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## The MAP kinase phosphatase, MKP-1, regulates BDNF-induced axon branching

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### Abstract

The refinement of neural circuits during development depends upon a dynamic process of branching of axons and dendrites that leads to synapse formation and connectivity. The neurotrophin BDNF plays an essential role in the outgrowth and activity-dependent remodeling of axonal arbors *in vivo*. However, the mechanisms that translate extracellular signals into axonal branch formation are incompletely understood. Here we report that the MAP kinase phosphatase-1 (MKP-1) controls axon branching. MKP-1 expression induced by BDNF signaling exerts spatio-temporal deactivation of JNK, which negatively regulates the phosphorylation of JNK substrates that impinge upon microtubule destabilization. Indeed, neurons from *mkp-1* null mice were unable to produce axon branches in response to BDNF. Our results indicate a heretofore-unknown signaling mechanism to regulate axonal branching and provide a framework for studying the molecular mechanism of innervation and axonal remodeling under normal and pathological conditions.

### Introduction

Growth of neuronal processes into axons and dendrites, critical to the development of neuronal connectivity, is highly modulated during development by many extrinsic factors and cues. In addition to semaphorins, ephrins and netrins, neurotrophins such as BDNF (brain-derived neurotrophic factor) highly influence neurite branching<sup>1,2</sup>. For example, increased BDNF-TrkB signaling enhances axonal arborization and synaptic connectivity of

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### Author contributions

F.J. and K.D. designed, performed and analyzed experiments. F.J., K.D. and M.V.C. wrote the manuscript. G.M. helped with the *in utero* electroporations. G.M. and A.M.B. provided mice and reagents.

retinal<sup>3</sup> and trigeminal axons<sup>4</sup> in the *Xenopus* tadpole, and of rat hippocampal explants<sup>5</sup>. Additionally, mice lacking TrkB show reduced axonal arborization in the hippocampus<sup>6</sup>. BDNF can elicit axon branching within two hours of treatment<sup>1</sup>, but the mechanisms that translate BDNF signaling into cytoskeletal rearrangements are not well understood. Rho family GTPases mediate actin reorganization, with Rac and Cdc42 promoting dendritic branching<sup>7</sup>. Other intracellular mechanisms of neurite arborization include activity-dependent calcium transients<sup>8</sup> and the mitogen-activated protein kinases (MAPKs) Erk1/2<sup>9</sup>.

The MAPK family integrates signals from many extracellular factors, such as neurotrophins, to create specific outcomes, including differentiation, survival and synaptic plasticity. The strength of MAPK signaling frequently determines not only the duration of biological responses, but also provides distinctive physiological results<sup>10,11</sup>.

Because the magnitude and duration of a MAPK signal is a critical determinant of different cellular outcomes, the ability to negatively regulate MAPK activities enables finely tuned responses of individual family members, including Erk, P38 and JNK. Dual specificity MAPK phosphatases (DUSP or MKP) inactivate specific MAPKs through dephosphorylation at threonine and tyrosine residues (TXY motifs)<sup>12</sup>. Phosphorylation at both residues is required for full MAPK activity. Eleven different MKP enzymes have been identified, and many are expressed in the nervous system<sup>12</sup>. Some MKP members are highly regulated and are induced as immediate early genes after stimulation with growth factors, oxidative stress or heat shock<sup>13</sup>.

Multiple tissues express the phosphatase MKP-1, and in the brain challenges such as long-term potentiation and visual cues robustly induce *mkp-1* mRNA<sup>14,15</sup>. While MKP-1 activity has been well studied in the context of cell cycle and immune responses, its physiological functions in the central nervous system are unknown. Here we report that ectopic expression of MKP-1 leads to aberrant axon branching through a mechanism that involves negative regulation of JNK and microtubule destabilization. Interestingly, the MKP-1 protein is highly regulated by BDNF in a spatio-temporal fashion. In the absence of MKP-1, there is an inability to produce axon branches in response to BDNF and neuronal activity. These findings reveal a critical control mechanism that lies between signaling by extracellular factors and a specific intrinsic mechanism to control axon branching events and ultimately, neuronal connectivity.

## Results

### MKP-1 expression alters neuronal morphology *in vivo*

Both *bdnf* and *mkp-1* mRNA are detectable in the central nervous system throughout embryonic development<sup>16,17</sup>. To test the hypothesis that MKP-1 downstream of BDNF can influence neuronal morphology, we first established its developmental protein expression pattern in the rat brain *in vivo*. MKP-1 protein level was low during embryogenesis and early postnatal days, but increased gradually from postnatal day 5 (P5) onward in the prefrontal cortex (Fig. 1a) as well as the hippocampus and striatum (not shown). In contrast, expression of another family member, MKP-2, remained unchanged (Fig. 1a).

To determine the functional significance of MKP-1 expression in the developing cortex, we modified MKP-1 levels during neural development *in vivo*. Using *in utero* electroporation at embryonic day 15, we targeted constructs to the neuronal lineage of the cortical plate that specifies future layer II–III excitatory neurons, and allowed the embryos to be born and develop to P7 before analysis. Expression (*MKP1::GFP*) and downregulation (*shRNA#7::GFP*; efficiency was verified in transfected HEK cells, Fig. 1b) constructs carried a GFP reporter cassette to monitor neuronal morphology in brain sections. At P7, when neuronal migration through the cortical plate is completed<sup>18</sup>, transfected LII–III neurons showed a polarized morphology with developing dendritic arbors and thin, straight axons. Ectopic expression of MKP-1 increased morphological complexity of dendritic and axonal arbors, whereas downregulation of MKP-1 resulted in less dendritic arborization compared to GFP-transfected cells (Fig. 1c–h). Axons from MKP-1 overexpressing cells entered the white matter (WM) with aberrant branching and innervation into the dorso-lateral striatum (str) and contralateral hemisphere when compared to the GFP and shRNA expressing cells (Fig. 1d–h). The complexity of axon terminals was increased by ectopic MKP-1 expression but decreased by MKP-1 knockdown, with notably less secondary branches (Fig. 1i). The number of total axonal branches within the contralateral cortical layers I–V was increased or decreased, respectively by upregulation or downregulation of MKP-1 (Fig. 1j). ShRNA incapable of silencing MKP-1 (*shRNA#6*; Fig. 1b,j), or overexpression of MKP-6, a related MKP family member (not shown), did not alter the neuronal morphology of transfected layer II–III neurons compared to the GFP control. Therefore, changing MKP-1 expression levels during neural development produced specific alterations of cortical neuron cytoarchitecture.

### JNK is the target of MKP-1

MKP-1 dephosphorylates and inactivates MAPKs with preference to P38 and JNK, and to a lesser extent, Erk1/219. To dissect the mechanism by which MKP-1 alters neuronal morphology, we used cortical neurons in culture. Both endogenous and exogenously expressed MKP-1 were detected throughout the cytoplasm, as indicated by the presence of the cytosolic protein Hsp90 and the absence of the nuclear protein MeCP2 after fractionation (Fig. 2a, endogenous MKP-1; **b**, transfected MKP-1). We found that MKP-1 expression in primary cortical neurons dephosphorylated JNK, P38 and Erk1/2. In contrast, Erk5 (Fig. 2c) and the parallel signaling pathways Akt, PLC $\gamma$  and GSK3 (not shown) remained unchanged.

Axon outgrowth of cultured cortical neurons decreased upon prolonged MKP-1 overexpression from DIV1–3 and concomitant dephosphorylation of pErk1/2, pP38 and pJNK (Fig. 2d,e). Among the three targets of MKP-1, JNK is constitutively phosphorylated along the axon<sup>20</sup>, while P38 and Erk1/2 are activated transiently in response to a stimulus (not shown). To test whether JNK is the MKP-1 substrate that controls axon outgrowth and therefore neuronal morphology, we used two independent approaches.

