Transient cerebral ischemia induces aberrant neuronal cell cycle reentry and Alzheimer’s disease-like tauopathy in female rats

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Abstract

Aberrant mitosis occurs in many tauopathy-related neurodegenerative diseases and is believed to precede the formation of neurofibrillary tangles (NFTs). In this study, we report for the first time that transient cerebral ischemia induces aberrant mitotic proteins and hyperphosphorylation of tau protein with NFT-like conformational epitopes in adult female rat cortex. Following transient cerebral ischemia in rats, initiation of apoptosis precedes and is potentially integrated with subsequent aberrant mitosis and tau hyperphosphorylation. Further, inhibition of mitosis-related cyclin-dependent kinases (Cdks) by roscovitine significantly reduced the hyperphosphorylation of tau. Administration of the female sex steroid and potent neuroprotective agent, 17 β-estradiol (17β-E2), reduced ischemia-reperfusion induced cerebral damage and the subsequent aberrant mitosis and tauopathies. These results provide a neuropathological basis for the higher prevalence of dementia in stroke patients and support the hypothesis that apoptosis and aberrant mitosis are integrated pathological events in neurons that may play a critical role in the development of Alzheimer’s disease (AD) and other tauopathy-related neuropathology.
Introduction

The prevalence of dementia in ischemic stroke patients is nine-times higher than controls at 3 months (Tatemichi, Desmond et al. 1992) and 4-12 times higher than in controls at 4 years after a lacunar infarct (Loeb, Gandolfo et al. 1992). Many of these dementias developed progressively, and cerebral damage is believed to be the direct cause of cognitive decline in only half of these cases (Tatemichi, Paik et al. 1994). AD is the most prevalent dementia (Pasquier and Leys 1997) and shares common neuropathology features with stroke. Amyloid angiopathy can lead to cerebral hemorrhage (Vinters 1987), Alz-50-immunoreactive granules are found around cerebral infarction after a stroke (Ikeda, Akiyama et al. 2000), amyloid precursor protein (APP) accumulates following transient focal ischemia (Shi, Yang et al. 2000), and ApoE4 is a genetic risk factor for both AD and stroke (Contois, Anamani et al. 1996).

Neurofibrillary Tangles (NFTs), whose major component is hyperphosphorylated tau (Goedert 1993), are observed in many neurodegenerative diseases (Spillantini, Crowther et al. 1998; Spillantini and Goedert 1998). Recent reports indicate that aberrant mitotic activation, as well as DNA replication, in terminally differentiated neurons appears to be involved in the pathogenesis of AD (Vincent, Zheng et al. 1998; Yang, Geldmacher et al. 2001). Many cyclin-dependent kinases, including Cdc2 and cyclin B1, as well as many other mitotic markers, are elevated in nearly all tauopathy-related neurodegenerative diseases (Husseman, Nochlin et al. 2000). These protein kinases regulate cell cycle progression into mitosis in developing and proliferating cells, if cell cycle reentrance is forced by ectopically driving an oncogene with a neuronal-specific promotor, the targeted neurons will die rather than divide (al-Ubaidi, Font et al. 1992;
Feddersen, Ehlenfeldt et al. 1992; Enokido, Araki et al. 1996; Nuydens, de Jong et al. 1998). In AD and other tauopathy diseases, induction of Cdc2 and cyclin B1, as well as aberrant DNA replication, is observed in neurons containing NFTs (Yang, Geldmacher et al. 2001). However, completion of successful nuclear divisions in differentiated neurons has never been reported. These inappropriately induced mitotic protein kinases can phosphorylate tau, modify other cytoskeletal proteins, and further induce apoptosis in terminally differentiated cells (Yen, Liu et al. 1995).

Recent studies reveal an intricate interaction between apoptosis, mitosis and formation of NFTs in terminally differentiated neurons. Activation of programmed cell death is observed in AD brains. Apoptosis, as indicated by TdT-mediated dUTP Nick-End Labeling (TUNEL) (Katsuse, Iseki et al. 2001) positive neurons and activation of neuronal caspase-3 (Stadelmann, Deckwerth et al. 1999), is widespread in AD brains. Survivin, a member of the Inhibitor of Apoptosis Protein (IAPs) family, is induced under stress, as well as in the G2/M phase of the cell cycle and binds to spindle microtubules (Uren, Beilharz et al. 1999). Up-regulation of cyclin D1 and other cell cycle progression markers, is observed during apoptosis in post-mitotic neuronal cultures (Shirvan, Ziv et al. 1997; Sakai, Suzuki et al. 1999; Stadelmann, Deckwerth et al. 1999), and cell cycle inhibitors are protective against apoptotic stimuli in neurons (Farinelli and Greene 1996). All above mentioned evidence indicates that mitotic events may contribute to the formation of AD pathology (Vincent, Rosado et al. 1996; Husseman, Nochlin et al. 2000).

