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Deregulation of the Phosphatidylinositol-3 Kinase Signaling Cascade Is Associated with Neurodegeneration in Npc1−/− Mouse Brain

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Niemann-Pick type C (NPC) disease is caused by mutations to genes that encode proteins critical to intracellular lipid homeostasis. The events underlying NPC progressive neurodegeneration are poorly understood but include neurofibrillary tangles of the type found in Alzheimer’s disease. Here we investigated possible contributions of a phosphatidylinositol-3 kinase cascade [PI3K, Akt, glycogen synthase kinase-3β (GSK-3β)] that is linked to apoptosis and various degenerative conditions. Brain concentrations of phosphorylated Akt, which phosphorylates and inactivates GSK-3β, were significantly elevated in Npc1−/− mice relative to Npc1+/+ mice. Accordingly, levels of inactive GSK-3β were 50 to 100% higher in mutant brains than in controls. Increases in inactive GSK-3β occurred early in postnatal development, well before neuronal loss, and were most prominent in structures with intracellular cholesterol accumulation, suggesting a contribution to subsequent degeneration. Perturbations of nuclear factor (NF)-κB, which is regulated by GSK-3β, occurred in Npc1−/− mouse brains. Nuclear concentrations and DNA binding activity of NF-κB’s transactivation subunit, p65, were significantly reduced in Npc1−/− mice compared to Npc1+/+ mice. Cytoplasmic levels of the p50 subunit and its precursor, p105, were higher in Npc1−/− mice. These results suggest that excessive activity in the PI3K-Akt pathway depresses GSK-3β, thereby disrupting the formation and/or nuclear import of p50/p65 NF-κB dimers and contributing to neuronal degeneration. (Am J Pathol 2005, 167:1081–1092)

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Address reprint requests to Dr. Xiaoning Bi, Department of Psychiatry and Human Behavior, 101 Theory Dr., #250, UC Irvine, Irvine, CA 92617. E-mail: xbi@uci.edu.
regionally selective manner. Taken together, the results changes occur early in postnatal development and in a sectioned at 25 paraformaldehyde. Brains were removed and incubated with phosphate-buffered saline (PBS) followed by 4% analyses. For histological studies, animals were perfused histological studies or by decapitation for biochemical group) under deep anesthesia (200 mg/kg sodium pen-

weeks 1, 2, 4, and 8 (four to eight animals for each age

degeneration. In the present study, we tested for spatio-
temporal correlations between the activity of Akt, GSK-
3β, and NF-κB and cholesterol deposits or neuropathol-
yogy in a mouse model of NPC. We found evidence for increased levels of Akt activation and GSK-3β inactiva-
tion, and for NF-κB deregulation; moreover, these changes occur early in postnatal development and in a regionally selective manner. Taken together, the results provide the first evidence that NPC is accompanied by a profound disturbance of the PI3K/Akt-GSK-3β-NF-κB sig-
naling pathway, and strongly suggest that such distur-
ance is a contributor to, rather than a consequence of, degeneration.

Materials and Methods

Mice

Heterozygous breeding pairs of BALB/cNctr-npc1N mice (Npc1+/−) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility in accordance with National Institutes of Health guidelines and protocols approved by the Institutional Animal Care and Use Committee with care to minimize distress to the animals. Mouse breeding and genotyping were as previ-
ously described.31 Animals were killed at postnatal weeks 1, 2, 4, and 8 (four to eight animals for each age group) under deep anesthesia (200 mg/kg sodium pento-
tobarbital) by perfusion for immunohistochemical and histological studies or by decapitation for biochemical analyses. For histological studies, animals were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed and incubated with 15% sucrose followed by 30% sucrose before being sectioned at 25 μm with a freezing microtome. Coronal sections were stored in a cryoprotective solution (30% ethylene glycol, 20% glycerol in 0.025 mol/L phosphate buffer, pH 7.3) at −20°C before being processed for histological and immunohistochemical studies.

