabbit AlexaFluor 488) and nuclear dye (Hoechst; Invitrogen) were incubated with cells for 1 hr at room temperature.

Image acquisition and analysis

Cells were imaged and analyzed in an automated fashion by the ArrayScan[®] VTI (Cellomics, Pittsburgh, PA). Neuronal processes were traced according to user-defined parameters using the Extended Neurite Outgrowth and/or Neuronal Profiling bioapplication software (Cellomics; Blackmore et al., 2010). The mean total neurite length per neuron (the sum of the lengths of all the processes for a single neuron), the length of the longest process, the number of branch points (the sum of the instances where a single process diverged), and

the number of cells (valid neurons) were determined for cells with a minimum neurite length of 25 μm . For the MAG-CHO experiments, a blinded experimenter imaged randomly chosen fields (20X objective, Olympus IX81 inverted microscope). Total neurite length was assessed by manual tracing using Neurolucida (MicroBrightField). Each experiment was done using two separate, duplicate plates. Data were analyzed using either a t-test or a one-way ANOVA with the appropriate post-test (Dunnett's, Tukey's, or Bonferroni; GraphPad Prism). All data shown are mean $\pm$ SEM.

Results

The novel regeneration-promoting compound F05 induces changes in gene expression similar to those induced by piperazine phenothiazine antipsychotics (PhAPs)

The novel compound F05 can promote growth in a variety of *in vitro* assays in which neurons encounter glial inhibitory molecules, and it also promotes regeneration *in vivo*, but its mechanism of action is unclear (Usher et al., 2010). To learn more about mechanisms of growth inhibitory signaling, we took advantage of the Broad Institute Connectivity Map, which allows users to query a database containing gene expression signatures from human cells treated with FDA-approved drugs and other bioactive compounds (Lamb et al., 2006). We treated MCF7 breast cancer cells with F05, and identified gene expression changes compared to vehicle treatment after 6 hours, since this is the time point used to generate the Connectivity Map expression profiles. Only 21 genes were identified as changing significantly with F05 treatment under these conditions (Supplementary Table II). However, comparison of these changes with those in the Connectivity Map database revealed a number of compounds with gene expression signatures closely related to that of F05 (Table I). These "hits" (compounds whose signatures significantly and positively correlated with the F05 signature) included glucocorticoids, antihistamines, calcium channel blockers, antipsychotics, and antidepressants, among others (Tables I, II). Interestingly, 3 of the top 14 hits (prochlorperazine, fluphenazine, and trifluoperazine) were phenothiazine antipsychotic drugs (PhAPs; Table I). Indeed, this class was the number one-ranked group of signatures clustered according to the Anatomical Therapeutic Chemical [ATC] classifications (Table II). Since PhAPs are known to perturb a variety of cellular signaling processes (Mosnaim et al., 2006; Tajima et al., 2009), these results do not immediately suggest a mechanism of action that may be relevant to overcoming growth inhibition. However, they suggest that PhAPs may induce changes in cellular signaling like those produced by F05, and might, therefore, promote neurite outgrowth on inhibitory substrates.

PhAPs, but not other structural classes of antipsychotics, promote neurite outgrowth in cultured neurons challenged with an inhibitory CSPG substrate

To test the hypothesis that PhAPs promote neurite outgrowth, we screened a number of PhAPs, together with other types of antipsychotics, for their ability to promote neurite growth of neurons challenged with inhibitory CSPGs. We used dissociated hippocampal neurons because their morphological development is well understood (Barnes and Polleux, 2009), and because their neurite growth can be strongly inhibited by a mixture of brain-derived CSPGs (Ernst et al., 1995; Usher et al., 2010). In these experiments, 3 of 4 PhAPs tested, but none of the other antipsychotics, significantly enhanced neurite growth on the CSPG substrate (Figure 1A). In subsequent experiments, we found that all 4 PhAPs tested (trifluoperazine, prochlorperazine, perphenazine, and fluphenazine) were able to increase overall neurite length (Figure 1A and Supplemental Figure 1), axon length (length of the longest process) and neurite branching (Figure 1B, C) in hippocampal neurons on CSPGs. PhAPs did not affect neuronal survival at this concentration (Figure 1D).