In the present study, we observed an induction of tau hyperphosphorylation, and mitosis-related proteins, and assessed the temporal pattern and colocalization of markers of apoptosis, mitotic protein activation, and tau hyperphosphorylation in a rodent model for
transient cerebral ischemia. In this non-transgenic animal model, apoptotic signals precede both mitotic protein activation and formation of NFT-like conformational changes. Further, brain delivery of a potent cyclin-dependent kinase inhibitor, roscovitine, or systemic administration of the potent neuroprotective reagent, 17β-estradiol, significantly reduced the epitope of tau-hyperphosphorylation. These results suggest that therapies directed at apoptosis or aberrant cell cycle may be a valuable target in preventing the sequel of neuropathological events, observed in AD and other neurodegenerative diseases.
Materials Methods

Animals

Four-six week old female Sprague Dawley (SD) rats, were purchased from Charles Rivers (Wilmington, MA), and maintained in our animal facility in a temperature-controlled room (22-25°C) with 12-hour dark-light cycles. All rats have free access to laboratory chow and tap water. All animal procedures were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Animal surgeries

All experimental animals received ovariectomies (Ovx) at least 14 days before any further surgical procedure. For middle cerebral artery (MCA) occlusion and reperfusion, an intraluminal filament model with slight modification was used (Bederson, Pitts et al. 1986; Simpkins, Rajakumar et al. 1997). Briefly, the animal was anesthetized with ketamine (60mg/kg) and xylazine (10mg/kg), then the internal carotid artery (ICA) was exposed, and a 3-0 monofilament nylon suture was introduced into the ICA lumen through a puncture and gently advanced to the distal internal carotid artery (ICA) until proper resistance was felt. After 1 hr, the suture was withdrawn from the ICA and the distal ICA was immediately cauterized. At the desired time after the onset of reperfusion (2, 4, or 24 hr) animals were sacrificed and sampled for immunoblotting, immunohistochemistry, or TTC stain, as described below.

TTC staining:

Brains were removed, immediately after sacrificing the animal, and dissected coronally into 2mm sections using a metallic brain matrix (ASI Instruments, Inc., Warren,
MI), and stained by incubation in a 2% 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) in a 0.9% saline solution at 37 °C for 30 min. Photographs were taken with Kodak digital camera. Each treatment group included at least 3-4 animals.

**Roscovitine administration**

Rats were anesthetized with halothane and placed in a stereotaxic device. A surgical stainless steel microsyringe was temporarily inserted with following coordinates: antero-posterior -5; lateral 1.8 from bregma; and -7.9 ventral from the dura, so that the tip of the syringe needle would be inserted into the third ventricle (Paxinos, Watson et al. 1985). The insertion position was confirmed with post-mortem dissection. Animals were administered 50µl of 1mM roscovitine (Sigma-Aldrich, St Louis, WA) in 30% 2-hydroxypropyl-β-cyclodextrin (HPβCD) (equivalent to approximately 20µM initial final concentration of roscovitine in the brain) into the right lateral ventricle over a period of 5 min using a microinjection syringe. The needles were held in position for at least another 30 min to avoid leaking. Sham treated animals received an equal volume of 30% HPβCD. The animals were then allowed to rest at least 2 hr before further MCA surgery.

**Estrogen treatments:**

17β-Estradiol (Steraloids, Inc. Newport, RI) was dissolved in absolute ethanol and then diluted in corn oil (Penta Manufacturing airfield, NJ) at a concentration of 100µg/ml. Ethanol was evaporated by incubation at 60°C over night. A single subcutaneous injection of 100 µg/kg or vehicle was given to the rats 2 hr before MCA occlusion surgery.

**Immunoblotting**
For immunoblotting analysis with various antibodies, brain tissue was dissected into frontoparietal cortex (peri-infarct area) and subcortical basal ganglia fractions (complete infarct area), then homogenized in RIPA buffer (1x PBS, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, 10µg/ml Aprotinin, 100 µg/ml Phenylmethyl Sulphonyl Fluoride (PMSF)). Samples were centrifuged at 12,000g for 30 min, and supernatants were collected for analysis. Samples were probed with specific antibodies at proper dilutions with procedures according to the manufacturer’s instruction. The blots were developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL). In all immunoblots, each lane represented an independent experimental animal with described treatments.

**Protein kinase assay**

Cdc2-like kinase activity was examined with SignalTACT cdc2 Protein Kinase Assay System (Promega, Madison, WI), according to manufacturer’s instruction. Briefly 10µg brain extracts was incubated with cdc2-specific substrates in 20 µl of the protein kinase assay buffer together with 1µCi γ-32P ATP (ICN Biomedicals, Inc. Irvine CA). After 15 min of reaction at 30°C, the reaction was terminated by adding an equal volume of stop buffer, heated at 95°C. The result was obtained by counting in a liquid scintillation counter.

