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ANTI-CANCER DRUG INDUCED NEUROTOXICITY AND IDENTIFICATION OF RHO PATHWAY SIGNALING MODULATORS AS POTENTIAL NEUROPROTECTANTS

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Abstract

Many chemotherapy drugs are known to cause significant clinical neurotoxicity, which can result in the early cessation of treatment. To identify and develop more effective means of neuroprotection it is important to understand the toxicity of these drugs at the molecular and cellular levels. In the present study, we examine the effects of paclitaxel (taxol), cisplatin, and methotrexate on primary rat neurons including hippocampal, cortical, and dorsal horn/dorsal root ganglion neuronal cultures. We found that all of these anti-cancer drugs induce substantial neurotoxicity evidenced by neurite degeneration. The neurons are capable of recovering after treatment withdrawal, but taxol exerts a biphasic effect that results in the collapse of processes days after treatment is withdrawn. After cisplatin and methotrexate treatment, we observed the degeneration of neuronal processes including the reduction of dendritic branching, length, and altered growth cone formation, indicating an abnormal arrangement of the actin cytoskeleton consistent with the involvement of Rho family small GTPases. Inhibiting RhoA downstream effector p160^{ROCK}/Rho kinase using Y-27632, or activating p75 neurotrophin receptor (p75^{NTR}) using non-peptide mimetic LM11A-31, were able to reverse the degeneration caused by cisplatin and methotrexate. Therefore, the neurotoxicity resulting from exposure to the anti-cancer drugs cisplatin and methotrexate can be alleviated by inhibiting Rho signaling pathway.

Keywords

cancer drug; neurotoxicity; neuroprotectant; cisplatin; methotrexate; Rho GTPases

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1. Introduction

Neurotoxicity is a common and often dose-limiting complication of chemotherapy treatment (Beinert et al., 2000; Cavaliere and Schiff, 2006; Macdonald, 1991; Plotkin and Wen, 2003). It can involve acute alterations in consciousness, seizures, cerebral infarctions, paralysis, neuropathy, and ototoxicity. Subacute and delayed toxicities also occur and can greatly impact cancer patients' quality of life. However, despite intensive efforts on the management of the neurologic side effects of chemotherapy in patients and the development of chemo protective agents, there is no generally accepted therapy at present (Verstappen et al., 2003). Therefore, much research has been focused on the identification and understanding of the molecular and cellular mechanisms of neuroprotectants against such toxicities (Albers et al., 2007; Bianchi et al., 2006; Hilpert et al., 2005).

Recent studies have uncovered several potential neuroprotective pathways that may help decrease neurotoxicities induced by anti-neoplastic agents. Neurotrophic factors are known to enhance neuronal survival. Several studies report neurotrophin protection of spiral ganglion neurons in the primary auditory pathway from the neurotoxic effects of the anti-cancer drug, cisplatin (Bowers et al., 2002; Zheng et al., 1995). Nerve growth factor (NGF) is effective against the toxic sympathetic nerve injury induced by cisplatin, taxol, and vincristine (Fischer et al., 2001; Hayakawa et al., 1998; Hayakawa et al., 1999; Peterson and Crain, 1982). Anti-neoplastic agents attack cancer cells via different mechanisms; therefore, the actions of neuroprotectants would conceivably differ as well. For instance, it has been reported that NGF acts on the dynamics of tubulin polymerization, thus preventing neurite injuries caused by either taxol, a microtubule stabilizing factor, or vincristine, which destabilizes microtubules (Mekhail and Markman, 2002).

To identify consistent cellular events associated with neurotoxicity by anti-cancer drugs, we analyzed several common chemotherapeutic agents of different pharmacological mechanisms, including taxol, cisplatin, and methotrexate. Taxol stabilizes microtubules and prevents cell division in cancer cells but its major side-effect in treatment is neurotoxicity (Mielke et al., 2006). Cisplatin cross-links DNA which hampers DNA replication and has a long history of inducing peripheral neuropathy but also has been implicated in toxic leukoencephalopathy and ototoxicity (Filley, 1999). Methotrexate is a folic acid analog that has been shown to produce a number of cognitive deficits and other neurotoxicities in patients, including children (Asato et al., 1992; Mahoney et al., 1998; Mulhern et al., 1999). We applied these drugs directly to primary rat hippocampal and cortical neurons, as well as dorsal horn/dorsal root ganglion co-cultures, and quantified their effects on neurite number, length, branching capability, and nuclear integrity. We demonstrate an altered development of neurite arborization and growth cone formation following chemotherapeutic exposure. Importantly, the inhibition of Rho GTPase signaling can be used to rescue neurons from the anti-cancer drug cisplatin and methotrexate induced neurite degenerations.

2. Materials and Methods

2.1 Primary rat neuronal cultures and anti-cancer drug treatment

Primary hippocampal and cortical neuronal cultures were prepared as described by Banker and Goslin (Banker and Goslin, 1988) with minor modifications (Jones et al., 2002; Jones et al., 2004). Primary rat dorsal horn and dorsal root ganglion co-cultures were prepared according to (Labrakakis et al., 2000) with minor modifications as well. Briefly, 18-day timed pregnant rats were sacrificed, and the embryos were removed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Hippocampi, cortices, dorsal horns, and dorsal root ganglia were collected, and cells were dissociated by trypsinization and plated onto poly-L-lysine coated glass coverslips or cell culture plates. After neurons adhered to the substrate, the

medium was changed to B-27 supplemented Neurobasal (Invitrogen, Carlsbad, CA). This culture scheme allowed us to maintain viable low-density cultures for several weeks. Neurons were grown for 5 days *in vitro* (DIV) and then treated with one of the following anti-neoplastic agents for 2 days, 5 days, or 9 days: taxol, cisplatin, and methotrexate in 0.1% DMSO (Sigma Co, St. Louis, MO). To inhibit RhoA downstream effector Rho kinase/p160^{ROCK}, 10 μ M Y-27632 (Calbiochem, La Jolla, CA) was applied for 24 hours after cells were first incubated with anti-neoplastic drugs for 24 hours. LM11A-31, a small non-peptide p75^{NTR} ligand (Massa et al., 2006), was used at 100 nM to inhibit the activation of Rho GTPases and was applied in the same manner as Y-27632.

2.2 Immunofluorescence light microscopy

On 7 DIV, 10 DIV, or 14 DIV, neurons incubated with the anti-cancer drugs were fixed in 4 % paraformaldehyde for 15 minutes at 37°C. For double labeling experiments, neurons were treated with 0.2% Triton X-100 in PBS pH 7.4 for 15 minutes, blocked with 10 % BSA for 30 minutes, and stained with FITC phalloidin (Molecular Probes, Inc, Eugene, OR) and mouse monoclonal anti-MAP2 or mouse monoclonal anti-Tau-1 (Sigma, St Louis, MO). After PBS washes, nuclear morphology was assessed by staining with 0.2 μ g/ml Hoechst 33258 (Sigma, St Louis, MO). The coverslips were mounted and analyzed with a Zeiss Axiovert S100 (Carl Zeiss, Thornwood, NY). Primary dorsal horn/dorsal root ganglion neurons were fixed and permeabilized as previously described for hippocampal neurons and were subsequently stained with rabbit polyclonal anti- β -tubulin III (Sigma, St Louis, MO).