To determine the optimal concentrations of PhAPs for promoting neurite growth, we examined dose-response relationships for the PhAPs as well as several non-piperazine antipsychotics. For the 3 PhAPs tested, we obtained similar dose-response relationships, with maximal neurite promotion at 2.5 μM , and a failure to promote growth (prochlorperazine) or even growth inhibition (perphenazine and trifluoperazine) at 5 μM concentrations (Figure 2A and data not shown). Optimal (2.5 μM) PhAP concentrations robustly promoted neurite extension (Figure 2B–D). Curve fitting for the prochlorperazine data suggested an EC_{50} around 500 nM, and this range likely also applies to perphenazine and trifluoperazine. The reason for the U-shaped dose-response relationships is unknown, but these observations are consistent with the drugs' having two mechanisms of action that are mutually antagonistic. In contrast to results with the PhAPs, the non-PhAP antipsychotics we tested, including thioridazine and pimozide, failed to promote growth at any concentration tested. These drugs, however, also inhibited neurite growth at higher concentrations (data not shown).

PhAPs promote neurite growth on a myelin-derived inhibitor but not on a permissive laminin substrate

The novel compound F05 promotes neurite growth on a variety of inhibitory substrates, but does not affect growth on permissive substrates, suggesting that it selectively interferes with a general aspect of inhibitory signaling (Usher et al., 2010). To determine whether PhAPs might also promote growth on different inhibitory substrates, we tested prochlorperazine using neurons challenged with a protein derived from CNS myelin (myelin-associated glycoprotein, MAG; Filbin, 2003; McKerracher et al., 1994). Hippocampal neurons were cultured on a monolayer of MAG-CHO cells, which express MAG on their surfaces and cause growth inhibition in CNS neurons (Mukhopadhyay et al., 1994). We found that hippocampal neurite growth was inhibited by cell surface MAG (Figure 3A–C), and that prochlorperazine reversed this inhibition (Figure 3A, B, C, E). This reversal was comparable to that produced by dibutyryl-cyclic AMP (dbcAMP; Figure 3A, D, E), which is known to overcome inhibitory myelin signaling (Cai et al., 1999; Domeniconi and Filbin, 2005). Thus, at least one PhAP can overcome growth inhibition caused by multiple classes of regeneration-inhibitory proteins.

To test whether PhAPs, like F05, can promote neurite growth on inhibitory substrates but not on permissive substrates, we characterized hippocampal neurite growth on a permissive laminin (LN) substrate in the presence and absence of PhAPs. None of the 4 PhAPs tested was able to improve growth on a LN substrate, when used at the same concentrations that were effective on the CSPG and MAG substrates (Figure 3F). Because neurons grow long processes on LN substrates, which might make it difficult to increase growth rates above baseline, we tested whether it was possible to demonstrate growth promotion in this assay using other agents. Indeed, we found that LN-mediated growth could be significantly increased by treatment with the protein kinase C inhibitor G 6976. G 6976 has previously been shown to increase growth of cerebellar granule neurons on inhibitory substrates, but not on a permissive poly-D-lysine (PDL) substrate (Sivasankaran et al., 2004). In hippocampal neurons, G 6976 increased growth both on inhibitory substrates and on permissive substrates such as LN and PDL (Figure 3F and unpublished results). Taken together, our results suggest that PhAPs, like F05, promote growth on inhibitory substrates by selectively reducing inhibitory signals in neurons.