**Immunohistochemical and immunofluorescent staining**

For histological studies, we used paraffin-embedded brain sections (5-7µm thick) that received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation, then switched to 70% alcohol, for at least another 24-hr before paraffin-embedding. All single antigen detection was performed using an avidin-biotin
immune-peroxidase method with DAB substrate (dark brown) and Histostain plus kits (Zymed, Inc, South San Francisco, CA) according to the manufacturer’s instruction. Multi-antigen immuno-detection or colocalizations were achieved with either immunofluorescence with a variety of highly species-specific or isotype-specific non-overlapping Alexa-fluorophore conjugated secondary antibodies (Molecular Probes, Inc. Eugene, OR). Detection of fluorophore was achieved by fluorescent microscope or confocal microscopy. Some immunofluorescent stainings were counter-stained with DAPI, a nuclear marker.

**TUNEL assay and colocalizations with specific antigens**

DNA fragmentation was detected by a TUNEL method using a Dead END fluorescent kit (Promega, Madison, WI) according to the manufacturer’s instruction. Colocalization with TUNEL staining was achieved by confocal microscopy described above, with Alexa-Florophore conjugated secondary antibodies that do not overlap with FITC-labeled TUNEL stain.

Antibodies used in the immunohistological studies include GFAP (1:50) (Sigma, St. Louis, MO); NeuN 1:50 (Chemikon, Temecula, CA), Cyclin B1 (1:50), Cdc2 (1:50) (Santa Cruz, Santa Cruz, CA), and MPM-2 (DAKO, Carpinteria, CA). The following antibodies were used to stain hyperphosphorylation and NFT-like conformational tau epitopes: MC1 (unique PHF conformation) and TG3 (Phospho-tau 231/235 and AD conformation); Phosphorylated tau epitopes: CP13 (Phospho-tau 202/205), CP3 (Phospho-tau 214), PHF-1 (Phospho-tau 396/404), and CP9 (Phospho-tau 231), all at a 1:10 dilution. All of the above-mentioned phospho-specific antibodies for tau were the kind gift and characterized by Dr. Peter Davies (Albert Einstein College of Medicine).
Sarcosyl tau extraction

Sarcosyl extraction was performed as described previously with slight modification (Gotz, Chen et al. 2001). Briefly, brain tissues from ischemic and contralateral rat cortex were homogenized in 10 volumes of buffer consisting of 10 mM Tris-HCl (pH 7.4), 0.8M NaCl, 1 mM EGTA, and 10% sucrose. The homogenate was centrifuged for 20 min at 20,000g. The supernatant was brought to 1% N-lauroylsarcosinate (Sarcosyl) and incubated for 1 hr at room temperature with moderate shaking. Samples were then centrifuged at 100,000g for 1hr at 4°C and the sarcosyl-insoluble pellets were resuspended in 50 mM Tris-HCl (pH 7.4). Extracts are either temporally stored at 4°C, or at –80°C for long-term storage until use. This material was used for both immunoblotting and electron microscopy analysis.

Electron microscopy

Resuspended sarcosyl-insoluble materials obtained from ischemic cortical or contralateral extracts were placed directly on carbon-coated, 300-mesh grids, stained with 2% phosphotungstic acid, and analyzed by electron microscopy (Gotz, Chen et al. 2001). As negative controls, filament preparations from contralateral extracts were examined at the same time. Microphotographs were recorded at an operating voltage of 80–100 kV and at nominal magnifications of 160,000-400,000 on a Zeiss electron microscope.

Semi-quantitative cell counting

To estimate the density of cortical cells labeled with specific antibodies, methods were adapted from previous reports (Jiang, Gu et al. 2001). Briefly, ischemic brain sections from animals that received roscovitine (n=6) or sham treatment (n=3) were
randomly selected from paraffin-embedded sections. Each brain section was labeled for a specific antigen or double-labeled, together with DAPI based counter-stain.

Cell counting was performed in the ischemic boundary cortex under a CAST-Grid system (Nikon, Inc). The region was delineated in the ischemic brain cortex near the edge of the surface, where the highest density of TUNEL-positive cells was observed. The striatum was not included in this study, as few positive cells were observed in this region. Within these boundaries, optical dissectors were systematically randomly sampled and the number of positive cells, together with DAPI labeled nuclear counter-stain in each optical section was counted. The positive labeled cell numbers in the investigated region are normalized with the total number of cells, indicated by DAPI stain. The data are expressed as the percentage of DAPI nuclear counter-stained cells.
Results

Cerebral ischemia-reperfusion rapidly induces apoptosis and subsequent mitotic events

