The *Drosophila* Netrin receptor Frazzled guides axons by controlling Netrin distribution

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Netrin is a secreted protein that can act as a chemotropic axon guidance cue[1,2]. Two classes of Netrin receptor, DCC[3–5] and UNC-5 (refs 6–9), are required for axon guidance[3,4,6–11] and are thought to mediate Netrin signals in growth cones through their cytoplasmic domains[12,13]. However, in the guidance of *Drosophila* photoreceptor axons, the DCC orthologue Frazzled[3] is required not in the photoreceptor neurons but instead in their targets, indicating that Frazzled also has a non-cell-autonomous function[14]. Here we show that Frazzled can capture Netrin and 'present' it for recognition by other receptors. Moreover, Frazzled itself is actively localized within the axon through its cytoplasmic domain, and thereby rearranges Netrin protein into a spatial pattern completely different from the pattern of *Netrin* gene expression. Frazzled-dependent guidance of one pioneer neuron in the central nervous system can be accounted for solely on the basis of this ability of Frazzled to control Netrin distribution, and not by Frazzled signalling. We propose a model of patterning mechanism in which a receptor rearranges secreted ligand molecules, thereby creating positional information for other receptors.

In vitro chemotropic responses of growth cones to Netrin indicate that graded distribution of Netrin may be important for guiding axons *in vivo*[1,2,15–22]. A Netrin gradient could be produced by constant secretion followed by diffusion and degradation. However, we found that in the ventral nerve cord of the *Drosophila* embryo the distribution of Netrin protein cannot be explained by such a mechanism. *Drosophila* Netrin is encoded by two genes, *Netrin-A* and *Netrin-B*[17,18]. Although Netrin messenger RNA is abundant in the midline and the ventral region of the nerve cord, Netrin-A (data not shown) and Netrin-B proteins localize in the dorsolateral region, where no Netrin mRNA is detected (Fig. 1a–d). Even when *Netrin-B* transcription was artificially restricted to midline cells, Netrin-B still accumulated in the dorsolateral region as in wild-type embryos, rather than forming a gradient centred at the midline (Fig. 1e, f; arrow). This suggests that Netrin is either transported to the dorsolateral region or is selectively captured there after secretion.

Frazzled is a good candidate for a molecule that relocalizes Netrin. Its accumulation is most evident on axon stalks of the commissural region[3] (Fig. 1g), and its orthologue, DCC, is known to bind Netrin[5]. Moreover, the dorsolateral Netrin-positive region precisely matches Frazzled distribution (Fig. 1b, g). In the absence of Frazzled, Netrin did not accumulate dorsolaterally and Netrin-B was observed only on cell bodies that express *Netrin-B* mRNA (Fig. 1h). Moreover, when we misexpressed Frazzled in ventral unpaired median (VUM) cells, ectopic Netrin-B protein was found on their surface (Fig. 1j, k) even though these cells do not express *Netrin-B* (Fig. 1i–k). These data indicate that ectopic Frazzled can capture Netrin synthesized elsewhere, and suggest that Frazzled localizes Netrin in the dorsolateral region of ventral nerve cord. As expected, Frazzled distribution was unaltered in *Netrin-A, Netrin-B* double-mutant embryos (data not shown).

Frazzled itself is not found uniformly throughout the membrane, but is concentrated in specific regions of the axon (Fig. 2a, b),