Western Blots

Brain regions [cerebellum, brainstem (including diencephalon, midbrain, pons, and medulla), hippocampus, and cortex] from Npc1−/− and Npc1+/+ mice were dissected in ice-cold artificial cerebrospinal fluid, and homogenized in 50 mmol/L Tris-HCl (pH 7.4) buffer contain-
ing 1 mmol/L EDTA, 1 mmol/L EGTA, and a protease inhibitor cocktail (Sigma, St. Louis, MO). Electrophoresis and immunoblotting were performed following conven-
tional procedures. Briefly, proteins (40 to 60 μg) from each sample were denatured by boiling for 5 minutes in a sample buffer [2% sodium dodecyl sulfate, 50 mmol/L Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue], and separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (12%), after which proteins were transferred to nitrocel-
lulose membranes. Nitrocellulose membranes were incub-
ated with primary antibodies for 12 to 16 hours at room temperature; immunoreactivity was visualized by using enhanced chemiluminescence (ECL Plus kit and re-
agents; Amersham Pharmacia Biotech). Antibodies used were: anti-Akt1 (1:10,000), anti-Akt1-pThr308 (1:1000; both from Upstate, Charlotteville, VA), anti-Akt1-
pSer473 (1:500; Cell Signaling Technology, Beverly, MA), and anti-GSK-3βpSer9, (which also recognizes GSK-3α phosphorylated at Ser21, 1:1000; Cell Signaling Technol-
gy); anti-GSK-3β Tyr216 (1:10,000; Upstate), anti-

NF-κB Binding Assay

DNA binding activity of NF-κB (p65/p50) was determined by using the enzyme-linked immunosorbent assay-based nonradioactive NF-κB p65/p50 transcription factor assay kit following the manufacturer’s protocol (Chemicon, Te-
mcuca, CA). Nuclear extraction was done as previously described.32 Briefly, brain tissues were homogenized in ice-cold PBS with a Dounce homogenizer (10 strokes). Homogenates were centrifuged at 4°C for 30 seconds at 12,000 × g, and the supernatants discarded. Pellets were resuspended in lysis buffer (10 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonl fluoride, 2 μg/ml anti-
pain, 2 μg/ml leupeptin) and incubated on ice for 10 minutes with 10% Nonidet P-40 solution added. The mix-
tures were mixed vigorously for 30 seconds and recen-
trifuged for 30 seconds at 14,000 × g. Pelleted nuclei were resuspended in extraction buffer (20 mmol/L HEPES, 25% glycerc, 1.5 mmol/L MgCl2, 300 mmol/L NaCl, 0.25 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonl fluoride, 2 μg/ml leupeptin, 2 μg/ml antipain), incubated on ice for 20 minutes, then centrifuged again for 20 minutes at 14,000 × g. The supernatants containing the nuclear proteins were incub-
ated with biotinylated-specific DNA probes for 1 hour at room temperature. The mixture was then transferred to the streptavidin-coated 96-well plates. Binding of active NF-κB with DNA was revealed by incubation with anti-
p50/p65 antibodies, followed by the horseradish peroxi-
dase-conjugated secondary antibodies and a colorimet-
ric reaction, which was analyzed by a microplate reader (Molecular Devices, Sunnyvale, CA). Results obtained from brains of Npc1−/− and Npc1+/+ mice were analyzed by one-way analysis of variance and P values smaller than 0.05 were considered significant.

Filipin Staining

Filipin has been demonstrated to specifically stain free cholesterol because treatment with cholesterol oxidase results in a complete loss of fluorescence. Brain tissue sections were washed with phosphate-buffered saline and incubated in the dark with 50 µg/ml filipin in PBS for 3 hours under agitation at room temperature. After washing in PBS, some sections were further processed for immunostaining with anti-GSK-3βSer9 antibody (see below); others were mounted on slides and coverslipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Filipin fluorescence was detected by a Zeiss Axiocamt epifluorescence microscope.

Fluoro-Jade B (FJB) Staining

FJB is a fluorochrome that is a selective marker for neurodegeneration. Sections were mounted on pre-treated slides (Superfrost/plus; Fisher, Pittsburgh, PA), air-dried, and rehydrated in distilled water for 3 minutes. Then, slides were incubated sequentially in 1) 1% sodium hydrosol in 80% ethanol for 5 minutes, 2) 70% ethanol for 2 minutes, 3) 0.06% potassium permanganate for 10 minutes, and 4) FJB staining solution (Histo-Chem Inc., Jefferson, AZ), prepared according to the manufacturer’s instructions for 20 minutes. Some sections were further processed for immunostaining with anti-GSK-3βSer9 antibody. Slides were then washed with water, dehydrated in graded ethanol, and coverslipped with DPX mounting solution (BDH Laboratory Supplies, Poole, UK).