PhAPs improve neurite growth in postnatal retinal ganglion cells on CSPG substrates

Adult CNS neurons exhibit lower growth potential than their embryonic counterparts (Blackmore et al., 2010; Bregman, 1998). In particular, there is a rapid developmental decline in the ability of rat retinal ganglion cells (RGCs) to grow axons during the first week

after birth (Goldberg et al., 2002). To test whether PhAPs, like F05, can promote growth of postnatal RGCs on inhibitory substrates, we sought to determine whether they could increase neurite growth in postnatal day 6 RGCs cultured on CSPGs. For these experiments, we chose prochlorperazine, which was the least toxic in the dose response assay (Figure 2A and data not shown). Prochlorperazine significantly enhanced neurite growth on the CSPG substrate, despite the low intrinsic growth capacity of the RGCs (Figure 4). Thus the growth-promoting effects of PhAPs are not restricted to hippocampal or to embryonic neurons.

Neurite growth promotion by PhAPs is dependent on calmodulin inhibition and not dopamine receptor antagonism

To gain insight into the mechanisms underlying the ability of PhAPs to promote neurite growth, we first tested whether the well-known ability of antipsychotics to antagonize dopamine receptors, particularly D2 receptors (Nord and Farde, 2011), was involved. Cultured hippocampal neurons express both D1 and D2 dopamine receptors (Chen et al., 2008), and we confirmed that they express D2 receptors on their surfaces in our culture conditions (Supplemental Figure 2). If dopamine receptor blockade is involved in growth promotion by PhAPs, we predicted 1) that other dopamine receptor antagonists would stimulate growth, and 2) that dopamine receptor agonists might interfere with the growth-promoting ability of PhAPs. However, we found that neither the D1 antagonist SCH-23390 nor the D2 antagonist sulpiride were able to promote neurite growth of hippocampal neurons on CSPG substrates (Figure 5A, B) despite using concentrations at and well above their known IC_{50} values (Iorio et al., 1983; Larson et al., 1995). The D1 agonist SKF-38393 inhibited growth when provided alone (Figure 5C and Supplemental Figure 3), and in parallel, impaired the ability of trifluoperazine to promote growth. Because SKF-38393 inhibited trifluoperazine-induced growth only at concentrations $\geq 5 \mu M$ (Figure 5C), more than 100X the known EC_{50} value (Van Vliet et al., 1990), this inhibition may not be due to D1 agonist activity. These results are further complicated by the fact that SKF-38393 elicited growth inhibitory effects when used alone. A D2 agonist, apomorphine, had no effects on neurite growth when used alone, and did not block growth promotion by trifluoperazine (Figure 5D), even at concentrations above its EC_{50} value (6.8 nM; Gardner et al., 1998). Overall, these results are inconsistent with the hypothesis that dopamine receptor antagonism is responsible for the growth-promoting effects of PhAPs.

In addition to blockade of various neurotransmitter subtypes (Mosnaim et al., 2006; Tajima et al., 2009), some PhAPs bind to and inhibit signaling through calmodulin, a ubiquitously expressed calcium-binding protein (Weiss et al., 1980). In particular, trifluoperazine is a calmodulin antagonist (Vandonselaar et al., 1994), and is widely used for this purpose (Feldkamp et al., 2010; Tanokura and Yamada, 1986). To investigate whether calmodulin antagonism plays a role in the ability of PhAPs to promote growth on inhibitory substrates, we treated cells with agonists and antagonists of calmodulin, similar to our strategy for investigating the role of dopamine receptors. We found that a calmodulin antagonist, W7, significantly promoted neurite growth at a concentration of 5 μM (Figure 6A). Interestingly, the growth response profile was similar to that observed with increasing doses of PhAPs (compare the sharp drop-off in growth in Figures 2A and 6A). For a calmodulin agonist, we chose the calcium-mimetic peptide Calp1, which binds to and activates calmodulin with an EC_{50} in the 50 μM range (Manion et al., 2000; Villain et al., 2000). When hippocampal neurons were cultured on a CSPG substrate, 200 μM Calp1 was able to completely abolish trifluoperazine's growth-promoting effect, but did not alter levels of neurite growth when used alone (Figure 6B). Taken together, these results suggest that the ability of trifluoperazine to promote growth on inhibitory substrates depends at least partly on calmodulin antagonism.