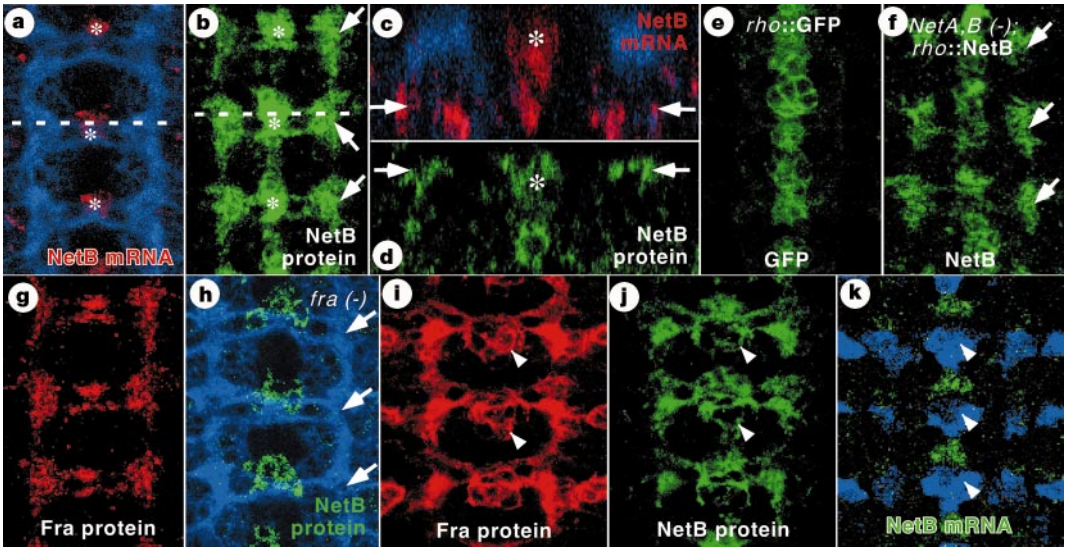

Figure 1 Frazzled relocates Netrin. Horizontal view (**a**, **b**, **e**–**k**) and cross-section (**c**, **d**) of the ventral nerve cord of stage 13 embryos. Axons were visualized using anti-HRP antibody (**a**, **c**, **h**; blue). **a**–**d**, Wild-type distribution of *Netrin-B* mRNA (**a**, **c**; red) and Netrin-B protein (**b**, **d**; green). Asterisk, midline cells. Dotted lines in **a**, **b** indicate the positions of vertical sections in **c**, **d**, respectively. *Netrin-B* mRNA is abundant ventrally (**c**; arrow), whereas Netrin-B protein accumulates dorsolaterally (**b**, **d**; arrows). This region corresponds to the point where commissural axons turn longitudinally. *Netrin-A* mRNA is expressed more dorsally than *Netrin-B* mRNA. Localization of Netrin-A is indistinguishable from that of Netrin-B in wild type and under experimental conditions described in **h**, **j** and Fig. 4b (data not shown). **e**, **f**, Expression of Netrin-B in midline cells in *Netrin-A, Netrin-B* double-mutant embryos (*Df (1) NP5; rhomboid–GAL4/+; UAS–Netrin-B/+*). Projections of a series of 2-μm confocal sections. Outlines of *rhomboid*$^{+}$ midline cells (**e**) visualized with membrane-targeted GFP (GAP–GFP). **g**, Frazzled in wild-type embryo. Compare with distribution of Netrin B (**b**). **h**, Netrin B (green) in *frazzled* (*fra*) mutant (*fra*^{3}/*fra*^{4}). Immunoreactivity is present only near cells that express *Netrin-B* mRNA (compare with **a**). **i**–**k**, Ectopic Frazzled (**i**; red) expressed in VUM cells (arrowhead, *engrailed–GAL4/UAS–lacZ/UAS–fra*). Compare Netrin-B protein (**j**, green) and mRNA (**k**, green). *engrailed–GAL4*$^{+}$ cells are visualized with β-galactosidase (**k**; blue).

indicating that its distribution may also be regulated. Localized distribution within the neuron has been observed for Roundabout (Robo), a transmembrane receptor for another guidance molecule, Slit[23], and the localization signal of Robo has been mapped to its cytoplasmic or transmembrane domain[13]. Similarly, we found that Frazzled lacking its cytoplasmic domain (Fra-ΔC) was distributed throughout the cell membrane (Fig. 2d). Furthermore, Robo–Fra, a chimaera with the extracellular and transmembrane domain of Robo and the cytoplasmic domain of Frazzled, was distributed in the same way as full-length Frazzled (Fig. 2e). This shows that the cytoplasmic domain of Frazzled is necessary and sufficient for proper localization. We also expressed Fra–Robo, a chimaera of the extracellular domain of Frazzled and the transmembrane and cytoplasmic domains of Robo, in *frazzled*$^{-}$ animals. In such embryos, the Fra–Robo fusion protein failed to distribute in the wild-type Frazzled pattern, and Netrin-B was mislocalized to many of the sites of Fra–Robo accumulation (Fig. 2f). These data show that Frazzled captures Netrin with its extracellular domain, whereas Frazzled distribution is controlled by a localization signal in the cytoplasmic domain.

