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Abstract
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Reference


DOI : 10.1242/jcs.090852
PMID : 21896646

Available at:
http://archive-ouverte.unige.ch/unige:25597

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Influence of matrix metalloproteinase MMP-9 on dendritic spine morphology

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Accepted 28 May 2011
Journal of Cell Science 124, 3369–3380
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doi: 10.1242/jcs.090852

Summary
An increasing body of data has shown that matrix metalloproteinase-9 (MMP-9), an extracellularly acting, Zn$^{2+}$-dependent endopeptidase, is important not only for pathologies of the central nervous system but also for neuronal plasticity. Here, we use three independent experimental models to show that enzymatic activity of MMP-9 causes elongation and thinning of dendritic spines in the hippocampal neurons. These models are: a recently developed transgenic rat overexpressing autoactivating MMP-9, dissociated neuronal cultures, and organotypic neuronal cultures treated with recombinant autoactivating MMP-9. This dendritic effect is mediated by integrin β1 signalling. MMP-9 treatment also produces a change in the decay time of miniature synaptic currents; however, it does not change the abundance and localization of synaptic markers in dendritic protrusions. Our results, considered together with several recent studies, strongly imply that MMP-9 is functionally involved in synaptic remodelling.

Key words: Brain, Extracellular matrix, Plasticity, Proteases, MMP-9

Introduction
It has been clearly established that experience modifies functional circuits in the brain (Chklovskii et al., 2004). Furthermore, it has been shown that changes in the morphology of dendritic spines, carrying postsynaptic domains of excitatory synapses, might be involved in synaptic plasticity, as well as in learning and memory (Holtmaat and Svoboda, 2009; Holtmaat et al., 2006; Moser et al., 1994; Xu et al., 2009). Moreover, induction of long-term potentiation (LTP) is associated with spine growth (De Roo et al., 2008b; Matsuzaki et al., 2004; Yang et al., 2008), whereas induction of long-term depression (LTD) is associated with spine shrinkage (Zhou et al., 2004).

Recent studies have indicated that spine structure can be regulated by extracellular matrix (ECM) proteins, such as reelin (Niu et al., 2008), as well as cell surface proteins [e.g. N-cadherin (Mysore et al., 2007), ephrin receptors (Moeller et al., 2006) and integrins (Shi and Ethell, 2006)]. Whereas the exact mechanism of this regulation is poorly understood, extracellularly acting proteases targeting ECM and/or surface proteins have recently been implicated in different forms of neuronal plasticity (Brown et al., 2009; Dityatev et al., 2010; Mizoguchi et al., 2007; Pizzorusso et al., 2002; Rivera et al., 2010).

Matrix metalloproteinases (MMPs) are predominantly secreted extracellular endopeptidases that can modify ECM components and control cell behavior (Mott and Werb, 2004; Sterllicht and Werb, 2001). Their expression and activity are tightly regulated; they are expressed (often in response to a cell activation) in an inactive form and require enzymatic processing in order to reveal the catalytic site. Once activated, they can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) but their activity is also regulated by glycosylation and internalization (Yong, 2005). Previously MMP-9 was believed to be associated mainly with pathologies of the brain, such as ischemia, gliomas or epilepsy (Asahi et al., 2000; Gu et al., 2002; Wilczynski et al., 2008; Yong, 2005); however, recently its involvement in brain physiology has been partially elucidated (Nagy et al., 2006; Szklarczyk et al., 2002).

An involvement of MMPs in modulation of morphology of dendritic spines has recently been observed. Bilousova et al. (Bilousova et al., 2006) showed that MMP-7, in an N-methyl-D-aspartate (NMDA)-dependent manner, appears to cause transformation of mature mushroom-shaped spines into long filopodia-like structures in cultures of dissociated neuronal cells. Tian et al. (Tian et al., 2007), also in dissociated cultures, showed that either MMP-2 or MMP-9, through cleavage of the intercellular adhesion molecule-5 (ICAM-5), can also cause elongation of dendritic filopodia. Furthermore, Wang et al. (Wang et al., 2008) showed that in acute hippocampal slices MMP-9 was...
necessary for the enlargement of spines associated with LTP induction. Notably, Bilousova et al. (Bilousova et al., 2009) showed that in the fragile X mouse model (Fmr1-knockout mice) there was an increase in the ratio of filopodia to mature spines; this effect could be reversed by minocycline, whose pleiotropic effects include the ability to inhibit MMP-9 expression. Furthermore, an incubation of a dissociated neuronal cell culture with recombinant MMP-9 caused transformation of dendritic spines from mushroom- to filopodia-like protrusions (Bilousova et al., 2009).

Here, we set out to verify directly the effects of MMP-9 on spine morphology. Recombinant autoactivating MMP-9 (Fisher et al., 2002) was either introduced into the rat brain in the form of a neuronally overexpressed transgene, or produced in a heterologous baculoviral expression system as a recombinant protein. Because MMP-9 has been shown to exert some effects independently of its enzymatic activity (e.g. through specific protein–protein interactions) (Ezhilarasan et al., 2009; Redondo-Munoz et al., 2010), we also produced a non-enzymatically-active form of the MMP-9 and tested its effects on spine morphology.

Results

Transgenic rats overexpressing an autoactivating mutant of MMP-9 display longer and thinner dendritic spines

In order to observe the influence of enzymatic activity of MMP-9 on dendritic spines in vivo, we compared spine shapes in transgenic rats overexpressing autoactivating mutants of MMP-9 under the control of the synapsin I promoter (MMP-9 transgenic rats; MMP-9 TR) with spines in wild-type (WT) rats (Wilczynski et al., 2008). Morphometric analysis of spines in neurons stained with a lipophilic dye, DiI was carried out in the CA1 area of the hippocampus (Fig. 1A). In our studies, we used a scale-free parameter, the length-to-width ratio (i.e. the length divided by the width), which reflects the spine shape (see Discussion) and thus effectively describes the spine form. MMP-9 TR rats displayed an larger average length-to-width ratio (2.845 ± 0.119, n=4 rats) than did WT rats (2.057 ± 0.112, n=4 rats) (Student’s t-test revealed that there was a significant difference in the spine shape parameter, length:width, of MMP-9 TR compared with WT rats; t=3.914, P=0.0021; Fig. 1B). Interestingly, the spine density in MMP-9 TR was unchanged in comparison with WT rats (Student’s t-test did not show a significant difference; t=0.4178, P=0.6907; Fig. 1C). We did not observe differences in spine length between WT rats (1.782 ± 0.0434 μm) and MMP-9 TR (1.826 ± 0.0258 μm) (t=0.8843, P=0.4106); however, there was a significant difference (t=2.497, P=0.0467) in the width of the spine head between WT rats (0.7292 ± 0.0280 μm) and MMP-9 TR (0.6443 ± 0.0193 μm).