First, we used a MKP-1 mutant that targeted only the dephosphorylation of JNK. When a conserved RRR motif within the MAPK-binding domain of MKP-1, which is required for selective binding of Erk1/2 and P38, is mutated to ASA (Fig. 2c), the resulting MKP-1<sup>ASA</sup> mutant no longer binds to Erk1/2 and P38, but is capable of retaining normal binding to

JNK21. Indeed, expression of MKP-1<sup>ASA</sup> in cortical neurons selectively dephosphorylated JNK, without affecting Erk1/2 or P38 (Fig. 2c). Similar to wild-type MKP-1, this mutant restricted axon outgrowth in culture (Fig. 2d,e).

Second, we engineered a JNK mutant (JNK<sup>R69S/D326N</sup>) by analogy to a previously published constitutively-active Erk1/2 mutant<sup>22,23</sup>, which displayed decreased sensitivity to MKP-1 (Supplementary Fig. 1). When co-expressed with MKP-1, JNK<sup>R69S/D326N</sup> reversed the negative effect of MKP-1 on axon outgrowth (Fig. 2f). Therefore, prolonged dephosphorylation of JNK by MKP-1 expression perturbed axon outgrowth, and restoration of JNK signaling was sufficient to rescue the observed axonal defects. Together, the effects observed with the MKP-1<sup>ASA</sup> and JNK<sup>R69S/D326N</sup> mutants suggest that JNK is the major neuronal MAPK substrate of MKP-1 that controls axonal shape.

### JNK inactivation leads to microtubule destabilization

JNKs have diverse substrates, including transcription factors and cytoskeletal proteins<sup>24</sup>. Since expression of MKP-1 led to defects in axon outgrowth, we tested the effects of the prolonged JNK inactivation on cytoskeletal substrates that maintain microtubule integrity<sup>25</sup>. Among these substrates, the stathmins (STMN) are structural modifiers involved in neurite branching and growth cone remodeling<sup>26,27</sup>. Activation of STMNs by dephosphorylation decreases microtubule stability through sequestration of free tubulin. Both MKP-1 and MKP-1<sup>ASA</sup> possessed similar abilities to suppress phosphorylation of STMN1 (Fig. 2g).

Because prolonged dephosphorylation of JNK through MKP-1 overexpression enhanced STMN activity, we also monitored microtubule stability by assaying post-translational modifications of tubulin. Expression of either MKP-1 or MKP-1<sup>ASA</sup> led to an increase of tyrosinated microtubules and a concomitant decrease of acetylated microtubules (Fig. 2h), changes that are associated with overall microtubule destabilization<sup>25</sup>. Therefore, MKP-1 expression leads to JNK dephosphorylation, and ultimately to decreased microtubule stability in cortical neurons.

### BDNF controls MKP-1 expression

Previous reports showed that MKP-1 expression is highly inducible by a variety of stimuli. Moreover, *mkp-1* mRNA is downregulated in the prefrontal cortex of conditional *bdnf*<sup>-/-</sup> mice<sup>28</sup>. To explore these observations further, we investigated whether BDNF acts upstream of MKP-1 expression in cultured cortical neurons. Three hours of treatment with BDNF robustly induced MKP-1 protein expression from a low basal level at different developmental stages *in vitro*, while MKP-2 levels remained unchanged (Fig. 3a). NT-3 and NT-4 also induced MKP-1 expression without altering MKP-2 or MKP-5 levels (Supplementary Fig. 2a). We focused our study on BDNF as it is widely expressed within the CNS.

Primary cortical cultures contain various neuronal subtypes, including approximately 11% GABAergic interneurons (125 GAD65 positive cells/ 1115 neurons counted). Since both inhibitory and excitatory neurons express the BDNF receptor TrkB<sup>29</sup>, we asked if both cell types expressed MKP-1. To this end, we isolated GFP expressing cortical interneurons

(Dlx5/6 positive<sup>30</sup>) using fluorescence-activated cell sorting, and subsequently cultured GFP-positive (inhibitory) and -negative (excitatory) cell populations. Treatment of these cells with BDNF induced MKP-1 in both excitatory and inhibitory populations (Fig. 3b). BDNF-dependent MKP-1 expression was blocked by pre-incubation with either the BDNF scavenger TrkB.Fc or the TrkB inhibitor K252a (Fig. 3c). Because BDNF is secreted in an activity-dependent manner<sup>31</sup>, we tested whether depolarization of cortical neurons with KCl also promoted MKP-1 expression. Like BDNF treatment, an increase in KCl was capable of upregulating MKP-1 protein levels, which was abrogated by TrkB.Fc (Fig. 3c) or K252a (not shown). Thus, an increase in neuronal activity with KCl resulted in an induction of MKP-1 that depended upon endogenous BDNF release and TrkB signaling. Additionally, the MEK1/2 inhibitor U0126 blocked the expression of MKP-1, whereas the JNK inhibitor SP60025 had no effect, and the protein and RNA synthesis inhibitors, cycloheximide (CHX) and actinomycin D (act D), both prevented MKP-1 induction (Fig. 3d).

Therefore, *mkp-1* behaved as an immediate early gene in response to BDNF, similar to *Arc*, a well-characterized immediate early gene downstream of BDNF (Fig. 3b–d). In addition, we observed that prolonged (3h) KCl or BDNF treatments led to a decrease of total pJNK in cortical neurons, both in the inhibitory and excitatory populations (Fig. 3e,f). Consistently, ectopic expression of MKP-1<sup>ASA</sup> both in the inhibitory and excitatory populations decreased pJNK, pSTMN1 and increased tubulin tyrosination (Supplementary Fig. 3), further supporting our idea that activity-dependent BDNF signaling via MKP-1 negatively influences JNK. The effect of KCl on pJNK was only partially blocked by TrkB.Fc, indicating that additional, BDNF-independent pathways also influence pJNK in response to neuronal activity.

BDNF has well-established positive effects on axonal growth and branching<sup>1</sup> that are at variance with the axonal defects observed upon a prolonged, global expression of MKP-1 (Fig. 2d,e). Therefore, we rationalized that BDNF effects must be transduced by MKP-1 in a tightly controlled spatio-temporal manner. In fact, BDNF elicited only transient expression of MKP-1 protein that peaked within the first 3 hours of treatment and subsequently declined (Fig. 4a). Previous reports described a molecular switch between phosphorylation and ubiquitination as a mechanism for prolonged MKP-1 protein stability<sup>32,33</sup>. Indeed, MKP-1 degradation was overcome by inhibition of the proteasome with MG132 (Fig. 4a; supplementary Fig. 4a,c). To address whether BDNF also affects MKP-1 phosphorylation and protein stability, we expressed MKP-1 in 293-TrkB cells using an inducible tet<sup>OFF</sup> system (Fig. 4b). In absence of doxycyclin (DOX), MKP-1 was expressed but no phosphorylation was observed (Fig. 4c). Addition of DOX to prevent MKP-1 expression led to a rapid loss of MKP-1 protein within 1 h (Fig. 4c, upper panel). However, if BDNF was added together with DOX, MKP-1 was phosphorylated and the turnover of the protein was delayed (Fig. 4c, lower panel; supplementary Fig. 5b). Further experiments showed that phosphorylation of some sites (S359, S364) mediated MKP-1 stability, whereas phosphorylation at other sites (S296, S323) enhanced turnover<sup>32,33</sup>. Mutation of all four residues (S296D, S323D, S359A, S364A) was required to render MKP-1 turnover insensitive to BDNF (supplementary Fig. 4b,5b). Therefore, MKP-1 ubiquitination and

phosphorylation are not mutually exclusive (supplementary Fig. 4d). Taken together, BDNF controls MKP-1 expression by mediating both induction and stabilization of the protein.

