Swimming against the Tide:
Mobility of the Microtubule Associated Protein Tau in Neurons

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Supplement

Mathematical Methods:

Estimation of diffusion constants from hydrodynamic properties: We used the Stokes-Einstein equation, $D = k_B T/6\pi\eta R_H$ (where k_B is the Boltzmann constant, T absolute temperature, η the viscosity of the medium, and R_H the hydrodynamic radius) to estimate the diffusion coefficients D of GFP, tau, and the GFP-tau fusion proteins. The viscosity of the axonal cytosol was assumed to be 3.4 cP, 5 times larger than that of water at 37°C [Popov & Poo, 1992]. The hydrodynamic radius for GFP (R_H = 2.4 nm, corresponding to D = 28 μm^2/s) was calculated from the formula for globular proteins, R_H/nm = 0.595 (MW/kDa)$^{0.427}$ (MW = molecular weight), in agreement with experimental values (Luby-Phelps, 2000). The hydrodynamic radius of tau protein (R_H =5.6 nm; corresponding to D = 12 μm^2/s) was taken from (Cleveland et al., 1977). This is larger than the value expected for globular proteins of equivalent mass because tau is a natively unfolded protein (Schweers et al., 1994). The empirical formula R_H/nm = 0.221 $N^{0.57}$ (N= number of amino acids) for highly denatured proteins (Wilkins et al., 1999) predicts R_H = 7.1 nm (corresponding to D = 9.4 μm^2/s) for a protein of the same number of amino acids as tau (N = 441), indicating that tau protein is not completely unfolded. The same formula can be used to calculate an upper limit for the hydrodynamic radius of the GFP-tau fusion protein (N = 679), assuming a completely unfolded protein of the same number of amino acid residues: $R_{H,max}$ = 9.1 nm (corr. to D_{min} = 7.4 μm^2/s). Considering that the GFP moiety of the fusion protein is compactly folded, and that the tau moiety also retains some residual structure, the true value is problably closer to $R_{H,min}$ = 5.6 nm (the value of tau protein alone) than to $R_{H,max}$. This is consistent with the diffusion constant measured for GFP-tau (D = 11 μm^2/s), which corresponds to R_H = 6.1 nm. Alternatively, the combined R_H of a fusion protein with two globular domains can be estimated from

$$(M_1+M_2)R_{tot}^2 = M_1R_1^2 + M_2R_2^2 + M_1M_2\,d^2/(M_1+M_2).$$

Since the values for R_H and MW of tau (5.6 nm, 48 kDa) are much larger than for GFP (2.4 nm, 28 kDa), the presence of GFP in the fusion protein makes only a minor contribution to the combined R_H (6.06 nm, +8%)

Modelling of free diffusion and diffusion with binding to microtubules: Experimental values for the diffusion coefficients were determined by FRAP from the half time of recovery, $t_{1/2}$, at the centre of an extended bleached region within the axon. To define the relation between $t_{1/2}$ and D, the axon was modeled as a cylinder (tube of constant cross section) and the diffusion of tau was described by solving the one-dimensional diffusion equation for free

diffusion or by numerical integration of the reaction-diffusion equations for diffusion with binding to microtubules.

Free diffusion: The one-dimensional diffusion equation

(1) $$\frac{\partial c(x,t)}{\partial t} = D\frac{\partial^2 c(x,t)}{\partial x^2}$$

with initial conditions

(2) $c(x,t=0) = c_0$ for $x < -L/2$ or $x > L/2$ (outside the bleached zone)

$c(x,t=0) = 0$ for $-L/2 < x < L/2$ (inside the bleached zone)

is solved by using the well-known solution of (1) for initial conditions corresponding to a step function (e.g. Segel, 1980). Using the notation

(3) $$erf(x) = \frac{2}{\sqrt{\pi}}\int_0^x e^{-s^2} ds \qquad \text{(error function)}$$

the solution of equation (1) with initial conditions (2) is given by

(4) $$c(x,t) = \frac{c_0}{2}\left[2 - erf\left(\frac{\frac{L}{2}+x}{2\sqrt{Dt}}\right) - erf\left(\frac{\frac{L}{2}-x}{2\sqrt{Dt}}\right)\right].$$