In addition, spine morphology was analysed using transmission electron microscopy and subsequent three-dimensional reconstruction of series of ultrathin sections, and we also performed analyses of synaptic densities and the categories of synapses. There were no differences in synaptic densities between WT rats (294.61 ± 19.883 synapses per 100 μm²) and MMP-9 TR (323.56 ± 27.53 synapses per 100 μm²) (F₁,₈=0.7263; P=0.4421). To determine whether there were changes in the morphology of mushroom and thin dendritic spines, we reconstructed 75 mushroom and 75 thin spines and their postsynaptic densities (PSDs) from WT and TR rats. Fig. 1D shows examples of these two categories of spines (the left-hand panel of Fig. 1D shows four electron micrographs, and the right-hand panel reconstructions of two mushroom and two thin spines). Fig. 1E shows that there was a statistically significant decrease in the proportion of mushroom spines in CA1 area of MMP-9 TR (10.62 ± 0.92) compared with WT rats (15.1 ± 1.17) (F₁,₈=9.0588; P=0.0395) with a corresponding increase in the proportion of thin spines in MMP-9 TR (86.69 ± 1.46) compared with WT rats.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Transgenic rats overexpressing an autoactivating mutant of MMP-9 have longer and thinner dendritic spines. (A) Examples of DiI-stained neurons in the CA1 area of rat hippocampus of wild-type (WT) and transgenic rats overexpressing the autoactivating mutant of MMP-9 under the control of the synapsin I promoter (MMP-9 TR). Pictures represent secondary apical dendrites. (B) Cumulative frequency of the shape parameter (length/width) of spines in WT and MMP-9 TR rats. Student’s t-test revealed a significant difference in the spine shape parameter of WT compared with MMP-9 TR rats (t=3.914; P=0.0021). (C) Mean (+ s.e.m.) spine density in WT and MMP-9 TR rats. Student’s t-test did not show significant differences (t=0.5225; P=0.6154). (D) Examples of mushroom and thin spines in the CA1 area: the left-hand panel shows four electron microscopic images of two mushroom (sp1, sp2) and two thin (sp3, sp4) dendritic spines; the right-hand panel shows a three-dimensional reconstruction of these four spines. PSD, post-synaptic density; head, spine head; neck, spine neck; den, dendrite; axon, presynaptic varicosity; SA, spine apparatus. (E) Redistribution of dendritic spines between the four main categories of spines and synapses (mean ± s.e.m.). One-way ANOVA showed that there is a significant decrease in the proportion of mushroom spines in MMP-9 TR compared with WT (F=9.0588, P=0.0395), whereas the proportion of thin spines increases (F=11.2548; P=0.0284).
(81.39 ± 0.66) (F1,8 = 11.2549; P = 0.02844). However, no changes were found in the proportion of stubby spines and shaft synapses (Fig. 1E). No changes were found in the size of mushroom and thin spines and their PSDs in MMP-9 TR compared with WT rats. To describe possible changes in spine shape in the active zone area, we analyzed the curvature of the PSD area of mushroom and thin spines [as described in the Materials and Methods and in Popov et al. (Popov et al., 2008)] but no significant changes were found in spine curvature. The curvature of mushroom spines in the PSD area was –4.66 ± 1.40° for WT rats and –0.19 ± 1.33° for MMP-9 TR (F1,8 = 0.6148; P = 0.4768); the curvature of thin spines in the PSD area was, respectively, –6.25 ± 0.53° for WT and –1.34 ± 0.62° for MMP-9 TR rats (F1,8 = 1.1313; P = 0.3474).

Production of autoactivating MMP-9 and its non-active analogue

In order to examine the influence of MMP-9 on the morphology of dendritic spines, we applied a recombinant autoactivating mutant of MMP-9 (Fisher et al., 2002). For protein expression we used the Bac-to-Bac baculovirus expression system. Recombinant baculovirus was used for infection of High-Five cells and conditioned medium was collected for analysis of expression by gel zymography. At 24, 48 and 72 hours after infection, medium was collected and equal volumes were subjected to gel zymography. The largest expression level was observed at 48 hours post infection (Fig. 2A). We then used the cell medium at 48 hours after infection with baculovirus for purification of the recombinant autoactivating mutant of MMP-9, using affinity chromatography on gelatine–Sepharose resin. The left-hand panel of Fig. 2B shows an SDS-PAGE gel of the recombinant MMP-9 after purification, and the protein identity was confirmed by western blotting and gel zymography (Fig. 2B middle and right-hand panel respectively). In order to obtain the non-enzymatically active form of MMP-9, glutamate 402 in the catalytic centre of the autoactivating mutant was replaced by alanine, so that enzyme activity was lost. To produce the recombinant inactive mutant (E402A) of autoactivating MMP-9 we again used the Bac-to-Bac baculovirus expression system. The expression and purification procedure was the same as for autoactivating MMP-9. We obtained protein with the same molecular mass as autoactivating MMP-9 and with the same immunoreactivity with anti-human-MMP-9 antibody, but without enzymatic activity, as determined by gel zymography (Fig. 2B).

We then checked the enzymatic activity of the obtained recombinant MMP-9 and MMP-9 E402A in solution using DQ-gelatine, a standard fluorescence substrate for gelatinases. As expected, MMP-9 displayed strong enzymatic activity in solution, whereas MMP-9 E402A did not (Fig. 2C). We also tested the potency of commercially available inhibitors of MMPs towards recombinant MMP-9 at standard concentration employed in our experiments (i.e. 400 ng/ml). Indeed the broad-spectrum MMP inhibitor GM6001 and the more specific Inhibitor-I were able to effectively decrease the activity of MMP-9. Importantly, 0.1% DMSO, which was a solvent for both inhibitors, in solution affected MMP-9 activity only slightly (Fig. 2C).

