Insulin and Insulin-like Growth Factor-1 Regulate Tau Phosphorylation in Cultured Human Neurons*

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Hyperphosphorylated tau is the major component of paired helical filaments in neurofibrillary lesions associated with Alzheimer’s disease. Hyperphosphorylation reduces the affinity of tau for microtubules and is thought to be a critical event in the pathogenesis of this disease. Recently, glycogen-synthase kinase-3 has been shown to phosphorylate tau in vitro and in non-neuronal cells transfected with tau. The activity of glycogen-synthase kinase-3 can be down-regulated in response to insulin or insulin-like growth factor-1 through the activation of the phosphatidylinositol 3-kinase pathway. We therefore hypothesize that insulin or insulin-like growth factor-1 may affect tau phosphorylation through the inhibition of glycogen-synthase kinase-3 in neurons. Using cultured human neuronal NT2N cells, we demonstrate that glycogen-synthase kinase-3 phosphorylates tau and reduces its affinity for microtubules and that insulin and insulin-like growth factor-1 stimulation reduces tau phosphorylation and promotes tau binding to microtubules. We further demonstrate that these effects of insulin and insulin-like growth factor-1 are mediated through the inhibition of glycogen-synthase kinase-3 via the phosphatidylinositol 3-kinase/protein kinase B signaling pathway.

Tau is a neuronal microtubule-associated protein found predominantly in axons (1). The function of tau is to promote tubulin polymerization and stabilize microtubules (2, 3). Tau, in its hyperphosphorylated form, is the major component of paired helical filaments (PHFs), the building block of neurofibrillary lesions in Alzheimer’s disease (AD) brain (4–6). Hyperphosphorylated tau is also found in neurofibrillary lesions in a range of other central nervous system disorders (7). In AD patients, the extent and topographical distribution of the neurofibrillary lesions correlate reliably with the degree of dementia (8, 9).

Hyperphosphorylation impairs the microtubule binding function of tau. PHF-tau does not bind to microtubules unless it is dephosphorylated (10, 11). It has been hypothesized that the reduced binding ability of PHF-tau to microtubules, coupled with reduced levels of normal tau, destabilizes microtubules in AD. This results in the disruption of vital cellular processes, such as rapid axonal transport, and leads to the degeneration of affected neurons.

Several serine/threonine protein kinases have been shown to phosphorylate tau in vitro. They include mitogen-activated protein kinase (MAPK) (12, 13), glycogen-synthase kinase-3 (GSK-3) (14–16), cyclin-dependent kinase 5 (17, 18), cAMP-dependent protein kinase, Ca2+/calmodulin-dependent protein kinase II (19), and a recently cloned 110-kDa protein kinase (MARK) (20). However, it is not clear which of these kinases phosphorylate tau in neuronal cells and are authentic regulators of tau phosphorylation in vivo. One of these kinases, GSK-3, has a possible physiological role in regulating tau phosphorylation, because recent studies have demonstrated that it induces cellular tau phosphorylation in Chinese hamster ovary cells and COS cells transfected with tau (21, 22).

Two isoforms of GSK-3, GSK-3α (51 kDa) and GSK-3β (46 kDa), are encoded by two different genes. They share 85% homology at the amino acid level (23). The activities of both isoforms can be down-regulated in response to insulin and growth factors, such as insulin-like growth factor (IGF-1) (23–26). This down-regulation involves phosphorylation of an N-terminal serine residue (serine 21 for GSK-3α and serine 9 for GSK-3β) (24–26). This phosphorylation is putatively mediated by several protein kinases, e.g. mitogen-activated protein kinase-activated protein kinase 1 (MAPKAPK1) or p90^rsk (46), which lies on the MAPK cascade (24), and the 70-kDa S6 kinase (p70 S6K) (25) and protein kinase B (PKB or Akt) (20), which lie downstream of the phosphatidylinositol 3-kinase (PI3/K) pathway.

GSK-3 is abundant in the brain (27). INSulin, IGF-1, and their receptors are also found throughout the developing and mature vertebrate brain (28–30). But it is currently unclear whether GSK-3 activity is regulated by insulin and IGF-1 in neurons. Recent studies have shown that insulin and IGF-1 promote survival by signaling through the PI3/K-PKB pathway in cultured cerebellar neurons (31). This led to our speculation that through the activation of PKB, insulin or IGF-1 may induce inhibition of GSK-3 and affect tau phosphorylation in neuronal cells.

