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Antillatoxin-Stimulated Neurite Outgrowth Involves the Brain-Derived Neurotrophic Factor (BDNF) - Tropomyosin Related Kinase B (TrkB) Signaling Pathway

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Abstract

Voltage-gated sodium channel (VGSC) activators promote neurite outgrowth by augmenting intracellular Na$^+$ concentration ($[Na^+]_i$) and upregulating N-methyl-D-aspartate receptor (NMDAR) function. NMDAR activation stimulates calcium (Ca^{2+}) influx and increases brain-derived neurotrophic factor (BDNF) release and activation of tropomyosin receptor kinase B (TrkB) signaling. The BDNF-TrkB pathway has been implicated in activity-dependent neuronal development. We have previously shown that antillatoxin (ATX), a novel lipopeptide isolated from

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the cyanobacterium *Moorea producens*, is a VGSC activator that produces an elevation of $[Na^+]_i$. Here we address the effect of ATX on the synthesis and release of BDNF and determine the signaling mechanisms by which ATX enhances neurite outgrowth in immature cerebrocortical neurons. ATX treatment produced a concentration-dependent release of BDNF. Acute treatment with ATX also resulted in increased synthesis of BDNF. ATX stimulation of neurite outgrowth was prevented by pretreatment with a TrkB inhibitor or transfection with a dominant-negative Trk-B. The ATX activation of TrkB and Akt was blocked by both a NMDAR antagonist (MK-801) and a VGSC blocker (tetrodotoxin). These results suggest that VGSC activators such as the structurally novel ATX may represent a new pharmacological strategy to promote neuronal plasticity through a NMDAR-BDNF-TrkB-dependent mechanism.

Graphical Abstract
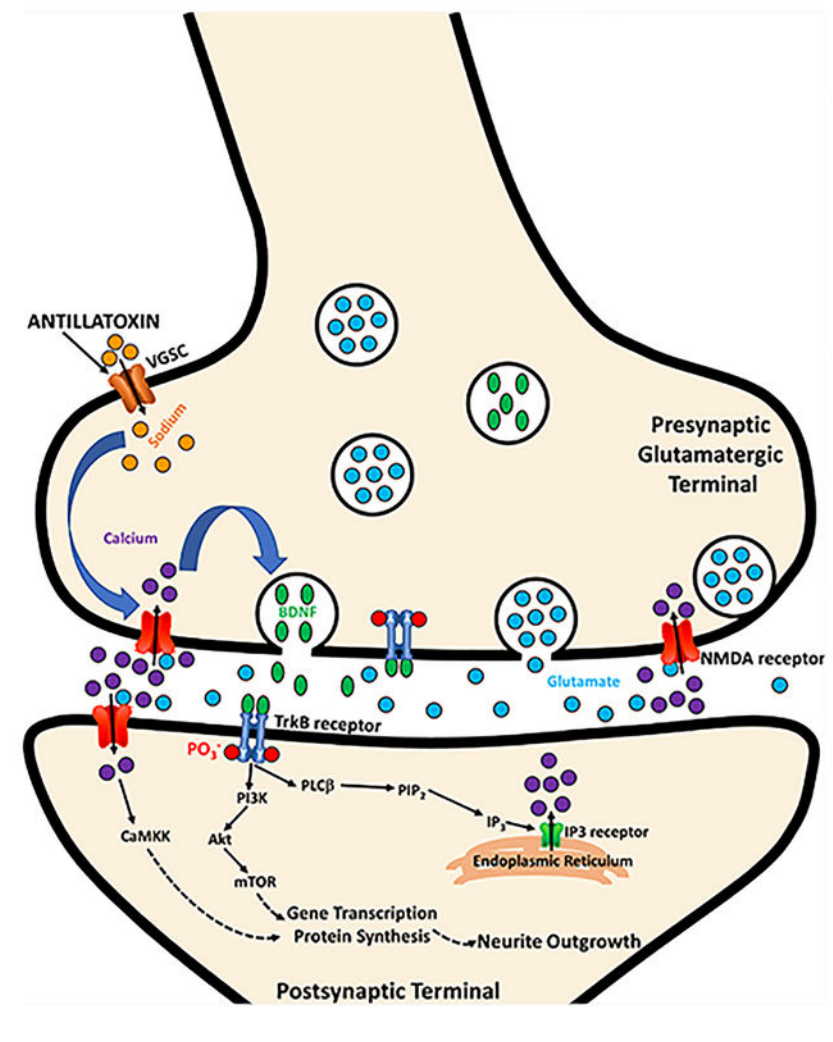
INTRODUCTION

Voltage-gated sodium channels (VGSCs) are principally responsible for the generation and propagation of action potentials in nerve, muscle, and other excitable cells. Mammalian

VGSCs are composed of a pseudotetrameric pore-forming α subunit and one or two β subunits.[48,49] Nine VGSC α subunits (Na$_v$1.1 to Na$_v$1.9) and four β subunits (Navβ1 to Navβ4) have been identified in the human genome.[9,10,41] VGSC α subunits display tissue specific expression patterns in the central nervous system (Na$_v$1.1, Na$_v$1.2, Na$_v$1.3, and Na$_v$1.6), peripheral nervous system (Na$_v$1.7, Na$_v$1.8 and Na$_v$1.9), skeletal muscle (Na$_v$1.4), and cardiac muscle (Na$_v$1.5).[42] VGSCs represent the molecular targets for a broad range of potent natural product toxins that bind to as many as eight distinct neurotoxin sites on the sodium channel α-subunit that affect ion permeation and gating of sodium channels.[19,48,49]

Development of the nervous system involves both a predetermined genetic program that controls the general organization of the brain and activity-dependent mechanisms that modulate several developmental processes, including dendritic arbor elaboration, spine formation, synapse formation, and synapse elimination.[12,30] Dendritic arbor shape determines the extent of neuronal connectivity and integration of synaptic signals: both are essential for the proper formation of neural circuits and function of nervous system. The neuronal-activity-dependent influence of the central nervous system development primarily involves calcium-dependent and neurotrophin signaling mechanisms.

Activity-dependent calcium signaling involves various calcium influx pathways including ionotropic glutamate receptors (*N*-methyl-D-aspartate receptor) and voltage-gated Ca^{2+} channels (VGCCs).[13,14,43] Intracellular calcium acts as a signaling molecule largely through binding to calmodulin, a calcium-binding protein that engages downstream Ca^{2+}/calmodulin-dependent protein kinase (CaMK). One such CaMK is CaMKII, an important downstream regulator of dendritic remodeling and synaptic activity.[17,46] Moreover, previous studies have demonstrated that activity-dependent neurite outgrowth[15] and synaptogenesis[22] are regulated by *N*-methyl-D-aspartate receptor (NMDAR)-dependent CaMKK/calmodulin kinase I-signaling cascades. Therefore, NMDARs play a critical role in activity-dependent development and plasticity,[43] dendritic arborization, spine morphogenesis,[18] and synapse formation[22] by stimulating these calcium-dependent signaling pathways.

Neurotrophins play an important role in regulating neuronal survival, development, and plasticity. These proteins are secreted in small quantities and regulate both local and global effects, for example, growth cone motility and gene transcription, respectively. Brain-derived neurotrophic-factor (BDNF) is one of the best characterized neurotrophins and plays a critical role in neuronal development and plasticity. BDNF has been demonstrated to increase dendritic arbor complexity of cerebrocortical pyramidal neurons by increasing total dendritic length, the number of branch points and the number of primary dendrites.[29,47] Moreover, activity-dependent increases in [Ca^{2+}]$_i$ trigger the release of BDNF,[38,39] which in turn mediates activity-dependent dendritic development and synaptic plasticity.[39] BDNF release is dependent on extracellular Ca^{2+}[36] whose entry may be due to influx through either NMDARs or VGCCs. Calcium activates CamKII leading to the fusion of BDNF containing secretory granules with the plasma membrane with subsequent release of BDNF into the extracellular milieu.[52] Neurotrophins activate receptor tyrosine kinases (Trks) and the Trk family is composed of three related receptors TrkA, TrkB, and TrkC:[35] these in turn regulate signaling pathways involved in neuronal survival, proliferation, neurite outgrowth, and dendritic arborization. The effects of BDNF on dendritic morphology are due to

the activation of signaling mechanisms downstream of its receptor, TrkB, that influence neuronal development.