At the center of the bleached region ($x = 0$) this equation simplifies to

(5) $$c(x=0,t) = c_0\left(1 - erf\left(\frac{L}{4\sqrt{Dt}}\right)\right)$$

which leads to

(6) $$D = 0.275\frac{L^2}{t_{1/2}}.$$

Diffusion with binding to microtubules: Equations (1) and (6) should apply to freely diffusing molecules like GFP and the 4KXGE-tau mutant. For wildtype tau and the 8R-tau mutant, reversible binding to MTs must be taken into account. Instead of a simple diffusion equation for a single species, two equations for the bound and for the detached molecules (concentrations c_{bound} and c_{free}) are required to describe diffusional motion and MT binding kinetics. Assuming that the total concentration of tau ($c_{total} = c_{bound} + c_{free}$) is far below saturation of MTs everywhere in the axon, association and dissociation can both be described by (pseudo) first order rate equations (rate constants k_{on} and k_{off}), leading to:

(7a) $$\frac{\partial c_{free}}{\partial t} = +k_{off}c_{bound} - k_{on}c_{free} + D\frac{\partial^2 c_{free}}{\partial x^2},$$

(7b) $$\frac{\partial c_{bound}}{\partial t} = -k_{off}c_{bound} + k_{on}c_{free}.$$

The system of reaction-diffusion equations (7a, b) with initial conditions (2) does not have an analytical solution, but two limiting cases can be discussed easily. In the case $L \to 0$ (small bleached zone) the diffusion is "fast", relative to MT binding, and therefore the experiment yields information on MT binding. Conversely, in the case $L \to \infty$ the diffusion is "slow" compared to MT binding so that the experiment yields information on diffusion. Specifically, if L is sufficiently small, such that diffusion of the free molecules can be considered fast

compared to the MT binding kinetics, then diffusional equilibration of c_{free} is virtually instantaneous over all relevant distances. Thus c_{free} is the same at every point, and equations (7a, b) with initial conditions (2) reduce to a set of three ordinary differential equations for the concentrations c_{free}, $c_{bound,1}$, and $c_{bound,2}$ where indices 1 and 2 refer to the bleached and the unbleached region:

(8a) $$\frac{dc_{free}}{dt} = +k_{off} c_{bound,1} - k_{on} c_{free}$$

(8b) $$\frac{dc_{bound,1}}{dt} = -k_{off} c_{bound,1} + k_{on} c_{free}, \qquad c_{bound,1}(t=0) = 0,$$

(8c) $$\frac{dc_{bound,2}}{dt} = -k_{off} c_{bound,2} + k_{on} c_{free}, \qquad c_{bound,2}(t=0) = c_0.$$

Equations (8a) and (8b) (or (8a) and (8c)) are the rate equations describing reversible association and dissociation of tau and MTs. At equilibrium ($dc_{free}/dt = dc_{bound}/dt = 0$) the concentrations obey the relationship

(9) $$\frac{c_{free}^{eq}}{c_{bound}^{eq}} = \frac{k_{off}}{k_{on}} = \frac{\tau_{free}}{\tau_{bound}}.$$

Here, $\tau_{free} = k_{on}^{-1}$, $\tau_{bound} = k_{off}^{-1}$ denote the mean time periods the molecules spend in the free or the bound state before binding or unbinding, respectively.

Equations (8a,b,c) can be solved by first considering a cylinder of finite length $> L$, then increasing its length to infinity. The result for the bleached region is

(10a) $$c_{free}(t) = c_{free}^{eq,0} = \text{const},$$

(10b) $$c_{bound,1}(t) = c_{bound}^{eq,0}\left(1 - e^{-k_{off}t}\right)$$

where $c_{free}^{eq,0}$ and $c_{bound}^{eq,0}$ are the equilibrium concentrations before bleach, $c_{free}^{eq,0} + c_{bound}^{eq,0} = c_0$. By combination of (10a) and (10b), the total concentration at the bleached region is:

(10c) $$c_{total,1}(t) = c_{free}^{eq,0} + c_{bound}^{eq,0}\left(1 - e^{-k_{off}t}\right) = c_0\left(1 - \frac{k_{on}}{k_{off} + k_{on}} e^{-k_{off}t}\right).$$

Thus, recovery after bleaching of a small region within the axon follows an exponential time dependence with rate constant k_{off}. Equation (10b) can be obtained more easily by considering the bleached instead of the unbleached molecules, i.e. by solving reaction-diffusion equations (7a, b) for initial conditions complementary to (2), that is $c(x, t=0) = c_0$ for $-L/2 < x < L/2$, and zero elsewhere. Since the diffusion is assumed to be fast, c_{free} in this case drops immediately to zero, $c_{free} = 0$ for all x and for all $t > 0$. Thus, $c_{bound}(t) = c_{bound}^{t=0} e^{-k_{off}t} = c_{bound}^{eq,0} e^{-k_{off}t}$. If we assume that bleaching does not change the fundamental properties of the molecules (but only their visibility), and if the system was at equilibrium before the bleach, then it will stay at equilibrium all the time, and any change in the concentration of the bleached molecules must be compensated by redistribution of the unbleached molecules. Thus the solutions for the bleached and the unbleached molecules follow the same (complementary) time dependence. Since this is a general argument that

does not rely on the special geometry of the system, the result in equation (10c) is valid for any geometry and can be applied, in principle, to the axon (one-dimension case) or the cell body (three-dimension case) as long as the bleached region is sufficiently small.

If the bleached region is large, and if we disregard local details at the boundaries between the bleached and the unbleached regions, but only consider the long-term behavior at large scales (characteristic time $t_c \propto L^2/D >> \tau = \tau_{free} + \tau_{bound}$, where τ is the mean time a molecule needs to cycle through the bound and the unbound state), then the reaction-diffusion equation (7a) can be simplified: If $t_c >> \tau$, each molecule attaches and detaches many times during all relevant time intervals; thus, the long-term molecular motions are the same as those of freely diffusing molecules, if only the fraction of time the molecules are freely diffusing is taken into account. Hence, the behavior of molecules interacting with MTs can be described with a simple diffusion equation by substituting the real time, *t*, by the effective time, t_{diff}, i.e. the time during which the molecules are actually diffusing:

(11) $$\frac{\partial c}{\partial t_{diff}} = D\frac{\partial^2 c}{\partial x^2} \qquad \text{with} \qquad t_{diff} = \frac{\tau_{free}}{\tau_{bound} + \tau_{free}} t = \frac{k_{off}}{k_{on} + k_{off}} t \ ,$$

or

(11') $$\frac{\partial c}{\partial t} = \frac{k_{off}}{k_{on} + k_{off}} D\frac{\partial^2 c}{\partial x^2} .$$

Thus, at very long time scales ($t >> k_{on}^{-1} + k_{off}^{-1}$) molecules that bind to MTs reversibly behave like freely diffusing molecules with an apparent diffusion coefficient

(12) $$D_{app} = \frac{k_{off}}{k_{on} + k_{off}} D \ .$$

Again, this conclusion holds for any geometry.

By numerical integration of the reaction-diffusion equations (7a, b) for a range of parameters we found that our bleaching experiments can by analyzed in terms of the limiting cases discussed above (see Fig. S1). For the long-range bleaching experiments, approximation by an apparent diffusion coefficient is excellent. Interpretation of the experiments with small bleach regions to determine k_{off} by using equation (10c) is more critical. At very short and very long times, the approximation inevitably breaks down. At medium time scales, the difference to the limiting exponential curve stays nearly constant (Fig. S1b). As a consequence, k_{off} values determined by exponential fitting of the data in the medium time range are within a factor of 2 close to the true value as long as bleaching zone does not exceed ~4 µm (see Fig. S1b for details).