Next, we investigated how axons are guided by the Netrin that is captured by Frazzled. We focused on an identified pioneer neuron, dMP2, that requires *Netrin-A/Netrin-B* and *frazzled* function. dMP2 axons extend laterally and then turn posteriorly to form the initial longitudinal axon pathway[24,25]. Precisely at the turning point, the medial edge of dorsolateral Netrin accumulation abuts the dMP2 pathway (Fig. 3d). dMP2 axons make pathfinding errors in both *Netrin-A, Netrin-B* double mutants and *frazzled* mutants (Fig. 3b, c, h, i), and such defects are often accompanied by severe disorganization of longitudinal tracts[3,17,18]. These data may indicate that dMP2 axon guidance by Frazzled and Netrin is essential for the formation of the longitudinal axon pathway.

To investigate how Frazzled functions in the guidance of dMP2, we tested whether *frazzled* is required in the dMP2 neuron itself. Contradictory to the idea that Frazzled is a Netrin sensor in dMP2 growth cones, Frazzled protein was not detected in dMP2 (Fig. 3e, f). Moreover, expressing Frazzled in dMP2 in a *frazzled*$^{-}$ background did not rescue the defects in dMP2 axon guidance (Fig. 3k, l). In contrast, when we expressed Frazzled in many central neurons in the *frazzled*$^{-}$ background, the defects of dMP2 axon guidance were rescued (Fig. 3m), even though Frazzled was not expressed in dMP2 (Fig. 2b). These data indicate that, for this guidance decision, Frazzled acts as a pathway marker and not as a sensor in growth cones.

The ability of Frazzled to capture Netrin raises the possibility that Frazzled guides dMP2 by capturing and presenting Netrin to dMP2.

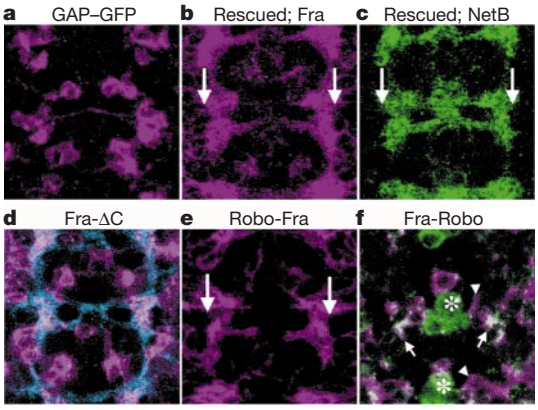

Figure 2 Functional domains of the Frazzled molecule. Frazzled and its derivatives were misexpressed using *GAL4* line G4-605. Confocal images of stage 13 embryos at the level of commissures. **a**, Outlines of GAL4^{+} cells visualized using GAP–GFP. **b**, **c**, Frazzled (**b**) and Netrin-B (**c**) distribution in an embryo in which full-length Frazzled expression was driven in *frazzled*$^{-}$ background (*fra*^{3}, *G4-605/fra*^{4}*; UAS–fra/+*). Frazzled does not distribute throughout the axon, but concentrates at specific regions (arrows, compare with **a**). Netrin-B (**c**) colocalizes with Frazzled in the lateral region, and is also present on the midline where it is independent of *frazzled* function (see Fig. 1h). **d**, **e**, Myc-tagged Frazzled derivatives expressed in wild-type background. Fra-ΔC (**d**; purple) distributes uniformly along the cell membrane, whereas Robo–Fra (**e**; purple) localizes to a specific region of the axon, as does endogenous Frazzled (blue). **f**, Expression of Fra–Robo in a *frazzled*$^{-}$ background (*fra*^{3}, G4-605/*fra*^{4}; *UAS–fra–robo/+*). Fra–Robo (purple) is localized by the localization signal of Robo[13]. Netrin-B (green) accumulates in a subset of the Fra–Robo-positive region (arrow). Some of the highly Fra–Robo-positive regions (arrowhead) fail to accumulate Netrin-B even though they are close to the Netrin-B source at the midline (asterisk).