To test this hypothesis, we conducted our study in human NT2N neurons, which are derived from a human teratocarcinoma cell line (NTera2/D1 or NT2) after treatment with retinoic acid (32, 33). These NT2N cells resemble late embryonic central nervous system neurons and express the fetal isoform of tau (33–35). Using this system, we demonstrate that insulin and IGF-1 reduce tau phosphorylation and promote tau binding to microtubules. This effect is mediated through the inhibition of GSK-3 via the PI3/K-PKB signaling pathway.

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§ The abbreviations used are: PHF, paired helical filament; AD, Alzheimer’s disease; GSK-3, glycogen-synthase kinase-3; MAPK, mitogen-activated protein kinase; IGF-1, insulin-like growth factor-1; MAPKAPK1, mitogen-activated protein kinase-activated protein kinase 1; p70 S6K, 70-kDa S6 kinase; PKB, protein kinase B; PI3/K, phosphatidylinositol 3-kinase; SFV, Semliki forest virus; DMEM-HG, Dulbecco’s modified Eagle’s medium with high glucose; MES, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; WT, wild type.
Insulin and IGF-1 Regulate Tau Phosphorylation

EXPERIMENTAL PROCEDURES

Materials—Recombinant GSK-3β and PD98059 were purchased from New England Biolabs; IGF-1 was from Promega; LY294002 was from BIOMOL Research Laboratories; protein kinase A inhibitor was from Upstate Biotechnology; γ-32P-ATP and 125I-labeled goat anti-mouse IgG were from NEP Life Science Products; and the Semliki Forest Virus gene expression system was from Life Technologies, Inc. Taxol was obtained from Dr. V. Narayanan of the NCI, National Institutes of Health. Other chemicals were purchased from Sigma.

Cell Culture—NT2 cells were grown and maintained as described (33). Briefly, NT2 cells were treated with retinoic acid for 5 weeks before the neuron-like NT2N cells were separated from the parent NT2 cells and replated on 6-well plates previously coated with poly-x-lysine (10 μg/mL) and matrigel. These NT2N neurons were maintained in Dulbecco’s modified Eagle’s medium with high glucose (DMEM-HG), supplemented with 5% fetal bovine serum, penicillin/streptomycin, and mitotic inhibitors (1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM uridine). 2–4-week-old NT2N cells were used for experiments.

Overexpression of GSK-3β in NT2N Cells Using the Semliki Forest Virus (SFV) Gene Expression System—Three GSK-3β/SFV viral constructs carrying C-terminal c-Myc tags (EQKLISEEDL) were made. GSK-3β WT is the wild type GSK-3β; GSK-3β S9A is a constitutively active form of GSK-3β, in which the serine 9 residue was mutated to alanine; and GSK-3β KM is an inactive mutant engineered by mutating lysines 85 and 86 to methionine and isoleucine. Another SFV construct expressing β-galactosidase (LacZ) was used as a control. To overexpress these constructs, NT2N cells were incubated for an hour with viral stocks diluted in DMEM-HG (MOI = 10) and then replenished with fresh medium. Cells were incubated overnight. For experiments that examined the effects of insulin on infected cells, cells were incubated for 6 h after infection, serum-starved (in DMEM-HG with 0.1% fetal bovine serum) for another hour, and then treated with insulin (100 ng/mL) for 5 min.

Treatment of NT2N Cells—To study the effects of insulin and IGF-1, NT2N cells were serum-starved in DMEM-HG with 0.1% fetal bovine serum. In some experiments in which insulin expression was high, non-serum fed cells were treated with 100 ng/mL of insulin or 100 ng/mL of IGF-1 for 5 min. Alternatively, NT2N cells were pretreated with PD98059 (50 μM, 60 min), LY294002 (100 μM, 15 min), wortmannin (100 nM, 15 min), 8-Br-CAMP (2 mM, 15 min), or rapamycin (100 nM, 15 min) before insulin or IGF-1 treatment. Pervanadate was prepared by incubating one part of 500 mM H2O2 with five parts of 10 mM Na3VO4 at room temperature for 10 min (36). This stock was added to NT2N cells at 1:100 dilution for 2 h.

