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CHANGES IN MICROTUBULE TURNOVER ACCOMPANY SYNAPTIC PLASTICITY AND MEMORY FORMATION IN RESPONSE TO CONTEXTUAL FEAR CONDITIONING IN MICE

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Abstract—Synaptic plasticity plays a crucial role in learning, memory, and cognitive disorders. Cytoskeletal reorganization underlies neuronal synaptic plasticity, but little is known about the regulation of cytoskeletal dynamics in living animals. We used stable isotope labeling to measure the turnover of tubulin in defined microtubule (MT) populations in murine brain. Neuronal MTs generally exhibited low turnover rates *in vivo*. Basal turnover was highest in tau-associated MTs, intermediate in microtubule-associated protein 2 (MAP2)–associated MTs, and lowest in cold-stable MTs. Labeling of MTs in mature neurons in cell culture yielded similar turnover results. Intracerebroventricular glutamate injection stimulated, via *N*-methyl-D-aspartic acid receptors, label incorporation (turnover) in cold-stable, tau-associated, and MAP2-associated MTs, the last of which was shown to be dependent on cyclic adenosine-3′, 5′-monophosphorothioate–protein kinase A. Contextual fear conditioning, a hippocampus-mediated form of memory formation, was accompanied by increased turnover of hippocampal MAP2-associated and cold-stable MTs. Treatment with the MT-depolymerizing drug nocodazole reversed the conditioning-induced increase in label incorporation in MAP2-associated MTs, reduced dendritic spine density, and impaired memory formation. The effects of nocodazole on MT turnover were prevented by the MT-stabilizing agent Taxol (Sigma-Aldrich, St. Louis, MO, USA) and by brain-derived nerve growth factor, both of which also restored dendritic spine density and memory formation in this model. In conclusion, these results suggest that changes in hippocampal MT turnover are required for, and are a biomarker of, the synaptic plasticity that is involved in memory formation.

Key words: cytoskeletal plasticity, stable isotope labeling, biomarker, spine density, tubulin, NMDA receptors.

The ability of neurons to form chemical synapses via axons and dendrites is critical to neurotransmission. Dynamic rearrangement and strengthening of these connections are important elements of learning and memory in response to stimuli, particularly at dendritic spines, which represent most glutamatergic postsynaptic sites (Yuen et al., 2005; Gu et al., 2008; Jaworski et al., 2009). Connections are maintained structurally in neurons over the long term by cytoskeletal elements, comprising primarily microtubules (MTs). MT assembly and disassembly are regulated largely by microtubule-associated proteins (MAPs) (Avila et al., 1997; Slaughter and Black, 2003). These MAPs generally use a conserved MT-binding domain to associate with MTs, and this interaction is regulated by post-translational modifications and Ca^{2+} binding (Avila et al., 1997; Brandt et al., 2005). Recent data have provided evidence that rearrangement of MTs is associated with changes in spine morphology, suggesting that MTs play a direct role in spine morphology and function (Hu et al., 2008).

MTs are polymers composed of tubulin dimers. In most non-neuronal cells, tubulin dimers and MTs exist in rapid dynamic exchange (Fanara et al., 2004). Axonal and dendritic MTs in neurons, however, are thought to be less dynamic because of their interactions with MAPs (Brandt et al., 2005; Fanara et al., 2007). *In vitro*, MAPs modulate the dynamic exchange of tubulin with MTs and lower the minimal or "critical" concentration of tubulin dimers required for MT polymerization (Heald and Nogales, 2002). A central role for MT stabilization in the maintenance of normal neuronal morphology and function has been inferred from the effects of MT-disrupting agents in neuronal cell cultures (Chuckowree and Vickers, 2003) and of mutant MAPs expressed in transgenic mice, which cause brain atrophy and cognitive deficits (Brandt et al., 2005; McGowan et al., 2006). Direct observations of neuronal MT turnover kinetics, however, have been limited to a few studies in cell culture, tracking the incorporation of microinjected, fluorescently tagged tubulin into MTs or measur-

