Neurite outgrowth mediated by the heat shock protein Hsp90\(\alpha\): a novel target for the antipsychotic drug aripiprazole

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Aripiprazole is an atypical antipsychotic drug approved for the treatment of psychiatric disorders such as schizophrenia, bipolar disorder, major depressive disorder and autism. The drug shows partial agonistic activity at dopamine D₂ receptors and 5-hydroxytryptamine (5-HT) 5-HT₁A receptors, and antagonistic activity at 5-HT₂A receptors. However, the precise mechanistic pathways remain unclear. In this study, we examined the effects of aripiprazole on neurite outgrowth. Aripiprazole significantly potentiated nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells, in a concentration-dependent manner. The 5-HT₁A receptor antagonist WAY-100635, but not the dopamine D₂ receptor antagonist sulpiride, blocked the effects of aripiprazole. Moreover, specific inhibitors of several common signaling pathways phospholipase C-\(\gamma\) (PLC-\(\gamma\)), phosphatidylinositol-3 kinase (PI3K), mammalian target of rapamycin, p38 MAPK, c-Jun N-terminal kinase, Akt, Ras, Raf, ERK, MAPK also blocked the effects of aripiprazole. Using proteomic analysis, we found that aripiprazole significantly increased levels of the heat shock protein Hsp90α in cultured cells. The effects of aripiprazole on NGF-induced neurite outgrowth were significantly attenuated by treatment with Hsp90α RNA interference, but not by the negative control of Hsp90β. These findings suggest that both 5-HT₁A receptor activation and Ca\(^{2+}\) signaling via IP₃ receptors, as well as their downstream cellular signaling pathways play a role in the promotion of aripiprazole-induced neurite outgrowth. Furthermore, aripiprazole-induced increases in Hsp90α protein expression may form part of the therapeutic mechanism for this drug.

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Introduction

Aripiprazole is an atypical antipsychotic drug approved for the treatment of psychiatric disorders such as schizophrenia, acute manic or mixed episodes associated with bipolar I disorder, as an adjunct treatment for major depressive disorder, and for irritability in children with autism.¹⁻⁹ Aripiprazole shows high affinity at dopamine D₂, and D₃ receptors, 5-hydroxytryptamine (5-HT) 5-HT₁A, and 5-HT₂A receptors, and moderate affinity at dopamine D₃ receptors, 5-HT₂C receptors, 5-HT₄ receptors, α₁ adrenergic receptors and histamine H₃ receptors.¹⁰⁻¹¹ While it is known that aripiprazole is a partial agonist at dopamine D₂ and 5-HT₁A receptors and an antagonist at 5-HT₂A receptors, the precise mechanisms underlying its clinical efficacy in psychiatric disorders are unknown.¹²⁻¹⁴

Accumulating evidence suggests that at the cellular level, neuronal plasticity, such as neurite outgrowth and neuroprotection, underlie the therapeutic effect of atypical antipsychotic drugs.¹⁵⁻¹⁸ PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, are widely used as a model system for nerve growth factor (NGF)-induced neurite outgrowth.¹⁹⁻²⁵ It is reported that atypical antipsychotic drugs such as olanzapine, quetiapine and clozapine could enhance neurite outgrowth in PC12 cells.¹⁵ A number of signaling molecules, including phospholipase C-\(\gamma\) (PLC-\(\gamma\)), phosphatidylinositol-3 kinase (PI3K), the mammalian target of rapamycin (mTOR), p38 MAPK, c-Jun N-terminal kinase (JNK), Akt and the Ras/Raf/ERK/MAPK pathways plays a role in the NGF-induced neurite outgrowth in PC12 cells.¹⁹⁻²⁵ To date, there are no reports on precisely how aripiprazole induces neurite outgrowth, although it is reported that this drug protects rat cortical neurons against glutamate toxicity.²⁶