2.3 Morphological data analysis

Morphometric analyses were performed using MetaMorphTM 4.6/7.5 imaging software system (Universal Imaging Corp, West Chester, PA) as described before (Jones et al., 2004). Neurite numbers were determined by applying the MEASURE COUNT OBJECT function of the software system. For hippocampal and cortical neurons, dendritic processes which extended directly from the cell body were designated as primary dendrites, and only scored when longer than 10 μ m. Secondary dendrites were designated when processes branch off primary dendrites, and they were scored when they were longer than 10 μ m. Processes shorter than 10 μ m were considered sprouts and not counted. The imaging system was calibrated so that all neurite length measurements were true values. One-way ANOVA with Tukey post-test were performed using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA), and *p*-values were designated for each experiment, which was repeated at least three times. Any null hypothesis with the probability level less than 95 % was rejected.

2.4 MTT cytotoxicity assays

Cortical neurons were plated onto poly-L-lysine coated 96 well culture trays, and neurons were treated as described earlier. Neurobasal media with 1mg/ml of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was added to the cultures and allowed to incubate for 3 hours at 37°C. All media was removed from the cells and replaced with DMSO (99.9%). The plates were placed on a shaker and agitated at 60 rpm for 5 minutes. Then the 540 nm absorbance measurements were taken in a BioTek SynergyTM HT multidetection microplate reader. Statistical analysis was conducted using KC4TM software (BioTek Instrument, Inc, Winooski, VT).

2.5 Glutathione S-transferase pull-down assays

Glutathione S-transferase (GST) pull-down assays were performed as described previously (Jones et al., 2004). Briefly, 5 DIV cortical or hippocampal cultures were treated with DMSO, taxol, cisplatin, or methotrexate for 48 hours with or without co-incubation of 100 nM LM11A-31. GTP-bound RhoA was affinity precipitated, using the RhoA binding domain of

Rhotekin (GST-RBD) (Cytoskeleton, Denver, CO). Bound proteins were washed, resolved on 8–16% SDS-PAGE gels, transferred to PVDF membranes (Millipore, Bedford, MA), and immunoblotted with mouse monoclonal anti-RhoA (Cytoskeleton, Denver, CO), using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) for detection.

3. Results

3.1 Taxol, cisplatin, and methotrexate cause deterioration of MAP2 immunoreactive neuronal morphogenesis and abnormalities in the actin cytoskeleton

Taxol, cisplatin, and methotrexate have all been implicated to have neurotoxic effects during and after clinical treatment. To characterize the effect they exert on neuronal cells we first utilized developing primary hippocampal neurons as a model system. The development of primary hippocampal neurons in culture is a well characterized process that begins with the establishment of the axon followed by dendrite development and maturation (Dotti et al., 1988). Neurites begin to extend from the cell body at 0.5 DIV, with one of these developing into the axon by 3 DIV. Neurons establish major dendritic processes by 7 DIV. We first treated the cells from 5 DIV–7 DIV, the period of active dendrite extension and branch formation. These experiments were performed to provide initial insight into any effect the drugs would have on process formation and extension.

The neurons were stained with the dendrite specific marker MAP2 (Fig 1), axon specific marker Tau-1 (Fig 2), and FITC phalloidin to label filamentous actin (F-actin). Control neurons treated with DMSO (vehicle control) exhibited the normal morphology and polarity expected at 7 DIV. The dendrites showed the clear pattern of branching (Fig 1A, arrow), while F-actin clustered along the dendrites and at the tips (Fig 1B and 2B, arrowheads). The actin clusters along the dendrites represent the site of future branch formation, while the clusters at the tips demonstrate the active extension of the developing dendrites. Tau-1 immunoreactivity was as expected, with staining largely isolated to the established axon (Fig 2A, arrow). In normal, developing neurons, MAP2 is increasingly segregated into the dendrites while microtubule-associated protein Tau localizes to the axon (Jones et al., 2004; Kosik and Finch, 1987). However, taxol effects on neurons were quite distinct. The MAP2 staining showed prominent primary dendrites but fewer numbers of secondary dendrites (Fig 1C, arrow). Also, taxol treatment caused a reduction of clustered F-actin along the dendrites and at the tips of branches (Fig 1D and 2D, arrowheads). The stabilization of microtubules results in a disruption in the axon-dendrite polarity of these neurons that is exhibited in the Tau-1 localization. Taxol treated neurons have an abnormally higher amount of Tau-1 staining in the cell body and throughout all of the processes (Fig 2C, asterisks). Cisplatin treatment clearly caused adverse effects to developing dendrites, primarily retarding dendritic branching (Fig 1E, arrow) and reducing the amount of F-actin at the leading edge of the processes (Fig 1F and 2F, arrowheads). Changes in Tau-1 immunoreactivity were not obvious (Fig 2E, arrow). Methotrexate treatment produced unique cellular morphology; often only one major apical-like dendrite (Fig 1G, arrow) was present with fewer basal processes (Fig 1G, small arrow). This morphology was present in ~ 40 percent of treated neurons. In contrast to other drug treatments, F-actin was heavily concentrated at the growth cone of the apical-like dendrite (Fig 1H, arrowhead). Changes in Tau-1 immunoreactivity were not evident (Fig 2G, arrow). Hoechst staining revealed no severe incidents of nuclear condensation or other signs of cell death (Fig 1 and 2, inserts).

3.2 Taxol, cisplatin, and methotrexate cause significant changes in the number and length of neuronal dendrites

MAP2 immunoreactivity was used to quantify the changes seen in dendrite morphology. For taxol treated neurons, there was a significant reduction in the total number of dendrites and the primary dendrite length (Fig 3A). Cisplatin and methotrexate treatment caused a significant

reduction in both the number of primary and secondary dendrites and the length of primary dendrites (Fig 3A).

To determine whether the dendrite degeneration was accompanied by the loss of viability of drug treated neurons, we performed MTT cytotoxicity assays. The results of the assay confirmed that the drug treatments were not causing death in cultured neurons (Fig 3B). The same drug concentrations resulted in significant cell death in prostate cancer cell line CWR22Rv-1 (Fig 3C) (Pretlow et al., 1993). Thus, the effects seen on neuronal process formation is more likely a specific effect of the drug than the induction of cell death.

3.3 Recovery of neuronal dendrogenesis following anti-neoplastic drug withdrawal

Another indication that the neurons were not experiencing extreme cytotoxicity was their response to withdrawal of the anti-cancer drugs. We found that when the neurons were treated for 48 hours and then allowed to recover in drug-free media for an additional 72 hours, they could recover from the effects of the drug treatment. At 10 DIV, there were no significant differences between treated neurons and the control neurons with respect to the number of primary dendrites and the primary dendrite length (Fig 4A and B). Treated neurons were also allowed to culture for even longer periods to determine if any long-term effects of drug treatment would appear. At 14 DIV, all treated neurons were capable of maintaining dendritic recovery with the exception of 1 μ M taxol treated neurons. After their initial recovery to near normal condition at 10 DIV, they suddenly lost their ability to maintain dendrite number and length, with some neurons dying after this loss (Fig 4A and B).