Discussion

Using a comparative microarray analysis of compound-induced changes in gene expression, we have demonstrated an unexpected similarity between piperazine phenothiazine antipsychotics and F05, a novel regeneration-promoting compound. PhAPs, but not antipsychotics of other structural classes, shared F05's ability to enhance CNS neuronal outgrowth in an assay in which growth was restricted by CSPGs. PhAPs were also able to overcome growth inhibition in response to the myelin-derived inhibitor MAG, but had no effect on neurite growth in the context of a permissive laminin substrate. The ability of PhAPs to promote growth over glial-inhibitory molecules was dependent on antagonism of calmodulin, and at least one other calmodulin antagonist, W7, is able to mimic the growth-promoting abilities of PhAPs.

These results highlight the dual utility of the comparative microarray approach. The Connectivity Map is a hypothesis-generating tool that can be used to identify signaling pathways affected by a compound of interest as well as to discover new properties of clinically prescribed drugs. The comparative microarray findings presented here led to the hypothesis that inhibition of calmodulin signaling might allow neurons to alleviate substrate-derived neurite growth restriction. These findings suggest that calmodulin could be exploited as a novel target for promoting CNS regeneration in the face of environmental barriers. In addition, our results suggest a previously unrecognized potential for piperazine phenothiazine antipsychotics to induce axonal regeneration when neurons are challenged with glial-derived inhibitory molecules. Thus, although antipsychotics are primarily prescribed to alleviate psychosis, our work suggests that they may be repurposed to improve regrowth after CNS injury.

The possibility of repurposing PhAPs for treatment of CNS injury raises questions concerning dosage and therapeutic index. In our *in vitro* assays, there was a narrow concentration window between efficacy (growth promotion) and toxicity (cell death), for the PhAPs tested. Presumably this reflects the existence of multiple targets for these drugs; future efforts could include structure-activity relationship studies to identify compounds that promote growth without killing cells. Alternatively, dose response studies *in vivo* could determine an optimal window for promotion of regeneration by existing PhAPs.

Prochlorperazine is typically prescribed at doses between 20 and 150 mg per day (~0.3–2.1 mg/kg for a 70 kg adult), depending on the indication and symptom severity. In one study, patients chronically treated with 120 mg trifluoperazine/day showed brain levels of 1.0 µg/mL (1.65 µM; Karson et al., 1992). Thus, PhAP concentrations that we find to be effective for growth promotion *in vitro* can be achieved with dosing schemes currently used in therapy. While PhAPs produce serious side effects, including extrapyramidal disorder, these would need to be balanced against the possibility of functional recovery from CNS trauma.

Other notable hits from the Connectivity Map include several agents that act on aspects of calcium signaling, including tetrandrine and fendiline. This reinforces our findings that targeting downstream calcium signaling pathways allows cells to overcome growth-inhibitory signals. In addition to PhAPs, there were several other clinically prescribed classes of drugs that emerged from the comparative microarray analysis. Antihistamines, vasodilators, antidepressants, glucocorticoid agonists, and antibiotics could all be studied further in assays of neuronal growth and regeneration. Future studies using any of these Connectivity Map hits could elucidate novel mechanisms of regenerative failure in the CNS and also point towards potential therapies for CNS injury.

Our studies show that PhAPs promote neurite growth from cells growing on a mixture of inhibitory CSPGs. We chose this mixture, which likely consists mainly of neurocan,

phosphacan, versican, and aggrecan (Ernst et al., 1995; Monnier et al., 2003), in order to mimic the CSPGs that accumulate at lesion sites after CNS injury (Asher et al., 2000; Levine 1994; McKeon et al., 1999; Monnier et al., 2003). Future studies could assess whether PhAPs are more active against some individual CSPGs as opposed to others, which might provide additional insight into the mechanism of growth promotion and the effects of shared domains among different CSPGs (e.g., immunoglobulin domains, hyaluronic acid-binding domains, chondroitin sulfate side chains).