Diffusion with active transport by microtubules: To account for the contribution of active transport of tau by attachment to moving fragments of microtubules, equations (7a, b) were expanded into three equations for c_{free}, $c_{mobile} = m \cdot c_{bound}$, and $c_{stat} = (1-m) \cdot c_{bound}$, where *m* is the fraction of the bound molecules transported by moving fragments of MTs, and an advection term $-v\frac{\partial c_{mobile}}{\partial x}$ was added to the equation of the mobile fraction (*v,* velocity of moving MT fragments).

(13a) $$\frac{\partial c_{free}}{\partial t} = +k_{off}(c_{mobile} + c_{stat}) - k_{on}c_{free} + D\frac{\partial^2 c_{free}}{\partial x^2} ,$$

(13b) $$\frac{\partial c_{mobile}}{\partial t} = -k_{off} c_{mobile} + m \cdot k_{on} c_{free} - v \frac{\partial c_{mobile}}{\partial x},$$

(13c) $$\frac{\partial c_{stat}}{\partial t} = -k_{off} c_{stat} + (1-m) \cdot k_{on} c_{free}.$$

For the simulation of tau dispersal in axons by diffusion and active transport (see Fig 6d), equations (13a,b,c) were numerically integrated with parameters m = 0.004 and v = 1 µm/s, corresponding to slow axonal transport with an average velocity of 0.003 µm/s.

Model calculations of Fig. 6d: The figure shows the long-term (30 days, top) and short term (16 hours, bottom) distribution of tau with different conditions. The blue solid line represents the linear movement of a pulse of labeled tau observed experimentally in slow axonal transport (~0.003 µm/s or ~0.2-0.4 mm/d, Mercken et al., 1995). The long-term movement of this tau is determined by its piggy-backing on MT fragments which move slowly by dynein dependent sliding along other stationary microtubules or microfilaments (Wang & Brown, 2002; He et al., 2005), but otherwise are almost non-diffusible. If diffusion is taken into account (red solid line, top), the front would arrive ~1-2 days earlier at a particular point along the axon, but the slopes (= mean velocities) are nearly the same because long term behavior is dominated by active transport of tau on microtubule fragments. However, up to ~10 days or ~3 mm diffusion is more efficient, as seen from the dashed curve (top) for the KXGE-tau mutant (which does not bind to MTs). In the case of reversible binding of wildtype tau to MTs (black solid line, bottom), the spreading is slowed down because tau molecules spend part of their time on slowly-moving MTs, but even here diffusion exceeds slow transport up to ~3 days or ~1 mm down the axon (intersection of blue and black lines, top). By contrast, the tightly binding construct 8R-tau spends most of its time on the immobile MT cytoskeleton and therefore has a severely restricted diffusion, but even here it advances faster than the MT fragments during the first day. The thick short line (green, bottom) represents the spreading of the labeled tau front reported by Utton et al. (2004) in cortical neurons. From these experimental data one cannot determine the relative contributions of diffusion vs. movement by sliding.

Ratio of cytoplasmic to MT-bound tau: To be sure that tau and its mutants are really bound to MTs in the imaged axons the ratio of MT-bound to cytoplasmic tau was determined. CFP-4KXGE-tau and mRFP-tau were co-transfected. CFP-4KXGE-tau has a very low affinity to microtubules because it is pseudo-phosphorylated and therefore and fills the entire volume of the axon, including regions of axonal protrusions where microtubules are not present. mRFP-tau (wildtype) has a high affinity for microtubules, it is thus found mainly in the microtubule rich areas and does not show strong additional signals in axonal protrusions (Fig. 4e). The ratio of the signal intensities of the two tau mutants (mRFP:CFP) in areas without MT (Fig. 4e, arrows) was used to normalize the intensities. The ratio of soluble to MT-bound tau can then be calculated because the intensity of CFP-4KXGE is proportional to cytosolic unbound mutant tau and the mRFP-tau signal reflects the sum of microtubule and cytoplasmic wildtype tau. The microtubule-bound fraction was calculated by subtracting the CFP-4KXGE-tau from mRFP-tau. In 9 separate experiments the ratio of microtubule-bound to unbound cytosolic tau was found to be 3:1. The ratio is consistent with the K_d of Tau (~1 µM).