MCA occlusion is a widely used ischemic stroke model (Bederson, Pitts et al. 1986). This in vivo model for neuronal death has the distinct advantage that apoptosis is rapidly induced and synchronized in a large number of neurons (Li, Chopp et al. 1997). Initially, we examined the effects of transient cerebral ischemia on apoptosis and the expression of mitotic markers. As early as 2hr after the initiation of reperfusion following a 1hr ischemia, positive TUNEL-staining appeared in the core ischemic region within the basal ganglion, but no mitotic markers were observed in the neurons in the ischemic region (Fig. 1A). At 4hr of reperfusion, the brain areas and number of cells displaying TUNEL-positive staining had increased. At the same time, expression of mitotic markers, cyclin B1 and MPM-2, appeared in the ischemic core (Fig. 1B, 1D), and these markers extended into parts of the cortex around the infarct area (data not shown). Cyclin B1 is the activator of Cdc2 protein kinase, and accumulates during mitosis (Fang and Newport 1991), and the MPM-2 monoclonal antibody recognizes a set of M-phase specific phosphoproteins, most of which are the substrates of Cdc2/cyclin B1 (Davis, Tsao et al. 1983). No phospho-epitopes of tau were detected at 4hr of reperfusion after MCA occlusion in either the ischemic region or the contralateral brain side (data not shown).

Aberrant mitotic proteins associate with delayed cell death

The ischemia-reperfusion injury showed that both density and intensity of TUNEL-positive staining progressed with time and was evident throughout the infarcted brain region at 24hr of reperfusion (Fig 1). The TUNEL signals were not present in the
contralateral hemisphere (Fig. 2A), but were abundant in both the core region (Fig 1C) and the cortical areas (Fig. 2B, 2C). In parallel, we also observed a marked induction of both the mitotic epitopes (MPM-2) and the mitotic proteins cyclin B1 in the ischemic area. In the hemisphere contralateral to the occlusion, these mitotic epitopes were absent (Fig. 2A). However, on the ischemic side, these mitotic epitopes were widespread and intensely stained (Fig. 2). Most of the mitotic epitopes had cytosolic distributions with a few instances of nuclear localization. This observation is consistent with previous research in AD patients (Husseman, Nochlin et al. 2000).

Further, in the peri-ventricular areas, where the lateral ventricle separates the ischemic from the fully perfused brain areas, TUNEL-positive cells formed a sharp line of demarcation across, and almost perfectly colocalize with the mitotic marker MPM-2 (Fig. 2D). Cyclin B1 (data not shown) or MPM-2 (Fig. 2D) colocalized with TUNEL in about 30-90% of cells, depending on the brain location and the severity of ischemic damage. Semi-quantitative co-localization assay indicates that most MPM-2 immunoreactivity associates with TUNEL. Most of the cyclin B1 and MPM-2 positive cells in the frontoparietal cortex had a neuronal morphology (Fig. 2C).

To further evaluate the co-localization of TUNEL and mitotic markers and to identify cell types containing these markers, we performed semi-quantitative counting (Fig 2E-F). More than 30% of cells in the cortex showed co-localization of MPM-2 and TUNEL. About 29% of the MPM-2 positive cells in the cortex stained positive of a neuronal marker, NeuN. Most unlabeled cells in cortex had neuronal morphology, while most unlabeled cells in subcortex were astrocytes and glia. Also it appeared that there was a reduction of GFAP immunoreactivity in the ischemic reason.
Transient cerebral ischemia-reperfusion induces tau hyperphosphorylation and NFT-like conformational epitopes.

We further assessed ischemic brain sections of animals that had undergone transient ischemia with a variety of antibodies that detect tauopathies in neurons affected with human AD. These monoclonal antibodies were directed against distinct phosphorylation sites and/or conformational states of tau protein present in AD brains (Goedert, Jakes et al. 1994). The transient cerebral ischemia induced not only hyperphosphorylation, indicated by PHF-1 (Fig. 3C) and CP13 (Fig. 3D), but also the unique tauopathy conformational change, indicated by MC1 (Fig. 3A) and TG3 (Fig. 3B). The NFTs were expressed throughout the ischemic area, with the most intense immunoreactivity observed in the frontoparietal cortex (Fig. 3 A-D), in a pattern similar to cyclin B1/MPM-2 staining. Although there was evidence of cell shrinkage and nuclear condensation, these NFT-positive cells still had an apparent neuronal morphology, and most of the immunoreactivity was localized in the cytoplasm (Fig. 3 A-D). Immunoblot analysis of cortical extracts revealed a marked induction of NFT-specific epitopes in cortical areas affected by MCA occlusion-induced ischemia compared with the control areas of the contralateral cortex (Fig.3 E). Further analysis of sarcosyl-extracted tau extracts by electronic microscopic analysis showed evidence of aberrant paired-helical filaments similar to that found in AD patient brain extracts. However, defined tangles and senile plaques, as identified with modified Gallyas silver and thioflavin-S staining were not detected.
Previous studies have demonstrated that tau can be phosphorylated by a number of protein kinases (Pelech 1995). We examined some candidate protein kinases involved in tau hyperphosphorylation. We observed an increase in Cdc2 protein kinase levels (Fig. 4A), as well as an increase in its kinase activity (Fig 4B). The corresponding activator of Cdc2, Cyclin B1 also showed a marked induction (Fig. 4C). Cdk5, GSK3-β, and pERK levels in cortical extracts from animals at 24 hr after MCA occlusion were not significant changed.