Previous studies have suggested that antipsychotics could modulate neurite outgrowth, but none have implicated piperazine phenothiazines as a class. Antipsychotics such as olanzapine, quetiapine, and clozapine have been shown to enhance growth factor induced neurite outgrowth in PC12 cells, at concentrations of 10–40 μ M (Lu and Dwyer, 2005). In neuroblastoma cells, high (100 μ M) concentrations of haloperidol had the opposite effect, disrupting neuritic cytoskeletal organization (Benitez-King et al., 2010). Interestingly, clozapine, and to some extent fluphenazine, were able to increase axon lengths from mechanosensory neurons in *C. elegans*, when made available to larvae at high (160 μ M) concentrations (Donohoe et al., 2008). These drugs also inhibited neuronal migration, and it is unclear to what extent the effects on axons were direct or reflected abnormal positioning. In rats, haloperidol reduced the density of dopaminergic axon terminals when administered immediately after a lesion to the substantia nigra, but improved dopamine terminal sprouting when administered after a delay (Tripanichkul et al., 2003), suggesting that the growth response of neurons to haloperidol can vary dramatically under slightly different circumstances. This parallels our observations that PhAPs can have either no effect or a robust growth-promoting effect when added to cells that are growing under permissive versus inhibitory conditions, respectively. The findings presented here are the first to show that a specific class of antipsychotics can improve growth of primary neurons in the face of inhibitory molecules that prevent CNS regeneration after injury, and do so at low micromolar concentrations.

All clinically effective antipsychotics inhibit dopamine receptors, primarily D2 type receptors (Nord and Farde, 2011). Dopamine receptor signaling has been linked to transcriptional regulation of axon guidance molecules (Jassen et al., 2006), suggesting that modulation of dopamine pathways could affect neurite growth. Indeed, treatment with the D1 agonist SKF-38393 increased neurite length and arborization of dissociated rat striatal cells cultured on polyornithine (Schmidt et al., 1996). The D2 agonist quinpirole can potentiate cortical neurite length and branching on a polylysine substrate (Todd, 1992), and dopamine itself has been shown to increase neurite growth in striatal neurons (Schmidt et al., 1998). Thus dopamine receptor agonism is generally associated with increases in neurite growth. Since antipsychotics act as dopamine antagonists, we hypothesized that their growth-promoting effects were independent of their effects on dopamine signaling. Accordingly, our results suggest that the growth-promoting effect of PhAPs did not depend on antagonism of dopamine receptors. Similarly, the ability of antipsychotics to cause excessive axon growth of mechanosensory axons in *C. elegans* appears to be independent of dopamine receptor antagonism (Donohoe et al., 2008). Overall, the results indicate that the ability of PhAPs to increase neurite growth is independent of their ability to antagonize dopamine receptors.

In addition to their effect on dopamine receptors, antipsychotics are known to target a variety of cell surface receptors and intracellular signaling cascades (Miyamoto et al., 2005). Some of these targets, including serotonin receptors (Dudok et al., 2009; Homma et al., 2006), histamine receptors (Munis et al., 1998), NMDA receptors (George et al., 2009; Kuo et al., 2010), adrenergic receptors (Kwon et al., 1996), and muscarinic receptors (VanDeMark et al., 2009), have been implicated in the modulation of neuronal growth and

differentiation. We chose to investigate the calcium binding protein calmodulin, since phenothiazine-based compounds, and in particular the PhAP trifluoperazine, are known to bind to and inhibit calmodulin (Tanokura and Yamada, 1986; Vandonselaar et al., 1994).