Recent studies have demonstrated that neuronal-activity-mediated increases in neuronal $[Na^+]_i$ augment NMDAR function and may contribute to activity-dependent synaptic plasticity.[23] Inasmuch as neuronal activity-induced increments in cytoplasmic sodium may augment NMDAR-mediated currents, we reasoned that intracellular Na^+ may function as a signaling molecule to positively regulate neuronal development in immature cerebrocortical neurons. In the present study, we used antillatoxin (ATX), a voltage-gated sodium channel activator to elevate $[Na^+]_i$ in immature cerebrocortical neurons. Antillatoxin is a structurally novel cyclic lipopeptide from the marine cyanobacterium *Moorea producens*.[36,55] We have previously demonstrated that in cerebrocortical neurons VGSC activators (antillatoxin and brevetoxin-2) elevated $[Na^+]_i$ and augmented NMDAR function.[36,45] These VGSC activators also enhanced neurite outgrowth with a hormetic concentration-response relationship. The inverted-U response to antillatoxin and brevetoxin-2 (PbTx-2) on neurite outgrowth mimics that of NMDAR activation.[36,45] Moreover, BDNF also displays an inverted-U concentration–response for retinal ganglion survival following optic nerve transaction[34] and for promoting serotonergic axonal growth and remodeling in the brain.[32]

We therefore hypothesized that sodium channel activators may stimulate neuronal development by elevating $[Na^+]_I$, enhancing NMDAR function, in turn stimulating BDNF release with activation of downstream BDNF-TrkB signaling pathways. Here, we demonstrate that antillatoxin increased BDNF synthesis and release. Antillatoxin moreover enhanced neurite outgrowth, and this response was dependent on TrkB receptor signaling. Furthermore, inhibition of TrkB receptors or its downstream signaling targets, phosphoinositide 3-kinase (PI3–K) and mammalian target of rapamycin (mTOR), inhibited antillatoxin-stimulated neurite outgrowth. Acute treatment with antillatoxin-stimulated phosphorylation of TrkB and its downstream effectors Akt and mTOR. These data provide support for the hypothesis that novel bioactive natural products are capable of mimicking activity-dependent neuronal development through potentiation of neurotrophin signaling pathways.

RESULTS

Antillatoxin Enhances BDNF Synthesis and Release in Immature Cerebrocortical Neurons.

Neurotrophins are expressed and released by neurons in an activity-dependent manner and act in an autocrine/paracrine mode to induce morphological and functional changes in neurons. Activation of NMDARs and elevation of $[Ca^{2+}]_i$ are critical for the expression and release of neurotrophins. We predicted that sodium channel activation would increase the synthesis and release of BDNF. The influence of antillatoxin on the release of endogenous BDNF was quantified using an *in situ* BDNF ELISA (Figure 1A,B). Three hours postplating neurons were exposed to various concentrations of antillatoxin ranging from 1 to 3000 nM for 96 h. Additional plates of neurons were exposed to 50 mM KCl as a positive control. Antillatoxin produced a concentration-dependent increase in BDNF release with 3000 nM ATX producing increases in BDNF release comparable to that of 50 mM KCl (Figure 1B). Antillatoxin in concentrations of 100 nM (***, $p < 0.03$), 1000 (**, $p <$

0.004), and 3000 (*, $p < 0.0001$) produced significant increases in BDNF release compared with control. We next investigated BDNF synthesis in immature cerebrocortical neurons following antillatoxin exposure. DIV-1 cerebrocortical neurons were exposed to 30 nM antillatoxin in the presence or absence of TTX (1 μM) or MK-801(1 μM). Antillatoxin treatment caused a robust increase in BDNF synthesis, which was blocked by both TTX and MK-801 (Figure 1C). These results demonstrate that the antillatoxin-induced increase in BDNF synthesis in immature cerebrocortical neurons was dependent on VGSCs and NMDARs.

Exposure of cerebrocortical neurons beginning on day 5 in culture to either 30 or 100 nM antillatoxin also produced significant increases in glutamate release following a 2 h treatment. Basal glutamate concentration in culture media after vehicle exposure was 1.61 μM (95% CI, 1.51–1.70 μM). Antillatoxin at 30 and 100 nM elevated glutamate release to respectively 2.32 μM (95% CI, 1.82–2.32.70 μM) and 2.07 μM (95% CI, 1.72–2.42 μM). These results parallel those previously described for another bioactive marine natural product brevetoxin, which like antillatoxin is an activator of voltage-gated sodium channels.[1] The modest increases in aspartate release evoked by antillatoxin were not statistically significant. The basal aspartate concentration was 1.31 μM (95% CI, 1.03–1.60 μM), while 30 and 100 nM antillatoxin exposures increased the aspartate concentrations, respectively, to 1.81 μM (95% CI, 1.56–2.05 μM) and 1.73 μM (95% CI, 1.46–2.00 μM).

Antillatoxin-Induced Neurite Outgrowth Involves TrkB Receptors.

Previous studies have indicated that activity-dependent neurtitogenesis and neuronal development involve Ca^{2+} influx through NMDAR with subsequent engagement of BDNF-TrkB signaling. Hence, we assessed the role of the TrkB receptor in sodium channel activator-induced neurite outgrowth. Co-incubation of K252a (200 nM), a TrkB inhibitor, with 30 nM antillatoxin inhibited the stimulation of neurite outgrowth in immature cerebrocortical neurons (**, $p < 0.05$ ANOVA) (Figure 2A,B), demonstrating the requirement for TrkB receptor activation. These data were confirmed with a genetic approach in which neurons were transfected with either a dominant negative isoform of TrkB (DN-TrkB), the full-length isoform of TrkB (TrkB-FL) or the backbone/empty vector (Empty). Consistent with the involvement of TrkB receptors, dominant-negative TrkB completely abolished antillatoxin-induced neurite outgrowth, whereas neurons expressing TrkB-FL in the presence of antillatoxin showed a robust increase in neurite length (Figure 2C,D; ***, $p < 0.05$ ANOVA).

To assess the ability of antillatoxin exposure to produce an activation of TrkB receptors, we determined the level of phosphorylation of the Tyr516 residue on TrkB receptors using an antiphospho-Tyr516 antibody. DIV-1 cerebrocortical neurons were exposed to 30 nM antillatoxin in the presence or absence of TTX (1 μM) or MK-801(1 μM). Antillatoxin treatment caused robust phosphorylation of the TrkB receptor which was blocked by both TTX and MK-801 (Figure 3) demonstrating that antillatoxin-induced phosphorylation of TrkB receptors is dependent on signaling through VGSCs and NMDARs.

Antillatoxin-Induced Neurite Outgrowth Involves PI3-Kinase Activity.