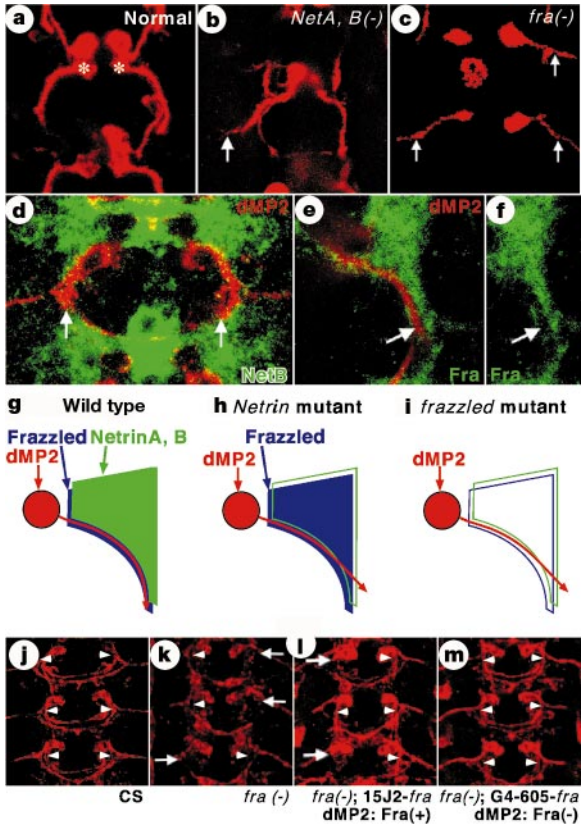

Figure 3 Axonal guidance of dMP2. dMP2 axons were visualized by driving tau-β-galactosidase expression using 15J12 GAL4 driver (**a**–**c**; **e**), or by staining with monoclonal antibody 22C10 (**d**, **j**–**m**). **a**–**c**, dMP2 axons in wild-type embryos, (**a**) in *Netrin-A*, *Netrin-B* double mutants (**b**; *Df(1)T9B118*; 7.1%, $n = 269$) and *frazzled* mutants (**c**; *fra*^{3}/*fra*^{4}; 30.3%, $n = 135$). In these mutants dMP2 axons often extend laterally (arrows), turn anteriorly or stall (not shown). Asterisk, dMP2 cell body. **d**–**f**, In early stage 13 wild-type embryos, dMP2 growth cones (red, arrow) track along the medial surface of the Netrin-B-positive region (**d**; green). dMP2 does not express Frazzled (**e**, **f**; green), and the axon path (**e**, **f**; arrow) is devoid of Frazzled. **g**–**i**, Spatial relationships between dMP2 axon trajectory (red) and domains of Netrin (green) and Frazzled (blue). In wild-type embryos, dMP2 growth cones track the medial surface of the dorsolateral Netrin-A and Netrin-B region created by Frazzled. In *Netrin-A*, *Netrin-B* double mutants (**h**) and *frazzled* mutants (**i**), neither Netrin-A nor Netrin-B is present in the dorsolateral region and dMP2 axons make pathfinding errors. It is not clear why the *Netrin-A*, *Netrin-B* double mutant produces less penetrant phenotypes than *frazzled* mutants. We note, however, that this is also true for the commissural phenotypes of these mutants. It is possible that Frazzled localizes additional guidance molecules. **j**–**m**, dMP2 axonal defect in *frazzled* mutant is non-cell-autonomous. Arrowheads, dMP2 axons extending along the correct pathway; arrows, those making pathfinding errors in late stage 13 embryos. **j**, Wild type (CS). In *frazzled* mutant (**k**; *fra*^{3}/*fra*^{4}), many dMP2 axons make pathfinding errors (25%, $n = 112$). Restoration of Frazzled expression in dMP2 (**l**; *fra*^{3}/*fra*^{4}*; UAS–fra*/15J2) does not rescue the axonal phenotype (18.8%, $n = 144$), but expression in the dMP2 path (**m**; *fra*^{3}/*fra*^{4}; G4-605/*UAS-fra*) does (0.8%, $n = 128$).