Our results implicate calcium/calmodulin signaling in growth-inhibitory responses to extracellular cues after CNS injury. This is consistent with findings that both CSPGs and myelin proteins induce a local influx of calcium in growth cones that can affect turning behavior (Hasegawa et al., 2004; Henley et al., 2004; Snow et al., 1994), and with studies in invertebrates linking calmodulin to growth inhibition (Polak et al., 1991). However, since calcium influx can also potentiate neurite outgrowth (Homma et al., 2006; Kater and Mills, 1991), the relationship between calcium/calmodulin signaling and neurite growth is highly dynamic, and depends on the growth state of individual neurons. It is possible that the drop-off in neurite growth seen with high concentrations of both PhAPs and the calmodulin antagonist W7 reflects this dynamic relationship.

Since calmodulin appears to be a relevant target for the effects we observe, it is curious that pimozide, an antipsychotic known to inhibit calmodulin (Levin and Weiss, 1976), did not promote neurite outgrowth on CSPGs. It may be that pimozide affects additional signaling pathways that negate potential growth promotion resulting from calmodulin inhibition. Alternatively, pimozide and PhAPs could have differing effects on calmodulin's interaction with downstream effector proteins. These distinct possibilities should be addressed in future mechanistic studies.

In addition to the relevance of these findings to CNS regeneration, our results also have implications for the mechanisms through which antipsychotics alleviate symptoms of psychosis. Schizophrenia is characterized by deficits in neuronal growth and connectivity (Lynall et al., 2010; Skudlarski et al., 2010; Zalesky et al., 2011), and genes associated with schizophrenia, such as Disrupted in Schizophrenia 1 (DISC1), are known to play significant roles in neurite growth (Hattori et al., 2010; Miyoshi et al., 2003; Ozeki et al., 2003). In particular, there is evidence that glial-derived growth inhibitors are associated with schizophrenia. For example, levels of both CSPGs and the myelin-derived inhibitor Nogo are increased in postmortem schizophrenic brains (Novak et al., 2002; Novak and Tallero, 2006; Pantazopoulos et al., 2010). Therefore, the ability of antipsychotics to promote growth/sprouting in the presence of glial-derived inhibitory molecules may represent a mechanism for improving neuronal connectivity in schizophrenic patients. Similar considerations apply to other neurodevelopmental disorders for which antipsychotics are used, including autism and depression (McPheeters et al., 2011; Pae et al., 2011).

A major goal in both neuropsychiatric research and neuroregeneration studies is to understand and overcome the mechanisms that contribute to maladaptive pathology. Using a unique tool to compare gene expression profiles, we have uncovered a novel ability of PhAPs to improve neurite outgrowth from CNS neurons grown on glial-derived inhibitory molecules, and have identified calmodulin as a novel therapeutic target that could be manipulated to improve axonal regrowth. In addition, our results highlight an underappreciated link between CSPG and myelin inhibitory signaling and the developmental deficits associated with schizophrenia. Importantly, since piperazine antipsychotics are already clinically prescribed to alleviate psychosis, our work suggests that they could be repurposed to induce neuronal growth and restore connectivity after CNS injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	Central Nervous System
SCI	Spinal Cord Injury
CSPGs	Chondroitin Sulfate Proteoglycans
MAG	Myelin Associated Glycoprotein
PhAPs	Phenothiazine Antipsychotics
Tri	Trifluoperazine
Pro	Prochlorperazine
LN	Laminin
PDL	Poly-D-Lysine
RGC	Retinal Ganglion Cells

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Figure 1.

Piperazine phenothiazine antipsychotics (PhAPs) promote neurite outgrowth in hippocampal neurons grown on inhibitory substrates. **A**) Neurons were treated with various antipsychotics at 1 μ M (trifluoperazine, TRI; prochlorperazine, PRO; perphenazine, PER; fluphenazine, FLU; thioridazine, THI; quetiapine, QUE; chlorprothixene, CHL; pimozide, PIM; clozapine, CLO; and aripiprazole, ARI) and cultured for 2 days on a mixture of inhibitory CSPGs before assessment of neurite outgrowth (N=3 independent experiments). **B–E**) PhAPs particularly increase the length of the longest process (**B**) as well as the total number of branch points of the processes (**C**) without significantly affecting neuronal cell numbers (**E**). Asterisks indicate a significant difference from vehicle control (white bars, mean $\pm$ SEM, * <0.05 , ** <0.01 , *** <0.001).