Live imaging of neurons in organotypic hippocampal cultures reveals the influence of active MMP-9 on dendritic spines

To observe the influence of MMP-9 on spines in a simpler model than live animals, and one which allows easier manipulation and temporal observations, we employed an organotypic hippocampal culture and live imaging of the dendrites. After transfection with a pcDNA3-eGFP plasmid using a biolistic method, hippocampal pyramidal neurons of CA1 area, which expressed eGFP, were visualized in a confocal microscope. Slices were treated with either recombinant MMP-9 or MMP-9 E402A, and images were taken following 30 and 90 minutes of incubation (Fig. 3A–C). The length-to-width parameter was used to evaluate the spine shape and a one-way ANOVA showed a significant difference between groups in cultures incubated with the MMP-9 (F = 8.206, P = 0.0023; n = 8 cells) and post-hoc Tukey test reached significance for t = 0 (2.930 ± 0.100) compared with the MMP-9 incubation for 30 minutes (3.433 ± 0.1564) (P = 0.05) and for
90 minutes (3.713 ± 0.152) (P = 0.001). We observed no changes in the shape parameter (length/width, L:W) in cultures treated with inactive protease MMP-9 E402A (one-way ANOVA failed to reach significance; $F = 0.04065, P = 0.9602; n = 9$ cells), which was found to be: L:W$_{t=0}$ = 3.214 ± 0.196; L:W$_{t=30}$ = 3.197 ± 0.124 and L:W$_{t=90}$ = 3.264 ± 0.188.

The enzymatic activity of MMP-9 causes changes in dendritic spine morphology producing longer and thinner spines in dissociated cultures

To investigate further the influence of MMP-9 on morphology of dendritic spines, and to extend the results obtained with the aforementioned models, we used dissociated hippocampal cultures. Neurons were transfected with plasmid vector carrying eGFP under the control of the β-actin promoter and after 15 days in vitro the cells were incubated with recombinant autoactivating form of MMP-9 for either 30 or 90 minutes. As a control, we used hippocampal cultures incubated with either the non-active mutant of MMP-9 or a buffer devoid of recombinant proteins. After fixing the cultures, we utilized immunofluorescence labelling of neurons expressing eGFP and analysed the density and morphology of protrusions (Fig. 4B–E). Notably, MMP-9 treatment did not affect the overall morphology of the cultures and there was no visible change in neurites (Fig. 4A). Moreover, incubation with MMP-9 did not affect the total density (mean density in buffer after 90 minutes, 1.058 ± 0.106 μm$^{-1}$; mean density with MMP-9 E402A after 90 minutes, 1.025 ± 0.101 μm$^{-1}$; mean density with MMP-9 after 30 minutes, 1.103 ± 0.04120 μm$^{-1}$; mean density MMP-9 after 90 minutes, 0.9819 ± 0.06754 μm$^{-1}$; $F = 0.3787, P = 0.7694, n = 6$ cells) of dendritic protrusions (Fig. 4C).

However, a one-way ANOVA of the shape parameter revealed differences between groups ($F = 15.61, P < 0.0001, n = 6$ cells) and a post-hoc Tukey test showed that by 30 minutes of incubation with MMP-9 there were significant increases in the length/width parameter, which by 90 minutes of incubation were increased further (L:W with buffer for 90 minutes, 1.883 ± 0.05692; L:W with MMP-9 for 30 minutes, 2.509 ± 0.1255; L:W with MMP-9 for 90 minutes, 2.940 ± 0.2199) (Fig. 4D). This effect can be visualized more easily by a cumulative frequency distribution of analysed spines in each group (Fig. 4E). Furthermore, we were interested to determine whether the enzymatic activity of MMP-9 is necessary to induce changes in morphology of spines. Hence, we incubated cultures for 90 minutes with the inactive mutant MMP-9 E402A; this did not influence either the shape of dendritic protrusions or their overall density (L:W MMP-9 E402A, 1.806 ± 0.1153) (Fig. 4B–E). We did not observed significant changes either in the length of protrusions (length in buffer after 90 minutes, 1.165 ± 0.06998 μm; length with MMP-9 E402A after 90 minutes, 1.301 ± 0.1114 μm; length with MMP-9 after 30 minutes, 1.419 ± 0.1047 μm; length with MMP-9 after 90 minutes, 1.746 ± 0.3431 μm; $F = 1.685, P = 0.2023, n = 6$ cells) or in the width of spine heads (width in buffer after 90 minutes 0.6208 ± 0.04934 μm; width with MMP-9 E402A after 90 minutes, 0.7742 ± 0.06082 μm; width with MMP-9 after 30 minutes, 0.5818 ± 0.02733; width with MMP-9 after 90 minutes, 0.6580 ± 0.07878 μm; $F = 2.110, P = 0.1310, n = 6$ cells).

Enzymatic activity of MMP-9 does not change the abundance of the presynaptic marker protein bassoon or its localization in the protrusions

To characterize further the influence of MMP-9 on synaptic morphology and to check whether the longer and thinner protrusions are potentially functional, we analysed the abundance of the presynaptic marker protein bassoon and the postsynaptic marker homer 1, which are often used to distinguish mature spines and synapses (Grabrucker et al., 2009; Konopka et
MMP-9 influences dendritic spines

Fig. 4. MMP-9 enzymatic activity makes dendritic spines in dissociated hippocampal culture longer and thinner but does not change the dendritic arbor.
(A) Live imaging of 15 DIV hippocampal neurons, expressing eGFP, incubated with MMP-9 does not reveal changes in the structure and number of neurites. (B) Maximal projections of confocal scans of a dissociated hippocampal culture transfected with a plasmid carrying eGFP under the control of the β-actin promoter. After reaching an age of at least 15 DIV, cells were incubated for 30 and 90 minutes with 400 ng/ml of active MMP-9, or for 90 minutes with inactive MMP-9 E402A or the buffer used for elution of recombinant protein from the affinity column. (C). Mean (± s.e.m.) protrusion density. One-way ANOVA did not reveal significant differences between groups of mean protrusions density calculated per cell (F = 0.936; P = 0.4353). (D) Mean (± s.e.m.) shape parameter (length/width). One-way ANOVA revealed significant differences between groups of mean shape parameter calculated per cell (F = 15.61; P < 0.0001). Post-hoc Tukey’s tests reached significance for buffer compared with MMP-9 at 30 minutes (*P < 0.05) and 90 minutes (**P < 0.001), for MMP-9 E402A compared with MMP-9 at 30 minutes (**P < 0.01) and for MMP-9 E402A compared with MMP-9 at 90 minutes (**P < 0.001). (E) The cumulative frequency of the shape parameter (length/width).