Supplement figure legends

Fig. S1: Simulation of the fluorescence recovery after photobleaching by diffusion of tau in axonal segments. The curves in panel a and b show the time dependence of c_{cen}, the concentration of total unbleached tau at the center of a bleached region (relative to the initial concentration before bleach, c_o) for various lengths *L* of the bleach zone (*L* = 1, 2, 4, 8, 16, 32 µm). The curves were obtained by numerical integration of the reaction-diffusion equations (7a, b) with *D* = 10 µm²/s, τ_{free} = 1 s, $\tau_{bound} = t_{1/2}/\ln 2 = k_{off}^{-1}$ = 3 s. Since the relevant time scales vary over three orders of magnitude (increasing quadratically with L), the over-all recovery in panel **(a)** is plotted with an *L*-dependent time scale, $t^* = (32\mu m/L)^2 \cdot t$ (e.g. *t** = *t* for *L* = 32 µm, *t** = 1024 *t* for *L* = 1 µm, i.e. the end point of the x-axis corresponds to shorter times as *L* decreases). For comparison, panel **(b)** shows only the initial phase of the recovery, using the same fixed time scale for all curves (from 0 to 20 seconds).

Panel a: The dashed curve corresponds to the analytical solution for infinitely fast binding and unbinding of tau (equation 5 with D_{app} = 0.25*D*). It represents the limiting curve that is approached in the case of very long bleached segments (where binding/unbinding is rapid compared with diffusion along the axon). This curve is almost indistinguishable from the *L* = 32 µm curve when one uses the above realistic rates for binding/unbinding. For *L* = 32 µm, it takes 600 s or 10 min to recover to 78% ($t^* = t$, in this case). The dotted line represents the exponential recovery expected for an infinitely small bleach area (small *L* limit, equation 10c) over the time range of the 1 µm curve (0 to ~0.6 s, $t^* = 32^2 \cdot t = 1024 \cdot t$). Only the initial, almost linear part of the exponential recovery is visible. The curve starts with a finite value, $\tau_{free}/(\tau_{bound} + \tau_{free}) = 0.25$, which corresponds to the concentration of free molecules, since bleached molecules in solution are instantaneoulsy replaced by unbleached molecules in the limit of zero bleach length. Approach of the 1 µm curve to the dotted line is extremely slow (see also panel b). Theoretically, 100% recovery at $t^* \to \infty$ is expected only for infinitely long cylinders (axons). For finite geometries, the maximum recovery is always smaller than 1 (unless new molecules are created) assuming that the absolute number of fluorescent molecules is irreversibly reduced by bleaching. Note that the gap between the 1 µm curve and the dotted line that remains even at longer times (panel b) is *not* due to the finite size effect; the boundary conditions used for numerical simulations avoid this effect.

Panel b: Curves corresponding to those of panel a are shown within the same time range (0 to 20 s) for all curves. The exponential curve representing the limiting case of an infinitely short bleach zone (dotted line, "0 µm") recovers to virtually 100% over the time of 20 s (time constant of recovery: $\tau_{bound} = k_{off}^{-1} = 3$ s). For very long bleach length *L*, recovery starts slowly and follows a pure diffusion curve with apparent diffusion constant D_{app}, reflecting the fact that diffusion is so slow that free and bound molecules are always close to equilibrium. For short segments (*L* < ~4 µm) recovery after photobleach displays three phases. During a short initial phase, from $t = 0$ to a fraction of a second, the concentration of unbleached molecules increases rapidly, since diffusion over short distances is fast and the concentration gradient of the diffusing molecules is high at the beginning. Once the concentration in the bleach zone has almost reached the equilibrium concentration of free molecules everywhere in solution, the recovery curve switches over to the second phase. This is governed by the kinetics of unbinding of bleached molecules and their substitution by unbleached molecules. In this phase, recovery follows a curve similar in shape to the limiting exponential curve. However, the recovery curve does not reach the same level as the exponential curve at realistic time scales accessible in experiments. Even for bleaching zones as short as 1 µm, approach to full recovery is extremely slow compared to the exponential curve, due to the fact that with increasing time the relevant distance to be covered by diffusion increases (i.e. the partially depleted region is always expanding, and unbleached molecules have to be supplied from more and more distant regions); thus, the assumption of fast diffusion becomes more and more inadequate. Nevertheless, approximation of the second phase by