The PI3-kinase-Akt signaling pathway is downstream from the BDNF-TrkB receptor pathway and plays an important role in neurotrophin-stimulated neurite outgrowth.[6] Given that antillatoxin stimulated both the synthesis and release of BDNF, we assessed whether PI3-kinase was involved in antillatoxin-induced neurite outgrowth. Co-incubation of LY294002 (10 μM), a PI3-kinase inhibitor, with 30 nM antillatoxin abrogated the stimulation of neurite outgrowth in immature cerebrocortical neurons (***, $p < 0.001$ ANOVA) (Figure 4A,B), suggesting the involvement of PI3-kinase. A major effector downstream of PI3-kinase involved in neurite outgrowth is Akt (protein kinase B). To assess the ability of antillatoxin to activate Akt, we determined the phosphorylation of the Ser473 residue on Akt using an antiphospho-Ser473 Akt antibody. Neurons were exposed 3 h after plating to varying concentrations of antillatoxin (1–300 nM) for 24 h, and cell lysates were collected for Western blot analysis. The results revealed that the 10 and 30 nM antillatoxin concentrations produced a robust ~3-fold activation of Akt as reflected by the increase in the phosphorylation of Ser473, while the concentration–response profile again displayed an inverted-U pattern with diminished responses at both lower and higher concentrations (Figure 4C).

Antillatoxin-Stimulated Akt Phosphorylation Involves PI3K, VGSCs, NMDARs, and TrkB Receptors.

To determine the involvement of PI3-kinase in antillatoxin-stimulated Akt phosphorylation, DIV-1 cultures were preincubated with the PI3-kinase inhibitor LY294002 (10 μM) for 15 min and then exposed to 30 nM antillatoxin for 30 min. Cell lysates were collected and probed for activation of Akt (Ser473) by immunoblotting. Western blot analysis revealed that LY294002 completely inhibited antillatoxin-stimulated Akt activation indicating the requirement of PI3-kinase (Figure 5A).

To further confirm involvement of additional signaling mechanisms involved in antillatoxin-induced Akt activation, we utilized a pharmacological approach using the VGSC blocker TTX (1 μM), the NMDAR antagonist MK-801 (1 μM) and the TrkB receptor inhibitor K252a (200 nM). These compounds were preincubated for 15 min prior to the addition of antillatoxin. All three compounds attenuated antillatoxin-stimulated Akt activation indicating the requirement for VGSC, NMDAR, and TrkB activation (panels B, C, and D, respectively, of Figure 5). These findings establish a role for the TrkB-PI3-kinase-Akt signaling pathway in the stimulatory effect of antillatoxin on neurite outgrowth.

Antillatoxin-Induced Neurite Outgrowth Involves the PI3K-Akt-mTOR Pathway.

Downstream of of TrkB, the mTOR signaling complex is critical for protein synthesis in dendrites and participates in activity-dependent dendritic arborization. Through its activation of mTOR, AKT signaling promotes the growth of multiple populations of neurons and plays a critical role in both brain developmental and synaptic plasticity.[5] To determine the involvement of mTOR signaling in antillatoxin-stimulated neurite outgrowth, cerebrocortical neurons were coincubated with antillatoxin in the presence or absence of rapamycin, an inhibitor of mTOR, for 24 h, and total neurite length was determined. Consistent with the involvement of mTOR, rapamycin inhibited antillatoxin-stimulated

neurite outgrowth (Figure 6A,B). To assess the ability of antillatoxin treatment to activate mTOR, we determined the phosphorylation of the Ser2448 residue on mTOR using an antiphospho-Ser2488 mTOR antibody. Cerebrocortical neurons were exposed to 30 nM antillatoxin and cell lysates were collected at various time periods for Western blot analysis. The results revealed that 30 nM antillatoxin produced a ~2 fold activation of mTOR as reflected by the increase in phosphorylation of Ser2448 at 3 h post exposure (Figure 6C). Taken together, these results indicate that mTOR activation participates in sodium channel activator-stimulated neuronal neurite outgrowth.

DISCUSSION

The electrical signals of neurons are fundamentally dependent on Na^+ influx through VGSCs. Sodium channels are primarily responsible for the rising phase of the action potential and hence supply the current that drives the membrane potential to peak depolarization.[16] VGSCs' activity has been shown to regulate neurotransmitter release in the developing cerebral cortex and to mediate neuronal firing dependent synaptic plasticity.[27,51] Previous research has shown that changes in intracellular sodium concentration ($[Na^+]_i$) produced in the soma and dendrites as a result of neuronal activity may play a role in activity-dependent synaptic plasticity. Synaptic stimulation elevates $[Na^+]_i$ to 10 mM in dendrites and up to 35–40 mM in dendritic spines.[23] In hippocampal neurons, such intracellular $[Na^+]$ increments have been demonstrated to increase NMDAR-mediated whole-cell currents and NMDAR single-channel activity by increasing both channel open probability and mean open time.[4,44] In immature cerebrocortical neurons, VGSCs' activators enhanced neurite outgrowth through potentiation of NMDAR signaling pathways that influence neuronal morphology.[36,44] Thus, sodium channel activators appear to be capable of mimicking activity-dependent neuronal development. Neuronal activity also cooperates with neurotrophins to influence neuronal development and plasticity.[11,12,24] Although activity-dependent neurotrophin influences on neuronal development are well characterized, little is known regarding the influence of VGSC activation on neurotrophin signaling. Toward this goal, we used the VGSC activator antillatoxin to assess the influence of VGSC activation on BDNF synthesis, release, and stimulation of TrkB signaling pathways. Although the biophysical mechanism for antillatoxin-induced stimulation of VGSCs remains to be described, it likely alters the gating properties of the channel (activation and/or inactivation). Our findings confirm an earlier report that exposure to the VGSC gating modifier PbTx-2 increased gene expression of BDNF in mouse cerebrocortical neurons.[3] We found that antillatoxin increased BDNF release and synthesis, and it activated BDNF-TrkB signaling pathways, including the downstream PI3-kinase-Akt-mTOR cascade (Figure 7). The antillatoxin stimulation of BDNF release and synthesis mimicked activity-dependent regulation of neuronal development inasmuch as BDNF plays an important role in regulating neural survival and development.[2] Antillatoxin produced a concentration-dependent increase in BDNF release (secretion) in immature cerebrocortical neurons. The released BDNF then binds to its cognate receptor TrkB in an autocrine/paracrine fashion to activate downstream signaling pathways and thereby influence neuronal development.

The antillatoxin-enhanced BDNF synthesis was TTX- and MK-801-sensitive, demonstrating the requirement for VGSC and NMDAR signaling. Transcription of BDNF from its

promoters (I and IV) is highly regulated by neuronal-activity-dependent Ca^{2+} influx[26,35] and by the pattern of phosphorylation of the transcription factor CREB.[13] Activation of L-type Ca^{2+} channels or the NMDA subtype of glutamate receptor have been demonstrated to lead to an enhancement of *BDNF* mRNA levels and stimulation of release of BDNF protein.[8,43] Given the inability of 30 nM antillatoxin to significantly alter membrane potential in cerebrocortical neurons, the involvement of voltage-gated calcium channels is unlikely.[36] The relevant Ca^{2+} influx pathway in the present results is therefore likely through NMDARs. We have previously shown that antillatoxin-induced depolarization of immature cerebrocortical neurons is also insufficient to influence the voltage-dependent Mg^{2+} block of NMDARs suggesting that the antillatoxin-induced neurite outgrowth in cerebrocortical neurons is a result of elevation of cytoplasmic $[Na^+]$ with attendant potentiation of NMDAR function.[36]

