The Ubiquitin Ligase MYCBP2 Regulates Transient Receptor Potential Vanilloid Receptor 1 (TRPV1) Internalization through Inhibition of p38 MAPK Signaling

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The E3 ubiquitin ligase MYCBP2 negatively regulates neuronal growth, synaptogenesis, and synaptic strength. More recently it was shown that MYCBP2 is also involved in receptor and ion channel internalization. We found that mice with a MYCBP2-deficiency in peripheral sensory neurons show prolonged thermal hyperalgesia. Loss of MYCBP2 constitutively activated p38 MAPK and increased expression of several proteins involved in receptor trafficking. Surprisingly, loss of MYCBP2 inhibited internalization of transient receptor potential vanilloid receptor 1 (TRPV1) and prevented desensitization of capsaicin-induced calcium increases. Lack of desensitization, TRPV internalization and prolonged hyperalgesia were reversed by inhibition of p38 MAPK. The effects were TRPV-specific, since neither mustard oil-induced desensitization nor behavioral responses to mechanical stimuli were affected. In summary, we show here for the first time that p38 MAPK activation can inhibit activity-induced ion channel internalization and that MYCBP2 regulates internalization of TRPV1 in peripheral sensory neurons as well as duration of thermal hyperalgesia through p38 MAPK.

Whereas growth regulation of cortical axons by MYCBP2 does not involve p38 MAPK (8), MYCBP2-dependent axonal overgrowth of spinal cord motor neurons and sensory dorsal root ganglion (DRG) neurons was regulated by p38 MAPK-mediated alterations in microtubule stability (11).

Besides its role in the regulation of neuronal growth, also a function of MYCBP2 in neuronal transmission has been demonstrated. In C. elegans and drosophila loss-of-function mutations in the MYCBP2 orthologs decreased the number of synaptic vesicles at cholinergic and GABAergic synapses in a p38 MAPK-dependent manner (9) and reduced strength of synaptic transmission at neuromuscular junctions (5, 12, 13). More recently, it was shown that the MYCBP2 ortholog in C. elegans, RPM-1, prevents in central neurons activity-dependent internalization of AMPA receptors by inhibiting p38 MAPK signaling through ubiquitylation of MAPK kinase kinase 12 (MAPKKK12), (14). Loss of RPM-1 caused constitutive activation of p38MAPK leading to an increased internalization of the AMPA receptor ortholog GLR1.

Interestingly, in mammals down-regulation of MYCBP2 had an opposite effect on neuronal transmission. Here, down-regulation of MYCBP2 in the spinal cord of adult rats increased pain-like (nociceptive) behavior in a model for acute and inflammatory pain, suggesting an enhanced neuronal signaling (3). Consistent with the finding that MYCBP2 is a potent inhibitor of adenylyl cyclases (15–17), MYCBP2 knockdown in rat spinal cords facilitated G-protein-coupled receptor (GPCR)-induced cAMP synthesis, which plays a key role in central sensitization (3, 15).

In this study, we investigated the consequences of the loss of mammalian MYCBP2 on neuronal functions in peripheral sensory neurons. We found that MYCBP2-deficiency causes constitutive p38 MAPK activation which, surprisingly, prevented activity-induced internalization of the transient receptor potential vanilloid receptor 1 (TRPV1). This finding contrasts the role of MYCBP2 and p38 MAPK in AMPA receptor internalization in C. elegans (14) as well as the previously described role of p38 MAPK in promoting receptor internalization in mammalians (18–20). Thus, our findings suggest that p38 MAPK fulfills receptor-dependent versatile roles in the regulation of receptor internalization.

EXPERIMENTAL PROCEDURES

Materials—Mouse anti-calcitonin gene-related peptide (CGRP) and Griffonia simplifolia isoelectric B4 (IB4) were
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purchased from Sigma. Antibodies against phospho-(Thr-180/Tyr-182) p38 MAPK, total p38, α-p38, γ-p38, myosin Va, and myosin Vι were from Cell Signaling (Danvers, MA). The TRPV1 antibody showing the partial colocalization was from Dianova (Hamburg, Germany), the antibody showing the TrpV1-internalization was from Osenses (Flagstaff Hill, Australia). The antibody against HSP90 was from Santa Cruz Biotechnology. SB203580 was from LC Laboratories (Woburn, MA).