To address the spatial regulation of MKP-1 expression, we investigated whether distal BDNF signaling from the axon is sufficient to induce MKP-1 expression in neurons. To this end, we grew cortical neurons on porous membranes (Fig. 4d) to physically separate axons that cross through the pores to the lower side of the chamber (“axons”) from cell bodies, dendrites and proximal axons, that grow in the top compartment of the setup (“total”)<sup>34</sup>. To confirm the purity of the axonal preparation, we validated that the axonal marker tau was found in both compartments, while the dendritic marker MAP2 was restricted to the total compartment (Fig. 4e).

Administration of BDNF to the axonal compartment was sufficient to induce MKP-1 expression in both compartments (Fig. 4e). Neither the addition of TrkB.Fc nor of K252a to the total compartment abolished MKP-1 induction upon distal BDNF stimulation. In contrast, MKP-1 protein expression was blocked by pretreatment of the total compartment with U0126, suggesting that the signal relayed from the distal axon to the soma in order to upregulate MKP-1 protein involves pErk. Therefore, BDNF stimulation of axons induced MKP-1 expression throughout the neuron. Continuous distal BDNF signaling might locally stabilize MKP-1 through specific phosphorylation. Indeed, stabilization of neuronal MKP-1 protein strongly correlated with its phosphorylation, which was also sensitive to U0126 (supplementary Fig. 4a). Thus, BDNF can regulate MKP-1 in a spatio-temporal manner by controlling the induction of the protein and subsequently its local stabilization at the sites of BDNF signaling.

To test whether transient induction of MKP-1 is sufficient to inactivate JNK signaling and alter microtubule stability in cultured cortical neurons, we used an inducible *tet<sup>ON</sup> mkp-1<sup>ASA</sup>* construct. We found that treatment with DOX produced transient induction of MKP-1<sup>ASA</sup>, which resulted in dephosphorylation of JNK and stathmin, and tyrosination of microtubules (Fig. 4f), confirming that temporally controlled MKP-1 activity was sufficient to destabilize cortical microtubules. However, transient (6h) and prolonged (48h) expression of MKP-1<sup>ASA</sup> differed in the amplitude of changes, which is reflected in the morphological outcome and therefore may reconcile the fact that BDNF induces both MKP-1 and axonal growth.

### **MKP-1 is required for BDNF-dependent axonal branching**

Microtubule destabilization has been implicated in axonal branching<sup>25</sup>. To test whether transient MKP-1 activity downstream of BDNF induces axonal arborization, we uncoupled MKP-1 activity from BDNF signaling by using a *tet<sup>OFF</sup>* system that allowed inducible MKP-1 expression, while constitutive GFP expression was used as a reporter (Fig. 4b). We electroporated cortical neurons with plasmids encoding *mkp-1*, *mkp-1<sup>ASA</sup>* or *GFP* and cultured them with DOX from DIV0 onward. MKP-1 expression was induced at DIV4 by withdrawal of DOX overnight and subsequent re-addition. During the time of MKP-1 expression, TrkB.Fc was applied to quench any endogenous BDNF. For the GFP control cells, BDNF was applied during the DOX washout to induce BDNF signaling. Finally, cells were grown for an extra day to consolidate newly formed branches (Fig. 5a). These



conditions mimicked the transient nature of MKP-1 expression when induced by BDNF, and allowed us to compare BDNF signaling with brief MKP-1 expression independent of BDNF. The experimental timeline is consistent with previous studies investigating growth factor-induced axonal branch formation<sup>1,9</sup>. We found that activation of BDNF signaling increased the number of primary axonal branches when compared to the TrkB.Fc control (Fig. 5b). This is consistent with previous findings, where BDNF induced axon branching in the optic tectum *in vivo*<sup>1</sup>. Moreover, we observed that transient MKP-1 induction increased axonal branching to a similar extent as BDNF (Fig. 5b), despite the total loss of BDNF signaling by application of TrkB.Fc (Fig. 3c). This suggests that MKP-1 acts downstream of BDNF in axon branching. The MKP-1<sup>ASA</sup> mutant phenocopied the effect of wild-type MKP-1 (Fig. 5b), suggesting that axon arborization is mediated by deactivation of JNK signaling. To address the role of BDNF-dependent stabilization of MKP-1, we coupled BDNF treatment with the ectopic expression of wild type MKP-1 and mutant MKP-1<sup>DDAA</sup>, which lacks BDNF-dependent stabilization (supplementary Fig. 5b). Stimulation with BDNF increased axon branching on top of MKP-1 ectopic expression, an effect lost with the MKP-1<sup>DDAA</sup> mutant (supplementary Fig. 5c). Therefore, both the induction and stabilization of MKP-1 activity play a key role in shaping axon arbors and instructing branch formation.

To ensure MKP-1 acts downstream of BDNF in axon branching instead of in a parallel pathway, we prepared cortical neurons from *mkp-1*<sup>-/-</sup> embryos<sup>35</sup>. We transfected these neurons with GFP, added KCl, BDNF or TrkB.Fc overnight (see Fig. 5a), and analyzed the number of primary axonal branches. In the presence of TrkB.Fc, there was no difference in axonal branching between cortical neurons derived from *mkp-1*<sup>-/-</sup> mice and those derived from their wildtype littermates (Fig. 5c,e). However, in contrast to wildtype neurons, *mkp-1*-deficient neurons showed no increase in primary branch formation in response to BDNF (Fig. 5c–e) or KCl (not shown). Moreover, unlike neurons derived from wildtype embryos, prolonged exposure to BDNF did not decrease pJNK in *mkp-1*<sup>-/-</sup> cells (Fig. 5f), further corroborating that MKP-1 inactivates JNK downstream of BDNF. Together, these results confirm that MKP-1 is a specific downstream target of BDNF signaling that is required for axonal branching decisions.

## Discussion

In this study, we describe evidence for an intracellular mechanism of BDNF-dependent axonal branch formation (supplementary Fig. 6a,b). Our results indicate that BDNF, through TrkB-Erk signaling, induces the expression of the immediate early gene *mkp-1*. Distal stimulation of the axonal compartment of cortical neurons with BDNF was sufficient to induce MKP-1 in both the soma and axon. The ability of neurotrophins to signal over long distances is well established in peripheral neurons. Both NGF and BDNF are capable of inducing nuclear responses via long-range retrograde signaling. Ample evidence suggests that this signal is conveyed in the form of a signaling endosome, containing the activated ligand-receptor complex as well as associated signaling molecules such as MAPK<sup>36</sup>. Both retrograde and anterograde neurotrophin transport have been described for central neurons<sup>37</sup>, with anterograde BDNF transport from the cortex to the striatum being critical for maintaining striatal integrity<sup>38</sup>. Here we show that in addition to its anterograde

function, a retrograde axonal BDNF signal in cortical neurons, involving pErk1/2, induces a nuclear response in form of immediate early gene expression.

Following induction, MKP-1 protein accumulates in the neuronal cytosol, but undergoes rapid turnover and degradation. However, sustained levels of MKP-1 exist at the site of BDNF-MAPK signaling by increasing its half-life. With no basal expression, high inducibility, rapid turnover (< 1h) and opportunity for stabilization, MKP-1 displays the necessary attributes to fine-tune the duration and amplitude of MAPK signaling at defined locations.