Our results moreover demonstrate ATX-increased neurite outgrowth was blocked by K252 a (TrkB inhibitor) and DN-TrkB. Marini et al.[31] have demonstrated that NMDA exerts a neuroprotective activity by increasing BDNF release (acute effect, 2–5 min) and synthesis (chronic effect, ~3 h) leading to an activation of TrkB signaling. They proposed an integral relationship between NMDAR activation and BDNF-TrkB signaling occurring in a bidirectional manner, consonant with the bidirectional (hormetic) pattern shown by NMDA and antillatoxin on neurite outgrowth.[36] The relationship between NMDA receptor activity and neuronal survival and growth displays an inverted-U concentration–response relationship.[33] In both cases, this inverted-U concentration–response relationship has primarily, but not exclusively, been attributed to $[Ca^{2+}]_i$ regulation. An optimal window for $[Ca^{2+}]_i$ is required for activity-dependent neurite extension and branching, with lower levels stabilizing growth cones and higher levels stalling them, in both cases preventing extension.[37,40]

The released BDNF regulates neuronal development by activating its cognate receptor TrkB. Our results show that antillatoxin exposure increases phosphorylation of TrkB at Tyr516, which indirectly activates the PI3-kinase pathway by interacting with adaptor proteins[24] that regulate the PI3-kinase-Akt pathway. Our results further demonstrate that antillatoxin increases phosphorylation of Akt at Ser473 and the enhanced neurite outgrowth was blocked by the PI3-kinase inhibitor LY294002. Akt has also been implicated in several aspects of neurite outgrowth. One of the major upstream regulators of Akt is PI3-kinase, which facilitates the conversion of membrane phospholipid phosphatidylinositol (4, 5)-diphosphate (PIP_2) to phosphatidylinositol (3, 4, 5)- trisphosphate (PIP_3). The accumulation of PIP_3 promotes the translocation of Akt to the plasma membranes, where Akt binds to PIP_3 via its pleckstrin homology (PH) domain. Akt binding to PIP_3 leads to the phosphorylation of Akt at Thr308 and Ser473 by phosphoinositide-dependent kinase1 and 2 respectively.[25] Our results demonstrate that Akt is phosphorylated at Ser473 following antillatoxin exposure, and this phosphorylation is blocked by the PI3-kinase inhibitor LY294002. Both TTX and MK-801 also inhibited the stimulation of Akt phosphorylation suggesting that antillatoxin activates VGSCs, which in turn leads to augmentation of NMDAR signaling with attendant activation of the Akt-PI3-kinase pathway.

The Akt-mediated neurite outgrowth has been shown to involve the mTOR signaling pathway, a major regulator of protein synthesis.[20,21] Once activated Akt can subsequently phosphorylate a number of substrates (GSK3β, MAP2, mTOR) that are involved in cell survival, neurite outgrowth, dendritogenesis, and synaptic plasticity.[11,25] Akt activation of mTOR pathway involves phosphorylation and inhibition of tuberous sclerosis complex proteins: hamartin (TSC1) and tuberin (TSC2). The TSC1/2 complex is a GTPase-activating protein for Ras homologue enriched in brain (Rheb), an immediate upstream activator of mTOR. Moreover, the Akt-mTOR pathway has been shown to be the primary mediator of PI3-kinase regulated dendritic branching.[35] Rapamycin, an inhibitor of mTOR activity,[28] blocked antillatoxin-induced neurite outgrowth, suggesting a dependence on mTOR signaling.

Together, these data demonstrate that the sodium channel activator antillatoxin appears to be capable of mimicking activity-dependent and neurotrophin-dependent neurite outgrowth. VGSCs are molecular targets for a wide range of bioactive natural product toxins that alter their voltage dependence, or kinetics of activation and inactivation. Antillatoxin has been shown to produce concentration-dependent increases in intracellular Na^+ and Ca^{2+} in immature and mature primary cerebrocortical neurons resulting in the enhancement of NMDAR signaling.[36,50] Here we show the involvement of BDNF, TrkB, PI3-kinase, Akt, and mTOR in the antillatoxin stimulation of neurite outgrowth. Bioactive natural products, such as antillatoxin, are valuable tools for understanding chemical biology as well as serving as potential lead compounds for drug discovery. Hence, marine natural product VGSC activators may represent a novel pharmacological strategy to stimulate neuronal plasticity in the treatment of neurodegenerative and neurological disorders.[7]

EXPERIMENTAL SECTION

Cerebrocortical Neuron Culture.

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Creighton University Institutional Animal Care and Use Committee (Protocol 0801). Primary cultures of cerebrocortical neurons were harvested from Swiss Webster mice on embryonic day 16 and cultured as described previously.[50] Cells were plated onto poly L-lysine-coated (Sigma-Aldrich, St. Louis, MO) 96-well clear-bottomed, black-well culture plates (MidSci, St. Louis, MO) at a density of 1.5×10^5 cells/ml (150 μL/well), 24-well (15.6 mm) culture plates at a density of 0.05×10^6 cells/ml (0.5 mL/well), or 6-well (35 mm) culture dishes at a density of 2.25×10^6 cells/ml (2 mL/well), respectively, and incubated at 37 °C in 5% CO_2 and 95% humid atmosphere.

BDNF Sandwich ELISA *in Situ*.

This method was performed as previously described.[54] A sandwich ELISA quantifies antigens between two layers of antibodies (i.e., capture and detection antibody). Briefly, 96-well ELISA plates (Nunc Maxisorp) were UV-sterilized for 30 min and then incubated overnight at 4 °C with anti-BDNF monoclonal capture antibody diluted in carbonate coating buffer. Next, plates were blocked for 1 h and then washed 2× with plating

media. Dissociated immature cerebrocortical neurons were plated and cultured for 4 days. Beginning 3h post plating cells were treated with various concentrations of antillatoxin or 50 mM KCl. BDNF standards were also added to plates at the start of the culture. After 4 days, the plates were washed vigorously with Tris-buffered saline detergent (TBST) to remove cells and debris. Subsequently antihuman BDNF polyclonal detection antibody was added and incubated with shaking at RT for 2h. Sandwich BDNF ELISA was performed to measure BDNF protein using a BDNF E_{max} Immuno-assay System (Promega, Madison, WI) according to manufacturer's specifications.

Measurement of Excitatory Amino Acid Release.

Cerebrocortical neurons cultured in 96-well plates at 5 DIV were used for excitatory amino acid (EAA) release assay. Briefly, growth medium was collected and saved, and the neurons were washed using Locke's buffer consisting of (in mM) HEPES (8.6), KCl (5.6), NaCl (154), glucose (5.6), $MgCl_2$ (1.0), $CaCl_2$ (2.3), and glycine (0.1), pH 7.4. The neurons were then exposed to different concentrations of antillatoxin for 2 h at 22 °C. After exposure, the neurons were washed three times in fresh Lock's buffer and replaced with 100 μL of collected growth medium at 37 °C with 5% CO_2 and 95% humidity. After incubation for an additional 22 h, the medium was collected for EAA release assays. The amino acids were subjected to derivatization with o-phthaldialdehyde (OPA) and assayed for EAA content by HPLC with fluorescence detection as described by Berman and Murray.[1]

Diolistic Labeling and Neurite Length.