The purpose of this study was first to examine the effect of aripiprazole on NGF-induced neurite outgrowth in PC12 cells. We found that aripiprazole significantly potentiated NGF-induced neurite outgrowth. Second, we examined the role of intracellular Ca\(^{2+}\) and the endoplasmic reticulum (ER) protein inositol 1,4,5-triphosphate (IP₃) receptors, on the potentiation of NGF-induced neurite outgrowth by aripiprazole, since Ca\(^{2+}\) signaling via IP₃ receptors plays an important role in NGF-induced neurite outgrowth.¹⁹⁻²⁵ Third, we investigated the role of cellular signaling pathways on aripiprazole potentiation of NGF-induced neurite outgrowth. Finally, using proteomic analysis, we observed significantly increased levels of heat shock protein Hsp90α, one of the most abundant

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proteins in cells, after treatment with aripiprazole and speculate that this protein may promote NGF-induced neurite outgrowth. Precise neurobiological functions of Hsp90 in the brain are currently unknown although Hsp90 protein is a ubiquitous and essential molecular chaperone that plays central roles in many signaling and other cellular pathways.

Materials and methods

Drugs. Drugs were obtained from the following sources: aripiprazole (Toronto Research Chemicals, North York, ON, Canada); xestospongion C (Wako Pure Chemicals, Tokyo, Japan); WAY-100635, and LY294002 (Sigma-Aldrich, St Louis, MO, USA); NGF (Alomone Labs, Jerusalem, Israel); 8OH-DPAT ((1-8-hydroxy-2-propylaminotetralin hydrobromide), and sulpiride (Tocris Bioscience, Bristol, UK); lovastatin, PD98059, GW5074, SB203580, MEK1/2 inhibitor (SL327), SP600125, U0126, U0124, 2-aminoethoxydiphenyl borate (2-APB) and rapamycin (Calbiochem-Novabiochem, San Diego, CA, USA); Akt inhibitor (Bio Vision, CA, USA); and BAPTA-AM, 1,2-bis (2-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid tetrakis) acetoxymethyl ester, (Dojindo Molecular Technologies, Kumamoto, Japan). Other drugs were also purchased from commercial sources.

Cell culture and quantification of neurite outgrowth. PC12 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum and 1% penicillin. Medium was changed two to three times a week. PC12 cells were plated onto 24-well tissue culture plates coated with poly-D-lysine/laminin. Cells were plated at relatively low density (0.25 x 10⁴ cells/cm²) in DMEM medium containing 0.5% FBS, 1% penicillin-streptomycin. Medium containing a minimal level of serum (0.5% FBS) was used as previously reported. Previously, we examined the optimal concentration of NGF needed to induce neurite outgrowth in PC12 cells, and found that NGF (2.5, 5, 10, 20, 40 ng/mL) increased the number of cells with neurite outgrowth in a concentration-dependent manner. In this study, 2.5 ng/mL of NGF was used to study the potentiating effects of aripiprazole on neurite outgrowth. Twenty-four hours after plating, the medium was replaced with DMEM medium containing 0.5% FBS and 1% penicillin-streptomycin with NGF (2.5 ng/mL), with or without aripiprazole (0.001, 0.01, 0.1, or 1.0 µM). Immunoactivity was detected by ECL Prime Western Blotting Detection Reagent (GE Healthcare Bioscience). Images formed for 1 h, at RT. After extensive washing, immunoreactivity was detected using a Fuji LAS3000-mini imaging system (Fujifilm, Tokyo, Japan) with Multi Gauge software (Ver.3.0; Fujifilm), β-Actin immunoreactivity was used to monitor equal sample loading.

RNAI transfection. RNA interference (RNAi) gene expression knockdown studies were performed using the TriFECTa RNAi kit (Integrated DNA Technologies, Coralville, CA, USA) and protocol. Each 27 mer RNAi duplex was transfected into
cells using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer’s guidelines. RNAi was purchased from Integrated DNA Technologies.