MKP-1 is a well-characterized phosphatase that can deactivate the three major classes of MAPK19. Here we show that the morphological and cytoskeletal effects of MKP-1 in neurons, which are phenocopied by the MKP-1<sup>ASA</sup> mutant, are mediated by dephosphorylation of JNK. The unique polarized localization of activated JNK along the axon despite its expression in every neuronal compartment<sup>20</sup> makes it an attractive target for dynamic regulation through phosphatases. JNK signaling controls many features of the nervous system including apoptosis, regeneration and neuronal architecture<sup>39</sup>. Thus, dephosphorylation of axonal JNK represents a master molecular switch for many pleiotropic effects. The decision of where, when and for how long to deactivate JNK may provide functional specificity. Suppression of JNK activity by pharmacological inhibitors, dominant negatives or genetic ablation in various models suggests that JNK controls the formation and the maintenance of axons<sup>20,39,40</sup>. For example, axon outgrowth is impaired following inactivation of JNK by the reversible inhibitor SP600125, an effect that is annulled upon washout<sup>20</sup>. Therefore, the transient nature of MKP-1 induction ensures temporally controlled deactivation of JNK signaling that is compatible with BDNF-induced axonal remodeling.

BDNF induces axon branching and outgrowth, which are supported by stable microtubules. However, formation of nascent axon branches relies upon transient destabilization of microtubules. Several lines of evidence suggest that spatio-temporal regulation of a select group of JNK substrates, including stathmins and neurofilaments, can produce cytoskeletal remodeling. First, *jnk1* knockout impairs the integrity of microtubules<sup>40</sup>. Second, stathmins are direct substrates of JNK<sup>27</sup>. Third, dephosphorylation of stathmins acts as an activation switch to destabilize microtubules<sup>41</sup>. Fourth, local dephosphorylation of SCG10, a stathmin family member, in the axon was observed in response to the morphogens ephrin B and laminin<sup>42</sup>. Fifth, SCLIP, another stathmin family member, regulates axon branching<sup>26</sup>. The transient induction of MKP-1 opens a signaling window during which BDNF trophic effects converge to controlled microtubule destabilization, and ultimately, axonal branch initiation (supplementary Fig. 6).

Ectopic expression of MKP-1 *in vivo* produced aberrant axon branching and innervation into the dorso-lateral striatum. Pyramidal LII–III neurons from the sensory cortex normally innervate the contra-lateral cortical areas<sup>43</sup>, and MKP-1 overexpressing neurons displayed a gain of collateral branches. BDNF levels in the cortex are low during embryonic and early postnatal development (Fig. 1a), nevertheless, TrkB loss-of-function during late embryogenesis impairs cortical progenitor function *in vivo*<sup>44</sup>. Therefore, we speculate that



limited amounts of ectopic MKP-1 protein are stabilized during this time *in vivo*, which accounts for increased axonal branching rather than the drastic outgrowth defects observed *in vitro*.

Both endogenous BDNF and MKP-1 expression are upregulated in the brain at later stages of development, when neuronal networks are refined in an activity-dependent manner. The formation and maturation of axonal arbors is tightly coupled with increased synaptic connectivity, and both processes are positively modulated by BDNF45. Furthermore, plasticity of neuronal networks is relayed by MAPK signaling46 and neuronal activity can induce MKP-1 expression. Therefore, MKP-1 downstream of BDNF may be an important regulator of synaptogenesis, and misregulation of this phosphatase may have deleterious effects on plasticity-dependent processes such as learning and memory. Altogether, our results provide a framework for studying the molecular mechanisms of cytoskeletal remodeling under normal and pathological conditions.

## Materials and Methods

### Animals

Time-pregnant Sprague-Dawley rats, CD1 mice (Charles River Laboratories), *Dlx5/6-Flpe* and RCE:FRT transgenic mice30 and *mkp-1<sup>-/-</sup>* mice47 were allowed ad libitum access to food and water, and maintained on a 12h light/dark cycle. Genotyping was performed as previously described47. All protocols complied with the National Institute of Health guide for the Care and Use of Laboratory Animals.

### Reagents

Trophic factors were obtained from PeproTech. Small molecule inhibitors were purchased from: U0126 (Cell Signaling), K252a and SP600125 (Calbiochem), cycloheximide and MG132 (Sigma). Doxycycline and TrkB.Fc were from Clontech and R&D Systems, respectively. Antibodies used were as follows: Arc (H300), MKP-1 (M18), Erk 1/2, JNK, STMN1 and pSTMN1 (S16-P or S25-P or S63-P) were from Santa Cruz Biotechnology; pMKP-1 (S359/S364-P), pJNK (T183/Y185-P), pERK (T202/Y204-P), pP38 (T180/Y182-P), were from Cell Signaling; Flag, actin, tubulin, pErk5 (T218/Y220-P), tyrosinated-tubulin, acetylated-tubulin were from Sigma–Aldrich; GFP and pMKP-1 (S296-P) were from Abcam; HSP90, MKP2, GAD65 and Tau1 were from BD Bioscience; MeCP2 and detyrosinated-tubulin were from Millipore; MAP2 was from Covance; GAPDH was from Biodesign; and pTrkB antibodies were described elsewhere48.

### Plasmids

The coding sequences for the mouse MKP-1 (obtained from Y.S. Lee) was fused to a N-terminal 3×Flag tag. Flag-MKP-1 was further subcloned into FCIV1, a lentiviral plasmid with a GFP reporter. For the Tet<sup>Off</sup> system, the plasmid ratio for co-transfection was 1:10 (pUHD15.1:pTRE2, from Addgene). The pUHD15.1 was modified by inserting a CMV-GFP reporter cassette (tTA::GFP) and the Flag-MKP-1 sequence was subcloned into the pTRE2 vector (tet<sup>OP</sup>MKP-1). For the Tet<sup>ON</sup> system, the MKP-1 sequence was first subcloned into the pENTRY and further recombined into the pSLIK-venus plasmid

according to manufacturer's instructions (ATCC). Flag-JNK isoforms were purchased from Addgene. Two mutations (R65S and D319N) previously described<sup>23</sup> in Erk1/2 as providing insensitivity to MKP-3 were introduced into the JNK sequence (R69S and D326N). To restrict MKP-1 substrate specificity, the RRR motif in the MAPK binding domain of MKP-1 was replaced by ASA triple mutation<sup>21</sup>. Alanine residues were introduced in place of Ser359 and Ser364 whereas glutamate residues were inserted in place of Ser296 and Ser323 to produce phospho-mimicking mutations. Site-directed mutagenesis was performed using the QuickChange kit from Stratagene. All constructs were verified by sequencing.

Short hairpin (ShRNA) sequences were inserted into the pLentiLox3.7 vector (ATCC) using 5'-ttcaagaga-3' as a loop sequence. Although *ShRNA #6* and *ShRNA #7* anneals to a similar region in the *mkp-1* gene, only the *ShRNA #7* produced knockdown. Therefore, we used *ShRNA #6* as a control. The *ShRNA* sequences used are as following: *Sh#3*, 5'-gctgctcttattatatta-3'; *Sh#4*, 5'-gctccactcaagtcttctt-3'; *Sh#5*, 5'-ccaattgtcctaaccactt-3'; *Sh#6*, 5'-gtcttctctccaaggagga-3'; *Sh#7*, 5'-gctccactcaagtcttcttcc-3'.

### Electroporation

*In utero* electroporation methodology has been previously described<sup>49</sup>. Briefly, DNA was injected in the ventricles of E15–E15.5 CD1 mouse embryos and the mice developed to P7, when neuronal migration in the cortical plate is accomplished. *In vitro* electroporation of the tet<sup>OFF</sup> plasmids *tet<sup>OFF</sup> MKP-1:ITA::GFP* with a 10:1 ratio was achieved with the AMAXA system according to manufacturer's instructions.

### Immunohistochemistry

Following transcardiac perfusion with 4% ice cold PFA, the brains were post-fixed for 24h and equilibrated in 30% sucrose solution for another day. Free-floating coronal sections rinsed in PBS were blocked in 5% normal goat serum /5% normal horse serum/ PBS/ 0.1% TritonX-100 for 1h at 25°C. Primary antibodies (anti-GFP, 1:2000) were incubated for 48h at 4°C with shaking. Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were incubated for 1h at 25°C. Sections were imaged using a LSM510 laser-scanning confocal microscope equipped with 4×, 10× and 20× objectives (Carl Zeiss Microimaging). Images were processed using LSM510 software (Zeiss) and ImageJ (NIH).