Immunohistochemistry

Sagittal sections from cerebellum and coronal sections from the rest of the brains of Npc1+/+ and Npc1−/− mice at different ages were simultaneously processed for immunostaining. Immunohistochemistry was performed using the avidin-biotin horseradish peroxidase complex (ABC) method. Briefly, free-floating sections were first incubated in 3% normal goat serum diluted in PBS with 0.1% Triton X-100 for 1 hour at room temperature, followed by incubation with anti-GSK-3βSer9 (1:500; Cell Signaling Technology) or anti-p65 (1:1000; Santa Cruz) overnight at 4°C. After three washes in PBS, sections were incubated with corresponding biotinylated secondary antibodies (1:400; Vector Laboratories) in 1.5% normal goat serum solution for 2 to 3 hours, then in ABC diluted in PBS for 45 minutes. Peroxidase reaction was performed with 3,3’-diaminobenzidine tetrahydrochloride (0.05% in 50 mmol/L Tris-HCl buffer, pH 7.4) as chromogen and 0.03% H2O2 as oxidant. Free-floating sections were mounted on precoated slides and air-dried. Sections were then dehydrated in graded ethanol and finally covered with Permunt. For double staining, rabbit anti-GSK-3βSer9 and mouse anti-cathepsin D antibodies (Santa Cruz Biotechnology) were used; binding of antibodies against antigens was revealed by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse antibodies (Molecular Probes, Eugene, OR). For tissues that were first stained by filipin or FJB followed by anti-GSK-3βSer9 antibody, Alexa Fluor 555 goat anti-rabbit IgG was used, and slides were coverslipped with Vectashield mounting media.

Results

Akt Phosphorylation in Npc1−/− Mouse Brain during Postnatal Development

To test the status of the PI3K-Akt pathway in the developing central nervous system of Npc1−/− mice, immunoblotting analysis was performed with anti-Akt1-pThr308, anti-Akt1-pSer473, or anti-Akt1 antibodies. At postnatal week 2, significant increases in levels of Akt1-pThr308 were found in cerebellum and neocortex (155% and 124% of values found in Npc1+/+ mice, respectively), whereas at postnatal week 4 a marked increase was found in brainstem (278% of Npc1+/+ mice) and cerebral cortex (172% of Npc1+/+ mice) and a moderate increase in hippocampus (133% of Npc1+/+ mice; Figure 1A). Immunoblots labeled with anti-Akt1-pSer473 antibodies indicated that marked increases (167% of Npc1+/+ mice) in active Akt1 were present in cerebral cortex at postnatal week 2; this effect expanded to all four brain regions analyzed by postnatal week 4 (Figure 1B). Levels of total Akt1 estimated from Western blots labeled with anti-Akt1 antibodies did not differ between the two types of mice [Figure 1, C and D (bottom)]. The ratios of Akt1-pThr308/Akt1 and Akt1-pSer473/Akt1 in Npc1−/−...
mice were significantly elevated in all brain regions at postnatal week 4 and in some brain regions at postnatal week 2 (Tables 1 and 2). Furthermore, in vitro experiments indicated that phosphorylation of GSK-3 fusion proteins mediated by Akt1 immunoprecipitated from Npc1−/− mouse brains using an anti-Akt1 immobilized antibody was 125% higher than that from Npc1+/+ mouse brains (n = 3, P < 0.05). Together, these results clearly indicate that levels of active Akt1 were increased in Npc1−/− mouse brain during postnatal development as compared to those found in Npc1+/+ mice.