The enzymatic activity of MMP-9 affects spine physiology

Because it is known that alterations of spine morphology associated with plasticity phenomena, such as LTP and LTD, are accompanied by profound functional changes at glutamatergic synapses, we wanted to test whether MMP-9 activity, besides affecting spine geometry, also influences the electrophysiological properties of synapses in our model. To this end, we used patch-clamping and recorded glutamatergic miniature excitatory synaptic currents (mEPSCs) in the whole-cell mode (V_m = −70 mV) from dissociated hippocampal neurons. Neurons were incubated in the presence of autoactivating MMP-9, MMP-9 E402A or protein buffer for up to 90 minutes and the impact of treatment was checked after 30, 60 and 90 minutes. We found that such treatment did not significantly affect the mean current amplitude of minis (31.38 ± 4.02 pA, n = 6; 34.08 ± 4.05 pA, n = 7; 28.53 ± 3.07 pA, n = 7 for MMP-9, E402A and buffer, respectively; P > 0.05, unpaired Student’s t-test, Fig. 6A,B). However, the functional impact of synaptic currents depends not only on amplitude but also on the timecourse. In particular, synaptic current duration (typically assessed as the weighted decay time constant) can strongly affect synaptic integration and this parameter is known to undergo a developmental increase that is strictly correlated with alterations in synapse geometry (Cathala et al., 2005; Wall et al., 2002). Treatment of neurons with MMP-9, MMP-9 E402A or buffer had no significant effect on the mEPSC decay time course in neurons treated for 30 or 60 minutes (data not shown). However, 90 minutes of treatment with MMP-9, resulted in a significant increase in the decay time constant with respect to buffer- or MMP-9-E402A-treated neurons (5.96 ± 0.91 ms, n = 7; 3.89 ± 0.53 ms, n = 6; 3.61 ± 0.51 ms, n = 6 for MMP-9, MMP-9 E402A and buffer,

al., 2010; Petrini et al., 2009; Verpelli et al., 2010). We incubated dissociated hippocampal cultures at 15 days in vitro (DIV) with buffer, recombinant MMP-9 or MMP-9 E402A for 90 minutes, then fixed the specimens and stained for the synaptic markers using immunofluorescence. One-way ANOVA did not reveal any changes in localization of synaptic markers in dendritic spines between the examined groups, measured as the percentage of bassoon- or homer-1-positive protrusions: bassoon with buffer, 62.67 ± 1.138%, n = 13 cells; bassoon with MMP-9-E402A, 66.41 ± 1.340%, n = 8 cells, bassoon with MMP-9, 63.19 ± 1.564%, n = 11 cells; F = 1.904, P = 0.1671 (Fig. 5A–C); homer with buffer, 62.28 ± 1.66%, n = 13 cells, homer with MMP-9-E402A, 66.67 ± 1.71%, n = 8 cells, homer with MMP-9, 63.61 ± 1.60%, n = 11 cells; F = 1.605, P = 0.2183 (Fig. 5D–F). We also did not observe any changes in the density of synaptic markers after incubation with recombinant MMP-9, measured as number of clusters per μm of examined dendrite (bassoon with buffer, 1.041 ± 0.0513 μm⁻¹, n = 13 cells, bassoon with MMP-9-E402A, 1.090 ± 0.0609 μm⁻¹, n = 8 cells, bassoon with MMP-9, 0.9791 ± 0.09308 μm⁻¹, n = 11 cells; F = 0.518, P = 0.5875; homer with buffer, 1.028 ± 0.0658 μm⁻¹, n = 13 cells, homer with MMP-9-E402A, 1.053 ± 0.0810 μm⁻¹, n = 8 cells, homer with MMP-9, 0.8091 ± 0.0760, n = 11 cells; F = 2.792, P = 0.0778).
P, unpaired Student’s t-test, Fig. 6A,C). The MMP-9-induced slowdown of the mEPSC decay phase with respect to buffer or MMP-9-E402A-treated groups is particularly clear in the cumulative distribution of the decay time constants (Fig. 6D). Treatment with MMP-9 (or with MMP-9 E402A) for up to 90 minutes had no effect on mEPSC onset kinetics (data not shown).

The MMP-9 enzymatic activity influences spine morphology through engagement of integrin β1 subunit

It has been shown previously that MMP-9 can exert its function in neurons through engaging the integrin β1 subunit (Michaluk et al., 2009; Nagy et al., 2006; Wang et al., 2008) and that integrins are involved in spine regulation (Bourgin et al., 2007; Shi and Ethell, 2006). Therefore, we decided to block the function of integrin β1 with a specific antibody and determine whether this would affect the action of MMP-9 on spine morphology. Dissociated hippocampal cultures were pretreated with either anti-integrin-β1 antibody or an isotype antibody (IgM), as a control, and were later incubated with either MMP-9 or MMP-9 E402A for 30 minutes (Fig. 7A). We did not observe any significant differences in spine density between the analysed groups (mean with IgM plus MMP-9 E402A, 0.7981 ± 0.0362 μm−1, n=16 cells; mean with IgM plus MMP-9, 0.733 ± 0.041 μm−1, n=17 cells; mean with anti-integrin-
Fig. 6. MMP-9 enzymatic activity influences the kinetics of the synaptic current. (A) Example of normalized and superimposed typical mEPSCs recorded in control conditions (black) and after 90 minutes of treatment with MMP-9 (red). (B) Mean (absolute; ± s.e.m.) values of mEPSCs amplitudes recorded after 90 minutes of treatment with buffer, MMP-9 E402A or MMP-9. (C) mEPSC deactivation time constant ($\tau_{\text{decay}}$) (mean ± s.e.m.) for currents recorded after 90 minutes of treatment with buffer, MMP-9 E402A or MMP-9. (D) Cumulative distribution of mEPSC time constants for currents recorded after 90 minutes of treatment with buffer, MMP-9 E402A or MMP-9. Cumulative distributions were constructed with data obtained from all cells included in the statistics but each cell is represented by an equal number (100) of mEPSCs.