Immunoblot Analysis of Tau Phosphorylation—NT2N cells were lysed in ice-cold high salt RAB buffer (0.1 M MES, 0.5 mM MgSO4, 1 mM EGTA, 2 mM diethiothreitol, and 0.75 mM NaCl, pH 6.8) supplemented with 0.1% Triton X-100 and a mixture of protease inhibitors (37). After centrifugation for 20 min at 50,000 × g at 4°C, the protein concentrations of the supernatants were determined by the bicinchoninic acid method. One microgram of total protein was resolved on 10% SDS-polyacrylamide gel electrophoresis for immunoblotting analysis with anti-tau antibodies. The blots were developed using enhanced chemiluminescence (ECL) or 3,3′-diaminobenzidine methods. Alternatively, for the quantitation of the relative levels of tau protein, 125I-labeled goat anti-mouse IgG was used as secondary antibody, and the blots were exposed to PhosphorImager plates as described (37). 10 μg of total protein was loaded for the detection of tau on immunoblots using the monoclonal antibodies T14/46, 25 μg for the antibodies T1 and PHF1 and 50 μg for the antibodies AT8, AT270, and PHF6.

Determination of Tau Binding to Microtubules—NT2N cells were treated with 100 ng/mL of insulin or infected with GSK-3β S9A/SFV overnight before they were lysed in 37°C RAB buffer (essentially high salt without NaCl) supplemented with 1% Triton X-100, 20 μM taxol, 2 mM GTP, and a mixture of protease inhibitors (38, 39). Cell lysates were Dounce homogenized 20 times and centrifuged for 20 min at 50,000 × g at 25°C. The protein concentrations of the soluble fractions were adjusted to be the same. The insoluble cytoskeleton fractions (pellet) were resuspended in ice-cold RAB buffer of the same volumes as the corresponding soluble fractions. Equal volumes of both fractions (approximately 5 μg of total protein from each fraction) were resolved on 10% SDS-polyacrylamide gel electrophoresis for immunoblot analysis with a monoclonal anti-α-tubulin antibody. To enrich tau, the samples were boiled for 5 min, incubated on ice for 5 min, and clarified by centrifugation for 15 min at 4°C. Equal volumes (containing approximately 10 μg of total protein) were resolved on 10% SDS-polyacrylamide gel electrophoresis for immunoblot analysis with T14/46. 125I-labeled goat anti-mouse IgG was used to detect the immunoreactivities, and quantitation was performed with the Image-Quant software (Molecular Dynamics). The ratios of microtubule-bound tau (in cytoskeletal fraction) versus soluble tau (in the soluble fraction) were calculated according to T14/46 immunoreactivities.

Insulin and IGF-1 Induce Tau Dephosphorylation and Promote Tau Binding to Microtubules in NT2N Cells—To determine whether insulin or IGF-1 stimulation alters tau phosphorylation, NT2N cells were treated with or without insulin or IGF-1 for 5 min and lysed for immunoblot analysis of tau phosphorylation with a panel of anti-tau antibodies. T14/46, a mixture of two phosphorylation-independent antibodies, detects total tau protein, whereas T1 detects dephosphorylated tau at residues 189–207 (numbering according to the largest isoform of central nervous system tau). With insulin and IGF-1 treatment, T1 immunoreactivity increased, indicating a reduction of tau phosphorylation. This was confirmed by the decrease in AT8 immunoreactivity, which is specific for phosphorylated serine 202 and threonine 205 (Fig. 1, A and B). The reduction of tau phosphorylation was induced by 100 ng/mL of insulin and 10 ng/mL of IGF-1. At these concentrations, insulin and IGF-1 bind with high affinity to their cognate receptors, because the affinity for cross-reaction is 100–1000 times lower (60). Therefore it is possible that both insulin and IGF-1 receptors mediate this effect.

We then examined whether insulin or IGF-1 stimulation...
affects tau binding to microtubules. Soluble and cytoskeletal fractions of cell lysates were prepared from control and insulin-treated NT2N cells. The tau protein and α-tubulin in each fraction were detected by immunoblot analysis (Fig. 1C). The ratio of microtubule-bound tau (P) versus soluble tau (S) was determined and plotted in Fig. 1D. This ratio was significantly higher in cells treated with insulin than in cells that received no treatment (n = 6; *, p < 0.01). This effect of insulin was completely blocked by LY294002.