**Statistical analysis.** Data are expressed as mean values ± standard error of the mean (s.e.m.). Statistical analysis was performed using one-way analysis of variance and the post hoc Bonferroni/Dunn test. P-values < 0.05 were considered statistically significant.

**Results**

**Role of 5-HT1A receptors in aripiprazole-mediated potentiation of NGF-induced neurite outgrowth.** Aripiprazole (0.001, 0.01, 0.1 or 1.0 μM) in conjunction with NGF (2.5 ng ml⁻¹), significantly increased the number of cells with neurites, in a concentration-dependent manner (Figure 1a). The selective 5-HT₁A receptor antagonist WAY-100635 (10 μM), significantly blocked the potentiation of NGF-induced neurite outgrowth by aripiprazole (1.0 μM), although this inhibition was partial (Figure 1a). MAP-2 immunocytochemistry, which identifies neuronal markers, showed that aripiprazole (1.0 μM) increased the number of cells with NGF (2.5 ng ml⁻¹)-induced neurites (Figure 1b). The selective 5-HT₁A receptor agonist 8OH-DPAT (10 μM) significantly increased the number of cells with neurites induced by NGF, although the effect was less potent than that of aripiprazole (Figure 1c). In contrast, dopamine D₂ receptor antagonist sulphide (10 μM) failed to potentiate neurite outgrowth in the presence of NGF and aripiprazole (Figure 1d). Furthermore, sulphide (10 μM) alone did not alter NGF-induced neurite outgrowth in PC12 cells (Figure 1d). Collectively, these findings suggest that activation at 5-HT₁A receptors, as opposed to dopamine D₂ receptors, may play a partial role in the mechanisms of aripiprazole-induced neurite outgrowth.

**Role of IP₃ receptors and intracellular Ca²⁺ on the potentiation of NGF-induced neurite outgrowth by aripiprazole.** IP₃ receptors on the ERs are part of the signaling pathway that promotes NGF-induced neurite outgrowth in PC12 cells.¹⁹⁻²⁵ To investigate the involvement of IP₃ receptors in aripiprazole’s action on this outgrowth, we examined the effects of xestospongin C (a selective, reversible and membrane-permeable inhibitor of IP₃ receptors)³⁵ and 2-APB³⁶,³⁷ on our culture system. Addition of xestospongin C (1.0 μM) or 2-APB (100 μM) with aripiprazole (1.0 μM) significantly blocked the potentiation of NGF-induced neurite outgrowth (Figures 2a and b). Neither xestospongin C (1.0 μM) nor 2-APB (100 μM) in the absence of aripiprazole promoted NGF-induced neurite outgrowth in PC12 cells (Figures 2a and b).

To assess how intracellular Ca²⁺ impacts on this system, we examined the effects of the BAPTA-AM, a Ca²⁺ chelator,²⁵,³⁸,³⁹ on the potentiation of NGF-induced neurite outgrowth by aripiprazole (1.0 μM). BAPTA-AM (5.0 μM) significantly blocked the potentiation of NGF-induced neurite outgrowth by aripiprazole (1.0 μM) (Figure 2c). In addition, BAPTA-AM (5.0 μM) also produced the same effect. A lactate dehydrogenase assay showed that BAPTA-AM (5.0 μM) was
not cytotoxic to PC12 cells (data not shown). These findings demonstrate that intracellular Ca\(^{2+}\) signaling is necessary for NGF-induced neurite outgrowth in PC12 cells, with or without aripiprazole.