MYCBP2-deficient Mice—A targeting vector was constructed from a 8.7-kb PCR product from a 129/SV BAC clone and subsequently used to generate the MYCBP2-targeted allele mice (genOway, Lyon, France). In the verified fragment was a LoxP site cloned in the EcoRI site between exon 11 and 12. Then a LoxP-FRT-neo-FRT site was inserted with AvrII between exons 10 and 11. Thus, exon 11 was flanked by loxP sites (Fig. 1A). Exon 11 encodes 13 amino acid N-terminal of the RCC1-like domain, a highly conserved domain necessary for MYCBP2 function (6, 8, 17). Deletion of exon 11 leads to a shift in the open reading frame and results in the translation of 23 new amino acids after exon 11 before to reach a premature stop codon. As a result a deletion of the whole RHD1 domain and all C-terminally located amino acids is reached (8, 21). The construct was electroporated into ES cells and homologous recombinants containing both loxP sites were confirmed by Southern blot. ES cells were injected into 105 mouse blastocysts to generate germline chimeras. Germline chimeras were bred to C57/bl6 mice to produce heterozygotes for the floxed allele (genOway, Lyon, France). In the verified allele mice (genOway, Lyon, France). In the verified allele mice after formalin injection. Thermal hyperalgesia was determined following baseline measurements 4, 6, and 8 h after formalin injection. The cut-off time was 90 s. Hanging wire test: For the hanging wire test animals were placed in the middle of a cage wire-lid for 5 s. Then the wire-lid was inverted, hold at about 30 cm height and the fall-off latencies were recorded (24). The cut-off time was set at 90 s. Thermal thresholds were determined using the tail-flick test (25), the hot-plate test (26) and the radiant heat (Hargreaves) test (27) as described previously.

For cold plate testing the mice were placed on a cold steel plate (5 + 0.5 °C) and the pain related behavior (licking, flinching, rearing, jumping) was assessed during a 2 min interval. Mechanical thresholds of the plantar side of a hind paw were determined using a plantar aesthesiometer (Dynamic Plantar Aesthesiometer, Ugo Basile) or von Frey hairs. Here, a steel rod (2 mm diameter) was pushed against the paw with ascending force (0–5 g over a 10-s period, time resolution 0.1 s) until a strong and immediate withdrawal occurred. The paw withdrawal latency was taken to be the mean of at least four consecutive trials with at least 20 s in-between. Von Frey hair testing was performed as described previously (28). Formalin test: 20 μl of a 5% formaldehyde solution (formalin) was injected subcutaneously (s.c.) into the dorsal surface of one hindpaw. The time spent licking the formalin-injected paw and the number of flinches was recorded in 5 min intervals up to 45 min after formalin injection. Thermal hyperalgesia was determined following baseline measurements 4, 6, and 8 h after formalin injection.

Implantation of Lumbar Catheters—For intrathecal (i.th.) delivery of drugs, a spinal catheter was constructed by insert-
ing a slim polytetrafluoroethylene tube (OD 0.2 mm, ID 0.1 mm, length 2 cm, SUBL-60, Braintree Scientific, Braintree, USA) for 1 cm into a wider polyethylene tube (OD 0.61 mm, ID 0.28 mm, length 9 cm, neoLab, Heidelberg, Germany) and fixation of both tubes with cyanoacrylate glue (Stabiliplast, Renfert, Hilzingen, Germany). A 2 cm longitudinal skin incision was made in anesthetized mice above the pelvic girdle and the muscles were bluntly dissected from the vertebrae to expose the L4 and L5 spinous processes. The L5 spinous process and the intervertebral ligament were cut carefully, and a small hole was made on the dura with a 27-gauge needle. Then the slim part of the catheter was inserted 1 cm, so that the tip reached about L1. The sign of a sudden tail or hind limb movement verified the proper localization of the catheter. Using cyanoacrylate glue, the hole on the dura was covered and the catheter was fixed on the bone. After muscle suture, the catheter was tunneled under the skin through a trocar and pulled out from the dorsal neck area. Finally, the skin incision was sutured and the end of the catheter was sealed by melting. The catheter was pulled out from the dorsal neck area. Finally, the skin incision was sutured and the end of the catheter was sealed by melting. After surgery, mice were caged individually for 3 days to recover. Only mice without disturbances of neurological functions were used for behavioral experiments.

