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RAPID REPORT

GENETIC DELETION OF *NOGO*/*RTN4* AMELIORATES BEHAVIORAL AND NEUROPATHOLOGICAL OUTCOMES IN AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE

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Abstract—The cognitive impairment in Alzheimer's disease (AD) is associated with synaptic loss, neuritic sprouting and altered neuroplasticity. Compensatory neuritic sprouting might be beneficial, while aberrant sprouting could contribute to the neurodegenerative process. Nogo (or Rtn4) is a major myelin-derived inhibitor of axonal sprouting in adult CNS. Recent evidence has implicated both the Reticulon family of proteins and a receptor for Nogo, NgR, in reducing amyloid-β production, a key step in AD pathogenesis. To test the hypothesis that Nogo, as an inhibitor of axonal sprouting, modulates disease progression in a mouse model of AD, we introduced an *APP* transgene (a human *APP* minigene carrying the Swedish and Indiana mutations under the *platelet-derived growth factor subunit B (PDGFB)* promoter) into a *Nogo* null background and characterized the behavioral and neuropathological consequences. We found that deleting *Nogo* ameliorates learning and memory deficits of *APP* transgenic mice in the Morris water maze at an early/intermediate stage of the disease. Furthermore, deleting *Nogo* restored the expression levels of markers for synapto–dendritic complexity and axonal sprouting including synaptophysin, MAP2, GAP43 and neurofilament that are otherwise reduced in *APP* transgenic mice. Other aspects of disease progression including neuronal loss, astrogliosis, microgliosis and, importantly, Aβ levels and amyloid deposits were not significantly altered by *Nogo* deletion. These data support the hypothesis that Nogo-mediated inhibition of neuritic sprouting contributes to the disease progression in an *APP* transgenic model of AD in a way that is mechanistically distinct from what has been proposed for Rtn3 or NgR.

Key words: Alzheimer's disease, central nervous system, myelin-derived axon growth inhibitors, neuritic sprouting, amyloid deposition, reticulon.

Alzheimer's disease (AD) is characterized by widespread neurodegeneration throughout the association cortex and limbic system, with formation of the Amyloid-β (Aβ) plaques and neurofibrillary tangles being the neuropathological hallmarks of the disease (Selkoe, 2005). One interesting aspect of disease progression is the cycles of synapse loss and aberrant sprouting (Troy et al., 1997; Hashimoto and Masliah, 2003). During the early stages of AD, the synapse loss in the limbic system appears to be partially compensated by sprouting of the cholinergic system in the neocortex and inner molecular layer of the hippocampus. However, as the disease progresses and synaptic damage is accentuated, aberrant sprouting of axons and dendrites may eventually contribute to the neurodegenerative process (Geddes and Cotman, 1991; Hashimoto and Masliah, 2003). Therefore, neuritic sprouting may be either beneficial or detrimental in disease progression. However, how neuritic sprouting modulates disease progression in AD remains poorly understood.

Axonal sprouting in the adult mammalian CNS has been extensively studied in the context of CNS injuries (Gonzenbach and Schwab, 2008). There is evidence that CNS myelin contains inhibitory molecules that restrict axonal sprouting following injury, thus limiting functional recovery. Nogo is a major myelin-derived inhibitor of axonal growth. The *Nogo* gene encodes three isoforms, Nogo-A, B and C, via alternative splicing and alternative promoter usage (Chen et al., 2000; GrandPre et al., 2000). The three isoforms share a C-terminal segment that contains two transmembrane domains separated by a 66 amino acid loop region (Nogo-66) that possesses potent inhibitory activity on neurite growth *in vitro* (GrandPre et al., 2000). The same C-terminal segment shares homology with a family of proteins called Reticulons (or Rtns), with Nogo (or Rtn4) being the 4th and last member of the family. Although Nogo alone cannot explain the very limited regeneration of injured axons in the CNS (Lee and Zheng, 2008; Lee et al., 2009), there is consensus for a role of Nogo in inhibiting compensatory sprouting of uninjured axons and in local axonal plasticity (Buffo et al., 2000; Raineteau et al., 2001; McGee et al., 2005; Cafferty and Strittmatter, 2006; Lee et al., in press).

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; *APP tg*, *APP* transgenic; BACE1, beta-site APP cleaving enzyme 1; DG, dentate gyrus; GAP43, growth associated protein 43; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NgR, Nogo receptor (also known as NgR1); *Non tg*, non transgenic; PDGFB, platelet-derived growth factor subunit B; Rtn, reticulon; Rtn3, reticulon 3; Rtn4, reticulon 4 (also known as Nogo).

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The presence of neuritic sprouting in AD pathology raised the possibility that inhibitors of CNS axonal sprouting such as Nogo may influence the disease outcome. Interestingly, recent studies indicate that both Reticulons (especially Rtn3) and NgR (also known as NgR1), a receptor for the Nogo-66 inhibitory domain (Fournier et al., 2001), can reduce the production of Aβ by interacting with beta-site APP cleaving enzyme 1 (BACE1) and amyloid precursor protein (APP) respectively (He et al., 2004; Park et al., 2006). However, the proposed mechanisms by which Reticulons or NgR influences neurodegeneration appear to be independent of the role of Nogo/Rtn4 as an inhibitor of axonal sprouting. Indeed, whether Nogo/Rtn4 plays a role in mouse models of AD remains unknown. We explored this possibility by generating and characterizing mice that express a mutated human *APP* transgene in a *Nogo* null background. We found that deleting *Nogo* ameliorates disease progression in this *APP* transgenic model of AD as assessed by behavioral and neuropathological measures. In particular, *Nogo* deletion restored the reduced expression of markers for axonal sprouting and synapto–dendritic complexity in *APP* transgenic mice. These date support the hypothesis that Nogo modulates disease progression in AD as an inhibitor of neuritic sprouting.