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Abbreviations: ^2H, two atoms of hydrogen; ^2H_2O, heavy water; AMPA, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid; AP5, D-(–)-2-amino-5-phosphonopentanoic acid; BDNF, brain-derived nerve growth factor; cAMP, cyclic adenosine-3′, 5′-monophosphorothioate; CFC, contextual fear conditioning; CS, cold-stable; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ICVC, intracerebroventricular cannulated; LTM, long-term memory; LTP, long-term potentiation; MAP, microtubule-associated protein; MAP2, microtubule-associated protein 2; MSB, microtubule-stabilizing buffer; MT, microtubule; NaCl, sodium chloride; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline; NMDA, *N*-methyl-D-aspartic acid; PBS, phosphate-buffered saline solution; PKA, protein kinase A; Rp-cAMP, Rp isomer of adenosine-3′,5′-monophosphorothioate.

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ing MT turnover by fluorescence recovery after photo bleaching (Edson et al., 1993; Joshi, 1998).

We recently developed a stable isotope labeling technique, using heavy water (^2H_2O) administration, to measure the dynamic exchange of newly synthesized tubulin dimers with MT polymers in dividing cells, neurons, and peripheral nerves (Fanara et al., 2004; Fanara et al., 2007). Using this approach, we previously detected the effects of MT-polymerizing agents such as taxanes on the modulation of tubulin exchange with MTs and observed marked alterations in MT turnover in motoneurons from rodent models of amyotrophic lateral sclerosis (Fanara et al., 2007).

In this study we apply this approach to mature neurons in cell culture and in the rodent brain *in vivo*, with the goal of exploring the role of MT turnover in synaptic plasticity and memory formation in response to contextual fear conditioning (CFC). CFC represents a form of associative learning and memory formation that has been well characterized in many species, as well as in transgenic mouse models of Alzheimer disease (Comery et al., 2005; Kim and Jung, 2006).

EXPERIMENTAL PROCEDURES

Cell culture

Rat brain hippocampal neurons were obtained from Cambrex Bio Science (Rockland, ME, USA). Neurons were plated at nominal densities of 2,000 cells/cm^2 and grown in neurobasal medium supplemented with 2-mM glutamine, 100-U/ml penicillin/streptomycin, and 2% B27 in a humidified atmosphere of 5% carbon dioxide at 37 °C on a 5-cm^2 tissue culture dish coated with poly-D-lysine and laminin. For labeling studies, culture media, as well as the humidified incubator, were adjusted to 5 mol % ^2H_2O by the addition of greater than 99% ^2H_2O (Cambridge Isotope Laboratory, Andover, MA, USA) and were maintained at this ^2H enrichment for up to 24 h. Hippocampal neurons were maintained in culture over a period of 14 d, during which time maturation occurred, with extended, well-defined axons and elaborate dendritic arbors (Santama et al., 1996; Goslin et al., 1998). Where indicated, potassium L-glutamate and Rp isomer of adenosine-3′,5′-monophosphorothioate (Rp-cAMP; Biolog, Bremen, Germany), or both, were added to cultures at the beginning of the labeling period.