3.4 Rho GTPase signaling in modulating dendrogenesis following anti-neoplastic drug treatment

The alterations in dendritic arborization and the disorganized actin cytoskeleton of drug treated neurons suggested the potential involvement of Rho GTPases. Several studies show that RhoA mediated neurite retraction can be prevented by inhibition of Rho kinase/p160^{ROCK} activity, a downstream effector of Rho A (Bito et al., 2000; Hirose et al., 1998; Nakayama et al., 2000). STI571 is an Abl tyrosine kinase inhibitor used to inhibit the constitutive active Abl tyrosine kinase activity which causes chronic myelogenous leukemia (CML). This anti-cancer drug caused impairments in hippocampal neuron dendritic formation, but treatment of neurons with Y-27632, a Rho kinase/p160^{ROCK} specific inhibitor (Uehata et al., 1997), reversed the inhibitory effect of STI571 on dendrogenesis (Jones et al., 2004). These data indicated that Rho kinase plays an important role in promoting dendrogenesis.

To determine whether anti-neoplastic drug induced dendritic alterations could be reversed by inhibiting Rho kinase/p160^{ROCK} activity, Y-27632 was added to the neuronal cultures 24 hours after they were treated with taxol, cisplatin, or methotrexate (Fig 5A and B). Neurons were fixed 24 hours later for MAP2 immunofluorescent light microscopy followed by quantification analyses. Neurons treated with DMSO at 7 DIV showed an elaborate, MAP2 positive, dendrite formation. Y-27632 promoted a significant elaboration of dendrites in neurons treated with cisplatin and methotrexate (Fig 5A and B), suggesting that Y-27632 can aid in the retention of normal neuronal process development following drug exposure. However, neuronal cultures in the presence of taxol did not display a strong response to Y-27632 treatment (Fig 5A and B), which would be expected because taxol acts on microtubule dynamics and not actin reorganization.

Growth factors and neurotrophins can alter Rho GTPases to mediate neurite extension and retraction. Ligand activation of p75^{NTR} neurotrophin receptor is known to suppress Rho GTPase activity (Yamashita et al., 1999). LM11A-31 is a recently described small molecule ligand of p75^{NTR} that induces survival signaling and inhibits proNGF-induced neuronal death

(Massa et al., 2003; Massa et al., 2006). To determine whether LM11A-31 can reduce cisplatin and methotrexate induced dendrite degeneration, we treated cells with 8 $\mu\text{g/ml}$ cisplatin or 10 μM methotrexate in the presence or absence of 100 nM LM11A-31. Dendritic analysis revealed that LM11A-31 provides a significant protective effect similar to that obtained with Y-27632 (Fig 5C and D). These data suggest that modulation of Rho signaling and/or other signaling events through p75^{NTR} activation can promote neuronal dendrogenesis during drug treatment.

3.5 Rho inhibition correlates with improved dendrogenesis following anti-cancer drug treatment

Since LM11A-31 binding to p75^{NTR} can potentially elicit other effects on neuronal dendrogenesis, we performed a Rho GTPase pull-down assay to determine if LM11A-31 modulates RhoA activity. Neuronal cultures were treated with taxol, cisplatin, or methotrexate and the relative amounts of Rho-GTP, the active form of Rho, were measured by Rhotekin-GST pull-down. We found that taxol, cisplatin, or methotrexate treatment resulted in increased RhoA activity (Fig 6, top panel). When neurons treated with anti-cancer drugs were co-incubated with LM11A-31, the RhoA activity was reduced (Fig 6, top panel). However, taxol treated neurons only displayed a minimal reduction of Rho activity with LM11A-31 treatment whereas cisplatin and methotrexate treated neurons showed a clear inhibition of Rho activity when co-incubated with LM11A-31. Therefore, LM11A-31 causes a decrease in RhoA activity, which corresponds with an increase in dendrogenesis in cisplatin and methotrexate treated neurons.

3.6 Dorsal horn/Dorsal root ganglion neurons undergo process retraction and branch reduction during cisplatin and methotrexate treatment which can be overcome by Rho pathway inhibition

The most common neurotoxicity exhibited in clinical practice associated with anti-cancer drugs is peripheral neuropathy, as many drugs do not easily cross the blood-brain barrier. We therefore investigated whether primary sensory neurons experience degeneration during cisplatin and methotrexate treatment and whether it can also be overcome by Rho pathway inhibition. Isolated dorsal horn/dorsal root ganglion (DRG) neurons extend long neurites with branches by 5 days in culture (Fig 7A, DMSO, upper panel). Class III β -tubulin is an isotype of tubulin which is neuron specific and known to be highly expressed in DRG neurons. Expression of β -tubulin III has been found to increase during neurite outgrowth (Luduena, 1993). Using β -tubulin III immunostaining, we found similar morphological changes in the sensory neurons as compared with the hippocampal cultures when they were exposed to cisplatin and methotrexate (Fig 7A, upper panel and Fig 7B). Most neurons maintained viability during cisplatin treatment but demonstrated significantly shorter processes with fewer branches extended. Methotrexate treatment caused a similar reduction in process length and branch number. The morphology of these neurons was restored towards control values when Y-27632 or LM11A-31 was used to inhibit Rho GTPase signaling (Fig 7A-E). However, taxol treated neurons did not respond to Rho inhibition.

4. Discussion

In our experiments, we used different types of primary rat neurons in an attempt to better characterize anti-cancer drug induced neurotoxicity. Peripheral neuropathy is considered the most common form of neurotoxicity associated with chemotherapy treatment, thus many studies focus on peripheral and sensory neurons. However, central nervous system tumors are the leading cause of death in pediatric oncology, which has created the necessity to more aggressively treat these tumors with chemotherapeutics in children (Zustovich et al., 2007). Recent studies have also identified a large number of long-term behavioral issues in pediatric oncology patients including depression, anxiety, and antisocial behavior. These effects appear

to be most prevalent in patients treated for CNS tumors or neuroblastoma (Schultz et al., 2007). Therefore, our current studies give a more complete picture of anti-cancer drug induced neurotoxicity by analyzing both CNS and PNS neurons.

Drug treated neurons were immunostained with both a well-established dendrite specific marker, MAP2, and an axonal specific marker, Tau-1 (Binder et al., 1986; Kosik and Finch, 1987). From our analyses, the MAP2 staining was the most sensitive marker for anti-cancer drug induced neurotoxicity in CNS neurons. As reported in Jones et al. 2004, the MAP2 marker has a distribution similar to other dendritic markers such as the transferrin receptor and the glutamate receptor. Tau-1 staining alone did not show significant differences in axon morphology between treatments, with the exception of polarity disruption evident with taxol treatment. Although Tau-1 and F-actin staining of the various drug treatments was affected, these effects could not be easily quantified or generalized. We found MAP2 to be a very sensitive and quantifiable marker for neurotoxicity associated with dendritic changes. Our studies suggest that MAP2 immunocytochemistry can be a potentially viable method of screening substances for neurotoxicity.