an exponential function is excellent at least for bleach lengths of 4 µm and below. The apparent time constants, however, differ fom the true value (3 s). The numerical simulations can be used to calculate correction factors for the apparent τ values obtained under the simplifying assumption of an exponential curve. Example: fitting the curves in the entire range from 0 to 20 s with a single exponential function leads to apparent τ values of 3.45 s (*L* = 1 µm), 3.98 s (2 µm), and 5.15 s (4 µm). Since the bleached spot in the "short L" experiments was between 2 - 4 µm, the correction factors for the k_{off} rates range between 1.3 and 1.8 . It should be mentioned that these correction factors apply for diffusion in a quasi 1-dimensional geometry (like a segment of a thin cylinder). For FRAP experiments in a really 3-dimensional environement (e.g. small area in the middle of a cell body), approximation of the limiting exponential curve is expected to be much better (thus, correction factors should be closer to 1), since the depletion zone is filled up more efficiently with unbleached molecules arriving from all directions.

The curves in panel a may be interpreted in an alternative way: They also represent the different time dependencies expected for varying time constants τ_{free} and $\tau_{bound} = 3\tau_{free}$ at a fixed bleach length, e.g. *L* = 32 µm. The curves labeled 16, 8, 4, 2, and 1 µm would then correspond to time constants τ_{free} = 4, 16, 64, 256, and 1024 s, respectively. Thus, even in the case of the strongly binding 8R-tau mutant (τ_{free} of the order of 4 s, corresponding to the curve labeled "16 µm"), the large *L* limit is a fairly good approximation for the experimental condition we used.

Figure S2: RGC neuron expressing CFP-KXGE and mRFP-tau. Cotransfected RGC neurons with mRFP-tau (red) and CFP-4KXGE-tau (green) were used to calculate the ratio of cytoplasmic tau to microtubule-bound tau. The CFP-KXGE-tau mutant does not bind to MTs and can be used as a volume marker because it just shows cytoplasmic localization. Arrows indicate axonal protrusions containing soluble tau but no microtubules. The signals of the two fluorophores were normalized by the signal intensities in these MT free areas. After that procedure the KXGE-tau intensities reflect the intensities of the cytoplasmic portion of mRFP-tau. The signal of the 4KXGE-tau mutant was subtracted from the signal of total tau leading to an image showing only MT-bound tau. The fraction of free tau was calculated by subtracting the signal of MT-bound tau from that of total tau. Arrowheads point to regions with MT-bound and free tau.
The procedure is described by the following equations (I = signal intensity, x = factor needed to normalize the signal intensities in the protrusions containing only tau):

(1a) $x = I_{CFP\text{-}KXGE,\ protrusion} / I_{mRFP\text{-}tau,protrusion}$

(1b) $I_{mRFP\text{-}tau,axon,\ MT\text{-}bound} = I_{mRFP\text{-}tau,\ axon} - (I_{CFP\text{-}KXGE,\ axon} / x)$

(1c) $I_{mRFP\text{-}tau,axon,\ unbound} = I_{mRFP\text{-}tau,\ axon} - I_{mRFP\text{-}tau,\ axon,\ MT\text{-}bound}$

Example:
$I_{mRFP\text{-}tau,\ axon} = 230$, $I_{mRFP\text{-}tau,protrusion} = 30$, $I_{CFP\text{-}KXGE,\ axon} = 105$, $I_{CFP\text{-}KXGE,\ protrusion} = 75$, yielding

(1a) $x = 75 / 30 = 2.5$

(1b) $I_{mRFP\text{-}tau,axon,MT\text{-}bound} = 230 - (105 / 2.5) = 188$

(1c) $I_{mRFP\text{-}tau,axon,unbound} = 42$

Thus, ~80% of the total tau is bound to microtubules, ~20% is unbound cytosolic tau.

Supplement References

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