**Role of signaling molecules proximal to TrkA, on the potentiation of NGF-induced neurite outgrowth by aripiprazole.** In cells, the signaling molecules PLC-γ, PI3K, mTOR, p38 MAPK, JNK and Akt are activated by NGF.\(^{19,21-23,40-47}\) We examined the effects of specific inhibitors for these molecules on neurite outgrowth. The PLC-γ inhibitor, U73122 (1.0 \(\mu\)M), PI3K inhibitor, LY294002 (10 \(\mu\)M), mTOR inhibitor (rapamycin; 5 \(\mu\)M), p38 MAPK inhibitor (SB203580; 10 \(\mu\)M), the JNK inhibitor (SP600125; 10 \(\mu\)M) and Akt inhibitor (1.0 \(\mu\)M) significantly blocked the effects of aripiprazole on NGF-induced neurite outgrowth. Data show the mean ± s.e.m. (\(n=12\)). ***P < 0.001 as compared with the control (NGF (2.5 ng ml\(^{-1}\)) alone group).
Role of the Ras/Raf/ERK/MAPK pathways in the potentiation of NGF-induced neurite outgrowth by aripiprazole.

The Ras/Raf/ERK/MAPK pathways are reported to be involved in NGF-induced neurite outgrowth. Therefore, we examined the effects of inhibition on this pathway in the presence of aripiprazole. Inhibitors of Ras, Raf, MEK, MEK1/2 and MAPK (lovastatin; 1.0 μM, GW5074; 5.0 μM, U0126; 10 μM, MEK1/2 inhibitor (SL327; 10 μM) and MAPK inhibitor (PD98059; 10 μM). In contrast, U0124 (10 μM), an inactive inhibitor of U0126, did not alter the number of cells with neurites after aripiprazole treatment. Data show the mean ± s.e.m. (n = 8–18). ***P < 0.001 as compared with the control (NGF (2.5 ng ml⁻¹) alone group).

Role of Hsp90α in the potentiation of NGF-induced neurite outgrowth by aripiprazole.

To investigate novel molecular targets for aripiprazole’s potentiation of NGF-induced neurite outgrowth, we performed two-dimensional gel electrophoresis and analyzed the proteomics. We identified the heat shock protein Hsp90α as being differentially expressed between control PC12 cells treated with NGF (2.5 ng ml⁻¹) and those treated with NGF (2.5 ng ml⁻¹) and aripiprazole (1.0 μM). Aripiprazole treatment significantly increased Hsp90α expression in PC12 cells (Figure 5a).

To interrogate the proposed link between aripiprazole treatment, elevated Hsp90α production and the potentiation of NGF-induced neurite outgrowth, we treated PC12 cells with Hsp90α RNAi. RNAi serves as a gene silencing mechanism, thereby reducing expression of Hsp90α protein. As shown in Figure 5a, increases in Hsp90α protein expression by aripiprazole (1.0 μM) were significantly blocked by treatment with Hsp90α RNAi, but not by the negative control of Hsp90α RNAi. In the absence of aripiprazole, treatment with Hsp90α RNAi or negative control of Hsp90α RNAi did not alter basal levels of Hsp90α protein (Figure 5a). Furthermore, the potentiating effects of aripiprazole (1.0 μM) on NGF-induced neurite outgrowth were significantly attenuated by treatment with Hsp90α RNAi, but not the negative control of Hsp90α (Figure 5b). Treatment with Hsp90α RNAi or the negative control of Hsp90α RNAi alone did not alter NGF-induced neurite outgrowth in PC12 cells (Figure 5b).