Taxol, cisplatin, and methotrexate all caused neurite degeneration and changes in the actin cytoskeleton of primary neurons in culture. The stabilization of the microtubule cytoskeleton and reduction in its dynamics are perhaps the major reason for the disruption of axon-dendrite polarity in taxol treated neurons (Mekhail and Markman, 2002). This lack of polarity may initiate the severe cellular stress that prevents neurons from maintaining long-term recovery. Further studies are needed to identify the molecular signals that are responsible for initiating the stress process. The morphological changes resulting from cisplatin and methotrexate toxicity can be partially reversed by Y-27632, a specific inhibitor of Rho downstream effector Rho kinase/p160^{ROCK}, indicating that signaling through Rho family of small GTPases is an underlying mechanism that can be utilized for protection against these neurotoxicities. Recently, the Rho family of small GTPases has proven to be integral in neuronal development and also is implicated in injury and inflammation. Rho GTPases are involved in the critical morphological changes that occur during development including neurite outgrowth, dendritic arbor maturation, and axon outgrowth (Davies, 2000; Schmidt and Hall, 2002). Rho activation has been shown to correlate with spinal cord injury in a number of studies (Dubreuil et al., 2006; Madura et al., 2004). Inhibition of Rho after neuronal injury encourages axonal outgrowth and regeneration of affected neurons (Ellezam et al., 2002; Madura et al., 2007). Cisplatin and methotrexate clearly retarded the normal development of neurite maturation in both hippocampal and dorsal horn/dorsal root ganglion neurons. Our data suggest this is due to an increase in GTP bound RhoA, which is detrimental to developing or regenerating neurons. Our studies further support the model that Rho GTPase signaling plays an important role in the regeneration of neuronal processes after injury.

Recent studies have also established that neurotrophic factors act upstream of the Rho GTPase cascade (Ozdinler and Erzurumlu, 2001) and are known to modulate neuronal survival (Fahnestock et al., 2004a; Fahnestock et al., 2004b). We found that LM11A-31, a small non-peptide p75^{NTR} ligand that promotes survival signaling and inhibits proNGF-induced neuronal death (Massa et al., 2006), reduced RhoA activity and was effective in controlling the neuronal degeneration induced by cisplatin and methotrexate. These studies further validate Rho signaling as an important element in the attenuation of anti-cancer drug induced neurotoxicity. Since Rho GTPases are now well established as a convergence point downstream of p75^{NTR} in the regulation of neurite outgrowth (Domeniconi et al., 2005; Yamashita and Tohyama, 2003; Yamashita et al., 1999; Yamauchi et al., 2004), we propose that the inhibition of p75^{NTR} activation of Rho GTPases is important to maintain normal neuronal morphology during anti-cancer drug exposure.

The signaling of neurotrophin receptor p75^{NTR} is rather complex and can lead to neurotrophic and/or apoptotic effects (for review, see Chao, 2003; Hempstead, 2002). It was shown that while small GTPase Ras was inactivated, Rho was activated by unstimulated p75^{NTR} (Blochl et al., 2004). However, in Schwann cells, endogenous RhoA was activated after stimulation with BDNF, whereas knockdown of p75^{NTR} inhibited this activation (Yamauchi et al., 2004). In spinal cord injury (SCI) model, blocking activation of Rho after SCI protects cells from p75^{NTR}-dependent apoptosis (Dubreuil et al., 2003). In our experiments, p75^{NTR} mimetic LM11A-31 stimulation led to inhibition of Rho GTPase in anti-cancer drug treated cells, supporting the notion that p75^{NTR} effects are context dependent. Therefore, our data supports the current literature but also demonstrates the potential efficacy of the Rho pathway as a therapeutic target to enhance regeneration after neurotoxin exposure.

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References

- Albers J, Chaudhry V, Cavaletti G, Donehower R. Interventions for preventing neuropathy caused by cisplatin and related compounds. *Cochrane Database Syst Rev* 2007;CD005228. [PubMed: 17253547]
- Asato R, Akiyama Y, Ito M, Kubota M, Okumura R, Miki Y, Konishi J, Mikawa H. Nuclear magnetic resonance abnormalities of the cerebral white matter in children with acute lymphoblastic leukemia and malignant lymphoma during and after central nervous system prophylactic treatment with intrathecal methotrexate. *Cancer* 1992;70:1997–2004. [PubMed: 1525778]
- Banker G, Goslin K. Developments in neuronal cell culture. *Nature* 1988;336:185–6. [PubMed: 3185736]
- Beinert T, Masuhr F, Mwela E, Schweigert M, Flath B, Harder H, Binder D, Oehm C, Behse F, Possinger K. Neuropathy under chemotherapy. *Eur J Med Res* 2000;5:415–23. [PubMed: 11076782]
- Bianchi R, Brines M, Lauria G, Savino C, Gilardini A, Nicolini G, Rodriguez-Menendez V, Oggioni N, Canta A, Penza P, Lombardi R, Minoia C, Ronchi A, Cerami A, Ghezzi P, Cavaletti G. Protective effect of erythropoietin and its carbamylated derivative in experimental Cisplatin peripheral neurotoxicity. *Clin Cancer Res* 2006;12:2607–12. [PubMed: 16638873]
- Binder LI, Frankfurter A, Rebhun LI. Differential localization of MAP-2 and tau in mammalian neurons in situ. *Ann N Y Acad Sci* 1986;466:145–66. [PubMed: 3089105]
- Bito H, Furuyashiki T, Ishihara H, Shibasaki Y, Ohashi K, Mizuno K, Maekawa M, Ishizaki T, Narumiya S. A critical role for a Rho-associated kinase, p160ROCK, in determining axon outgrowth in mammalian CNS neurons. *Neuron* 2000;26:431–41. [PubMed: 10839361]
- Blochl A, Blumenstein L, Ahmadian MR. Inactivation and activation of Ras by the neurotrophin receptor p75. *Eur J Neurosci* 2004;20:2321–35. [PubMed: 15525274]
- Bowers WJ, Chen X, Guo H, Frisina DR, Federoff HJ, Frisina RD. Neurotrophin-3 transduction attenuates cisplatin spiral ganglion neuron ototoxicity in the cochlea. *Mol Ther* 2002;6:12–8. [PubMed: 12095298]
- Cavaliere R, Schiff D. Neurologic toxicities of cancer therapies. *Curr Neurol Neurosci Rep* 2006;6:218–26. [PubMed: 16635431]
- Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* 2003;4:299–309. [PubMed: 12671646]
- Davies AM. Neurotrophins: neurotrophic modulation of neurite growth. *Curr Biol* 2000;10:R198–200. [PubMed: 10712898]
- Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, Chao MV, Filbin MT. MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* 2005;46:849–55. [PubMed: 15953414]