**GSK-3β Phosphorylates Tau and Reduces Tau Binding to Microtubules in NT2N Cells**—To determine whether GSK-3 phosphorylates tau in neuronal cells, we overexpressed wild type (WT) and two mutant forms (S9A and KM) of GSK-3β in NT2N cells using the SFV gene expression system. The S9A mutation abolishes phosphorylation-dependent regulation at serine 9 and results in a constitutively active enzyme. KM has a double mutation and is enzymatically inactive. All three overexpressed GSK-3β carried a c-Myc-tag at their C termini and were immunoprecipitated with polyclonal anti-c-Myc antiserum. GSK-3 kinase assay was performed in the presence of [γ-32P]ATP with human recombinant tau as substrate. GSK-3 activity was represented by 32P incorporation into tau. Note that GSK-3β S9A and WT were enzymatically active, whereas KM was not. Immunoblot analysis with anti-GSK3 showed the amount of immunoprecipitated GSK-3 present in each reaction. B, infected NT2N cells were harvested for immunoblot analysis of tau phosphorylation. In cells overexpressing GSK-3β S9A (S9A) and GSK-3β WT (WT), T1 (dephosphorylated tau) levels decreased, whereas PHF1 (phosphorylated S396/404), AT270 (phosphorylated T181), and PHF6 (phosphorylated serine 262) immunoreactivities remained unchanged. Cells infected with SFV/LacZ (LacZ) were used as a control. C, to determine tau binding to microtubules, soluble (S) and insoluble cytoskeleton fractions (P) of cell lysates were harvested. About 70% of α-tubulin was recovered in the microtubule fraction (Fig. 1E). The ratio of tau bound (P) versus unbound (S) to microtubules was plotted in D. This ratio was significantly higher in cells treated with insulin than in control cells. D, indicating an enhanced tau binding to microtubules.

**PHF1** (phosphoserine 396/404), AT270 (phosphothreonine 181), and PHF6 (phosphothreonine 231) levels increased, indicating elevations of phosphorylation at these sites. The phosphorylation levels also increased at sites recognized by AT8 (phosphoserine 202/phosphothreonine 205) and PHF13 (phosphoserine 396) (data not shown). However, the immunoreactivity to 12E8 (phosphoserine 262) was not affected by GSK-3 (Fig. 2B). Cells infected with LacZ/SFV were used as a control in which tau phosphorylation levels did not differ from noninfected cells (data not shown). The expression of GSK-3β KM also did not alter tau phosphorylation (data not shown).

We also examined the effects of GSK-3β on tau binding to microtubules. As shown in Fig. 2C (C and D), significantly less tau was bound to microtubules in cells infected with GSK-3β S9A/SFV, because the ratio of cytoskeletal tau versus soluble tau was decreased. These data suggest that in neuronal cells, GSK-3β phosphorylates tau at multiple sites and reduces the affinity of tau for microtubules.

**Insulin Regulates Tau Phosphorylation through the Inhibition of GSK-3**—It has been shown previously that in nonneuronal cells, insulin and IGF-1 down-regulate GSK-3 activity by inducing the phosphorylation of a serine residue (serine 9 in
Insulin and IGF-1 Regulate Tau Phosphorylation

GSK-3β and serine 21 in GSK-3α, 23–26). To determine whether insulin mediates its effects on tau phosphorylation through the inhibition of GSK-3 in neurons, we first examined whether or not in NT2N cells insulin inhibits GSK-3β activity through the phosphorylation of serine 9. To do this, the cells were infected with either GSK-3β S9A/SFV or GSK-3β WT/SFV and subsequently treated with or without insulin. Overexpressed GSK-3β was immunoprecipitated and kinase activities were determined quantitatively using the in vitro kinase assay and normalized to the amount of GSK-3 in each reaction. As shown in Fig. 3A, wild type GSK-3β activity (WT) was inhibited by insulin, whereas GSK-3β S9A activity (S9A) remained unchanged. The endogenous GSK-3β activity was also assayed in noninfected NT2N cells by immunoprecipitating both GSK-3α and GSK-3β with a monoclonal anti-GSK-3 antibody. The normalized relative endogenous GSK-3 activity was also decreased by insulin treatment (Fig. 3A, Endogenous). These results indicate that the phosphorylation of serine 9 is involved in the insulin-mediated inhibition of GSK-3β in neurons.