### Cell culture

Primary cortical neurons from embryonic day 18 rats or E15–E18 mice were prepared from timed-pregnant Sprague–Dawley rats and transgenic mice as described previously<sup>48</sup>. GFP-positive interneurons were separated from GFP-negative excitatory neurons using fluorescence-activated cell sorting, and both populations were cultured separately as described below. There were 11% of GFP-positive cells within a standard preparation of E18 primary cortical neurons. Primary neurons were cultured on glass coverslips coated with poly-D-lysine, maintained in Neurobasal media containing B27 supplement, 0.5 mM l-glutamine, 5-fluoro-uridine, and uridine (10 μM each). 293FT and 293-TrkB cells (Invitrogen) were grown in DMEM containing 10% FBS plus 200 μg/ml G418.

## Transfection and infection

Neurons were transfected at DIV1 using Lipofectamine2000 as described previously<sup>48</sup>. For all biochemical analysis, cells were infected with lentivirus. Viruses were produced by transfecting packaging plasmids into 293FT cells. The set of plasmids used were 8.9, pCMV-VSVG, and FCIV1. Media were collected after 48h and diluted 1:4 with regular culture medium to infect cells at DIV0.

## Cell lysis

Neurons were lysed in 10mM Tris-HCl, pH 8.0/ 150mM NaCl/ 1mM EDTA/ 10% glycerol/ 1% NP40/ 0.1% SDS complemented with proteases inhibitors, 1 mM  $\text{Na}_3\text{VO}_4$ , and 10mM NaF. Cleared lysates (14,000 rpm 10 min) were analyzed by Western blot. To purify nuclear extracts, neurons were rinsed in ice cold PBS and incubated for 15 min on ice with buffer A (10mM HEPES-KOH, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT with protease and phosphatase inhibitors) before harvesting. 0.5% NP40 was added to the cells for 3 min on ice and lysates were centrifuged for 1 min at 13000 rpm. The resulting supernatant was stored as the cytoplasmic fraction and the pellet was further rinsed in 1 ml of buffer A. The pellet was vortexed for 20 min in 50ml buffer B (20mM HEPES-NaOH, pH 7.9, 0.4mM NaCl, 1mM EDTA, 1mM EGTA with protease and phosphatase inhibitors) and cleared by centrifugation for 10 min at 13000 rpm. The resulting supernatant was collected as the nuclear fraction.

## Immunofluorescence and microscopy

Cells were fixed at indicated days in vitro (DIV) in 4% PFA, 20% sucrose in PBS for 10 min at room temperature (RT). Fixative was quenched with 50mM  $\text{NH}_4\text{Cl}$  in PBS, cells were permeabilized with 0.1% TritonX-100 in PBS for 3 min, blocked with 10% goat serum, 2% BSA, 0.25 % fish skin gelatin in TBS for 30 min and then incubated with the relevant antibodies (anti-FLAG, 1:3000; anti-GFP, 1:3000; secondary antibodies, 1:1000) for 30 min at RT in blocking solution. Cells were washed in TBS, 0.25% fish skin gelatin and mounted in Mowiol488. Images were taken on an EclipseE800 microscope equipped with a 20 $\times$ , Plan Apo NA 0.75 Phase 2 objective (both Nikon Instruments), FITC and TRITC filters (HQ96170M and HQ96171M, Chroma Technology Corp) and an Axioacam HR camera using Axiovision software (both Carl Zeiss Vision GmbH).

Images stacks were processed using ImageJ (NIH). Neurite length was quantified and axon branches traced using the NeuronJ plugin<sup>50</sup>, and axonal arborization was measured by counting the number of primary branches extending from the longest neurite. All image analysis was performed blinded to the condition.

## Statistics

Statistical analysis (GraphPad Prism software, version 4.0) was carried out using one-way analysis of variance with paired Student t-test. Statistical significance was defined at  $P < 0.05$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## Abbreviations

<b>MAPK</b>	Mitogen-activated protein kinase
<b>MKP</b>	MAPK phosphatase
<b>BDNF</b>	brain-derived neurotrophic factor
<b>Trk</b>	tropomyosin related kinase
<b>tet</b>	tetracycline
<b>ERK</b>	extracellular related kinase
<b>Arc</b>	activity-regulated cytoskeleton-associated protein
<b>STMN</b>	stathmin
<b>JNK</b>	c-jun N-terminal kinase

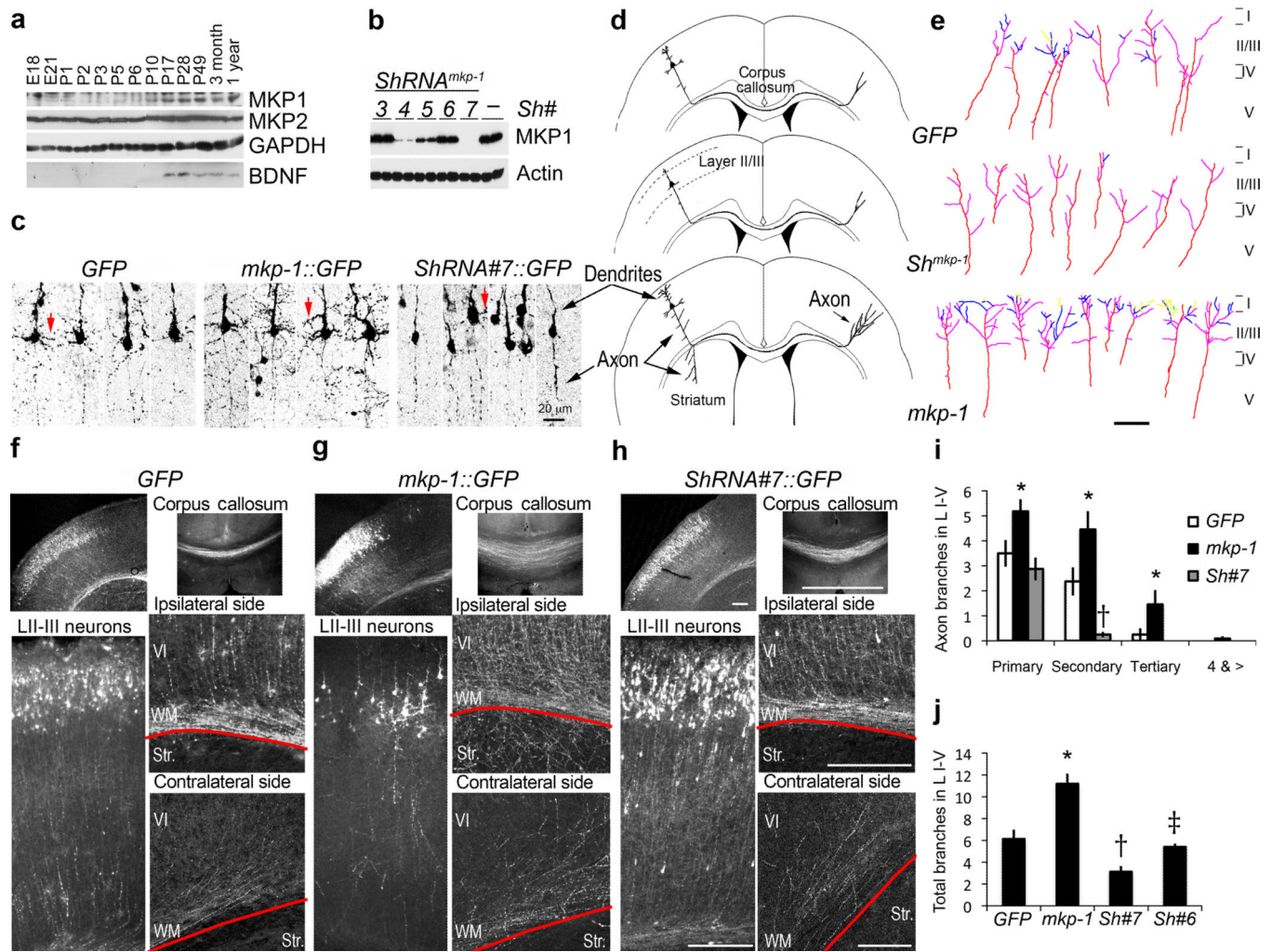
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**Figure 1. Gain- and loss-of-function of MKP-1 *in vivo* affect neural development**  
**(a)** Developmental expression of endogenous MKP-1 in the cortex. **(b)** Knockdown efficiency of short hairpin RNAs (*shRNA#3-7*; -, empty vector) against MKP-1. **(c)** Representative images of individual cortical layer II–III neurons from *in utero* electroporated brains expressing control (*GFP*), *mkp-1::IRES-GFP* (*mkp-1::GFP*) or *shRNA#7* (*shRNA#7::GFP*) plasmids. Arrows highlight differences in dendritic development. **(d)** Summary of the morphological effects of MKP-1 gain and loss-of-function on layer II/III cortical neurons. **(e)** Traces of axon terminals within the contralateral cortical layers of transfected brains. Primary (purple), secondary (blue), tertiary (yellow) and quaternary (green) branches from axons (red) are highlighted. **(f–h)** Representative images of sections from P7 brains electroporated at E15.5 with control (*GFP*) **(f)**, *mkp-1::IRES-GFP* (*mkp-1::GFP*) **(g)** or *shRNA #7* (*shRNA#7::GFP*) **(h)** plasmids. Red lines indicate the boundary between white matter (WM) and lateral striatum (str). Data from MKP-1 (n=23), GFP (n=14) and *shRNA#7* (n=11) animals were collected from at least 3 independent experiments. Scale bars, 200  $\mu$ m. **(i,j)** Number of axonal branches within the contralateral cortical layers were expressed as mean  $\pm$  s.e.m.. Data were collected from individual axons expressing *GFP* (n=8), *mkp-1::GFP* (n=11), *shRNA#7::GFP* (n=16), *shRNA#6::GFP*