Discussion

Using three independent neuronal models (one in vivo and two in vitro) we show that excessive MMP-9 drives dendritic spines to change their shape towards being longer and thinner, which was characterized by an increase in a shape parameter (length/width). Moreover, we show that this process is mediated through integrin β1, as its blockade with a specific antibody abolished the effect on spine morphology caused by MMP-9. Furthermore, we provide evidence that changes in spine morphology can be

β1 antibody and MMP-9 E402A, 0.8492 ± 0.04130 μm$^{-1}$, $n=16$ cells, mean with anti-integrin-β1 antibody and MMP-9, 0.8279 ± 0.05236 μm$^{-1}$, $n=15$ cells) (Fig. 7B). Two-way ANOVA did not reveal any significant differences for the antibody factor ($F=2.91$, $P=0.093$), for the MMP-9 factor ($F=1.02$, $P=0.32$) or for the interaction of both ($F=0.26$, $P=0.61$). Anti-integrin antibody did not influence spine shape parameter in the presence of inactive MMP-9 E402A in comparison with the presence of isotype antibody (mean with anti-integrin-β1 antibody and MMP-9 E402A, 2.14 ± 0.045, $n=16$ cells; mean with IgM plus MMP-9, 2.21 ± 0.041, $n=16$ cells); however, it completely abolished the increase in the shape parameter caused by MMP-9 (mean with IgM plus MMP-9, 2.51 ± 0.065, $n=17$ cells; mean with anti-integrin-β1 antibody and MMP-9, 2.18 ± 0.061, $n=15$ cells) (Fig. 7C,D). Two-way ANOVA reached significance for the antibody factor ($F=13.3$, $P=0.0006$), for the MMP-9 factor ($F=9.59$, $P=0.003$) and moreover for interaction of both ($F=6.05$, $P=0.017$). Post-hoc Tukey’s analysis revealed significant differences for IgM plus MMP-9 E402A compared with IgM plus MMP-9 ($P=0.0011$); anti-integrin-β1 antibody and MMP-9 compared with IgM plus MMP-9 ($P=0.00048$) and for anti-integrin-β1 antibody plus MMP-9 E402A compared with IgM plus MMP-9 ($P=0.0002$).

Fig. 7. MMP-9 enzymatic activity influences spine morphology through the integrin β1 subunit. (A) Representative images of two-week-old neurons in dissociated culture expressing eGFP preincubated with anti-integrin-β1 antibody (β1Ab) or isotype antibody as a control (IgM) and later treated for 30 minutes with either MMP-9 or MMP-9 E402A as a control. (B) Mean (± s.e.m.) of protrusion density. Two-way ANOVA did not reveal any significant differences for antibody factor ($F=2.91$; $P=0.093$), for MMP-9 factor ($F=1.02$; $P=0.32$) or for the interaction of both ($F=0.26$; $P=0.61$). (C) Mean (± s.e.m.) of the shape parameter (length/width). Treatment with MMP-9 in the presence of IgM causes an increase in the shape parameter, but preincubation with β1Ab completely abolishes this effect. Two-way ANOVA reached significance for antibody factor ($F=13.3$; $P=0.0006$), for MMP-9 factor ($F=9.59$; $P=0.003$) and, moreover, for the interaction of both ($F=6.05$; $P=0.017$). Post-hoc Tukey’s analysis revealed significant differences for IgM + E402A compared with IgM + MMP-9 ($P=0.0011$); β1Ab + MMP-9 compared with IgM + MMP-9 ($P=0.00048$) and for β1Ab + E402A compared with IgM + MMP-9 ($P=0.0002$). (D) Cumulative frequency graph of the shape parameter (length/width) of spines in all groups.
associated with modifications of the decay time of synaptic currents.

To study the influence of MMP-9 on spines in vivo we used transgenic rats overexpressing autoactivating MMP-9 under the control of the synapsin I promoter. In comparison with control (WT rats), intensified MMP-9 activity caused an increase in the spine length/width ratio. Furthermore, we confirmed our light microscopy data with electron microscopy, demonstrating clearly that there is an increase in the largest spine category, thin spines, at the expense of mushroom spines, which decrease as a percentage of the total spine complement. Interestingly, Bilousova et al. have observed a decreased spine-head area and increased length of spines in CA1 and CA3 regions of the hippocampus in Fmr1-knockout mouse, which among various pathological features overexpresses MMP-9 (Bilousova et al., 2009). This phenotype was rescued by treatment with minocycline, a tetracycline derivative that, among other effects, decreases the expression and activity of MMP-9 (Yao et al., 2004).

To avoid subjective discrimination between spines and filopodia we utilized a shape parameter defined as the ratio of the length to the maximal width of the spine. We decided to do this because there is no coherent definition of a filopodium and many research groups use different shape parameters to distinguish between these structures and, moreover, there is also no clear functional criterion of filopodia.

For our studies, we have produced a recombinant autoactivating mutant of MMP-9, as well as its inactive form MMP-9 E402A, and characterized both proteins, which were then used in hippocampal cultures. These new experimental tools allowed us to address, for the first time, directly the issue of whether enzymatic activity and not just protein–protein interactions are responsible for the effect of MMP-9 on spines. This was important because MMP-9 has several structural protein domains and has been shown to have biological activity in the absence of enzymatic activity (Ezhilarasan et al., 2009; Redondo-Munoz et al., 2010). Furthermore, it has been reported that MMP-9 is capable of binding many surface proteins, such as integrins (Bjorklund et al., 2004; Redondo-Munoz et al., 2008; Stefanidakis et al., 2003), LRP-1 (Van den Steen et al., 2006) and CD44 (Bourguignon et al., 1998). We demonstrated clearly that lack of enzymatic activity renders the MMP-9 inactive mutant MMP-9 E402A incapable of affecting either spine morphology or changing the shape parameter in hippocampal cultures.