Animal studies and CFC protocols

Each experimental group consisted of aged-matched drug-treated and untreated vehicle littermates. Animals in these studies were handled according to office of Laboratory Animal Welfare–National Institutes of Health guidelines. C57BL/6JBomTac mice (Taconic, Oxnard, CA, USA), aged 10 wk, were kept in a facility with controlled light-dark cycle, temperature, and humidity. All studies received prior institutional approval. After anesthesia (2%–2.5% isoflurane and 2% O_2), intracerebroventricular cannulated (ICVC) mice were infused with 6 μl of either 80-μM potassium L-glutamate (Sigma-Aldrich, St. Louis, MO, USA) or 80-μM potassium L-glutamate with 10-μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline (NBQX; Sigma-Aldrich), 80-μM potassium L-glutamate with 10-μM D-(–)-2-amino-5-phosphonopentanoic acid (AP5; Sigma-Aldrich), and 80-μM potassium L-glutamate with 6.6-pmol Rp-cAMP (Biolog), over a period of 6 min. Control animals were infused with 6 μl of sterile water. Other groups received 6-μl infusions of either nocodazole (30 nM in 20% Captisol; CyDex Pharmaceuticals, Inc., Lenexa, KS, USA) or nocodazole with brain-derived nerve growth factor (BDNF) in 20% Captisol (0.1 μg/μl; Biovision Inc., Mountain View, CA, USA) and nocodazole with Taxol (10 nM; Sigma-Aldrich). Control animals were infused with 6 μl of 20% Captisol. For ^2H_2O labeling, mice then received an i.p. priming bolus of 30 to 35 ml/kg of ^2H_2O (99.9 mol % ^2H_2O) containing 0.9% wt/vol sodium chloride (NaCl), resulting in 4% to 5% body water ^2H enrichment, and were maintained on 8% ^2H_2O in drinking water (to allow for dilution of label by metabolic water) for 24 h before they were killed. All animals tolerated the treatments well, and there were no differences in body weight among animals in different groups. ICVC mice were purchased from Taconic. For ICVC infusion, the internalized tip of a sterile cannula was stereotaxically placed in the lateral ventricle (anterior/posterior, −1.0 mm; medial/lateral, +1.0 mm (left side); and dorsal/ventral, −2.0 to 2.5 mm).

On the training day, C57BL/6JBomTac mice (Taconic), aged 10 wk, received ICVC infusion of vehicle or treatment (n=10 mixed gender) 4 h before training. Each group was then placed in the fear-conditioning chamber (Med Associates Inc., St. Albans, VT, USA) for 2 min before the onset of conditioned stimulus, a tone, which lasted for 30 s at 2,800 Hz (85 dB). The last 2 s of the conditioned stimulus was paired with the unconditioned stimulus, a 0.5-mA shock, all controlled by Med-PC computer software (Med Associates Inc.). The chambers were located in a sound-isolated room. We adopted 3 pairings of the conditioned with unconditioned stimuli with a 1-min intertrial interval. After an additional 30 s in the chamber, the mice were returned to their home cage. One day later, mice were returned to the same chambers in which training occurred (context), and freezing behavior was recorded by the experimenter using time sampling in 10-s intervals for 5 min (30 sample points). Freezing was measured as percent of freezing to context defined as complete lack of movement except for respiration. At the end of the 5-min context test, mice were returned to their home cage. Approximately 1 h later, freezing was recorded in a novel environment consisting of modifications including an opaque Plexiglas divider diagonally bisecting the chamber, a Plexiglas floor, and decreased illumination. Mice were placed in the novel environment, and the auditory cue was then presented for 3 min, and freezing was again scored. Freezing scores for each animal were expressed as a percentage for each portion of the test. Memory for the context (contextual memory) for each mouse was obtained by subtracting the percent freezing in the novel environment from that in the context (Comery et al., 2005). Mice were continuously labeled with 8% ^2H_2O in drinking water 1 day before and during CFC training and tested to measure the incorporation of newly synthesized tubulin dimers into tubulin polymers (MTs).

Locomotor activity

Locomotor activity was measured by stride-length analysis. To measure stride length, mice were trained to walk in a straight line on a 75-cm long narrow corridor. The mice's hind feet were painted with a dye, and the tracks left as they ran through the corridor were recorded on paper tape lining the floor of the corridor. The test was repeated until a mouse walked for 4 clear continuous strides. Stride length was measured as the distance between prints made by the same paw, taken from the center of one print to the center of the next (Azzouz et al., 2004; Fanara et al., 2007). In this model stride length and number of strides closely correlated during the mouse's continuous locomotion.

Assessment of dendritic spine density

Mice (10-wk-old males) were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffer (PFA in phosphate-buffered saline solution [PBS]), pH 7.4. Brains were removed, post fixed overnight at 4 °C in 4% PFA in PBS, and stored in PBS at 4 °C. For rapid Golgi staining, brains were stained as described earlier (Hundelt et al., 2009). Coronal sections (200 μm; bregma, −1.58 to −2.30 mm) (Paxinos and Franklin, 2001) were cut in 50% (vol/vol) glycerol with a Vibratome (VT1000S Leica Micro-