Levels of GSK-3 in Npc1−/− Mouse Brain during Postnatal Development

As noted, GSK-3β is inactivated via phosphorylation at its Ser9 residue by the PI3K-Akt system; the results described above would thus predict increases in the inactive form of GSK-3β in Npc1−/− mice. Brain homogenates prepared from 2- to 4-week-old animals were subjected to Western blot analyses using a polyclonal antibody that recognizes the inactive (phosphorylated) forms of GSK-3α and -3β, which migrate as two separate bands with an apparent molecular weight of 51 and 46 kd (Figure 2A). Levels of both GSK-3α-pSer21 and GSK-3β-pSer9 were significantly increased in Npc1−/− compared to Npc1+/+ mice (Figure 2A). Quantitative analysis showed that at postnatal week 2, increases in phosphorylated GSK-3α (166 ± 23%, P < 0.05; Figure 2B) and GSK-3β (142 ± 18%, P < 0.05; Figure 2C) were statistically significant in cerebral cortex, but not in other brain areas. By 4 weeks however, significant increases in levels of phosphorylated GSK-3α were observed in brainstem, cerebral cortex, and hippocampus but not in cerebellum (Figure 2B). Levels of phosphorylated GSK-3β were higher in all four brain regions of Npc1−/− as compared to those in Npc1+/+ mice (Figure 2C). Levels of total GSK-3β (Figure 2D) and of the active isoforms of GSK-3 (GSK-3βpTyr216, not shown) did not differ between the two genotypes.

Immunohistochemical Localization of GSK-3βpSer9 in Npc1−/− Mouse Brain

Week 1

Immunohistochemical studies revealed that there was virtually no detectable staining of GSK-3βpSer9 in Npc1−/+ mouse brains at postnatal week 1, whereas high levels of the phosphorylated kinase were present in Npc1−/− mouse brains at this age (Figure 3). Interestingly, the highest levels of staining were mostly distributed along the major sensory projection systems, including the medial geniculate nucleus and primary auditory cortex, the lateral geniculate nucleus and primary visual cortex, and the lateral thalamic complex and primary somatosensory cortex (Figure 3). In neocortex, high levels of GSK-3βpSer9 staining occurred mainly in neurons of layers IV and VI. Evident GSK-3βpSer9 staining was also observed in deep layers of primary motor cortex (not shown). High magnification (Figure 3, inset; and Figure 4) indicated that GSK-3βpSer9-immunopositive elements were mostly granules with different sizes, which were located in cell bodies around nuclei. From their morphology and subcellular location, these elements resembled endosomes/lysosomes. In some cells, these elements were either clustered at the polar end of cell bodies or accumulated in the initial segments of axons forming megal neurite-like structures (Figure 4, arrows). Occasionally, spines budding from megal neurites were observed (Figure 4, arrowheads), a peculiar feature known as ectopic dendritogenesis in lysosomal storage diseases.36

Weeks 2 to 4

By postnatal week 2, levels of GSK-3βpSer9 immunoreactivity decreased in layer IV of neocortex, but moderate levels of staining remained in layer VI (Figure 5, SS/2W). Staining for GSK-3βpSer9 also became obvious in several other brains areas, being particularly dense in several thalamic nuclei and in the molecular layer of cerebellum (Figure 5). By 4 weeks, immunoreactivity for GSK-3βpSer9 further increased in the aforementioned areas.

Table 1. Ratio of Akt1-Thr308/Akt1 (Percentage of Npc1+/+)

<table>
<thead>
<tr>
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<th>NPC−/− BS</th>
<th>NPC−/− Cere</th>
<th>NPC−/− CX</th>
<th>NPC−/− Hipp</th>
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<tr>
<td>2 weeks</td>
<td>110.7 ± 12.8</td>
<td>150.7 ± 13.6*</td>
<td>118.2 ± 5.2*</td>
<td>10.27 ± 6.2</td>
</tr>
<tr>
<td>4 weeks</td>
<td>278.1 ± 16.5†</td>
<td>125.4 ± 23.8</td>
<td>160.3 ± 7.9†</td>
<td>135.5 ± 0.8†</td>
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*p < 0.05, †p < 0.01.