EXPERIMENTAL PROCEDURES

Experimental mice

The *platelet-derived growth factor subunit B (PDGFB)-hAPP* transgene with Swedish (K670N/M671L) and Indiana (V717F) mutations (Line J9) (Mucke et al., 2000) was crossed with homozygous *Nogo* null mice (Lee et al., 2009). *APP tg*, *Nogo*+/− mice were then crossed to *Nogo*−/− mice to obtain the experimental mice of four different genotypes (see Results) as littermates in a C57BL/6, 129S7 and DBA mixed genetic background. All experimental procedures were approved by the Institutional Animals Care and Use Committee at UCSD. Experiments were designed and conducted in ways to minimize the number of animals used to obtain valid results and to minimize animal distress.

Behavioral studies

Mice were tested in the Morris water maze as described (Rockenstein et al., 2006) at 8–10 months of age. For this purpose, a pool was filled with opaque water and mice were first trained to locate a visible platform (days 1–3) and then a submerged hidden platform (days 4–7) in three daily trials 2–3 min apart. Mice that failed to find the hidden platform within 90 s were placed on it for 30 s. The platform location was fixed. The entry point into the water for each mouse was alternated randomly between two points at an equal distance from the platform. On day 8, another visible platform trial was performed to exclude differences in motivation and fatigue, and a probe trial was performed when there was no platform present to calculate percent time spent in the target quadrant. Time to reach the platform and path length were recorded with a Noldus Instruments EthoVision video-tracking system (San Diego Instruments).

Tissue processing

Mice were euthanized by deep anesthesia and brains removed and divided sagittally. One hemibrain was post-fixed in 4% paraformaldehyde at 4 °C for 48 h and sectioned at 40 μm with a Vibratome 2000 (Leica), while the other hemibrain was snap frozen and stored at −70 °C for protein analysis.

Immunohistochemistry

As described (Rockenstein et al., 2006), blind-coded Vibratome sections were immunostained with monoclonal antibodies against synaptophysin (1:20, Chemicon, now Millipore, Billerica, MA, USA), MAP2, (1:40, Chemicon), NeuN (1:1000, Chemicon), GFAP (1:500, Chemicon), Iba-1 (1:1000, Wako Chemicals, Richmond, VA, USA), GAP43 (1:100, Chemicon), neurofilament (SMI312, 1:1000, Abcam, Cambridge, MA, USA), APP (Mucke et al., 2000), and rabbit anti-Nogo (1:500) (Zheng et al., 2003). After overnight incubation with the primary antibodies, sections were incubated with fluorescein isothiocyanate (FITC)-conjugated IgG secondary antibodies, transferred to SuperFrost slides and mounted under glass coverslips with anti-fading media. Alternatively, sections were detected with peroxidase reaction with standard techniques. All sections were processed under the same standardized conditions. The immunostained blind-coded sections were serially imaged with the laser scanning confocal microscope (MRC1024, BioRad, Hercules, CA, USA) and analyzed with the Image 1.43 program (NIH). For each mouse, a total of three sections were analyzed and for each section, four fields in the frontal cortex and hippocampus were examined. The average of individual measurements was used to calculate group means. For GAP43, MAP2 and synaptophysin, results were expressed as percent area of the neuropil occupied by immunoreactive axons/dendrites/terminals; for neurofilament, GFAP and Iba-I, levels were expressed as optical density. For NeuN, the mean neuronal density was estimated using the optical disector method (Chana et al., 2003); briefly, for stereology, immunostained sections were counterstained with 1% Cresyl Violet and four 100-μm-wide fields from at least three sections (180-μm interval) per animal were analyzed and results averaged and expressed as total number per mm^3. The extent of the Aβ deposits were detected using the mouse monoclonal antibody 4G8 (1:600, Senetek, Napa, CA, USA) as described (Rockenstein et al., 2006) and percent area occupied by Aβ deposits plotted.

Western blot

Antibodies used: Rtn3 (R458, 1:1000, a generous gift from Dr. R. Yan, Cleveland, OH, USA) (He et al., 2004), NgR (1:1000, a generous gift from Dr. B. L. Tang, Singapore), LC3 (1:1000, MBL, Woburn, MA, USA), Beclin 1 (1:1000, Novus Biologicals, Littleton, CO, USA), Caspase 3 (1:1000 Cell Signaling, Danvers, MA, USA), Aβ (6E10, 1:1000, Covance, Princeton, NJ, USA).

Statistical analysis

Values in the figures were expressed as means±SEM. To determine the statistical significance, values were compared by using the one-way ANOVA with post-hoc Dunnet when comparing the *Non tg* to the *Nogo* null or *App tg* mice. Additional comparisons were done using Tukey–Krammer or Fisher post-hoc tests.

RESULTS

In order to evaluate the functional effects of deleting *Nogo* on disease progression in an *APP* transgenic model of AD, an *APP transgenic* line (*PDGFB-hAPP* with Swedish and Indiana mutations) (Mucke et al., 2000) were crossed with *Nogo* null mice. Unlike other *Nogo* mutants reported in the literature, the *Nogo* null mutant used in this study is fully viable and lacks the expression of all known Nogo isoforms including Nogo-A,B,C (Lee et al., 2009). The *PDGFB-hAPP* transgenic line (referred to as *APP tg* here) was chosen because this line has been extensively characterized for neuritic sprouting (Chin et al., 2004). Four groups