To further demonstrate that insulin regulates tau phosphorylation through the inhibition of GSK-3, we infected NT2N neurons with GSK-3β S9A/SFV or GSK-3β WT/SFV and subsequently treated the cells with insulin. The total tau levels were not altered by infection or by insulin treatment (T14/46, Fig. 3B). Similar to what was shown in Fig. 2, in cells not treated with insulin, overexpression of GSK-3β S9A and GSK-3β WT resulted in a decrease in T1 and an increase in PHF1. However, insulin treatment only reduced tau phosphorylation in cells expressing GSK-3β WT but not in cells expressing GSK-3β S9A, because an increase in T1 and a decrease in PHF1 can be detected only in cells infected with GSK-3β WT/SFV (Fig. 3B). These experiments demonstrate that tau phosphorylation cannot be regulated by insulin unless GSK-3 activity can be inhibited through serine 9. This strongly suggests that insulin regulates tau phosphorylation through the inhibition of GSK-3.

Insulin and IGF-1 Regulate Tau Phosphorylation and Microtubule Binding of Tau through the PI3K-PKB Pathway—Three different protein kinases have been proposed to phosphorylate and inhibit GSK-3. They are MAPKAPK1, which lies downstream of the MAP-kinase cascade, and p70 S6K and PKB, which lie on the PI3K/PKB pathway. All three have been shown to phosphorylate GSK-3β at serine 9 and GSK-3α at serine 21 (24–26).

To test which of these kinases and pathways mediate(s) the effects of insulin and IGF-1 on tau phosphorylation and microtubule binding, we utilized specific inhibitors of these pathways as tools. Wortmannin (48), which inhibits PI3K, blocked the insulin and IGF-1-induced dephosphorylation of tau (Fig. 4, A and C). LY294002, which is another specific PI3K inhibitor (49), produced the same results (Fig. 4, B and C). PD98095 (50), which specifically inhibits the activation of MAPKAPK1 by the MAPK cascade, had no effect; nor did the cAMP analogue...
Insulin and IGF-1 Regulate Tau Phosphorylation

**DISCUSSION**

Current hypotheses regarding the pathogenesis of AD include the deposition of β-amyloid as a result of aberrant amyloid precursor protein metabolism and formation of neurofibri
tilary lesions by aggregation of hyperphosphorylated tau protein.

However, it seems that amyloid deposits are not sufficient for dementia, because large numbers of amyloid deposits are found in some cognitively normal individuals (53). On the other hand, it has been confirmed that cognitive deficits do not occur until dystrophic neurites containing PHFs and neurofibrillary tangles have developed (8, 9, and 54). Thus hyperphosphorylation of tau is believed to be a key event in the pathogenesis of the neurofibrillary pathology and the dementia of AD.

Several kinases have been shown to phosphorylate tau in vitro. But they do not necessarily do so in neuronal cells in vivo. For example, MAPK phosphorylates tau and generates a number of PHF-tau epitopes in vitro (12, 13). However, activating MAPK in cultures of primary neurons or transfected COS cells expressing tau does not increase the levels of phosphorylation for any PHF-tau epitopes (22). Another study also showed that stimulation of MAPK by v-ras transformation of fibroblasts fails to induce hyperphosphorylation of transfected tau (55). Therefore it is important to identify the kinases that are truly responsible for tau phosphorylation in neurons in vivo.

GSK-3 is one promising candidate. It phosphorylates tau in vitro (14–16) and in non-neuronal cells transfected with tau (21, 22). Moreover, multiple phosphorylation sites of tau are reported to be affected, including the sites recognized by phosphorylation-dependent antibodies PHF1 (serines 396/404), T3P (serine 396), AT8 (serine 202/threonine 205), AT270 (threonine 181), and AT180 (threonine 231) (21). Our results from the current study confirmed the role of GSK-3 in tau phosphorylation in neuronal cells. We also demonstrated that besides the previously reported phosphorylation sites, overexpression of GSK-3β in NT2N cells also increases phosphorylation at the sites recognized by antibodies T1 (dephosphorylated 189–207), PHF13 (serine 396), and PHF6 (threonine 231). But the phosphorylation level at the site recognized by 12E8 (serine 262) is not affected.