- Dotti CG, Sullivan CA, Banker GA. The establishment of polarity by hippocampal neurons in culture. *J Neurosci* 1988;8:1454–68. [PubMed: 3282038]
- Dubreuil CI, Marklund N, Deschamps K, McIntosh TK, McKerracher L. Activation of Rho after traumatic brain injury and seizure in rats. *Exp Neurol* 2006;198:361–9. [PubMed: 16448651]
- Dubreuil CI, Winton MJ, McKerracher L. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. *J Cell Biol* 2003;162:233–43. [PubMed: 12860969]
- Ellezam B, Dubreuil C, Winton M, Loy L, Dergham P, Selles-Navarro I, McKerracher L. Inactivation of intracellular Rho to stimulate axon growth and regeneration. *Prog Brain Res* 2002;137:371–80. [PubMed: 12440379]
- Fahnestock M, Yu G, Coughlin MD. ProNGF: a neurotrophic or an apoptotic molecule? *Prog Brain Res* 2004a;146:101–10. [PubMed: 14699959]
- Fahnestock M, Yu G, Michalski B, Mathew S, Colquhoun A, Ross GM, Coughlin MD. The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. *J Neurochem* 2004b;89:581–92. [PubMed: 15086515]
- Filley CM. Toxic leukoencephalopathy. *Clin Neuropharmacol* 1999;22:249–60. [PubMed: 10516874]
- Fischer SJ, Podratz JL, Windebank AJ. Nerve growth factor rescue of cisplatin neurotoxicity is mediated through the high affinity receptor: studies in PC12 cells and p75 null mouse dorsal root ganglia. *Neurosci Lett* 2001;308:1–4. [PubMed: 11445271]
- Hayakawa K, Itoh T, Niwa H, Mutoh T, Sobue G. NGF prevention of neurotoxicity induced by cisplatin, vincristine and taxol depends on toxicity of each drug and NGF treatment schedule: in vitro study of adult rat sympathetic ganglion explants. *Brain Res* 1998;794:313–9. [PubMed: 9622663]
- Hayakawa K, Itoh T, Niwa H, Yamamoto M, Liang Y, Doyu M, Sobue G. Nerve growth factor prevention of aged-rat sympathetic neuron injury by cisplatin, vincristine and taxol--in vitro explant study. *Neurosci Lett* 1999;274:103–6. [PubMed: 10553948]
- Hempstead BL. The many faces of p75NTR. *Curr Opin Neurobiol* 2002;12:260–7. [PubMed: 12049931]
- Hilpert F, Stahle A, Tome O, Burges A, Rossner D, Spathe K, Heilmann V, Richter B, du Bois A. Neuroprotection with amifostine in the first-line treatment of advanced ovarian cancer with carboplatin/paclitaxel-based chemotherapy--a double-blind, placebo-controlled, randomized phase II study from the Arbeitsgemeinschaft Gynakologische Onkologie (AGO) Ovarian Cancer Study Group. *Support Care Cancer* 2005;13:797–805. [PubMed: 16025262]
- Hirose M, Ishizaki T, Watanabe N, Uehata M, Kranenburg O, Moolenaar WH, Matsumura F, Maekawa M, Bito H, Narumiya S. Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma NIE-115 cells. *J Cell Biol* 1998;141:1625–36. [PubMed: 9647654]
- Jones SB, Lanford GW, Chen YH, Morabito M, Kim K, Lu Q. Glutamate-induced delta-catenin redistribution and dissociation from postsynaptic receptor complexes. *Neuroscience* 2002;115:1009–21. [PubMed: 12453475]
- Jones SB, Lu HY, Lu Q. Abl tyrosine kinase promotes dendrogenesis by inducing actin cytoskeletal rearrangements in cooperation with Rho family small GTPases in hippocampal neurons. *J Neurosci* 2004;24:8510–21. [PubMed: 15456825]
- Kosik KS, Finch EA. MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum. *J Neurosci* 1987;7:3142–53. [PubMed: 2444675]
- Labrakakis C, Gerstner E, MacDermott AB. Adenosine triphosphate-evoked currents in cultured dorsal root ganglion neurons obtained from rat embryos: desensitization kinetics and modulation of glutamate release. *Neuroscience* 2000;101:1117–26. [PubMed: 11113360]
- Macdonald DR. Neurologic complications of chemotherapy. *Neurol Clin* 1991;9:955–67. [PubMed: 1758434]
- Madura T, Kubo T, Tanag M, Matsuda K, Tomita K, Yano K, Hosokawa K. The Rho-associated kinase inhibitor fasudil hydrochloride enhances neural regeneration after axotomy in the peripheral nervous system. *Plast Reconstr Surg* 2007;119:526–35. [PubMed: 17230085]
- Madura T, Yamashita T, Kubo T, Fujitani M, Hosokawa K, Tohyama M. Activation of Rho in the injured axons following spinal cord injury. *EMBO Rep* 2004;5:412–7. [PubMed: 15031718]

- Mahoney DH Jr, Shuster JJ, Nitschke R, Lauer SJ, Steuber CP, Winick N, Camitta B. Acute neurotoxicity in children with B-precursor acute lymphoid leukemia: an association with intermediate-dose intravenous methotrexate and intrathecal triple therapy--a Pediatric Oncology Group study. *J Clin Oncol* 1998;16:1712-22. [PubMed: 9586883]
- Massa SM, Xie Y, Longo FM. Alzheimer's therapeutics: neurotrophin domain small molecule mimetics. *J Mol Neurosci* 2003;20:323-6. [PubMed: 14501015]
- Massa SM, Xie Y, Yang T, Harrington AW, Kim ML, Yoon SO, Kraemer R, Moore LA, Hempstead BL, Longo FM. Small, nonpeptide p75NTR ligands induce survival signaling and inhibit proNGF-induced death. *J Neurosci* 2006;26:5288-300. [PubMed: 16707781]
- Mekhail TM, Markman M. Paclitaxel in cancer therapy. *Expert Opin Pharmacother* 2002;3:755-66. [PubMed: 12036415]
- Mielke S, Sparreboom A, Mross K. Peripheral neuropathy: a persisting challenge in paclitaxel-based regimes. *Eur J Cancer* 2006;42:24-30. [PubMed: 16293411]
- Mulhern RK, Reddick WE, Palmer SL, Glass JO, Elkin TD, Kun LE, Taylor J, Langston J, Gajjar A. Neurocognitive deficits in medulloblastoma survivors and white matter loss. *Ann Neurol* 1999;46:834-41. [PubMed: 10589535]
- Nakayama AY, Harms MB, Luo L. Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci* 2000;20:5329-38. [PubMed: 10884317]
- Ozdinler PH, Erzurumlu RS. Regulation of neurotrophin-induced axonal responses via Rho GTPases. *J Comp Neurol* 2001;438:377-87. [PubMed: 11559894]
- Peterson ER, Crain SM. Nerve growth factor attenuates neurotoxic effects of taxol on spinal cord-ganglion explants from fetal mice. *Science* 1982;217:377-9. [PubMed: 6124041]
- Plotkin SR, Wen PY. Neurologic complications of cancer therapy. *Neurol Clin* 2003;21:279-318, x. [PubMed: 12690653]
- Pretlow TG, Wolman SR, Micale MA, Pelley RJ, Kursh ED, Resnick MI, Bodner DR, Jacobberger JW, Delmoro CM, Giaconia JM, et al. Xenografts of primary human prostatic carcinoma. *J Natl Cancer Inst* 1993;85:394-8. [PubMed: 8433392]
- Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev* 2002;16:1587-609. [PubMed: 12101119]
- Schultz KA, Ness KK, Whitton J, Recklitis C, Zebrack B, Robison LL, Zeltzer L, Mertens AC. Behavioral and social outcomes in adolescent survivors of childhood cancer: a report from the childhood cancer survivor study. *J Clin Oncol* 2007;25:3649-56. [PubMed: 17704415]
- Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990-4. [PubMed: 9353125]
- Verstappen CC, Heimans JJ, Hoekman K, Postma TJ. Neurotoxic complications of chemotherapy in patients with cancer: clinical signs and optimal management. *Drugs* 2003;63:1549-63. [PubMed: 12887262]
- Yamashita T, Tohyama M. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat Neurosci* 2003;6:461-7. [PubMed: 12692556]
- Yamashita T, Tucker KL, Barde YA. Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* 1999;24:585-93. [PubMed: 10595511]
- Yamauchi J, Chan JR, Shooter EM. Neurotrophins regulate Schwann cell migration by activating divergent signaling pathways dependent on Rho GTPases. *Proc Natl Acad Sci U S A* 2004;101:8774-9. [PubMed: 15161978]
- Zheng JL, Stewart RR, Gao WQ. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. *J Neurosci* 1995;15:5079-87. [PubMed: 7623136]
- Zustovich F, Cartei G, Ceravolo R, Zovato S, Della Puppa A, Pastorelli D, Mattiazzi M, Bertorelle R, Gardiman MP. A phase I study of cisplatin, temozolomide and thalidomide in patients with malignant brain tumors. *Anticancer Res* 2007;27:1019-24. [PubMed: 17465237]