Table 2. Ratio of Akt1-Ser473/Akt1 (Percentage of Npc1+/+)

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<thead>
<tr>
<th></th>
<th>NPC−/− BS</th>
<th>NPC−/− Cere</th>
<th>NPC−/− CX</th>
<th>NPC−/− Hipp</th>
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<tr>
<td>2 weeks</td>
<td>99.3 ± 4.0</td>
<td>110.1 ± 3.0</td>
<td>160.2 ± 2.3*</td>
<td>106.1 ± 2.1</td>
</tr>
<tr>
<td>4 weeks</td>
<td>142.2 ± 11.4†</td>
<td>116.9 ± 4.4*</td>
<td>125.9 ± 4.9†</td>
<td>147.2 ± 6.1†</td>
</tr>
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</table>

*p < 0.05, †p < 0.01.
noncortical regions and expanded to other brain structures. Dense GSK-3βpSer9 immunoreactivity appeared inside Purkinje cells (Figure 5, PC). GSK-3βpSer9-immunoreactive cells were also evident in the hilus region of hippocampus (Figure 5). In most cases, the staining was clearly granular and double-immunostaining results showed that GSK-3βpSer9-immunopositive granules were also labeled with anti-cathepsin D antibodies, in particular in meganeurite-like structures (Figure 6; A to D). In the molecular layer of cerebellar cortex, stained granules were located in cell bodies, around 4,6-diamidino-2-phenylindole-labeled nuclei in small-sized cells (Figure 6E, arrowheads and inset). These cells were not labeled with antibodies against F4/80 antigen, a marker of microglia (Figure 6F). GSK-3βpSer9 immunostaining became weaker by 8 to 10 weeks, when numerous neurons were dying or had died (not shown). No obvious GSK-3βpSer9 immunostaining was found in brains of Npc1+/+ mice at any time during postnatal development (an example is shown in Figure 5).

Co-Localization of Inactive GSK-3β with Intracellular Accumulation of Cholesterol in Degenerating Brain Regions

Intracellular accumulation of cholesterol and glycolipids is the characteristic pathology of NPC.36–38 Cholesterol clusters were visualized by staining brain tissue sections with filipin, a natural fluorescent antibiotic that has a high affinity for cholesterol. In agreement with the literature, accumulation of cholesterol assessed with filipin staining was obvious only in axonal spheroids scattered along the corpus callosum at postnatal week 2, whereas clear somatic staining was seen in brain sections from animals older than 4 weeks of age; spatial distribution of intraneuronal filipin staining matched that of GSK-3βpSer9 immunostaining and of Gallyas silver staining (not shown). As shown in Figure 7, GSK-3βpSer9 immunostaining was specifically localized in filipin-labeled brain structures from 4-week-old Npc1−/− mice. Closer examination showed that inactive GSK-3β was located in cholesterol-enriched vesicles in filipin-labeled structures, eg, in the ventral lateral thalamic complex (Figure 7, middle). Double staining indicated that GSK-3βpSer9-immunopositive structures were also labeled with FJB, a dye widely used to label degenerating neurons. Images taken at higher magnification showed that GSK-3βpSer9-immunoreactive products often clustered at one pole of the cell body or accumulated in structures previously described as “meganeurites”36 in FJB-labeled neurons (Figure 7, arrows in bottom panels).

Changes in NF-κB in Npc1−/− Mouse Brains

In vitro studies have shown that GSK-3β phosphorylates NF-κB subunit p65.25 A more recent study showed that
GSK-3β can also affect nuclear levels of NF-κB by directly participating in nuclear transport of p65. Western blots labeled with anti-p65 antibodies showed that nuclear p65 levels were significantly lower in brains of 2- to 3-week-old Npc1−/− mice as compared to Npc1+/+ mice of the same age (70 ± 7% of Npc1+/+; mean ± SEM; P < 0.01); in contrast, whole homogenate p65 levels were not modified (Figure 8, A and B), suggesting that nuclear import of the factor might be impaired. GSK-3β can also phosphorylate and stabilize p105, the precursor of the p50 subunit. Immunosblots labeled with anti-p105/p50 antibodies indicated that levels of both p50 and its precursor were significantly higher in homogenate (154 ± 13% and 233 ± 11%, respectively) and cytosol fractions (134 ± 16% and 237 ± 3%, respectively) from brains of Npc1−/− mice as compared to Npc1+/+ mice (Figure 8, A and B). Finally, p50 levels in nuclear fractions from Npc1−/− mice were similar to those in Npc1+/+ mice. To further determine whether changes in levels of NF-κB proteins also affected the transcriptional activity of this nuclear factor, an enzyme-linked immunosorbent assay-based kit was used to compare the DNA binding ability of NF-κB in brain nuclear fractions from Npc1−/− and Npc1+/+ mice. A very large decrease in DNA binding ability of p65/p50 dimers was observed in nuclear fractions from Npc1−/− mouse brains as compared to those from Npc1+/+ mice [optical density = 344 ± 20 in Npc1+/+ and 205 ± 18 in Npc1−/− mice (mean ± SEM); n = 6 mice and P < 0.002; Figure 8C].