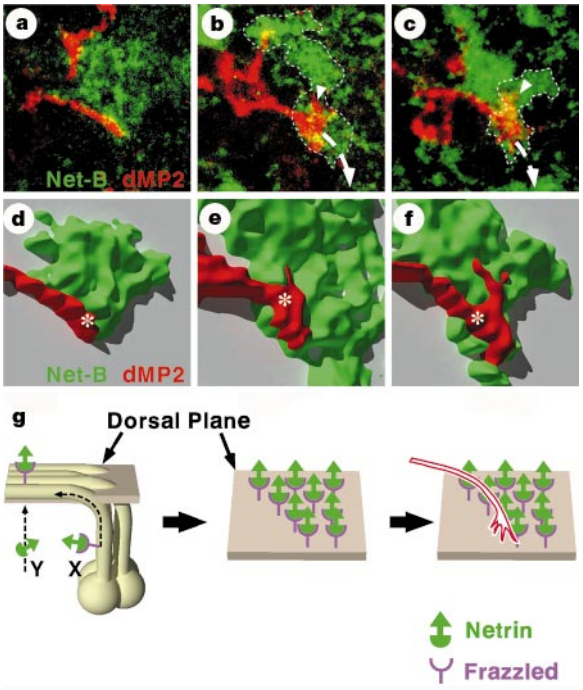

Figure 4 dMP2 growth cones respond to ectopic Netrin-B.The behaviour of the dMP2 growth cones in wild type (**a**) and upon ectopic expression of Fra-ΔC (**b**; *UAS–fra-ΔC*/G4-605) and Netrin-B (**c**; *UAS–Netrin-B*/G4-605). dMP2 growth cones (red) visualized by monoclonal antibody 22C10 in stage 12/0 embryos. Green, Netrin-B. Projections of series of 0.5-μm confocal sections. **b**, Expression of Fra-ΔC in a *frazzled* mutant background (*fra*[3], G4-605/*fra*[4], *UAS–fra-ΔC*) created an artificial Netrin-B-positive region (dotted line). dMP2 growth cone spreads abnormally (arrowhead) over the ectopic Netrin-B surface (55.8%, $n = 52$). Wild-type (**a**) and *frazzled* mutant (*fra*[3]/*fra*[4]) dMP2 growth cones never show such behaviour. Dotted arrow, normal path of the dMP2 axon (**b**, **c**). **c**, Misexpression of Netrin-B in the dMP2 axon pathway. dMP2 growth cone responded to ectopic Netrin-B (dotted contour) by spreading its growth cone anteriorly (arrowhead, 37.9%, $n = 108$). Occasional failure of dMP2 to show abnormal growth cone morphology under these experimental conditions is due to segmental variability in the timing of Netrin-B produced by the GAL4/UAS system. When the expression of ectopic Netrin-B occurs before the arrival of the dMP2 growth cone, dMP2 always responds (**b**, **c**). **d**–**f**, Three-dimensional views of samples shown in **a**–**c**, respectively, reconstructed from a series of confocal sections for 5-μm depth. dMP2 growth cones track the surface of Netrin-B-positive regions. Asterisks, corresponding positions in each sample. **g**, Capture/relocation model of Netrin and Frazzled. Plane indicates dorsal surface of ventral nerve cord, where commissures and longitudinal connectives lie. Left, Netrin is relocated to the dorsal region. This probably involves transport along axons with Frazzled (X) and diffusion (Y). Middle, Frazzled rearranges Netrin in a specific pattern. Right, dMP2 growth cone uses rearranged Netrin as a guidance cue.