**Co-localization of markers of apoptosis, mitosis and NFTs**

To further understand the spatial relationship of the apoptotic, mitotic, and NFTs, we examined the colocalization of these markers in the frontoparietal cortex in the penumbra areas. These markers highly colocalized with each other in cortical pyramidal neurons in the peri-infarct areas. Double staining of NFTs and TUNEL revealed that that TUNEL-positive signals colocalized with 30-70% NFT-positive cells (Fig. 5).

**Roscovitine, a potent mitotic Cdk inhibitor, reduces tau hyperphosphorylation in the ischemic cortex**

In order to further confirm the relationship of this tau hyperphosphorylation and aberrant cell cycle, we administered roscovitine, a potent cyclin-dependent kinase inhibitor ($K_i=0.2-0.7 \mu M$), into the lateral ventricle. The effect of this treatment was evaluated by examining the mitotic epitopes with MPM2 labeling (Fig. 6A). The treatment reduced the MPM2 epitope from an average of 38.4 ± 4.4% in sham-treated animals to an average of 9.9 ± 2.3% in roscovitine-treated animals in frontoparietal cortex (Fig 6B). We also noticed a reduction in average staining intensity, which was difficult to evaluate quantitatively.
In order to evaluate roscovitine’s effects on tau hyperphosphorylation, we performed semi-quantitative cell counting in the frontoparietal cortex. Roscovitine treatment reduced PHF-1-positive cell number in the ischemic cortical regions from an average of 23.24 ± 3.0% to in sham-treated to 5.9 ± 1.4% in roscovitine-treated animals (Fig. 7A, 7B).

17-β estradiol reduces ischemic damage, aberrant mitosis and NFT epitopes

To determine the correlation between ischemic damage and NFT formation, we assessed the responses to MCA occlusion in the presence of a known anti-apoptotic agent, 17 β-estradiol. Estrogen treatments have been demonstrated to be effective in protecting greater than 50% of ischemic brain tissues from MCA occlusion-induced apoptosis (Simpkins, Rajakumar et al. 1997; Behl 2002; Wise 2002). 2,3,5-Triphenyltetrazolium chloride (TTC) staining was used to reveal the extent of the infarcted area after ischemia-reperfusion (Fig. 8A). 17 β-estradiol treatments in this stroke model reduced the ischemic volume by about 50% (Fig. 8A) and caused a similar reduction in TUNEL staining (Fig. 8A) in the ischemic areas. We found that this treatment significantly reduced the expression of the mitotic protein, cyclin B1 (Fig. 8B) and of NFT epitopes in cortical brain extracts from the ischemic cerebral cortex (Fig. 8C).
Discussion

In the current study, we observed the early appearance of apoptosis and the subsequent induction of aberrant mitotic proteins and tau hyperphosphorylation by a transient focal cerebral ischemia, and further characterized the tau hyperphosphorylation with monoclonal antibodies that detect a variety of phosphorylation sites and NFT-like conformational epitopes. We also examined the sarcosyl-insoluble tau extracts with high magnification electromagnetic microscopy and identified tangle-like abnormal filaments. Further, we demonstrated that roscovitine (a potent Cdk inhibitor) and 17β-estradiol (a potent neuroprotective agent), significantly reduced the tau hyperphosphorylation induced by this transient ischemia. These results may help to establish an in vivo model for tau hyperphosphorylation and aberrant neuronal cell cycle re-entry without genetic alteration. Further, these results indicate that cell damage induced cell cycle re-entry is involved in the formation of tau hyperphosphorylation and NFT-like epitopes in damaged neurons.