**Immunohistochemistry**—12-μm tissue slices and neurons from adult DRG cultures were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 for 5 min and blocked for 1 h in 3% BSA in PBS. Primary and secondary antibody incubations were done in PBS containing 1% BSA for 1 h each. The samples were washed three times with PBS prior mounting. Costaining with TrpV1 and both myosins, all produced in rabbit, were possible using the TSA Fluorescence System from Perkin Elmer (Waltham, MA). For analysis a Zeiss ApoTom fluorescence microscope (Zeiss, Germany) was used. Colocalization was evaluated using the Intensity Correlation Analysis of the software Image I 1.43m. The Pearson correlation (Rr), the Mander’s colocalization coefficient (R) and split coefficients of the Mander’s overlap the (M1, M2) were calculated. The ratio of the positive pixels in both channels was between 0.87 and 1.02. TRPV1 internalization was assessed as described previously (16). Briefly, images for each experimental condition were taken and internalization occurrence was assessed by generating linear densitometric profiles of the cells using the ImageJ 1.60 software. Cells were judged to show internalization if the difference of TRPV1 signals of cytoplasm and plasma membrane decreased to less than double of the signal strength in the cytoplasm of unstimulated SNS-Cre-MYCBP2lox/lox cells.

**DRG Primary Cultures**—20–30 dorsal root ganglia (DRG) were prepared from adult mice and pooled in ice-cold Hanks’ balanced salt solution (HBSS). Then the DRGs were transferred to Neurobasal-Medium (Invitrogen, Carlsbad, CA) containing 500 units/ml collagenase (Biochrome AG, Berlin, Germany) and 2.5 units/ml dispase II (Roche, Mannheim, Germany) and incubated at 37 °C for 2 h. The dissociated tissues were washed twice with medium containing 10% FCS and once with HBSS. Afterward the cells were incubated in 0.05% trypsin-EDTA (Invitrogen) for 10 min at 37 °C and washed again three times in medium before plating poly-l-lysine-covered coverslips in Neurobasal-Medium containing B27, glutamine, and 10% FCS. After 2 h, the medium was changed to Neurobasal Medium without FCS. The neurons were used for calcium imaging the next day.

**Calcium Imaging**—Calcium-imaging experiments were performed with primary dorsal root ganglion cells after 1 day in culture. Prior determination of intracellular calcium levels ([Ca2+]i), cells were incubated with 5 μM of Fura-2-AM (Biotrend, Cologne, Germany) in Neurobasal medium containing 0.02% Pluronic F-127 (Biotium) for 30 min. Fura-2 loaded cells were transferred to the perfusion-chamber continuously superfused with Hepes-buffered ringer-modified saline containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM d-glucose, 1.25 mM CaCl2, and 10 mM Hepes, pH 7.3, at room temperature (20–22 °C). Images were taken with an Axioscope 2 upright microscope (Zeiss, Jena, Germany) using a 10× Achroplan water immersion objective (Zeiss). The microscope was equipped with an Imago CCD camera, a polychrome IV monochromator (all TILL Photonics, Gräfelfing, Germany). Images were acquired and processed using TILLvision software. [Ca2+]i was expressed as the ratio of the background-subtracted fluorescence emission at 510 nm (filter type LP 440) due to excitation at 340 nm and 380 nm using the Polychrom IV Monochromator (Till Photonics). For desensitization experiments cells were 4 times stimulated with 0.2 μM or twice with 0.5 or 1 μM Capsaicin/Ringer delivered via bath application for 10 s with a washing step of 12 or 15 min in between. Desensitization experiments using mustard oil included two stimulations for 30 s with 100 μM mustard oil with a washing step of 8 min. In all experiments included control stimulations with 50 mM KCl for 10 s were performed except when 4 capsaicin-stimulations were used. For the dose-response experiment the time of washing between the indicated stimulations were 15 min. The baseline (first 3 min of each measurement) was used to calculate the increase in ratio (Δratiom) for each cell.