Discussion

This study found that aripiprazole potentiated NGF-induced neurite outgrowth in PC12 cells, mediating this at the molecular level, at least in part, via an increase in Hsp90α protein. Aripiprazole, an atypical antipsychotic drug, licensed for use in a variety of psychiatric disorders, acts through partial agonism of dopamine D₂ and 5-HT₁A receptors, and antagonism of 5-HT₂A receptors. We found that aripiprazole potentiated NGF-induced neurite outgrowth in PC12 cells, and that this potentiation could be partially blocked by the selective 5-HT₁A receptor antagonist WAY-100635, but not the dopamine D₂ receptor antagonist sulpiride. The selective 5-HT₁A receptor agonist 8OH-DPAT also potentiated NGF-induced neurite outgrowth in PC12 cells, although to a lesser degree than aripiprazole. This result is in agreement with findings from human neuroblastoma SK-N-SH cells, which showed that 8OH-DPAT increased neurite outgrowth that could be blocked by the selective 5-HT₁A receptor antagonist WAY-100169. Thus, it is likely that activation of 5-HT₁A receptors plays a role in the mechanisms that lead to aripiprazole-driven enhancement of NGF-induced neurite outgrowth.
Figure 5  An increase in Hsp90α protein is required for aripiprazole-induced potentiation of nerve growth factor (NGF)-induced neurite outgrowth. (a) The potentiating effects of aripiprazole (1.0 μM) on Hsp90α protein levels were significantly antagonized by treatment with Hsp90α RNA interference (RNAi), but not the negative RNAi. In contrast, neither Hsp90α RNAi nor negative RNAi alone altered levels of Hsp90α protein in the control (NGF 2.5 ng ml−1)-treated group. Data show the mean ± s.e.m. (n = 6). ***P < 0.001 as compared with the aripiprazole (1.0 μM) group. (b) The potentiating effects of aripiprazole (1.0 μM) on NGF-induced neurite outgrowth were significantly antagonized by treatment with Hsp90α RNAi, but not negative RNAi. Neither Hsp90α RNAi nor negative RNAi alone altered NGF (2.5 ng ml−1)-induced neurite outgrowth. Data show the mean ± s.e.m. (n = 8). ***P < 0.001 as compared with the aripiprazole (1.0 μM) group.

Nagai et al.49 reported that aripiprazole ameliorates cognitive deficits in mice after repeated dosing with the N-methyl-D-aspartate receptor antagonist, phencyclidine, and that this effect is antagonized by co-treatment with WAY-100635, a 5-HT1A receptor antagonist, suggesting a role for 5-HT1A receptors. Given that 5-HT1A receptor function is implicated in schizophrenia related cognitive impairment,50–53 activation of this receptor by aripiprazole may confer a beneficial effect in these patients.54 Interestingly, aripiprazole attenuated established behavioral sensitization in rats after repeated administration of the stimulant methamphetamine.55 This effect was antagonized by pretreatment with the 5-HT1A receptor antagonist WAY-100169, suggesting that aripiprazole acts through 5-HT1A receptors in attenuating methamphetamine-induced behavioral sensitization.55 Since methamphetamine-induced behavioral sensitization is an animal model for both stimulant-induced psychosis and relapse vulnerability of schizophrenia, it is logical to reason that the therapeutic actions of aripiprazole are mediated by activation of 5-HT1A receptors.

IP3 is a ubiquitous second messenger responsible for the release of Ca2+ from the ER, a tightly controlled process which is critically important for maintaining cellular functions, including cell growth, and neurite outgrowth.56,57 In this study, we found that IP3 receptors antagonists (xestospongin C and 2-APB), and the intracellular Ca2+ chelator, BAPTA-AM, significantly blocked potentiation of NGF-induced neurite outgrowth by aripiprazole, indicating involvement of IP3 receptors and intracellular Ca2+ signaling on NGF-induced neurite outgrowth. Previously, we reported that several drugs utilize IP3 receptors to potentiate NGF-induced neurite outgrowth.19–25 It is reasonable to conclude that Ca2+ signaling via IP3 receptors plays a key role in the mechanism underlying aripiprazole’s potentiation of NGF-induced neurite outgrowth.