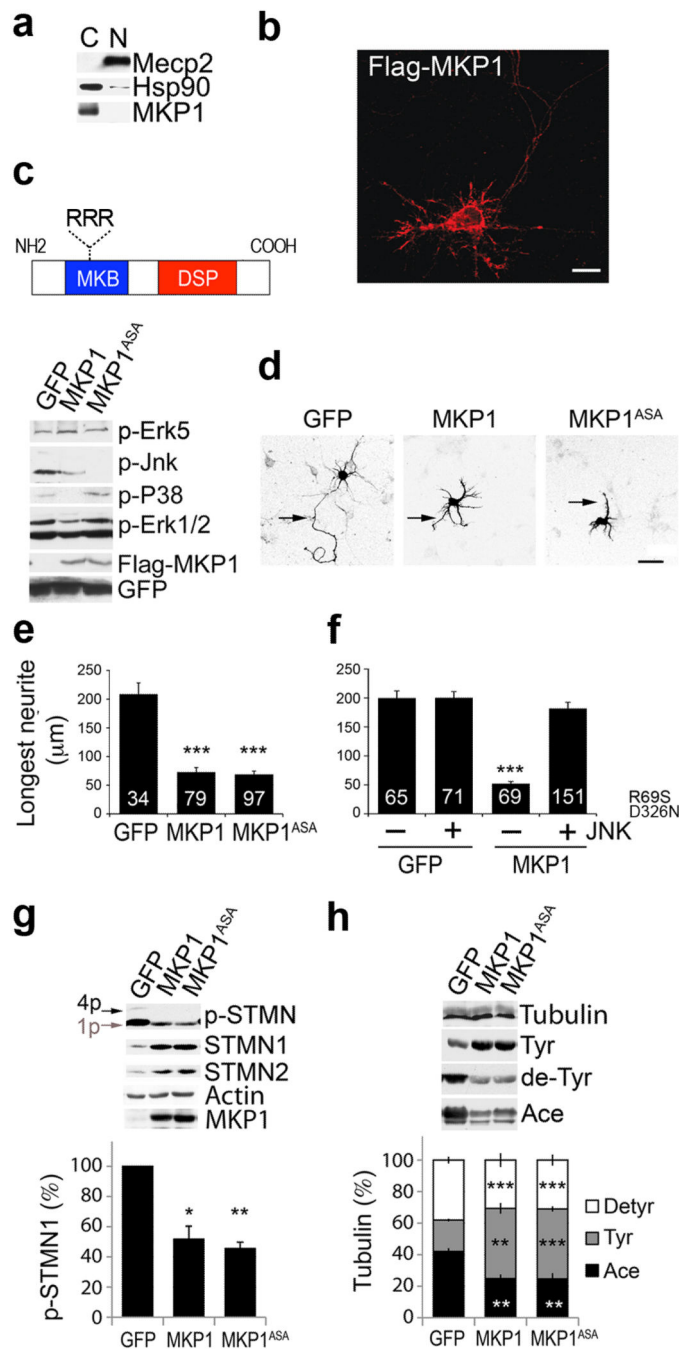
(n=10) from at least 5 different brains and at least 3 independent electroporations per condition. Significant differences are indicated as follows:  $p < 0.05$ , t-test \*, MKP1 vs. GFP; †, GFP vs. *shRNA#7*; ‡, *shRNA#7* vs. *shRNA#6*.

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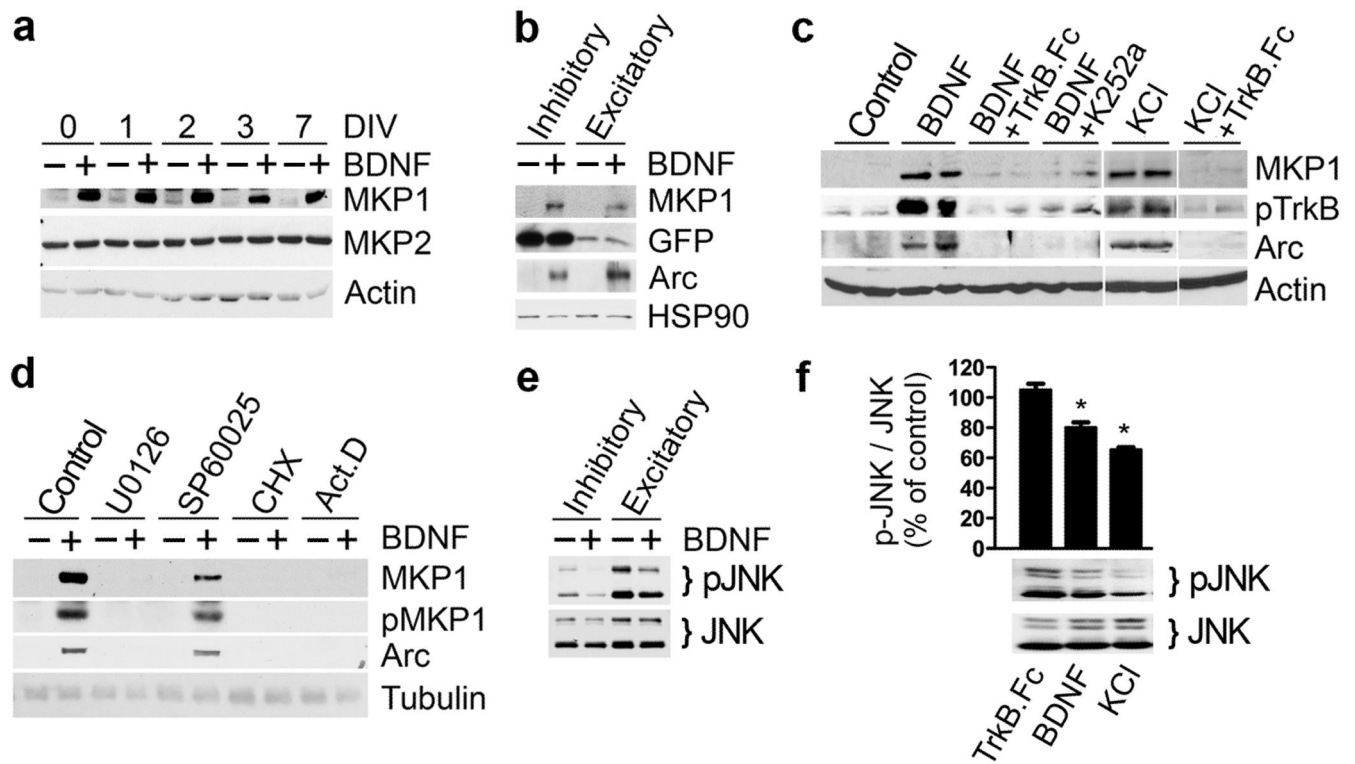
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**Figure 2. JNK is the MKP-1 substrate altering axonal morphology**