AD is characterized by pathological features that include senile plaques and NFTs, the latter of which is composed of intracellular aggregates of hyperphosphorylated tau protein. NFTs are a common feature of many neurodegenerative diseases, including Down syndrome, frontotemporal dementia linked to chromosome 17, and Parkinson-amyotrophic lateral sclerosis complex of Guam (Sanchez, Alvarez-Tallada et al. 2001). As such, NFTs could initiate and/or contribute to the progression of neurodegenerative diseases. How these markers are pathologically related and their roles in the initiation and progression of neurodegeneration remains controversial (Vincent, Rosado et al. 1996). Transgenic mice that host the familial disease mutations in amyloid
precursor protein (APP) and/or presenilins developed senile plaques, but lack NFTs and exhibit little neuronal loss (Goate, Chartier-Harlin et al. 1991; Sherrington, Rogaev et al. 1995; Holcomb, Gordon et al. 1998). This suggests that the development of senile plaques and NFTs may have independent causative mechanisms or require involvement of further factors during the pathogenesis of AD. It has been well documented that tauopathy-related neurodegenerative diseases involve the induction of aberrant mitotic proteins in affected neurons (Vincent, Jicha et al. 1997; Husseman, Nochlin et al. 2000; Raina, Zhu et al. 2000). The mitotic kinase and its activators, as well as other mitotic phosphoepitopes, accumulate in degenerating neurons that contain the characteristic structural lesions of AD (Vincent, Rosado et al. 1996; Husseman, Nochlin et al. 2000). The common appearance of mitotic proteins and NFTs in neurodegenerative diseases and their coincident appearance in cells suggest that mitotic proteins are involved in the hyperphosphorylation of tau and could be responsible for the initialization and progression of NFTs in affected neurons.

In the present study, we identified aberrant tau hyperphosphorylation and NFT-like conformational epitopes from ischemic rat brains. There is still insufficient evidence that the transient ischemia-induced NFTs are identical to those in AD brains. Rat tau proteins are not identical to the human tau (Kanai, Takemura et al. 1989), and our electronic microscopic analysis of the sarcosyl-extracted tau revealed only a small fraction with filamentous conformation. Furthermore, we failed to identify Gallyas silver and thioflavin-S positive plaques within 24hr of MCA occlusion. However, many aspects of the transient ischemia-induced tau hyperphosphorylation and PHF-like conformational changes are similar to that of tauopathy in AD, including the specific phosphoepitopes
and multiplicity in the migration of immunoblotting detection, induced by hyperphosphorylation of tau. The MCA occlusion model may be a useful non-transgenic animal model, with which to understand the biological features of tauopathies.

Our results suggest that the early cell damage induced by a variety of apoptotic/necrotic factors, including amyloid deposition, neurotrophin reduction, and sporadic hypo-perfusion, can result in aberrant cell cycle-related protein kinases, and cause further hyperphosphorylation of tau protein that eventually develop into neurofibrillary tangles. In such a case, formation of NFTs may represent a downstream marker (tombstones) of earlier pathologies in neurons, not an initiating factor during the neurodegenerative progression. Our findings are consistent with the current hypothesis in the pathogenesis of AD, which suggests that amyloid deposition and/or a variety of apoptotic factors induces neurotoxicity and formation of NFTs are the result of cell damage (Hardy and Selkoe 2002). This hypothesis is supported by many evidences, including the recent triple transgenic animal study that indicate that amyloid deposition and synaptic dysfunction precedes the formation of plaques that contains NFTs (Oddo, Caccamo et al. 2003).

Previous research indicates that cell cycle signaling mechanisms are involved in neuronal apoptosis (Shirvan, Ziv et al. 1997; Nuydens, de Jong et al. 1998; Sakai, Suzuki et al. 1999). This apoptosis-related induction of cell-cycle proteins in post-mitotic neurons has been studied in both in vitro and in vivo models (Nuydens, de Jong et al. 1998; Lin, Chong et al. 2001). In post-mitotic neurons of the adult brain, active mitotic kinase complexes can induce an abortive or a catastrophic M-phase and eventually lead to cell death. Ischemic neuronal death may involve cell cycle signaling. Cyclin D1, cyclin
G1, PCNA and p21 waf1/cip1 are reported to be activated in vulnerable neurons following transient global ischemia (Tomasevic, Kamme et al. 1998; van Lookeren Campagne and Gill 1998). Mitotic protein induction and DNA replication have been well established in AD patient neurons in many studies (Yang, Geldmacher et al. 2001; McPhie, Coopersmith et al. 2003).

The activation of mitosis-related protein complex in the neurons may be fundamental to the progression to neuronal death and to the expression of NFT pathology (Vincent, Rosado et al. 1996; Husseman, Nochlin et al. 2000). Tau protein is a high affinity substrate of Cdc2 (Paudel 1997). In differentiated neurons, Cdc2-induced phosphorylation of tau inhibits its binding to tubulin and reduces the stability of the cytoskeleton (Lu, Wulf et al. 1999). Nucleolin, another Cdc2 substrate, is crucial for ribosomal biogenesis and cytoplasmic-nuclear shuttling of RNA, and may also contribute to AD pathology (Dranovsky, Vincent et al. 2001). During mitosis in dividing cells, phosphorylation of these mitotic substrates is necessary for dynamic microtubule reorganization. However in differentiated neurons this mitotic protein activation may contribute to cell death signaling and initiate the formation of tau hyperphosphorylation. Indeed, DNA degradation and caspase activation are increased in both AD and other degenerative disorders and these events are suggested to play a key role in the pathogenesis of these diseases (Marx 2001).