Survey images from immunohistochemical studies indicated that in 2- to 4-week-old Npc1−/− mouse brains, p65 immunoreactivity was particularly low in the most vulnerable regions (Figure 9D), where levels of GSK-3βpSer9 were markedly elevated (Figure 9B). Closer examination revealed that in these areas, p65 immunoreactivity in neurons was markedly lower, whereas that in glia (Figure 9F, inset) was higher as compared to that in Npc1+/+ mice (Figure 9E). Interestingly, less vulnerable neurons, eg, pyramidal neurons in the hippocampus, exhibited no detectable levels of GSK-3βpSer9 (Figure 9H) and did not show significant decrease in NF-κB (Figure 9G).

**Discussion**

**Pathology and Abnormal Activity in the PI3K Signaling Cascade**

The results of the present study indicate that the PI3K signaling cascade exhibits major disturbance in the brains of Npc1−/− mice. Changes in each of three links in the pathway were evident in immunoblots by postnatal week 2 and in immunocytochemical analyses of phosphorylated GSK-3β staining by week 1. It is noteworthy that the degree of changes in levels of active Akt and inactive GSK-3 estimated by Western blots might be underestimated because immunohistochemical results indicated that only selective cell populations were GSK-3βpSer9-immunopositive in brains of Npc1−/− mice. The earliest signs of axonal degeneration described for the Npc1−/− mouse occur at postnatal day 9 in the corpus callosum and in the white matter of the cerebellum. Similarly, we found here that cholesterol deposits,
as detected by filipin staining, were infrequent at postnatal week 2 and only became widespread at postnatal week 4, as did FluroJade-labeled neurons. The changes in the PI3K pathway seen here thus occurred concurrently with disturbances in lipid storage and well in advance of widespread neuronal degeneration. Sustained increases in levels of inactive GSK-3β were most evident in a subset of brain structures, including thalamic sensory relay nuclei and cerebellar cortex, known from the literature and confirmed here, to be vulnerable to NPC.

These temporal and regional results suggest that the observed perturbations of the PI3K cascade are contributors to the brain pathology that characterizes the disease. On the other hand, the apparent inactivation of GSK-3β does not seem to accord with the idea that hyperphosphorylation of tau protein by this enzyme is an important step in the process leading to neurofibrillary tangles found in NPC patients, although we cannot exclude the possibility that tau is associated with levels of active GSK-3β beyond the sensitivity of our assays, which could then participate in tau phosphorylation in Npc1−/− mice. Activated GSK-3β has been localized to tangles in Alzheimer’s disease brains, leading to the hypothesis that the enzyme, through tau hyperphosphorylation, contributes critically to the assembly of helical filaments. Several kinases other than GSK-3β can phosphorylate tau and have been implicated in tangle formation; as previously reported, MAP kinase and cdk5 are likely to drive the process of tangle formation in NPC.

Elevated levels of phosphorylated Akt strongly suggest that PI3K is unusually active in Npc1−/− mouse brain. The NPC proteins are normally involved in transporting cholesterol from late endosomes/lysosomes to the plasma membrane and, related to this, NPC cells differ from controls with regard to membrane cholesterol content. Cholesterol levels, in turn, potently modulate the activation of PI3K by endogenous triggering agents and this is reflected in the phosphorylation of Akt. Activation of Akt could also be mediated by MAPK because activity of this kinase is increased in Npc1−/− mouse brain.