Cells were plated on poly lysine-coated 12- mm glass coverslips (Thermo Fisher Scientific, Waltham, MA) and placed inside 24-well culture plates at a low density of 0.05×10^6 cells/ml (0.5 mL/well). To assess the influence of antillatoxin on neuritogenesis, primary cultures of immature cerebrocortical neurons were exposed to 30 nM antillatoxin for 24 h beginning 3 h after plating, and total neurite outgrowth was measured. In some experiments, cells were pretreated with tetrodotoxin (TTX) a VGSC blocker (1 μM), MK-801 a NMDAR antagonist (1 μM), LY294002- a phosphoinositide 3-kinase inhibitor (PI3-kinase) (10 μM) (Sigma-Aldrich, St. Louis, MO), K252a- a Trk family receptor inhibitor (200 nM), or rapamycin an mTOR inhibitor (1 μM) (Calbiochem, San Diego, CA). At 24 h after plating, cultures were fixed at room temperature for 15 min using 1.8% paraformaldehyde in phosphate-buffered saline (PBS). After fixation, neurons were diolistically labeled with DiI.[45] The dye particles were allowed to spread across the neuronal membrane overnight and coverslips were then mounted for imaging on an Olympus IX 71 inverted microscope with a Himamatsu camera. Digital images of individual neurons were captured, and total neurite length was quantified.[45] To reduce the effect of paracrine neurotrophic factors on neurite growth, only those neurons that were separated from surrounding cells by a minimum of 150 μm were digitally acquired and analyzed. Digital images of individual neurons were captured and exported as 16-bit images. All neurites on a single neuron including those from secondary branches were semiautomatically traced, and the length was measured by using the Filament Tracer module of Imaris 6.4.0 software (Bitplane, South Windsor, CT). At least 25–30 randomly chosen neurons from two different cultures were evaluated for each treatment groups.

Plasmids and Nucleofection.

FL-TrkB and DN-TrkB (truncated TrkB) in pBluescript sk−/− vector were a generous gift from Dr. Tony Hunter (Salk Institute, San Diego). The truncated TrkB receptor lacks the catalytic tyrosine kinase domain and therefore functions as a dominant negative. The primary cultures of immature cerebrocortical neurons were transfected with Lipofectamine 2000 (Life Technologies, Grand Island, NY). Dissociated cortical neurons obtained from E16 pups were plated at density of 0.5×10^6 neurons/well. Two hours post plating, cells were transfected with either 0.5 μg plasmid containing the gene of interest or empty vector. Also, all transfected reactions contained a GFP plasmid (0.25 μg) bringing the total amount of plasmid to 0.75 μg per reaction. At 3 h post transfection; cells were treated with 30 nM antillatoxin or vehicle control. To allow sufficient time for the expression of genes of interest and to access the influence of antillatoxin on neuritogenesis, DIV-2 neurons were imaged in experiments involving transfection.

Western Blotting.

Western blot analysis was performed by using cells grown in six-well plates. For acute experiments, DIV-1 cells were exposed to 30 nM antillatoxin for 30 min at 37 °C. For pharmacological experiments, cultures were preincubated either in the presence or absence (vehicle) of specific antagonists for 15 min. At the end of the incubation period, cultures were transferred onto ice slurry to terminate drug exposure and washed three times with ice-cold PBS. Cells were lysed using ice-cold lysis buffer (50 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Nonidet P40, 0.1% SDS, 2.5 mM sodium pyrophosphate, and 1 mM sodium orthovanadate), phenylmethylsulfonyl fluoride (1 mM), and 1× protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) were then added, and the lysate was incubated for 30 min at 4 °C. Cell lysates were sonicated and then centrifuged at 13 000g for 15 min at 4 °C. The supernatant protein content was assayed by the Bradford method.[53] Equal amounts of protein were mixed with the Laemmli sample buffer and heated for 5 min at 75 °C. The samples were loaded onto a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to a PVDF membrane and immuoblotted with specific antibodies. Blots were developed with Pierce ECL kit (Thermo Fisher Scientific Rockford, IL) for 2 min. Blots were subsequently stripped (63 mM Tris base, 70 mM SDS, 0.0007% 2-mercaptoethanol, pH 6.8) and reprobed for further use. Western blot densitometry data were obtained by using ImageJ software (NIH, http://imagej.nih.gov/ij/).

Antibodies.

Phospho-TrkB (C35G9) (dilution used, 1:1000), TrkB (80E3) (1:2000), phospho-Akt (Ser473) (1:1000), Akt (1:2000), phospho-mTOR (Ser2481) (1:1000) and mTOR (7C10) (1:2000), β-Actin (1:5000) and antirabbit IgG HRP-linked antibody (1:5000) from Cell Signaling Technology (Danvers, MA). BDNF (1:500) from Santa Cruz Biotechnology (Dallas, TX).