Estrogens are potent anti-apoptotic agents against a variety of insults (Green and Simpkins 2000). Our ability to prevent apoptosis and reduce mitotic proteins and NFTs with an 17 β-estradiol pretreatment suggests that prevention of the generation of signaling components upstream of apoptosis can also prevent or diminish subsequent
mitotic and tau hyperphosphorylation responses to an ischemic event. Since initiation of apoptosis is an early event that eventually leads to tau pathology, anti-apoptosis therapeutic strategies are reasonable approaches to the prevention of NFT formation. By inference, therapies aimed at prevention of NFTs, that do not treat apoptosis and cell cycle protein activation may not be effective.

Our studies show that roscovitine treatment suppresses ischemia-induced tau hyperphosphorylation, suggesting the involvement of mitotic complex in this event. It is difficult to clearly identify a causative relationship between neuronal apoptosis, cell-cycle re-entry and hyperphosphorylation of tau, in part because neuronal apoptosis is not a single event, but a process that includes a number of signaling cascades and responds to a variety of stimuli. However, several of our observations indicate that these 3 processes are intimately connected and may be initiated by the ischemic event. First, neuronal apoptosis precedes aberrant cell-cycle protein induction (Fig. 1). Second, the anti-apoptotic agent, 17β-E2, reduces apoptosis and aberrant cell-cycle induction (Fig. 8). Finally, roscovitine, a cyclin-dependent kinase inhibitor, appear to reduce apoptosis (Fig. 6 & 7). These data support the hypothesis that aberrant cell cycles are involved is the neuronal apoptotic process.

In summary, the present data may help to establish an animal model to study pathological tau hyperphosphorylation in a relatively short period of time without genetic manipulation. Further, these findings support the hypothesis that apoptotic factors may initiate and be involved in the progression of neuropathology in non-familial AD patients and may provide the mechanisms of the long-observed correlation between the ischemic events and the increased prevalence of AD. As such, it is tempting to hypothesize that
prevention of ischemic episodes, inhibition of aberrant cell cycle, or neuroprotective agents may serve as an additional therapeutic approach to prevent the initiation and progression of AD.

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Reference


Figure Legends

Figure 1: Early induction of apoptosis and mitotic proteins following transient ischemia

Apoptotic marker (TUNEL staining) and mitotic proteins (cyclin B1 and MPM-2) are induced at different times after ischemia-reperfusion. (A) As early as 2hr post-reperfusion, TUNEL was detected in the striatum, the core region of the infarct, but cyclin B1 and MPM2 were not. (B) At 4hr of reperfusion TUNEL staining was more intense, and extended to the frontoparietal cortex; cyclin B1 and MPM2 immunoreactivity was observable in the core region. (C) At 24 hr, there was extensive TUNEL, cyclin B1 and MPM-2 staining throughout the core and frontoparietal cortex. (D) TUNEL, cyclin B1, and MPM2 positive neurons at 4hr reperfusion in the frontoparietal cortex. All photographs were taken from adjacent brain slices in representative experiments. Scale bar = 100µm.

Figure 2: Extensive expression of apoptotic and mitotic markers at 24h of reperfusion

At 24 hr of reperfusion, apoptosis and mitotic proteins were evident throughout the infarct area. (A) TUNEL as well as cyclin B1 and MPM2 staining was negative in the contralateral cortex. (B, C) TUNEL and cyclin B1/MPM2 staining was observed throughout the infarct area, including the core (B) and the peri-infarct cortex (C). (D) Immunofluorescence of DAPI, a nuclear counterstain (blue), TUNEL (green), MPM2 (red) and the merged image near the ipsilateral lateral ventricle, which separated the
ischemic from the fully perfused area. Scale bar = 100 µm. (E): Semi-quantitative analysis of MPM-2 and TUNEL co-localizations in different brain regions (n=4). (F): Semi-quantitative analysis on the identity of MPM-2 positive cells in different brain regions (n=4). In E-F, Error bar indicates SEM. Y-axis indicates the percentage of positively labeled cells of total cells labeled by DAPI.