Initially, the effects of the recombinant MMP-9 proteins were demonstrated in live organotypic hippocampal cultures. Wang et al. (Wang et al., 2008) also noted the effect of application of recombinant MMP-9 on spine morphology, namely on increasing spine-head volume. However, no detailed analyses of the spine geometry were provided in that study. To extend further our observations we also employed dissociated hippocampal cultures, which in this case are more amenable to experimental manipulation. Incubation of neurons at 15 DIV with recombinant MMP-9 also caused a significant increase in length/width parameter, which was notable after only 30 minutes of incubation. This result is in agreement with previous observations of Bilousova et al. (Bilousova et al., 2009), who also applied recombinant MMP-9 for 1 hour in dissociated hippocampal culture and demonstrated an increase in the number of filopodia-like long spines with small heads and a concomitant decrease in the number of mushroom-shaped short spines with large heads. Furthermore, it was recently shown that 6 hours of treatment with 5 μM NMDA caused an increase in spine number, and the appearance of new small spines and maturation of existing spines, which was blocked by both the MMP-2 and MMP-9 inhibitor, and by the knockout of ICAM-5, the presumed MMP-2 and MMP-9 substrate (Tian et al., 2007). The proposed model assumes that MMP-2 and/or MMP-9 enzymatic activity, upon neuronal stimulation, leads to maturation of spines and elongation of filopodia (Tian et al., 2007). Interestingly, the recent findings of Conant et al. (Conant et al., 2010) support the notion that ICAM-5 cleavage by MMPs (also including MMP-3 and MMP-7) can occur as early as 15 minutes after neuronal stimulation by either NMDA treatment or induction of LTP.

We have also shown that the changes in spine morphology induced by MMP-9 treatment are not accompanied by significant changes in the abundance and localization of the presynaptic marker bassoon, which suggests that MMP-9 activity does not affect synaptic contacts of dendritic protrusions. Moreover, we observed that incubation of neurons with MMP-9 caused a slowing of the mEPSC decay phase (Fig. 6A–D) without affecting the absolute amplitude. The increase in decay time is intriguing, as neuronal development comprises both conversion of spine shape (from filopodial into mushroom-like) and an increase in synaptic currents (Cathala et al., 2005; Wall et al., 2002). Thus, the appearance of filopodia and slowing of mEPSCs might suggest an MMP-9-induced ‘juvenilization’ of glutamatergic synapses in our model. However, the precise mechanism of mEPSC slowing described here is not clear. Several potential processes might underlie the change in the timecourse of mEPSCs, including a subunit switch (Kumar et al., 2002), lateral receptor mobility (Heine et al., 2008), endocytosis or exocytosis of receptors (Lee et al., 2004), changes in synapse geometry (Barbour et al., 1994; Cathala et al., 2005) and post-translational modulation of synaptic receptors (Lee, 2006). Notably, Cathala et al. (Cathala et al., 2005) have attributed the developmental speeding of glutamatergic EPSCs, to changes in the synapse geometry, a mechanism that might be consistent with the changes in spine shape and mEPSC kinetics that we observed here. However, it should be stressed that the induction of filopodial spine shape by MMP-9, as described here, need not be the only mechanism whereby these enzymes could affect the glutamatergic synapse. For instance, MMPs could change the synaptic cleft geometry, the relative localization of releasing sites and of postsynaptic densities and alter the immediate surrounding of the synapse (thus affecting the diffusion of the agonist to the receptors), giving rise to a change in synaptic glutamate transients, thereby altering mEPSC kinetics. Moreover, the fact that 30 minutes of treatment with MMP-9 was sufficient to induce a significant alteration in spine shape, whereas 90 minutes were needed for MMP-9 to induce a change in the mEPSC kinetics, suggests that additional factors, other than spine geometry, might also be involved. Further studies are needed to elucidate the extent to which MMP-9 treatment modulates the shape and function of glutamatergic synapses by either common or separate mechanisms.

Finally, we have shown that the influence of MMP-9 on spines depends on integrin β1 activation, which agrees with previous reports that MMP-9 acts through this integrin subunit (Michaluk et al., 2009; Nagy et al., 2006; Wang et al., 2008) and that MMP-9 can bind integrins (Redondo-Munoz et al., 2008; Rolli et al., 2003; Stefanidakis et al., 2003). The influence of integrins on spine morphology reported previously agrees with those results (Shi and Ethell, 2006). In particular, RGD peptides that mimic integrin ligands, and can stimulate integrin signalling, can cause elongation of spines and formation of new filopodia (Shi and
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Ethell, 2006). We suggest that all of these results could be interpreted in the following way: MMP-9, by virtue of its proteolytic activity, cleaves some, yet to be defined, ECM component or cell adhesion molecule and thus releases cryptic ligand-binding integrins. MMP-9 has been shown previously to produce such ligands in other tissues (Dityatev et al., 2010; Van den Steen et al., 2000; Xu et al., 2001).

In conclusion, our data, considered together with those from several recent studies, strongly indicate that MMP-9 can be functionally involved in synaptic remodelling. Such an activity might explain the physiological role of MMP-9 in LTP, as well as in learning and memory (Meighan et al., 2006; Nagy et al., 2006; Okulski et al., 2007) and in pathological conditions implicating neuronal plasticity (e.g. epilepsy and drug addiction) (see Kim et al., 2009; Mash et al., 2007; Rivera et al., 2010; Samochowiec et al., 2010; Takacs et al., 2010; Wilczynski et al., 2008; Yamada, 2008).