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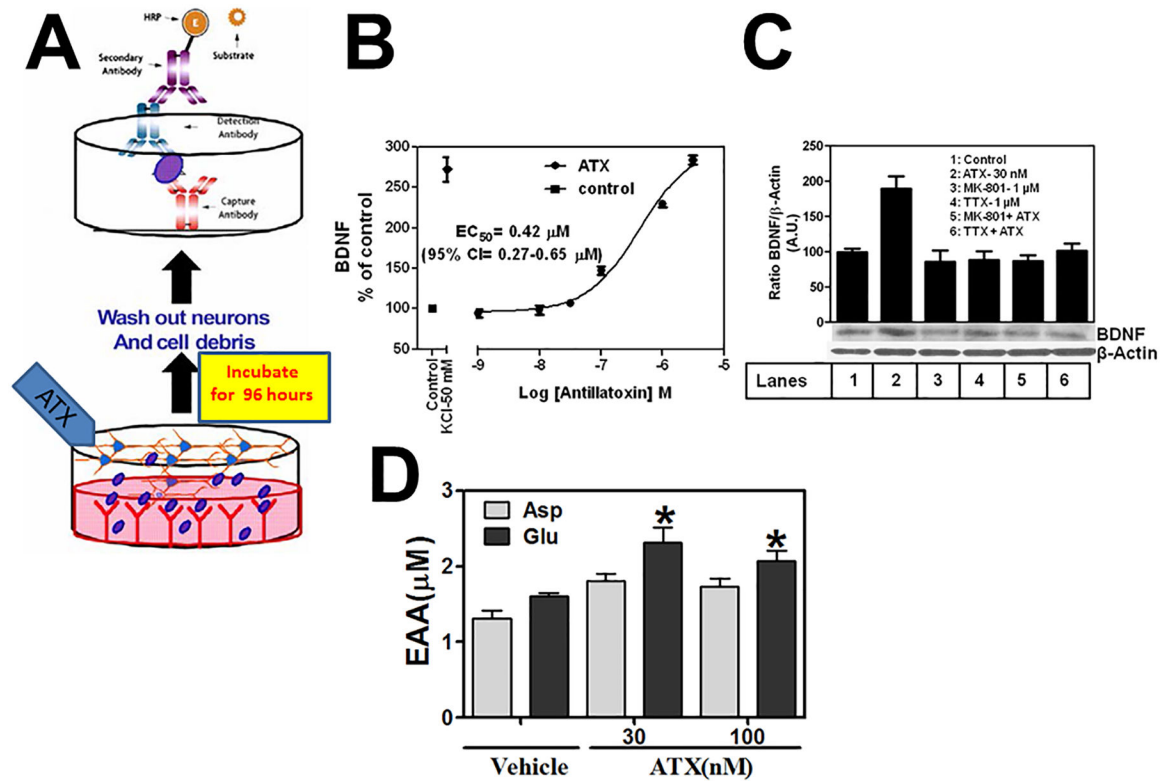
Figure 1.
Antillatoxin enhances BDNF and glutamate release in immature cerebrocortical neurons. Antillatoxin (ATX) increases *in situ* BDNF release as measured with a sandwich ELISA (A and B). Antillatoxin (ATX) enhances BDNF synthesis as measured by Western blotting (C) and glutamate release as measured by HPLC with fluorescence detection (D) in immature cerebrocortical neurons. The antillatoxin induced BDNF synthesis required VGSCs and NMDARs. Data shown represent the mean ± SEM of 2–3 experiments performed with 2–6 replicates each. Excitatory amino acids (EAA), aspartic acid (Asp), and glutamic acid (Glu).
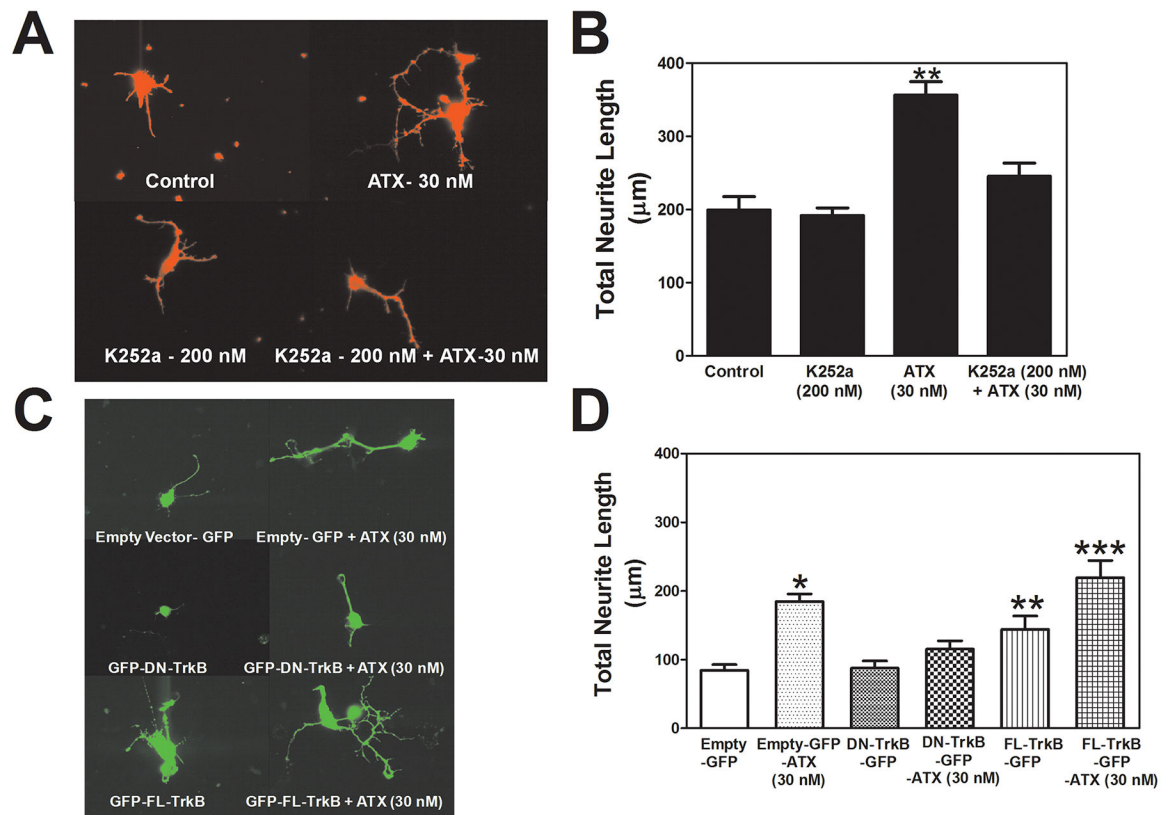
Figure 2.
TrkB is essential for antillatoxin-induced neurite outgrowth. Murine cerebrocortical neurons were exposed to antillatoxin (ATX) in the presence or absence of the TrkB inhibitor K252a. Treatment with K252a