Figure 2.

Dose-response relationships for growth promotion by PhAPs. **A)** Hippocampal neurons grown on laminin (LN, black bar) or on CSPGs (white and gray shaded bars) were treated with various doses of three PhAPs (perphenazine, prochlorperazine, trifluoperazine). The optimal dose to promote growth was 2.5 μ M, while at 5 μ M the drugs became toxic to the cells (cell count data not shown). Asterisks indicate significant differences from CSPG vehicle control (white bar; * <0.05 , ** <0.01 , *** <0.001 ; N=5). **B–D)** Representative images of cells on CSPGs treated with either vehicle or PhAPs shows the robust promotion of axonal outgrowth at 2DIV.

Figure 3.

PhAPs promote neurite growth on an inhibitory protein from myelin (MAG), but not on a permissive laminin substrate. **A)** The average total neurite length per neuron on either control or MAG-transfected CHO cells was assessed at 2DIV after treatment with either vehicle (0.01% DMSO), 100 nM dbcAMP as a positive control, or 1 μ M Pro. At least 100 neurons were analyzed per condition. Asterisks indicate significant differences from MAG-CHO vehicle control (* <0.05 , ** <0.01 , *** <0.001). **B–E)** Representative images show neurons stained for neuronal-specific β III tubulin (green) growing on the confluent monolayer of CHO cells (Hoechst nuclear stain, blue) under the various conditions. **F)** PhAPs (1 μ M) do not promote neurite outgrowth on a permissive laminin substrate, in contrast to the PKC inhibitor G 6976 (Go, 100 nM). Asterisks indicate a significant difference from DMSO vehicle control (** <0.01 , N=5).

Figure 4.

PhAPs also promote growth of mature RGCs on CSPG substrates. **A)** Postnatal day 6 RGCs were cultured on a mixture of inhibitory CSPGs and treated with various concentrations of Pro. Neurite lengths were assessed at 2DIV. Asterisks indicate significant differences from CSPG vehicle control (white bar, * <0.05 , ** <0.01 , $N=4$). **B–C)** Representative images show cells treated with vehicle (**B**) or 1 μ M Pro (**C**).

Figure 5.

Antagonism of dopamine receptors is not responsible for PhAP-induced growth promotion.

A) The D1 receptor antagonist SCH-23390 (SCH) does not mimic the growth-promoting effect of Tri. **B)** The D2 receptor antagonist sulpiride (Sul) also does not mimic trifluoperazine's ability to overcome CSPG inhibition. **C)** The dopamine D1 receptor agonist SKF-38393 (SKF) significantly inhibits PhAP-induced growth promotion (1 μM trifluoperazine). However, SKF by itself elicited parallel inhibitory effects on neurite growth compared to CSPG vehicle control. **D)** Apomorphine (APO, a D2 receptor agonist) did not block growth promotion by trifluoperazine and did not have effects on growth when used alone. Asterisks indicate a significant difference from CSPG vehicle control (white bars in A and B and gray dashed lines in C and D; * <0.05 , ** <0.01 , *** <0.001) and daggers denote a significant difference from 1 μM trifluoperazine (black bars in A and B and black dashed lines in C and D; † <0.05 , †† <0.01).

Figure 6.

PhAP induced neurite growth promotion involves calmodulin antagonism. **A)** The calmodulin antagonist W7 significantly enhances growth on CSPGs (N=3–6) in a dose-dependent manner, similar to PhAP effects. **B)** The calcium-like peptide Calp1 alone does not affect growth by itself but is able to completely negate trifluoperazine mediated growth promotion (N=4). Asterisks indicate significant differences from CSPG vehicle control. The black dashed line indicates treatment with 1 μM trifluoperazine alone, while the gray dashed line is the CSPG vehicle control. (*<0.05, ***<0.001).