Materials and Methods

Transgenic rats
All animal experiments were performed according to approved guidelines. Transgenic rats overexpressing autoactivating MMP-9 under the control of the synapsin I promoter (Wilczynski et al., 2008) were injected with pentobarbital (200 mg per kg of body weight; intraperitoneal injection) and perfused transcardially, first with PBS (15 ml per rat) and then with 4% paraformaldehyde (PFA, 60 ml per rat). Brains were cut out into 300-μm-thick vibratome slices. Random dendrite labelling with a gene gun, using 1.6 μm tungsten particles (Biorad) coated with propelled lipophilic fluorescent dye (DiI; Invitrogen), was performed for the spine classification. Slices were incubated in 1.5% PFA for 48 hours. Images of secondary apical dendrites (50 μm–200 μm) from the cell soma of the CA1 field of hippocampi were acquired under 561-nm fluorescent illumination using a confocal microscope (633 objective, 1.4 NA) at a pixel resolution of 1024 × 1024 with a 3.43 zoom, which resulted in a 72-nm pixel size. Slices were measured and analysed using ImagJ software (NIH). There were 4 animals (n=4 rats) in each group for the spine analyses. From each of the animals, at least 200 spines were analysed, which were made at least two neurons per animal and two dendrites of an overall dendritic length of 250 μm per neuron. This result was from more than 1200 spines measured for both wild-type and transgenic rats.

Transmission electron microscopy
Brains for three-dimensional analyses were cut into 50-μm-thick vibratome slices (VT1000; Leica, Milton Keynes, UK) and transferred to 2.5% glutaraldehyde in 0.1 M PBS. After washing with buffer, the tissue was osmicated in 2% osmium tetroxide (4 hours) and dehydrated in graded aqueous solutions of ethanol from 40% to 96% (each for 10 minutes) and then 100% acetone (three changes, each for 10 minutes). Specimens were infiltrated with a mixture of 50% epoxy resin and 50% pure acetone (30 minutes) and then 100% acetone (three changes, each for 10 minutes). Images of secondary apical dendrites (50 μm–200 μm) from the cell soma of the CA1 field of hippocampi were acquired under 561-nm fluorescent illumination using a confocal microscope (633 objective, 1.4 NA) at a pixel resolution of 1024 × 1024 with a 3.43 zoom, which resulted in a 72-nm pixel size. Slices were measured and analysed using ImagJ software (NIH). There were 4 animals (n=4 rats) in each group for the spine analyses. From each of the animals, at least 200 spines were analysed, which were made at least two neurons per animal and two dendrites of an overall dendritic length of 250 μm per neuron. This result was from more than 1200 spines measured for both wild-type and transgenic rats.

Digital reconstructive analysis
Serial sections were aligned as JPEG images (software available from http://synapses.clm.utexas.edu/). Alignments were made with full-field images. Stereological analysis was performed as described previously (Harris, 1994; Popov et al., 2004), with tissue volumes of ~500–800 μm³. Because synapse density is markedly influenced by elements occurring non-uniformly in the sample areas (i.e. myelinated axons, cell bodies, non-synaptic interneuron dendrites and large dendrites with section profiles that are >0.94 μm²), the areas of these elements were measured and subtracted from the sample areas to obtain the homogeneous neuropil area to avoid bias in the data obtained, as described previously (Harris, 1994; Popov et al., 2004). Synaptic densities were expressed as the number of synapses [identified through postsynaptic densities (PSD)] and the presence of at least two presynaptic vesicles] per 100 μm² of tissue. Synaptic densities were expressed as the number of synapses (identified through PSDs and the presence of at least two presynaptic vesicles) per 100 μm² of tissue. Spines and synapses were categorized as described previously (Harris et al., 1992; Peters and Kaiserman-Abramof, 1970). We thus distinguished three spine categories: mushroom, where the spine head is large and considerably in excess of the spine neck diameter; thin, where the height is usually several times in excess of the width; and stubby, where the spine protrudes only slightly from the dendritic shaft. Shaft synapses are a fourth category where the spine contacts directly the dendritic shaft. There were three animals in each group for the three-dimensional analyses, which were made from the stratum radiatum of CA1; 25 thin and 25 mushroom spines were reconstructed to analyse spine volume, surface area and curvature of the PSD, as described previously (Popov et al., 2008). The volume of the stratum radiatum area of PSDs on these reconstructed spines were also analysed. All data were averaged to receive 1 mean per animal for each parameter and all statistics for three-dimensional analyses were calculated using this data (one value per animal). The curvature of dendritic spines was measured as described previously (Popov et al., 2008). All data from digital reconstructive analysis was evaluated to give 1 value for each individual animal in each data set. Three-dimensional reconstructions were exported to 3D-Studio-Max software for rendering and subsequent rotation to display the optimal views of the reconstructed structures.

Recombinant autoactivating MMP-9 and inactive MMP-9 E402A
Expression of the previously described (Fisher et al., 2002) auto-activating mutant of MMP-9 was performed using the Bac-to-Bac Baculovirus expression system, according to the manufacturer’s instructions (Invitrogen). Briefly, the MMP-9 G100L mutant (a gift from Katherine Fisher, Pfizer, Groton, PA) was cloned into pFastBac1, and the resulting recombinant plasmid was used to transform DH10B competent cells. Colonies that performed transposition of recombinant plasmid fragment into bacmid DNA were identified by blue–white selection, and recombinant bacmid was isolated and verified by PCR. The S21 insect cells were transfected with recombinant bacmid with Cellfectin reagent (Invitrogen) to obtain recombinant baculovirus. After amplification and titration of the recombinant baculovirus, High-Five cells were infected and incubated in the SF-900ISFM serum-free medium (Invitrogen). Conditioned medium was collected for analysis of expression by gel zymography. At 24, 48 and 72 hours after infection, medium was collected and an equal volume was tested by gel zymography. The highest expression level was observed at 48 hours post infection (data not shown). Thus, the cell medium harvested 48 hours after the infection with baculovirus was used for purification of recombinant autoactivating mutant of MMP-9 by affinity chromatography with gelatin–Sepharose 4B (GE Healthcare) as previously described (Sadatmansoori et al., 2001). Protein concentrations in the collected fractions were measured using Bradford reagent (Sigma). The recombinant inactive mutant MMP-9 E402A was generated using QuickChange (Stratagene) according to the manufacturer’s instructions. The point mutation changing glutamate 402 to alanine in the catalytic centre of human MMP-9 was inserted by PCR using a pair of primers: 5′-TGCGCGCGCATGGGTCGGCGC-3′ and 5′-CGCTGCTGGAAAGTCGCCTGGCC-3′. Next, MMP-9 E402A plasmid was cloned into Sf9 insect cells. Western blotting
Samples were subjected to SDS-PAGE (8% gels) and electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), which were blocked for 2 hours at room temperature with 10% (w/v) dried non-fat milk powder in Tris-buffered saline with 0.1% Tween 20 (TBS-T). After blocking, the membranes were incubated at 4°C overnight with rabbit anti-human-MMP-9 antibody (Abcam, # ab52496) diluted in 5% (w/v) dried non-fat milk powder in TBS-T. Membranes were then incubated for 2 hours at room temperature with horseradish-peroxidase-labelled secondary antibody diluted 1:10,000 in 5% dried non-fat milk powder in TBS-T. After washing, the peroxidase activity was visualized with ECLplus reagent (GE Healthcare).