reduced antillatoxin-induced neurite outgrowth (A and B). The murine cerebrocortical neurons were transfected with either dominant negative (DN) or full length (FL) TrkB plasmids (C and D). The antillatoxin-induced neurite outgrowth was blocked by DN-TrkB, while FL-TrkB transfected neurons showed increased neurite outgrowth alone or when combined with 30 nM antillatoxin. These experiments were repeated twice, and 25 to 30 neurons were analyzed for each exposure condition.
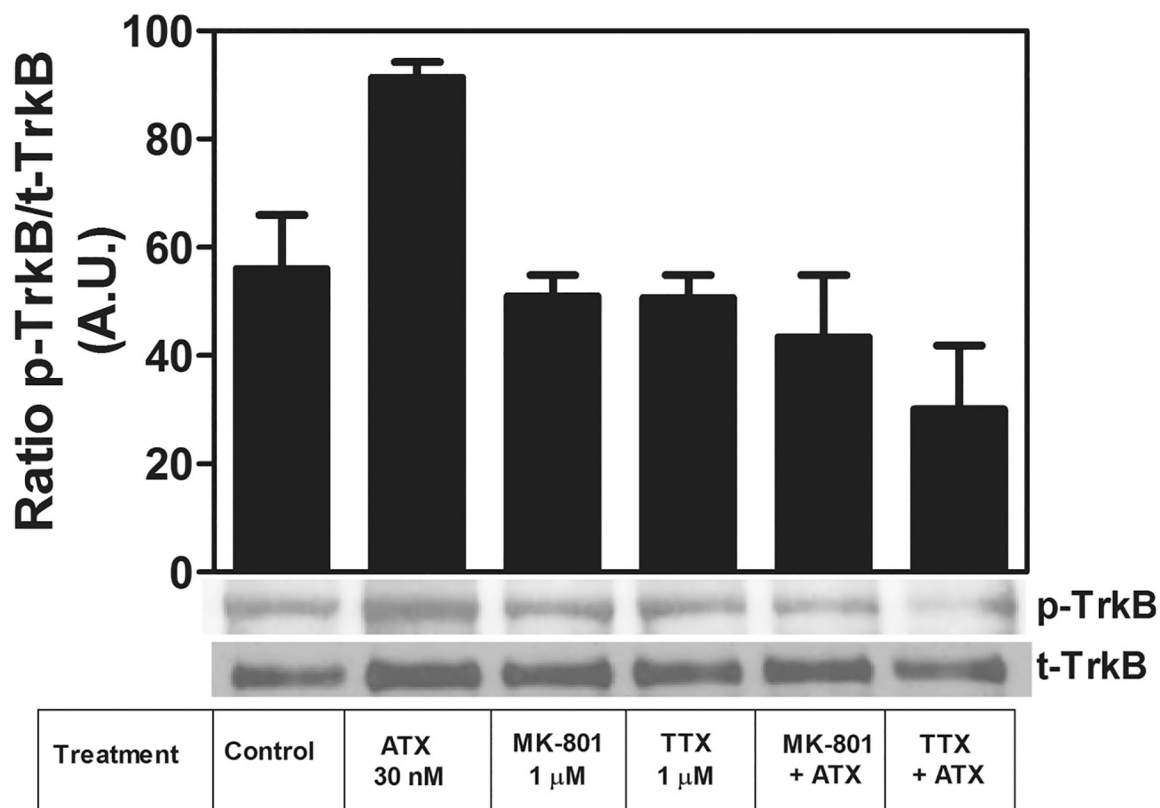
Figure 3.
Antillatoxin treatment leads to increased phosphorylation of TrkB receptors which was
blocked by the VGSC antagonist TTX (1 μM) and the NMDAR antagonist MK-801 (1
μM). A representative blot is shown. The experiment was performed three times with
independent cultures. Also depicted is the quantitative analysis of the relative band densities
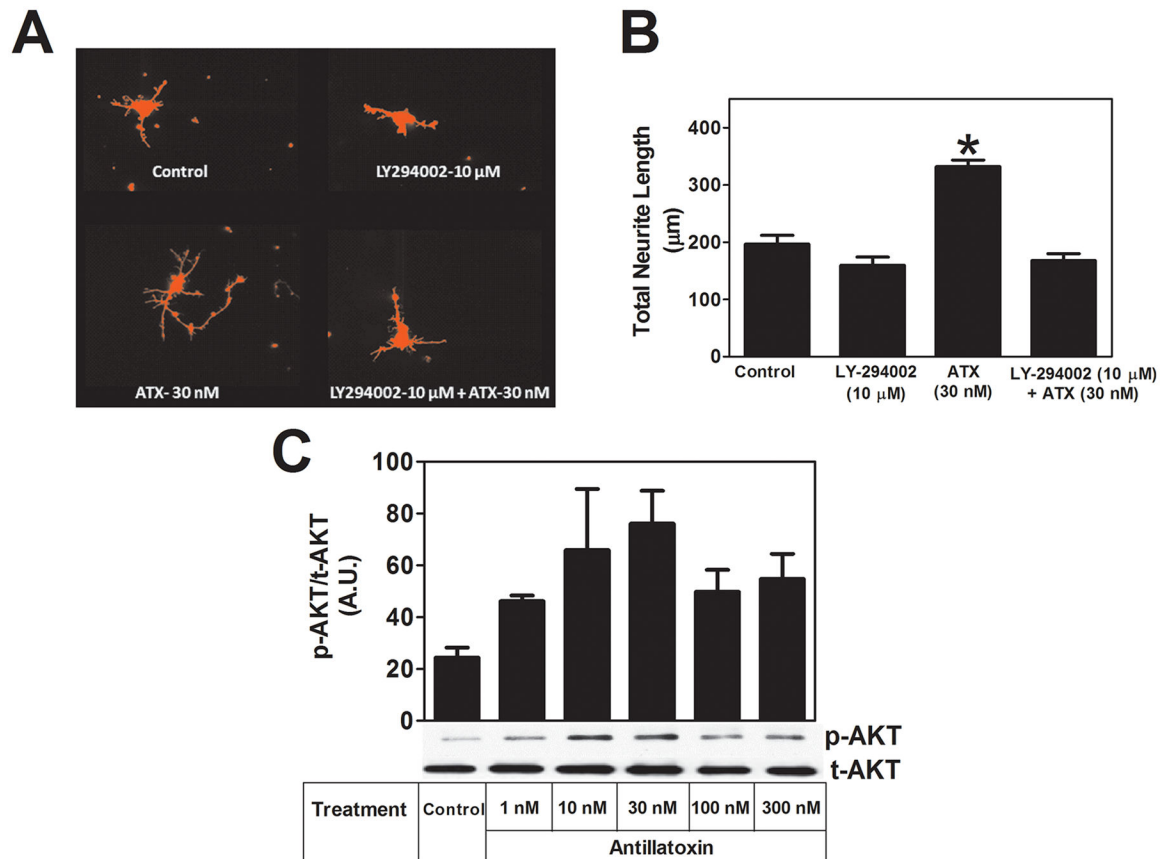of immunoblots. Each bar represents mean ± SEM of three values.