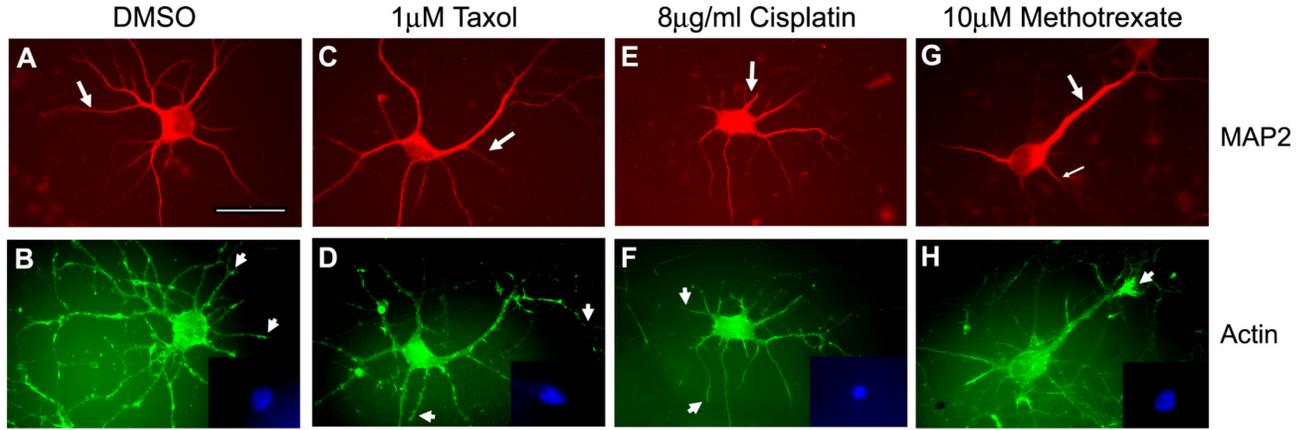


Figure 1. Anti-neoplastic drug treatment causes abnormal dendritic morphology in primary hippocampal neurons

MAP2, FITC phalloidin, and Hoechst (inserts) staining of hippocampal neurons treated with DMSO as a vehicle control, 1 μ M taxol, 8 μ g/ml cisplatin, or 10 μ M methotrexate for 48 hours (arrows point to MAP2 immunoreactive dendrites and arrowheads point to F-actin in neuronal processes) from 5 DIV to 7 DIV. In methotrexate treated neurons, arrow points to apical-like dendrite whereas smaller arrow indicates basal-like dendrites. Scale bar: 25 μ m.

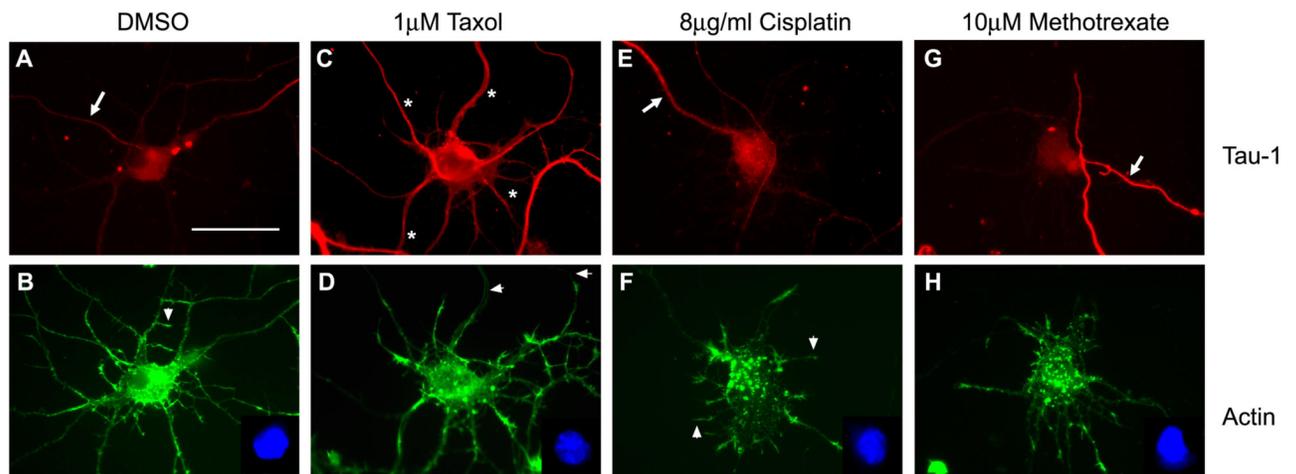


Figure 2. Taxol treatment causes a disruption in axon-dendritic polarity in primary hippocampal neurons

Tau-1, FITC phalloidin, and Hoechst (inserts) staining of hippocampal neurons treated with DMSO as a vehicle control, 1 μM taxol, 8 $\mu\text{g/ml}$ cisplatin, or 10 μM methotrexate for 48 hours from 5 DIV to 7 DIV. Arrows point to Tau-1 immunoreactive axons in DMSO, cisplatin or methotrexate treated neurons. Asterisks indicate multiple Tau-1 immunoreactive processes emanating from the same neuron in taxol treated cultures. Arrowheads point to the tips of the processes where F-actin is normally clustered in DMSO treated neurons, but often weakened in taxol and cisplatin treated neurons. Scale bar: 25 μm .