Gel zymography
Samples of culture medium were mixed with 33 SDS sample buffer without DTT and subjected to SDS-PAGE (8% gels containing 2 mg/ml gelatine) and electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), which were blocked for 2 hours at room temperature with 10% (w/v) dried non-fat milk powder in Tris-buffered saline with 0.1% Tween 20 (TBS-T). After blocking, the membranes were incubated at 4°C overnight with rabbit anti-human-MMP-9 antibody (Abcam, # ab52496) diluted in 5% (w/v) dried non-fat milk powder in TBS-T. Membranes were then incubated for 2 hours at room temperature with horseradish-peroxidase-labelled secondary antibody diluted 1:10,000 in 5% dried non-fat milk powder in TBS-T. After washing, the peroxidase activity was visualized with ECLplus reagent (GE Healthcare).

Hippocampal organotypic cultures and live imaging
Transverse hippocampal organotypic cultures were prepared as described previously (Stopnis et al., 1991). Briefly, 400-μm-thick hippocampal slices were cut from 6- to 7-day-old rats and maintained for 11–18 days at 33°C under an humidified 5% CO₂ atmosphere.
atmosphere on Millipore inserts in culture medium (MEM plus HEPES, 25% horse serum and 25% Hanks solution). After 8 DIV cultures were transfected with either pcDNA3.1-GFP or a pCX-mRFP1 using a biolistic method (Helios Gene Gun, Bio-Rad) and were used for experiments 3 days after transfection (De Roo et al., 2008a).

Brief imaging sessions were carried out on an Olympus Fluoview 300 system coupled to a single photon laser. We focused on secondary or tertiary apical dendrites from CA1 field of hippocampi using a 403 objective (0.8 NA) and 103 digital zoom (final resolution 25 pixel size; steps between scans of 0.4 μm). Z-stacks were analysed naturally using ImageJ software (NIH).

**Dissociated hippocampal cultures**

Dissociated hippocampal cultures from P0 (postnatal day 0) Wistar rats were prepared as described below. Brains were removed and hippocampi were isolated on ice in dissociation medium DM: 81.8 mM Na2SO4, 30 mM K2SO4, 5.8 mM MgCl2, 0.25 mM CaCl2, 1 mM HEPES pH 7.4, 20 mM glucose; 1 mM kynurenic acid; 0.001% Phenol Red). Hippocampi were then incubated for 15 minutes at 37°C with 100 units of papain (Worthington, NJ) in DM and rinsed three times in DM and subsequently three times in plating medium (MEM, 10% fetal bovine serum, and 1% penicillin-streptomycin). Hippocampi were triturated in plating medium until no clumps were visible and cells were diluted 1:10 in OptiMEM (Invitrogen), centrifuged for 10 minutes at room temperature, at 208.5 g. The resulting cell pellet was suspended in plating medium, cells were counted using a haemocytometer and 12 000 cells were plated in wells of 12-well plates in serum (FBS) and 1% penicillin-streptomycin. Cells were triturated in OptiMEM (Invitrogen) and subsequently three times in plating medium (MEM, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin). Hippocampi were triturated in OptiMEM until no clumps were visible and cells were diluted 1:10 in OptiMEM (Invitrogen), centrifuged for 10 minutes at room temperature, at 208.5 g. The resulting cell pellet was suspended in OptiMEM, cells were counted using a haemocytometer and 12 000 cells were plated in wells of 12-well plates in serum (FBS) and 1% penicillin-streptomycin. Cells were triturated in OptiMEM until no clumps were visible and cells were diluted 1:10 in OptiMEM (Invitrogen), centrifuged for 10 minutes at room temperature, at 208.5 g. The resulting cell pellet was suspended in OptiMEM, cells were counted using a haemocytometer and 12 000 cells were plated in wells of 12-well plates in serum (FBS) and 1% penicillin-streptomycin.

**Cell stimulation**

Cells were incubated for 30–90 minutes with 400 mM of recombinant MMP-9 or MMP-9 E402A in maintenance medium or the buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 10 mM CaCl2, and 2% DMSO) used for elution of recombinant proteins from the affinity column, which was diluted at least 1:125 in the maintenance medium, so the final concentration of DMSO in the culture did not exceed 0.016%. As indicated, cells were also incubated overnight with anti-CD29 (integrin β1 chain) antibody (BD Pharmingen, no. 555002) or isotype antibody (IgM; BD Pharmingen no. 553957) at a final concentration of 40 μg/ml.

**Immunostaining and confocal microscopy**

Cells were fixed for 10 minutes at room temperature with 4% PFA, washed with PBS, permeabilized for 7 minutes with 0.1% Triton X-100 in PBS and blocked for 1 hour at room temperature with 10% normal goat serum in PBS. After blocking, cells were incubated with mouse anti-GFP antibody (Millipore #MAB3850) and rabbit anti-basoon antibody (Synaptic Systems no. 141003), and guinea-pig anti-homer-1 antibody (Synaptic systems no. 160004), and then were washed and incubated with fluorescent (Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a helium-neon laser (for excitation of Alexa Fluor 488), the 633 nm line of a hel...
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