Our study also demonstrated that overexpression of GSK-3β reduces tau binding to microtubules in NT2N cells. This agrees with a recent study using transfected Chinese hamster ovary cells (56). Hyperphosphorylation of tau reduces its affinity for microtubules (10, 11), but it is currently unclear about the relative contributions made by individual phosphorylated residues. A study using Chinese hamster ovary cells transfected with tau has shown that phosphoserine residue 396, which lies right next to the microtubule-binding domain of tau, makes a significant contribution toward the reduced microtubule binding (10). However, the role of phosphoserine 262 (recognized by 12E8), which lies within the microtubule-binding domain, is controversial. One study suggests that the phosphorylation on this site alone eliminates microtubule binding of tau (44), whereas another study shows that this phosphorylation is not sufficient for abrogating such binding (47). Our current study supports the latter point of view, because overexpression of GSK-3 does not affect the phosphorylation of this site but still causes reduced microtubule binding of tau.

In this study, we first report that tau phosphorylation and function can be regulated through a signal transduction pathway by a hormone or a growth factor. We showed that insulin and IGF-1 induce inhibition of GSK-3, which results in tau dephosphorylation and increased microtubule binding of tau. Summarized in Fig. 6 are the putative signal transduction pathways through which insulin and IGF-1 might exert their effects on tau. We demonstrated that the PI(3)K-PKB pathway is involved in this action, but not the MAPK-MAPKAPK1 or the PI(3)K-p70 S6K pathways.

Insulin, IGFs and their receptors are found throughout the developing and mature vertebrate brain (28–30). Although the source of insulin in the brain remains a controversial issue,
Insulin and IGF-1 Regulate Tau Phosphorylation

Our results presented in the current study suggest phosphorylation of insulin receptor substrate-1 and PI(3)K is involved in the regulation of tau phosphorylation. The activation of certain protein phosphatases, e.g., protein phosphatase 1, affects observed in liver, muscle, and fat. Indeed, there is evidence that insulin and IGF-1 stimulate protein synthesis (57), increase neurite growth (58), induce tubulin mRNAs (59), and promote survival in neurons (31).

Insulin and IGF-1 receptors are structurally similar and belong to the same family of receptor protein tyrosine kinases. There is evidence that they have similar signaling modes: both phosphotyrosine insulin receptor substrate-1 and activate PI(3)K, MAPK, and c-fos (60). In non-neuronal cells, the cellular actions of insulin are mainly mediated via several signaling pathways involving changes in protein phosphorylation (61). It is likely that at least some steps of these pathways are shared among the classic target tissues and the nervous system. We have shown both insulin and IGF-1 receptors are expressed in NT2N cells, and upon stimulation by these ligands, tyrosine phosphorylation of insulin receptor substrate-1 and PI(3)K is induced. Our results presented in the current study suggest that the inhibition of GSK-3 via the PI(3)K-PKB pathway is a common event. This leads to reduced phosphorylation of tau induced by insulin is probably not mediated by the activation of phosphatases but by the inhibition of GSK-3, because the expression of GSK-3β S9A abolishes the effect.

The identification of a signal transduction pathway that regulates tau phosphorylation brings new insights to the study of the pathogenesis of AD. It raises the possibility that defects on this pathway may contribute to molecular pathology of the disease. Indeed, defects of glucose metabolism in AD brains have been well documented and are suggested to be the result of desensitized brain insulin receptors (63). This is also supported by the observation of increased insulin levels in cerebrospinal fluid of AD patients after an oral glucose tolerance test (64). If these observations indicate defects in the insulin receptor or its signaling pathways in AD, considering the effects of insulin on tau phosphorylation and function, long term malfunctioning may lead to the hyperphosphorylation of tau and impairment of tau function. Further studies need to be done to investigate whether in AD brains any abnormalities can be found in GSK-3, insulin, and IGF-1 receptors or their signaling pathways. This hypothesis should also be taken into consideration in future development of therapeutic strategies.

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