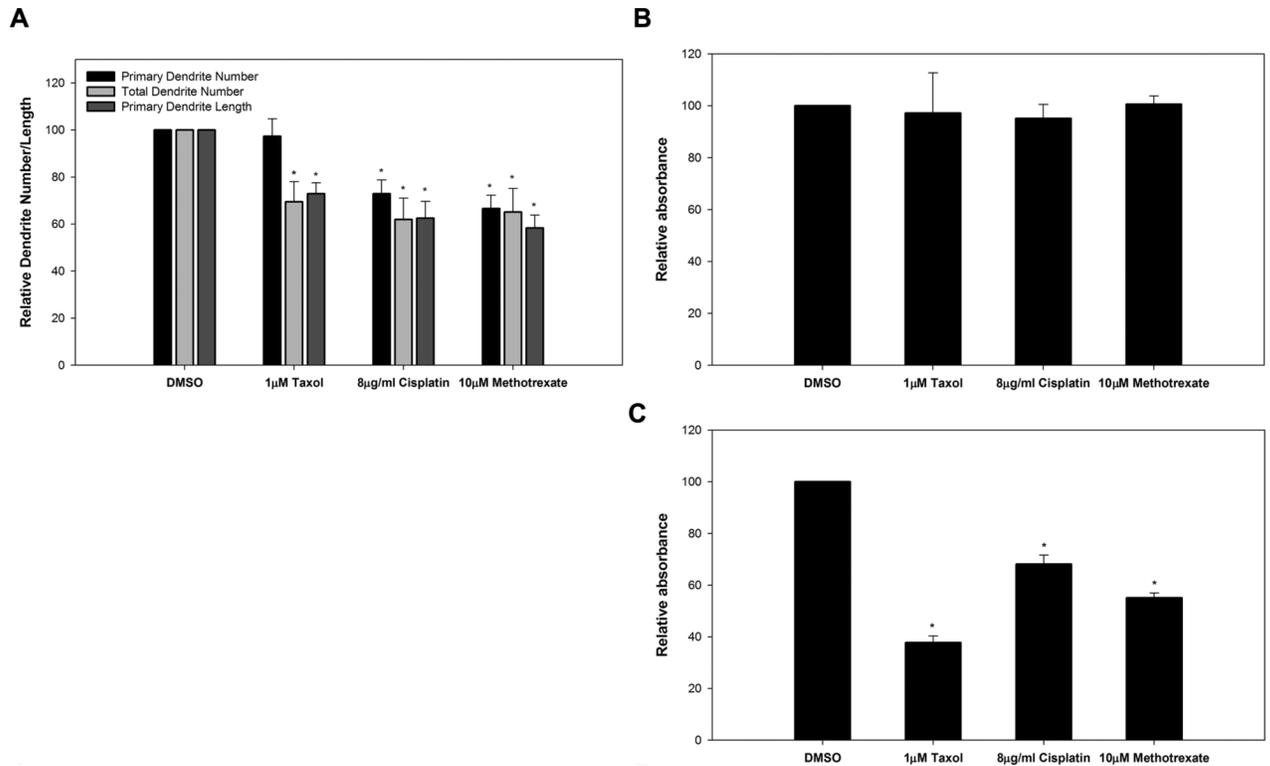


Figure 3. Anti-neoplastic drug treatment for 48 hours causes a reduction in total number of dendrites, number of primary dendrites, and dendrite length

Graphs representing the difference (mean \pm SEM) of the drug treated neurons from the DMSO control neurons in number of primary dendrites, total number of primary dendrites, and primary dendrite length (A). Here, dendrite numbers and length in DMSO treated neurons are designated as 100%. MTT assay using neurons (B) and CWR22Rv-1 prostate cancer cells (C). $p < 0.05^*$.

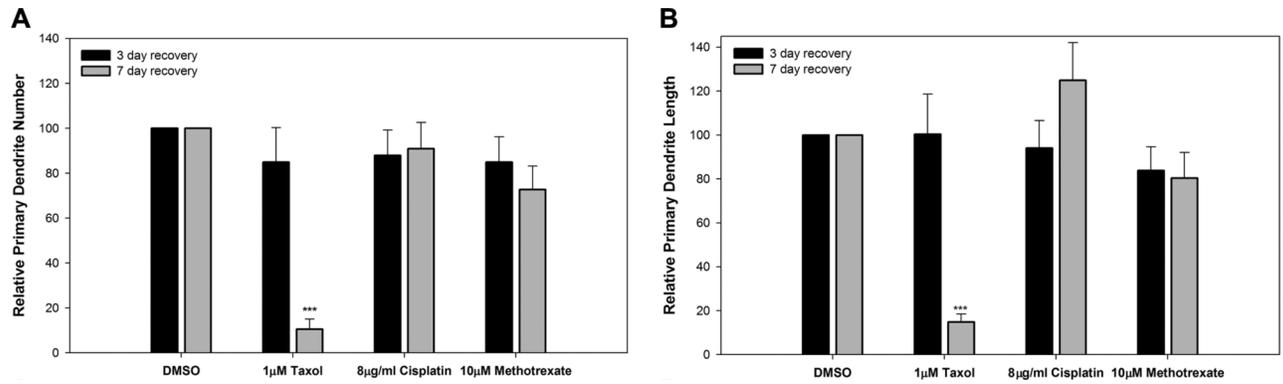


Figure 4. Neurons recover after anti-cancer drug withdrawal and remain viable during treatment
 Drugs were withdrawn from the neurons following a 48-hour treatment from 5 DIV to 7 DIV. Neurons were then cultured in drug-free media until 10 DIV or 14 DIV. The effects of the treatment withdrawal on primary dendrite number (A) and primary dendrite length (B) are shown. The measurements are graphed as a percentage of drug treated vs. control (DMSO). Here, dendrite numbers and length in DMSO treated neurons are designated as 100% (mean \pm SEM). $p < 0.001^*$.