From our results, we postulate that several common cellular, signaling pathways might be responsible for promoting aripiprazole’s potentiation of NGF-induced neurite outgrowth. NGF binds to the high-affinity tyrosine receptor TrkA, initiating several cellular signaling pathways, affecting both morphological and transcriptional targets.19,21–23,41,42 Signaling molecules, including PLC-γ, PI3K, Akt, mTOR, p38 MAPK and JNK, are activated upon the addition of NGF.40 PLC-γ catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol and IP3. Diacylglycerol activates protein kinase C, and IP3 promotes transient release of Ca2+ from the ER via stimulation at IP3 receptors. Thus, the pathway via PLC-γ is responsible for NGF-induced neurite outgrowth.19,21–23,56 Furthermore, PI3K stimulation promotes neurite outgrowth in PC12 cells.19,21–23,47 In this study, we found that selective inhibitors of these cellular signaling pathways significantly blocked potentiation of NGF-induced neurite outgrowth by aripiprazole. In addition, we found that specific inhibitors of the Ras/Raf/MEK/MAPK pathway also significantly blocked the potentiation of NGF-induced neurite outgrowth by aripiprazole. These combined findings suggest that common cellular pathways, including PLC-γ, PI3K, Akt, mTOR, p38MAPK and JNK and Ras/Raf/MEK/MAPK, contribute to the potentiation of NGF-induced neurite outgrowth by aripiprazole.

Considering the function of the aforementioned signaling molecules in regulating protein synthesis-dependent learning and memory,59 aripiprazole-driven changes in these pathways may promote synthesis of new proteins associated with neurite outgrowth. Through proteomic analysis, we identified differential expression of Hsp90α, a molecular cytoplasmic chaperone, as a novel protein linked to neurite outgrowth. To our knowledge, this is the first report demonstrating a requirement for aripiprazole-driven increases in Hsp90α.
protein for neurite outgrowth. Hsp90 is an essential molecular chaperone, ubiquitously active in many signaling and other cellular pathways.\(^3\)–\(^5\)\) Accumulating evidence suggests that Hsp90 plays a key role in the assembly of a number of protein complexes,\(^6\) which could include those required for anti-psychotic drug action. Previously, Ishimoto et al.\(^60\) reported that the Hsp90 protein promoted neurite formation in vitro, in chick telencephalic and spinal neurons. We found that another 5-HT\(_{1A}\) receptor agonist, 8OH-DPAT also increased Hsp90\(_x\) protein levels in PC12 cells, although its effect was less pronounced compared with aripiprazole (Supplementary Figure 1). This suggests that 5-HT\(_{1A}\) receptor activation contributes to increased Hsp90\(_x\) protein, although the precise mechanisms underlying this expression are not known. It would appear that aripiprazole-driven increases in Hsp90\(_x\) protein potentiate NGF-induced neurite outgrowth although it is unclear how enhanced Hsp90\(_x\) expression contributes to its therapeutic effect in psychiatric disorders. Taken together, it is likely that induction of Hsp90\(_x\) levels in the brain may have beneficial effects in patients with psychiatric disorders. It would, therefore, be of great interest to study the effect of aripiprazole on serum Hsp90\(_x\) levels, in patients with psychiatric disorders.

Induction of Hsp90\(_x\) in the hippocampal CA1 cells after transient global ischemia may suggest a neuroprotective role of Hsp90\(_x\) in ischemia-induced cell death.\(^5\)\(1\) It may be that molecules that increase Hsp90\(_x\) protein levels may confer a therapeutic effect in psychiatric and neurodegenerative conditions, with altered neurite outgrowth. Furthermore, it is reported that the antibody to HSP90 was detected in the serum of a subset of patients with schizophrenia, suggesting the role of the autoimmunity to HSP90 in the pathogenesis and development of schizophrenia.\(^6\) In order to confirm the role of HSP90 in the pathogenesis of schizophrenia, the detection of antibodies to HSP90 in the cerebrospinal fluid of patients would be needed.

In conclusion, our results suggest that aripiprazole potentiates NGF-induced neurite outgrowth in PC12 cells, by Ca\(^{2+}\) signaling, via the IP\(_3\) receptors and common cellular signaling pathways. Furthermore, the increased expression of Hsp90\(_x\) protein induced by aripiprazole, may drive potentiation of NGF-induced neurite outgrowth. This suggests that Hsp90\(_x\) may represent a novel effector protein for the therapeutic action of aripiprazole.

Conflict of interest
The authors declare no conflict of interest.

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