A comparative microarray of MCF7 cells using the Broad Institute Connectivity Map identifies small molecules that induce changes in gene expression similar to those seen with a regeneration-promoting compound (F05). The top 15 compounds in the Connectivity Map database were ranked according to how similar their genetic signatures were to that of F05. The list is ranked in order of Connectivity Score, which is the degree of similarity between the signatures ($1 =$ perfectly correlated, $-1 =$ perfectly anticorrelated; see Lamb et al., 2006). The Table also shows significance values (p-values, p). The scores are averaged over n individual microarrays performed for each respective compound. Phenothiazine antipsychotic (PhAP) compounds (prochlorperazine, fluphenazine, and trifluoperazine) represented 2 of the top 3 and 3 of the top 14 hits.

Table 1

Rank	Perturbagen and Cell Line	Connectivity Score	p	n	Remarks
1	astemizole - MCF7	0.997	0.00002	2	antihistamine
2	prochlorperazine - MCF7	0.724	0.00002	9	piperazine phenothiazine
3	fluphenazine - MCF7	0.705	0.00002	10	piperazine phenothiazine
4	tetrandrine - MCF7	0.994	0.00004	2	calcium channel blocker
5	clomipramine - MCF7	0.994	0.00004	2	antidepressant
6	corticosterone - MCF7	0.992	0.00006	2	glucocorticoid
7	terfenadine - MCF7	0.992	0.00006	2	antihistamine
8	geldanamycin - MCF7	0.639	0.00016	10	antibiotic
9	mometasone - MCF7	0.985	0.0004	2	glucocorticoid
10	desipramine - MCF7	0.982	0.00062	2	antidepressant
11	niclosamide - MCF7	0.978	0.00084	2	anthelmintic
12	fendiline - MCF7	0.974	0.00111	2	calcium channel blocker
13	dilazep - MCF7	0.973	0.00119	2	vasodilator
14	trifluoperazine - HL60	0.83	0.00127	4	piperazine phenothiazine
15	fluticasone - MCF7	0.963	0.00239	2	glucocorticoid

Results from the Connectivity Map analysis when reference compounds were grouped by the Anatomical Therapeutic Chemical (ATC) Code chemical classification system. In this analysis, PhAPs were the top hit.

Table II

Rank	ATC Code	ATC Code Description	Place of Known Drug Action	Compound Names
1	N05AB	antipsychotics (phenothiazines with piperazine structure)	nervous system	perphenazine, thiothoprazine, prochlorperazine, trifluoperazine, fluphenazine
2	D07XC	corticosteroids	dermatologicals	mometasone, betamethasone
3	R06AX	antihistamines for systemic use	respiratory system	cyproheptadine, antazoline, ketotifen, astemizole, terfenadine, deproprine, pimehixene, mebhydrolin, triprolidine
4	D07AD	corticosteroids, very potent	dermatologicals	halcinonide, clobetasol
5	C01DX	vasodilators used in cardiac diseases	cardiovascular system	heptaminol, molisodimine, prenylamine, trapidil, dilazep
6	C10AA	lipid modifying agents (hmg coa reductase inhibitors)	cardiovascular system	fluvastatin, simvastatin, lovastatin
7	C02AA	antihypertensives (antiadrenergic agents, centrally acting rauwolfia alkaloids)	cardiovascular system	reserpine, rescinamine
8	C08EA	phenylalkylamine derivatives (non-selective calcium channel blockers)	cardiovascular system	fendiline, bepridil
9	N05AF	antipsychotics, psycholeptics (thioxanthene derivatives)	nervous system	flupentixol, zuclopenthixol, chlorprothixene
10	S01EA	ophthalmologicals (antiglaucoma preparations and miotics 1, sympathomimetics in glaucoma therapy)	sensory organs	dipivefrine, clonidine
11	P01BC	antiprotozoals, antimalarials (methanolquinolines)	antiparasitic products	mefloquine