To test this, we expressed Fra-ΔC in a *frazzled*[−] mutant background to create an ectopic Netrin-B positive region near the axon pathway of dMP2 without changing the pattern of *Netrin* transcription. In such embryos, dMP2 growth cones spread abnormally over this surface of artificial Netrin accumulation (Fig. 4b). We also directly misexpressed Netrin-B in cell bodies located near the dMP2 axon pathway. Again, dMP2 growth cones responded to the ectopic Netrin-B-positive region (Fig. 4c). This strongly suggests that the response of dMP2 to the ectopic Frazzled extracellular domain is due to a response to the Netrin bound to the domain.

An implication of these data is that dMP2 uses a Netrin receptor other than Frazzled to respond to Netrin. Redirection of dMP2 growth cones to ectopic Netrin indicates that Netrin is perceived as an attractive cue to dMP2. As the *Drosophila* genome does not contain any other genes with significant homology to DCC, we expect that the Netrin receptor expressed in dMP2 is structurally different from the DCC class of Netrin receptors.

Our data indicate that Frazzled captures and rearranges Netrin, and presents it to other growth cones (Fig. 4d). The capture/relocation mechanism we have uncovered can create a precise Netrin distribution even in regions that are quite distant from the source of Netrin protein. Just as Frazzled presents Netrin to the dMP2 axon at its lateral turning point, the vertebrate Frazzled orthologue DCC also captures Netrins, and is localized to the point where the commissural axons turn longitudinally[5]. Presentation of Netrin may thus be a general feature of DCC proteins. How Netrin reaches its final location is not yet clear. As Netrin-B does not localize to all Fra–Robo positive regions even when they are close to a source of Netrin-B (Fig. 2f), relocation of Netrin is likely to involve transport along axons rather than diffusion alone (Fig. 4d). Perhaps active relocalization of receptors such as Frazzled or Robo[23] may be used to transport ligands to the final target area, where they are interpreted by other receptors. In addition to neuronal axons, extended cellular processes, such as the cytonemes of *Drosophila* imaginal discs and vertebrate limb buds[26], have been implicated in other patterning systems. It will be interesting to see whether such systems also use capture/relocation mechanisms to generate precise spatial patterns away from the source of the diffusible morphogen. □

Methods

Constructs and strains

The Fra-ΔC construct (Fig. 4) was made as follows. A *Not*I linker was inserted into Frazzled complementary DNA[3] at codon 1,097 within the cytoplasmic region. This plasmid was linearized by *Not*I, and a myc epitope (Invitrogen) and a stop codon were added, generating the sequence [1097]LAAAEQKLISEEDLNGAAstop. The resulting Fra-ΔC fragment was cloned into pUAST[28] for transformation into flies. Frazzled and Robo derivatives[13] used for the localization experiment (Fig. 2) all contain the transmembrane domain of Robo, and carry 6-myc epitopes at their carboxy termini. The following strains have been described[3,17,25,27,28]: *fra*[3], *fra*[4], *UAS–fra, Df (1) NP5, Df(1)T9B118, UA –Netrin-B, GAL4* line 15J2, *UAS–tau–lacZ, UAS–lacZ*. GAL4 line G4-605, *engrailed GAL4* and *UAS–GAP–GFP* strains were gifts from G. M. Technau, A. Brand and A. Chiba, respectively. The G4-605 line expresses GAL4 in many central neurons, but not in dMP2.