Abnormal Distribution of GSK-3β

The immunocytochemical studies showed that disturbances of the PI3K pathway go beyond abnormal levels

Figure 6. Subcellular and cellular localization of GSK-3βSer9 in brains of 4-week-old Npc1−/− mice. A–C: GSK-3βSer9 (A) and cathepsin D (B) were co-localized (C) in meganeurite-like structures in cortical neurons of Npc1−/− mice. D: Confocal microscopic image showing the co-localization (yellow granules) of GSK-3βSer9 (red) and cathepsin D (green). E: GSK-3βSer9 immunoreactivity in cerebellar cortex where it was localized in small-sized cells (arrowheads) and inset. F: GSK-3βSer9 (red)-immunopositive cells were not labeled by a microglial marker, F4/80 antigen (green). gl, granular layer; ml, molecular layer; pcl, Purkinje cell layer. Scale bar = 20 μm in E, 40 μm in F.
of activity to include abnormal distribution. Specifically, the phosphorylated (inactive) form of GSK-3β found in Npc1−/− mouse brains, but uncommonly in controls, was concentrated in granules surrounding nuclei. The size, number, and distribution of these puncta indicated that they were related to late endosome-lysosomes, a point that was confirmed by co-localization studies indicating that inactive GSK-3β accumulated in cholesterol- and cathepsin D-positive structures. Although the subject has not been extensively studied, it is reasonable to assume that glycolipid and cholesterol cellular accumulation substantially impairs lysosomal functioning, resulting in increased aberrant autophagocytosis. Indeed, the conversion of LC3-I to LC3-II was significantly higher in Npc1−/− mouse brains (X. Bi et al, unpublished results).

Although GSK-3β, a cytoplasmic kinase, would not normally be a target for lysosomal processing, the enzyme is found in mitochondria, and is inactivated there by phosphorylated Akt transported from the plasma membrane. Mitochondria are a principle target of macroautophagy, and thus could be the source of the lysosomal accumulation of inactive GSK-3β; the persistence of the kinase, or at least of its pertinent epitopes, would then reflect the compromised internal environment of the organelles and consequent reduced rates of proteolysis.

Interestingly, GSK-3β immunoreactivity was found in meganeurites, massive swellings located at the base of the cell body or in the initial segment of the axon. These structures are a concomitant of various storage diseases including NPC, and have been described in animal models of NPC. They are composed of large numbers of individual and fused lysosomes and can be experi-
in nuclear fractions from brains of Npc1 binding activity assay showed a marked decrease in p65/p50 binding activity. No significant difference was observed with levels of nuclear p50.

Kines48–52 trigger the PI3K/Akt system and thereby inactivate GSK-3, inhibiting the activity of NF-κB, whereas phosphorylation at Ser486 decreased the activity of NF-κB. Accumulating evidence indicates that GSK-3 regulates the activity of NF-κB, although the mechanisms involved are not clear. nor is it known whether the kinase increases or decreases the activity of this transcription factor. NF-κB-activating stimuli such as growth factors or cytokines,48–52 trigger the PI3K/Akt system and thereby activate GSK-3β. Moreover, suppression of GSK-3β with lithium or more specific inhibitors enhanced the transcriptional activity of NF-κB in renal medullary interstitial cells.60 In contrast, genetic depletion of GSK-3β resulted in embryonic lethality with similar pathological features as those produced by gene targeting of iκB kinase-β, an enzyme critical to NF-κB activation, or of the p65 subunit of NF-κB itself.28 These results strongly suggested that GSK-3β activates the transcription factor. The confusion between these opposite results suggests that GSK-3β interacts in multiple, and in some cases opposing, ways with NF-κB. Four potential GSK-3β phosphorylation sites have been identified on the transactivation domains of the p65 subunit and, as predicted from this, recombinant GSK-3β phosphorylated a fusion protein containing the relevant segments.25 Subsequent work indicated that phosphorylation of p65 at Ser536 increased NF-κB activity,61 whereas phosphorylation at Ser486 decreased basal NF-κB activity.52 GSK-3β can also affect nuclear levels and activity of NF-κB by directly affecting nuclear import of p65.22 In addition, GSK-3β phosphorylates the p105 precursor of the p50 member of the NF-κB dimer; this has the dual effect of slowing constitutive processing to p50 and accelerating the degradation of p50 on stimulation with TNF-α.24 Indeed, knocking out GSK-3β increases constitutive processing of p105 to p50 and leads to accumulation of p50. Knocking out GSK-3β also prevents the degradation of p105 in response to TNF-α and results in increased levels of p105 as GSK-3β phosphorylated p105 could be further phosphorylated by IKK, which targets p105 to proteasome-mediated degradation.24 It has been proposed that the absence of GSK-3β results in the accumulation of p50 and the formation of p50/p50 homodimers that compete with p50/p65 and inhibit NF-κB-mediated transcription.24,63