Figure 4.
Antillatoxin-induced increase in neurite outgrowth involves PI3-kinase activity. The experiment was repeated twice, and 25 to 30 neurons were quantified for each exposure condition (A and B). The antillatoxin-induced enhancement of neurite outgrowth was eliminated by the addition of 10 μM LY-294002 an inhibitor of PI3-kinase. Antillatoxin treatment also increased phosphorylation of Akt (C). The experiment was performed two times with independent cultures. Also depicted is the quantitative analysis of the relative band densities of immunoblots. Each bar represents mean ± SEM of two values (C).
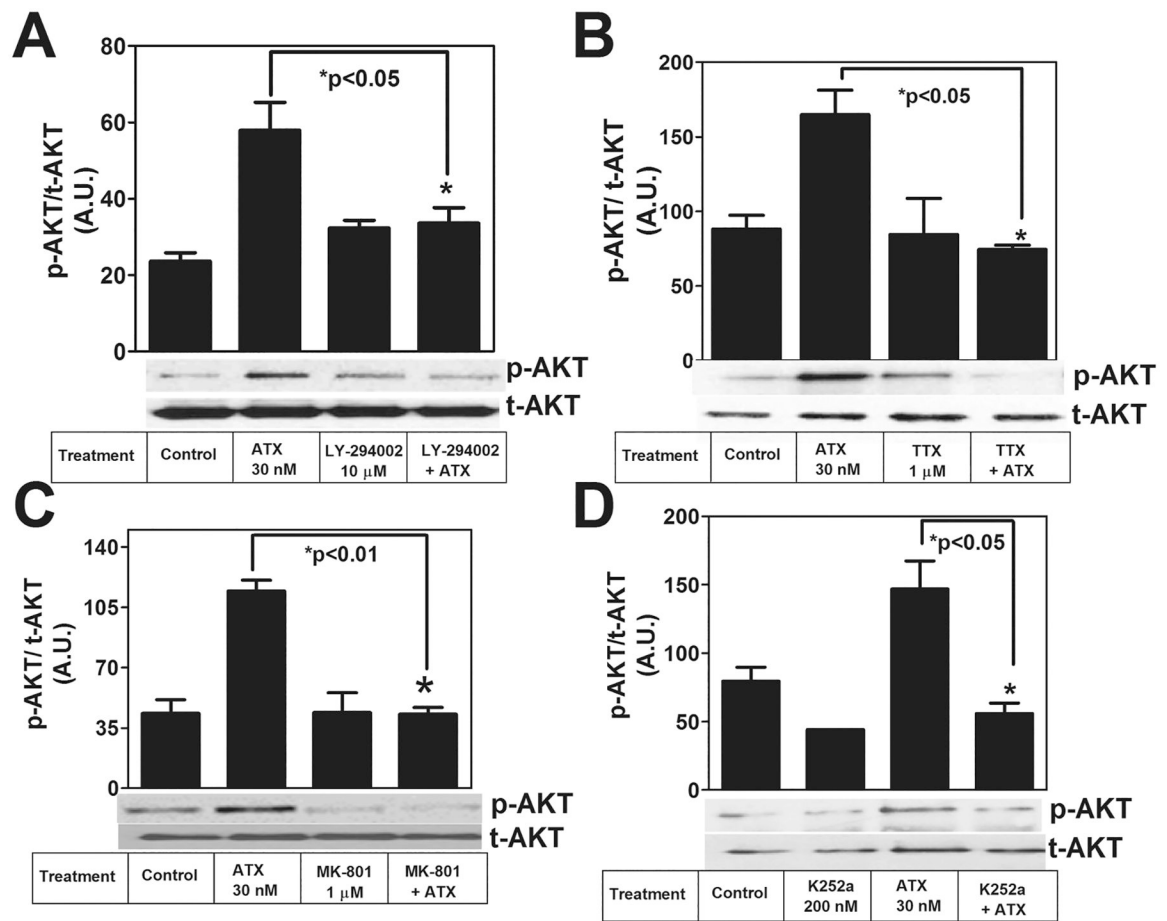
Figure 5.
The antillatoxin-stimulated Akt phosphorylation involves signaling through VGSCs, NMDARs, TrkB receptors, and PI3-kinase. (A) Antagonism by the PI3-kinase inhibitor LY-294002. (B) Antagonism by the VGSC blocker TTX. (C) Antagonism by the NMDA receptor blocker MK-801 (D) Antagonism by the TrkB inhibitor K252a. The experiment was performed two to three times with independent cultures. Also depicted is the quantitative analysis of the relative band densities of immunoblots. Each bar represents the mean ± SEM of two to three values.
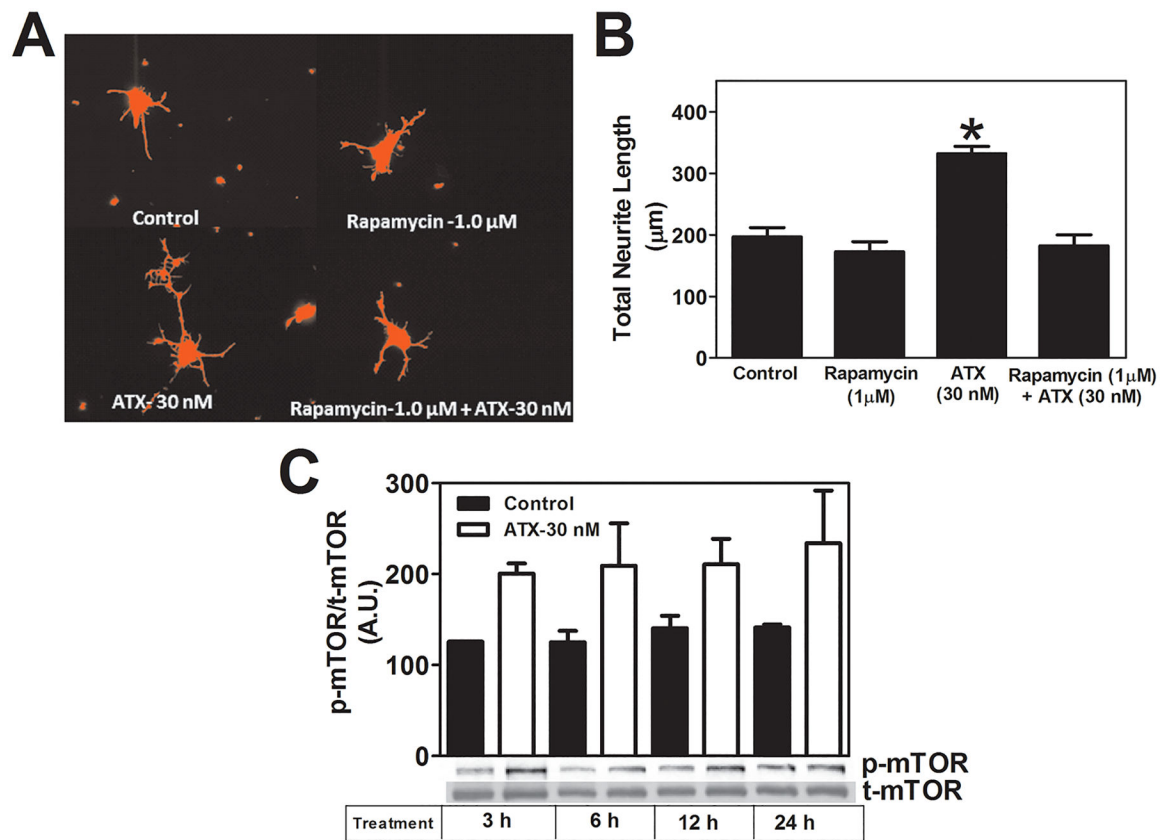
Figure 6.
Antillatoxin-induced neurite outgrowth involves the mTOR pathway. The experiment was repeated twice with independent cultures, and 25 to 30 neurons were analyzed for each exposure condition (A and B). Antillatoxin treatment leads to increased phosphorylation of mTOR. Also depicted is the quantitative analysis of the relative band densities of immunoblots. Each bar represents mean ± SEM of two values (C).
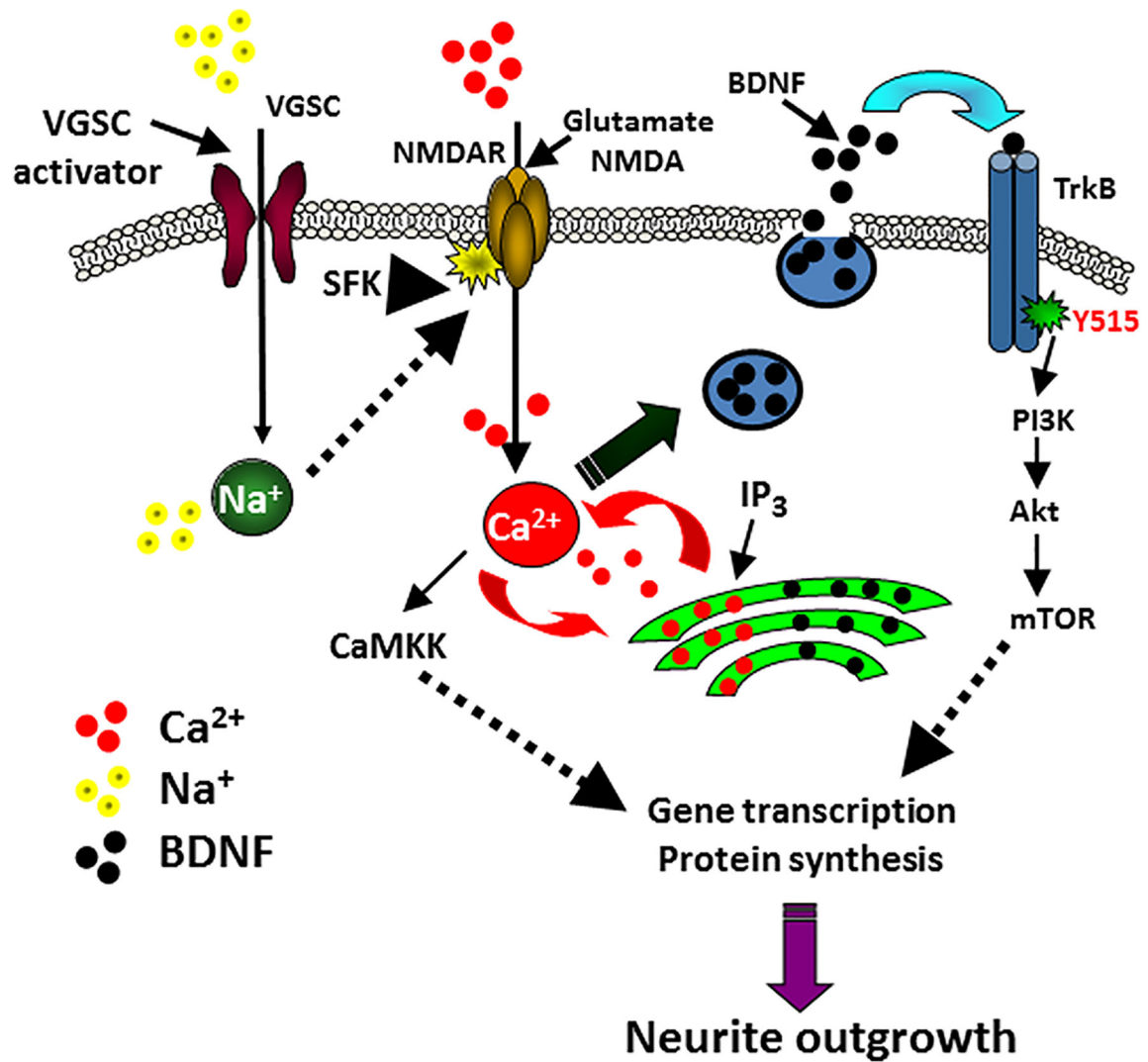
Figure 7.
Summary of antillatoxin-induced neurite outgrowth and signaling pathways involved downstream of voltage-gated sodium channels. Antillatoxin (ATX) acts as a voltage-gated sodium channel (VGSC) activator. Antillatoxin actions increase Ca^{2+} influx through NMDARs leading to release of BDNF from secretory vesicles. The released BDNF binds to its receptor TrkB and initiates the downstream signaling events causing activation of PI3K, Akt, and mTOR leading to increased neurite outgrowth. NMDAR (*N*-methyl-D-aspartate receptor), PI3K (phosphoinositide 3-kinase), Akt (protein kinase B), mTOR (mammalian target of rapamycin), BDNF (brain-derived neurotrophic factor).