Immunofluorescence staining

Embryos were fixed and processed for indirect immunofluorescence microscopy as described[29]. Confocal images were taken on a Biorad MRC-1024 imaging head, mounted on a Zeiss Axioplan II microscope. Images were processed using Bio-Rad LaserSharp 2.1A,

Adobe Photoshop 4.0, Adobe Streamline 3.0 and Strata Studio Pro 2.5.3. We used the following primary antibodies: rabbit anti-Frazzled[3] (1:500 dilution); goat anti-Netrin-B[18] (1:500); rabbit anti-β-galactosidase (1:2,000, Cappel), goat anti-HRP (1:2,000, Cappel), monoclonal antibody 22C10 (ref. 30; 1:20, from Developmental Studies Hybridoma Bank (DSHB)), rabbit anti-GFP (1:100, Clontech); monoclonal antibody anti-Myc 9E10 (1:10, developed by J. M. Bishop, obtained from DSHB). Mutant embryos were identified using balancer chromosomes carrying β-galactosidase genes, or by staining with anti-Frazzled antibody (Fig. 2f, 4b) or anti-Netrin-B antibody (Fig. 3b). The rescue of the dMP2 phenotype by Frazzled expression in and outside dMP2 (Fig. 3j–m) was judged using the following criteria. To show the presence of the mutant phenotype (Fig. 3k, l), we scored the dMP2 pathway as abnormal if the $22C10^+$ fascicle was discontinuous at the segmental boundary. To demonstrate phenotypic rescue when Frazzled was expressed outside dMP2 (Fig. 3m), we scored as phenotypically normal hemisegments that had both of the following properties: (1) $22C10^+$ fascicle was present at the segmental boundary; and (2) an axon with dMP2-specific morphology was present after it has passed the segmental boundary. These criteria were chosen because there are several $22C10^+$ axons within the dMP2 fascicle at the segmental boundary. Our criteria underestimate both the dMP2 defect in Fig. 3k, l and the rescue of the phenotype in Fig. 3m.

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Intracellular calcium dependence of transmitter release rates at a fast central synapse

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Calcium-triggered fusion of synaptic vesicles and neurotransmitter release are fundamental signalling steps in the central nervous system. It is generally assumed that fast transmitter release is triggered by elevations in intracellular calcium concentration ($[Ca^{2+}]_i$) to at least 100 μM near the sites of vesicle fusion[1–5]. For synapses in the central nervous system, however, there are no experimental estimates of this local $[Ca^{2+}]_i$ signal. Here we show, by using calcium ion uncaging in the large synaptic terminals of the calyx of Held, that step-like elevations to only 10 μM $[Ca^{2+}]_i$ induce fast transmitter release, which depletes around 80% of a pool of available vesicles in less than 3 ms. Kinetic analysis of transmitter release rates after $[Ca^{2+}]_i$ steps revealed the rate constants for calcium binding and vesicle fusion. These show that transient (around 0.5 ms) local elevations of $[Ca^{2+}]_i$ to peak values as low as 25 μM can account for transmitter release during single presynaptic action potentials. The calcium sensors for vesicle fusion are far from saturation at normal release probability. This non-saturation, and the high intracellular calcium cooperativity in triggering vesicle fusion, make fast synaptic transmission very sensitive to modulation by changes in local $[Ca^{2+}]_i$.

The rapidly decaying, local $[Ca^{2+}]_i$ signal that triggers vesicle fusion in presynaptic terminals cannot readily be resolved with current imaging techniques. To circumvent this problem, we applied Ca^{2+} uncaging in large presynaptic terminals, the calyces of Held[6,7], located in the brainstem medial nucleus of the trapezoid body. During Ca^{2+} uncaging, cytosolic $[Ca^{2+}]_i$ is elevated in a spatially homogenous manner[8]. Therefore, fluorescent Ca^{2+} indicators can be used to measure directly the biologically relevant $[Ca^{2+}]_i$ acting at the sensor for vesicle fusion[9,10].

Calyces were loaded through whole-cell patch pipettes with mixtures of the photolysable Ca^{2+} chelator DM-nitrophen (1 mM) and the low-affinity fluorescent Ca^{2+} indicator fura-2 FF (Fig. 1a). The transmitter release activity was monitored by recording excitatory postsynaptic currents (EPSCs) from principal neurons with a second patch pipette (Fig. 1a). Cyclothiazide (CTZ; 100 μM) was present during most recordings to suppress the desensitization[11,12] of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors, which mediate fast EPSCs in these synapses.