**Figure 3: Induction of tau hyperphosphorylation and NFT-like immunoreactivity in ischemic cortex**

(A, B) Representative immunohistochemical staining from frontoparietal cortex, in the contralateral (left), ipsilateral (middle), and higher magnification in the ipsilateral (right) of conformation dependent monoclonal antibodies MC1 (A) and TG3 (B). (C, D) Representative immunohistochemical staining of PHF-1 (C) and CP-13 (D). EXT: external; Cor: cortex; (E) Representative immunoblotting analysis of NFT-specific epitopes in the contralateral frontoparietal cortical extracts (lanes 1-4) and ischemic frontoparietal cortical extracts (lanes 5-8). Each lane in (E) represents an individual experimental animal. (F) Electron microscopy analysis of the sarcosyl-extracted tau shows filamentous conformation, which resemble the paired helical filament from AD patient brains, in the ischemic brain extract, but not in the contralateral extract. The width of the tangles was approximately 20nm. Scale bar = 100 µm in A-D.

**Figure 4: Relevant protein kinases level and activity in the ischemic cortex**
(A) Immunoblotting of Cdc2/P34 protein kinase. Lanes 1-3 were from the contralateral cortex and lanes 4-6 were from the ischemic cortex. (B) Neuronal cdc-2 like activity in the cortex, * indicates P<0.05 by unpaired student t-test. (C) Immunoblotting of Cdk5, GSK3-β, p-ERK, cyclin B1, and β-actin (loading control). Lanes 1-4 were from the contralateral cortex, and lanes 5-8 were from the ischemic cortex. Each lane represents a sample from an independent experimental animal.

Figure 5: Colocalization of markers of apoptosis, mitosis and NFTs
Colocalizations of multiple antigens detected by confocal microscopy with non-overlapping immunofluorescence DAPI, a nuclear counterstain (blue), TUNEL (green), and PHF-1 (red). Scale bar = 100µm.

Figure 6: Effects of roscovitine injection on MPM2 immunostaining
(A) Representative immunofluorescent staining of MPM2 in the ischemic cortex in sham (upper panel) and roscovitine treated (lower panel) animals. Blue indicates nuclear counterstaining with DAPI and red indicates mitotic epitope MPM2 staining. Scale bar = 100µm. (B) Semi-quantitative analysis of the effects of roscovitine injection on MPM2 immunostaining cell density. * indicates P<0.05 by unpaired student t-test. Y-axis indicates the percentage of positively labeled cells of total cells labeled by DAPI.

Figure 7: Effects of roscovitine injection on PHF-1 immunostaining.
(A) Representative immunofluorescent staining of PHF-1 and TUNEL in the
ischemic cortex of sham (upper panel) and roscovitine treated (lower panel) animals. Blue indicates nuclear counterstaining with DAPI, red indicates tau hyperphosphorylation detected by PHF-1 and green indicates apoptotic marker TUNEL. Scale bar = 100 µm.

(B) Semi-quantitative analysis on the effects of roscovitine injection on PHF-1 immunostaining cell density in frontoparietal cortex. * indicates P<0.05 by unpaired student t-test. Positive Y-axis indicates the percentage of positively labeled cells of total cells labeled by DAPI.

**Figure 8: Effects of 17 β-E2 treatment on the NFT phospho-epitope and cyclin B1**

(A): Representative TTC (left) and TUNEL (right) stained brain slices from ovariectomized (upper) or 17 β-E2 treated ovariectomized rats (lower) at 24 hr following MCA occlusion. The circle indicates the cortical area from which the TUNEL stained slices were obtained. Scale bar = 100 µm. (B) Immunoblot analysis of cyclin B1 of the brain extracts from ovx rat contralateral cortex (lanes 1-3), 17 β-E2 treated ipsilateral rat cortex (lanes 4-6) and ovariectomized ipsilateral rat cortex (lanes 7-9). β-actin was probed as a loading control (lower panel). (C) Immunoblot analysis of NFT epitopes in ovariectomized rat ipsilateral cortex (lanes 1-3) and 17 β-E2 treated ipsilateral cortex (lanes 4-6). Each lane represents a sample from individual experimental animal.
Figure 1

<table>
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<th>TUNEL</th>
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</tr>
<tr>
<td>B</td>
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</tr>
<tr>
<td>C</td>
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Figure 2

A

B

C

D

E

F

35
Figure 4

A

B

C
Figure 5
Figure 6

A

Sham

Roscovitine

B

Cell Numbers

sham  roscovitine

*
Figure 7

A

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</tbody>
</table>

B

![bar graph showing comparison between sham and roscovitine]
Figure 8

A

TTC

TUNEL

Vehicle

Estrogen

B

Cyclin B1

β-Actin

Ovx Contra | Ipsi | Ovx Ipsi

1 2 3 4 5 6

C

CP13

PHF-1

TG3

Tau

Ovx Ipsilateral | E2 Ipsilateral
Transient cerebral ischemia induces aberrant neuronal cell cycle reentry and Alzheimer's disease-like tauopathy in female rats
Yi Wen, Shaohua Yang, Ran Liu, Anne Marie Brun-Zinkernagel, Peter Koulen and James W. Simpkins

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