Results from the present study showed that nuclear p65 levels were reduced as was DNA binding activity of the transcription factor, which suggests an impaired nu-
clear import of NF-κB heterodimers from the cytoplasm. We also found that cytoplasmic concentrations of p105 and p50 were significantly elevated in the mutants, as would presumably occur under conditions in which GSK-3β-mediated phosphorylation of the subunit was deficient and TNF-α stimulation was also increased.64

**Deregulation of NF-κB in NPC Disease: Involvement in Trophic Signaling Impairment and Inflammation?**

NF-κB is constitutively active in hippocampus, neocortex, and other central nervous system structures; activation of NF-κB leads to transcription of many genes, such as growth factors, the anti-apoptotic factor Bcl-2, and the antioxidant enzyme Mn-SOD, which in turn promote neurite growth during development, and provide neuroprotection against various insults in adult central nervous system.65 Interestingly, a recent study has revealed a candidate NF-κB site in promoter 3 of the BDNF gene and showed that N-methyl-D-aspartate receptor-induced BDNF expression was mediated by NF-κB.66 Thus, activation of NF-κB by trophic factors would enhance BDNF expression, thereby providing a prosurvival-positive feedback loop. Disruption of this loop resulting from deregulation of NF-κB could be a molecular basis for impairment in trophic signaling in NPC disease as reported in embryonic striatal neurons from Npc1−/− mice.67 Lack of BDNF responsiveness seems to be a common feature of NPC, as embryonic striatal neurons prepared from Npc2-deficient mice also failed to respond to BDNF.68 Although this could be due to changes in the lipid environment of trophic factor receptors because depletion of membrane cholesterol interferes with NGF-mediated responses in primary neuronal cultures69 and PC12 cells.70,71 our results point to deregulation of NF-κB as an additional contributor.

Unlike neurons, glia in vulnerable regions of Npc1−/− mice brains exhibited increased expression of p65. Besides GSK-3β, activity of NF-κB in glia is regulated by multiple factors. For instance, extracellular ATP released from injured neurons is known to activate NF-κB in microglia via P2Y receptors that are only expressed in certain types of immune cells.72 Cytokines, such as TNF-α and IL-1β, activate NF-κB in glia,73,74 via an IκB kinase-dependent pathway.69,75 Activation of this transcriptional factor in turn activates microglia. Thus, both neuronal and glial changes could contribute to neurodegeneration in NPC disease: decreases in NF-κB activity in neurons, possibly induced by inactivation of GSK-3β-dependent pathway, would reduce the expression of neuroprotective factors, whereas increases in glial NF-κB, induced by ATP, TNF-α, or IL-1β, would induce inflammatory responses and promote neurodegeneration (see Figure 10 for a summary of the potential mechanisms of neurodegeneration in NPC).

Together, our results showed that the PI3K-Akt-GSK-3β signaling system was markedly disturbed during early postnatal development in Npc1−/− mouse brains, especially in regions that exhibit early neurodegeneration. Changes in GSK-3β were closely associated with accumulation of cholesterol and deregulation of NF-κB. Furthermore, the appearance of NF-κB in microglia was temporally and spatially associated with microglia activation, a result which suggests that deregulation of the GSK-3β/NF-κB pathway contributes to neurodegeneration in Npc1−/− mice not only by directly affecting neuronal function but also indirectly through microglia activation. Results from the present study could therefore provide novel therapeutic targets to develop new approaches for treating this devastating disease.

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