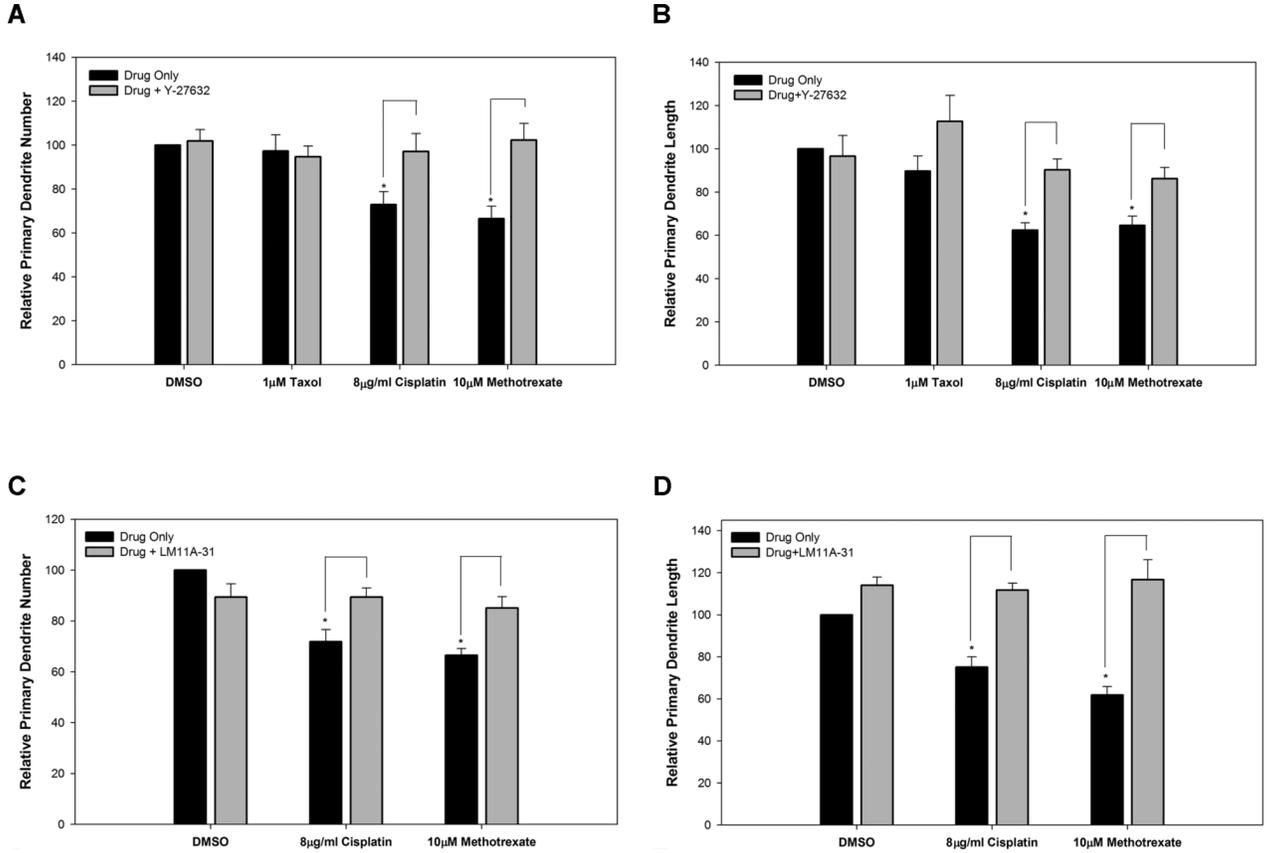


Figure 5. Inhibition of the Rho signaling prevents the degeneration of hippocampal neurons
 Effects of addition of the Rho kinase/p160^{ROCK} inhibitor Y-27632 on primary dendrite number (A) and length (B) of neurons treated with taxol, cisplatin, and methotrexate. Effects of non-peptide p75^{NTR} ligand LM11A-31 on primary dendrite number (C) and length (D) of neurons treated with cisplatin and methotrexate. The measurements are graphed as a percentage of drug treated vs. control (DMSO) neurons. Here, dendrite numbers and length in DMSO treated neurons are designated as 100% (mean \pm SEM). $p < 0.05^*$

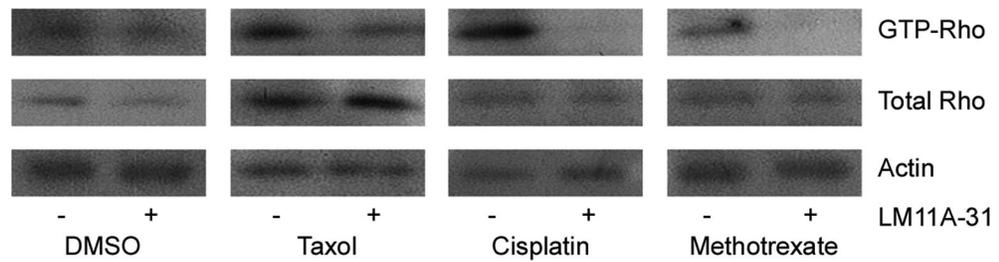


Figure 6. Changes in GTP-bound, active RhoA associated with taxol, cisplatin, and methotrexate treatment along with LM11A-31 incubation

The top panel shows the amount of Rho-GTP (active form of RhoA) with treatment of taxol, cisplatin, and methotrexate with (+) and without (-) the addition of LM11A-31. The middle panel shows the total amount of RhoA protein in the corresponding lysates. The bottom panel shows the total amount of actin in each lysate, which was used as a protein loading control.

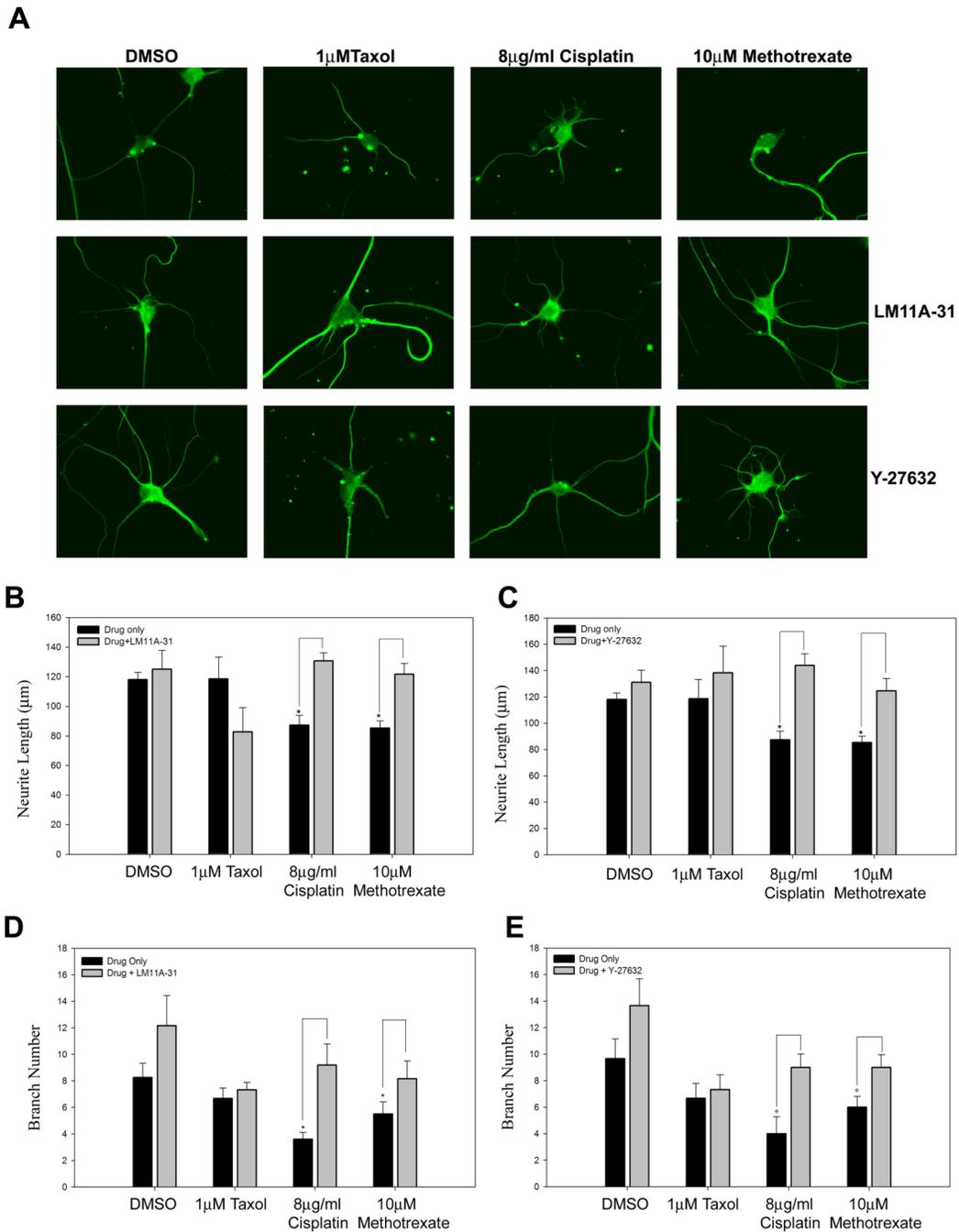


Figure 7. Sensory neurons demonstrate an abnormal morphology after anti-cancer drug treatment which is reversed by Rho pathway inhibition in cisplatin and methotrexate but not taxol treatment β -tubulin III staining reveals the morphological changes associated with taxol, cisplatin, and methotrexate treatment in dorsal horn/dorsal root ganglion co-cultures (A). Rho pathway inhibition using either LM11A-31 or Y-27632 results in the recovery of neurite morphology in cisplatin and methotrexate but not taxol treated neurons. Cisplatin and methotrexate treatment results in a significant decrease in process extension (B and C) and branch number (D and E). Treating the cells with LM11A-31 or Y-27632 after anti-cancer drug treatment, reverses degeneration and increases neuronal complexity (mean \pm SEM). $p < 0.05^*$