(a,b) Localization of MKP-1 in primary cortical neurons. (a) Cytosolic (C) and nuclear (N) extracts were analyzed for endogenous MKP-1. (b) Transfected Flag-MKP-1. Scale bar, 20 μm. (c) Cortical neurons were infected with wildtype MKP-1, MKP-1<sup>ASA</sup> or GFP between DIV1–5 and analyzed by Western blot. (d) Representative images of MKP-1, MKP-1<sup>ASA</sup> or GFP expressing neurons. Arrows indicate the longest neurite. Scale bar, 40 μm. (e) Length of the longest neurite per cell shown as mean ± s.e.m. Numbers in bars indicate the number of cells analyzed. \*\*\*, t-test p<0.0001, n=3 independent experiments. (f) Rescue of axon

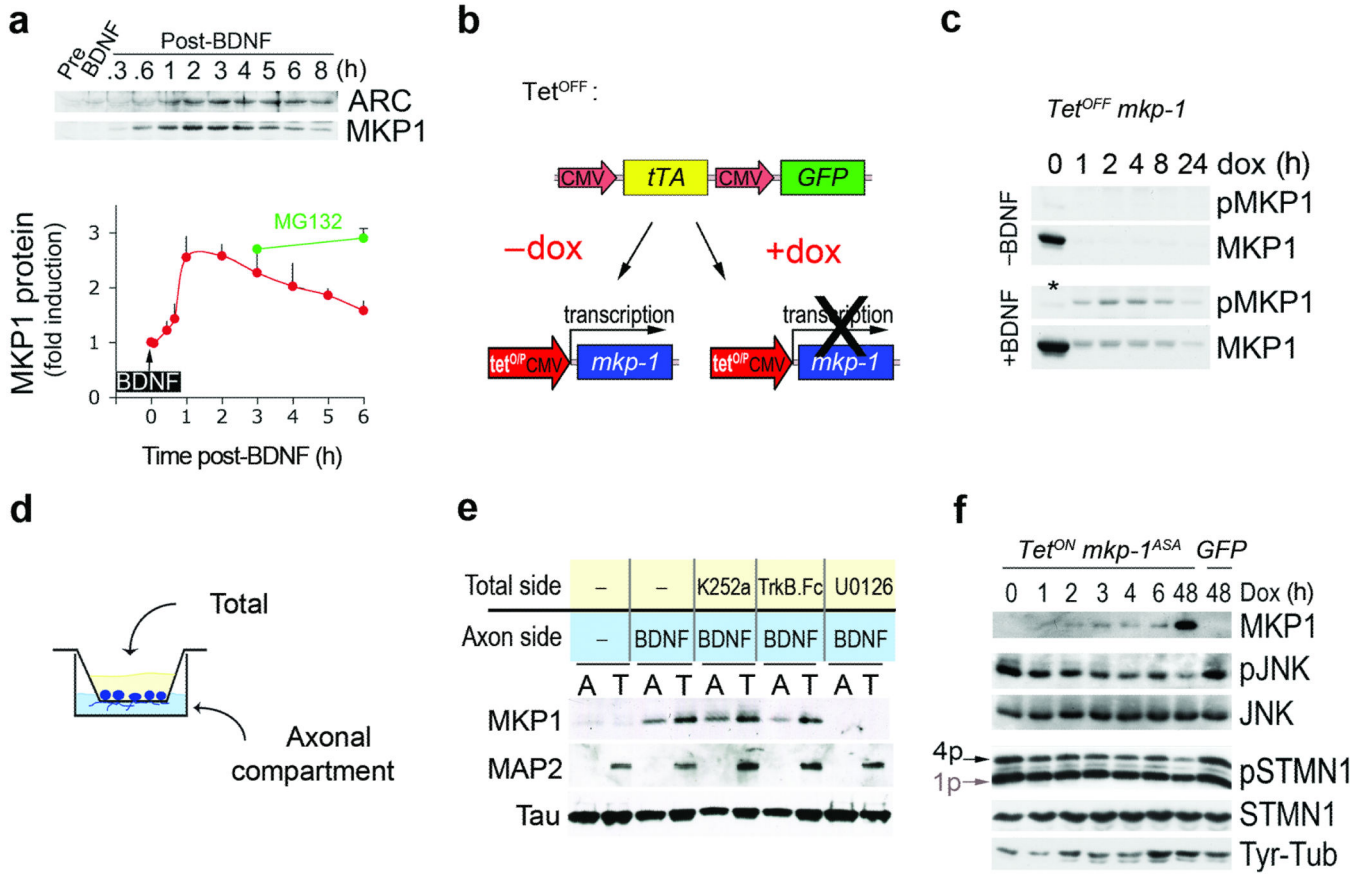
growth by a MKP1-insensitive JNK mutant (JNK<sup>R69S/D326N</sup>) when co-transfected with MKP-1. \*\*\*, t-test,  $p < 0.001$ ,  $n = 4$  independent experiments. **(g)** MKP-1 overexpression from DIV1–5 in cortical neurons affected the phosphorylation of stathmins, expressed as percentile of control GFP cells (mean  $\pm$  s.e.m.; \*,  $p < 0.015$ , MKP1 vs. GFP and \*\*\*,  $p < 0.001$ , MKP1<sup>ASA</sup> vs. GFP using t-test;  $n = 3$  independent experiments). Mono (1p) and multi (4p) phospho-isoforms are active and inactive, respectively. **(h)** Tubulin tyrosination and acetylation were analyzed to assess microtubule stability. Results are expressed as percentile of control (mean  $\pm$  s.e.m.,  $n = 3$  independent experiments). Significant differences are as follows: MKP-1 vs. GFP (\*\*, tyr,  $p = 0.03$ ; \*\*, ace,  $p = 0.006$ ; \*\*\*, de-tyr,  $p = 0.0001$ ) and MKP-1<sup>ASA</sup> vs. GFP (\*\*\*, tyr,  $p = 0.001$ ; \*\*, ace,  $p = 0.004$ ; \*\*\*, de-tyr,  $p < 0.0001$ ) using t-test.



**Figure 3. Induction of MKP-1 expression by BDNF**

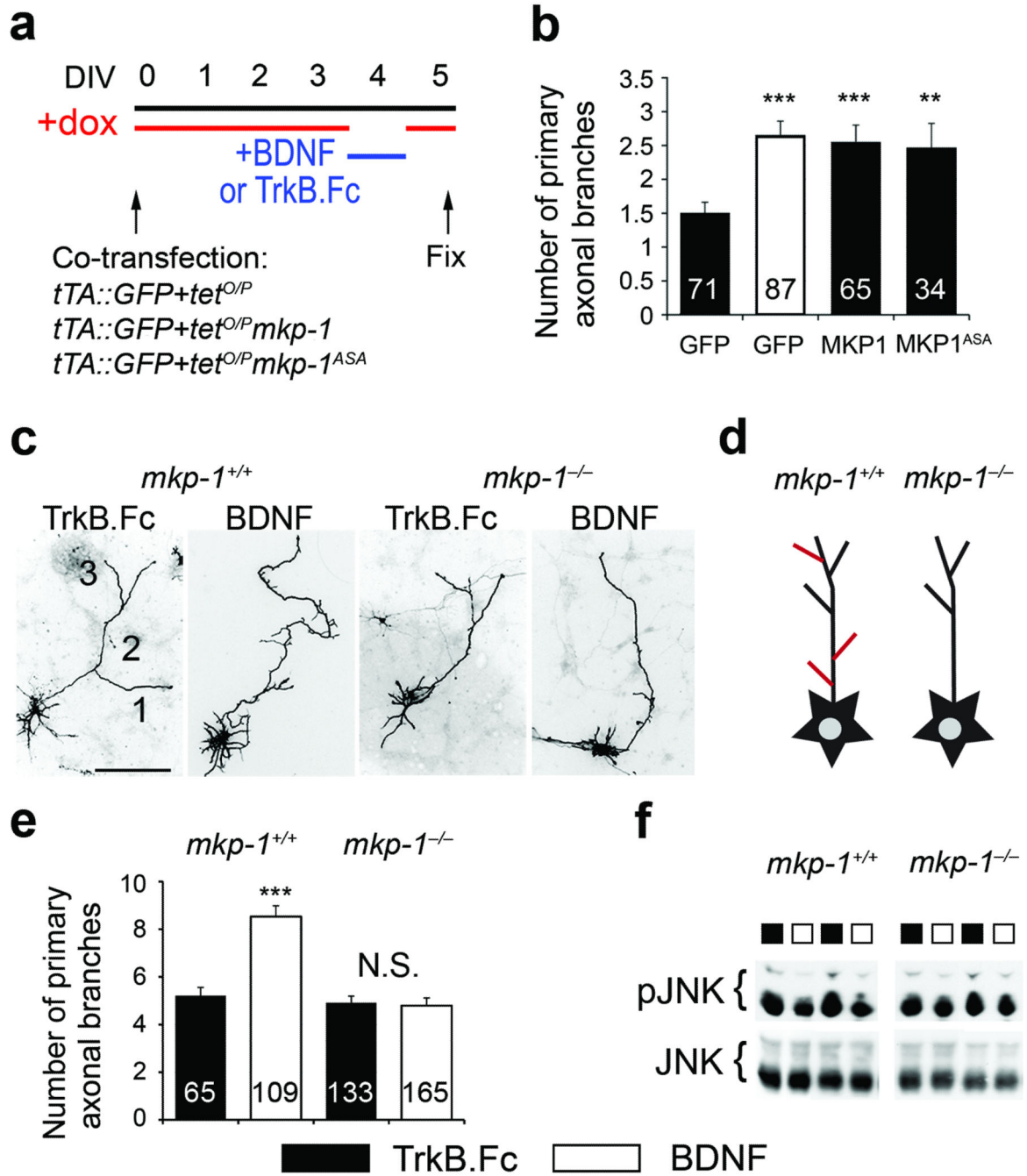
Dissociated cortical neurons were cultured for 7 days or the indicated DIV before pharmacological treatments. MKP-1 protein is highly inducible by BDNF (50 ng/ml) at any stage in culture (a) both in FACS sorted GFP-positive interneurons and GFP-negative excitatory neurons (b), as well as by depolarization (c) using 25 mM KCl for 3 hours. (c,d) Induction was prevented when TrkB signaling (TrkB.Fc, 100 ng/ml; K252a, 100 nM), the Erk pathway (U0126, 10 μM) or gene expression (CHX, 20 μg/ml; ActD, 5 μM) were pharmacologically blocked. (e,f) Treatment of inhibitory or excitatory neurons FACS sorted from transgenic mice expressing GFP in all cortical interneurons (e) and mixed cortical neuron cultures (f) with BDNF (50 ng/ml) or 45 mM KCl for 2 h leads to a decrease in pJNK immunoreactivity. Results from 4 independent experiments were quantified, normalized to JNK and expressed as percentile of control GFP cells (mean ± s.e.m., \*, t-test p=0.004).





**Figure 4. BDNF controls MKP-1 expression in a spatio-temporal manner**  
**(a)** A 5-min pulse treatment with 50 ng/ml BDNF induces transient expression of MKP-1. Arc is shown for comparison. MKP-1 protein levels were expressed as fold induction compared to pre-stimulation (mean + s.d., n=3). MG132 was added just after the BDNF pulse for the indicated times. **(b)** Scheme of the tet<sup>OFF</sup> system composed of 2 plasmids used to uncouple MKP-1 expression from BDNF signaling. **(c)** MKP-1 protein turnover was rapid but prolonged by BDNF-induced phosphorylation in 293-TrkB cells when MKP-1 induction was uncoupled from BDNF signaling. Addition of doxycyclin (DOX) turned off MKP-1 expression. The \* indicates that the sample was not treated with BDNF. **(d)** Schematic representation of the setup used to physically separate axons from the somatodendritic compartment. **(e)** Representative Western blots of the material scraped from both sides of the filter membrane. Pre-treatment of the total side with K252a, TrkB.Fc or U0126 exerted distinct effects on retrograde BDNF signaling and MKP-1 induction. Tau and MAP2 are markers of the axons and somatodendritic compartments, respectively. **(f)** To uncouple MKP-1<sup>ASA</sup> induction from BDNF signaling we used a tet<sup>ON</sup> system where DOX induced MKP-1 in cortical neurons infected at DIV1–5. 1p and 4p indicate the mono- and multi-phosphorylated forms of STMN1, respectively.





**Figure 5. MKP-1 mediates BDNF-induced axon branching**

(a) Timeline of the experimental procedure used to manipulate axonal branching *in vitro*. (b) Number of primary axonal branches expressed as mean ± s.e.m. GFP/TrkB.Fc is significantly different from MKP-1 (\*\*\*, p=0.0004, n=6 experiments), MKP-1<sup>ASA</sup> (\*\*, p=0.0046, n=3 experiments) and GFP/BDNF (\*\*\*, p<0.0001, n=5 experiments) using a t-test. (c,e) Images (c) and quantification (e) of the number of primary branches in GFP-transfected cortical neurons derived from *mkp-1<sup>-/-</sup>* mice and their wildtype littermates. Results from 3 *mkp-1<sup>+/+</sup>* and 6 *mkp-1<sup>-/-</sup>* embryos were expressed as mean±s.e.m. The

number of cells analyzed is indicated in the bars. BDNF induces branching in wildtype neurons only (wildtype, TrkB.Fc vs BDNF:  $p=0.0036$ ; *mkp-1*<sup>-/-</sup>, N.S, not significant, TrkB.Fc vs BDNF:  $p=0.547$  using a t-test). Scale bar, 50  $\mu\text{m}$ . **(d)** Scheme summarizing the effects on branching. Cultured neurons grow axons and branch (black outline). This branching can be enhanced by BDNF or KCl (red lines) in wildtype neurons, however, *mkp-1* null neurons fail to increase arborization in response to stimulation. **(f)** While incubation with BDNF for 3 h decreases pJNK levels in wildtype neurons (see also Fig. 3e,f), it fails to do so in *mkp-1*<sup>-/-</sup> cells.