**RESULTS**

**Generation of Mice with MYCBP2 Deficiency in Nociceptive and Thermoreceptive Neurons**—To investigate the consequences of a MYCBP2 deficiency in neurons of adult mice, we generated conditional MYCBP2-knock-out mice utilizing the Cre-Lox system to delete exon 11 as described under “Experimental Procedures.” The generation of these mice was necessary, since constitutive MYCBP2 knock-out mice die at birth because of incorrect formation of neuromuscular junctions at the lung diaphragm (7, 8). Conditional MYCBP2 knock-out mice were generated by mating MYCBP2-floxed mice to mice expressing Cre-recombinase under control of the Nestin promoter (23) or to mice expressing Cre-recombinase under control of the promoter of the sodium channel Na1.8 (SNS) (22) (Fig. 1, A and B; supplemental Figs. S1 and S2). In Nestin-Cre mice Cre-recombinase expression is found in all neurons, while in SNS-Cre mice Cre-recombinase expression is restricted to nociceptive and thermoreceptive neurons of the dorsal root ganglia, trigeminal ganglia as well as a small proportion of proprioceptive neurons (22, 23). However, because Cre-positive Nestin-MYCBP2lox/lox mice showed a lethal phe-
notype, only the adult SNS-MYCBP2lox/lox mice were used in the following studies.

Because MYCBP2 negatively controls neuronal growth, we stained terminals of the primary afferents in the superficial dorsal horn of the spinal cord using the lectin *Griffonia simplicifolia* IB4 to investigate morphological consequences of the MYCBP2-deficiency. IB4 recognizes small-diameter, non-myelinated DRG neurons, which includes the sensory and thermosensitive neurons, whose central axons terminate in inner lamina II of the spinal cord (29). In accordance with the known function of MYCBP2 in restricting neuronal growth, the medial region of lamina II was enlarged in spinal cords of SNS-MYCBP2-knock-out mice (Fig. 1, C and D). However, the neuronal overgrowth had no behavioral consequences in two tests addressing the motor abilities of mice, the rotarod (panel E) and the hanging wire test (panel F). Mechanical thresholds were determined using a plantar aesthesiometer (panel G). Data are shown as average of 7–18 animals ± S.E.

FIGURE 1. Generation and characterization of conditional MYCBP2 knock-out mice. A, scheme depicting the genomic region of the mouse MYCBP2 gene and the inserted LOX-P sites. B, PCR of exon 11 using genomic DNA of spinal cords and DRGs from 2 SNS-Cre-negative and 2 SNS-Cre-positive MYCBP2lox/lox mice. Arrows indicate the wild type (upper band) and the deleted (lower band) alleles. C, IB4 staining of spinal cords from Cre-negative and Cre-positive MYCBP2lox/lox mice. The white bar represents 50 μm. D, size of lamina I and II in Cre-negative and Cre-positive MYCBP2lox/lox mice according to the area stained with IB4. Data are shown as average of 4–8 animals ± S.E. Two-tailed Student’s t test; **, p < 0.003. E–G, motor coordination in Cre-negative (black bars) and Cre-positive MYCBP2lox/lox mice (gray bars) using the rotarod (panel E) test. Thermal thresholds were determined using the radiant heat test (panel F). Mechanical thresholds were determined using a plantar aesthesiometer (panel G). Data are shown as average of 7–18 animals ± S.E.

neither the overgrowth of the central terminals nor the loss of MYCBP2 causes fundamental changes in basal functions of the sensory neurons.

Regulation of TRPV1 Internalization by MYCBP2—To investigate physiological consequences of MYCBP2 deficiency in peripheral nociceptive neurons, we tested pain thresholds of SNS-MYCBP2-deficient mice in an inflammatory model. In the early phase of the formalin test, the nociceptive behavior did not differ between both animal groups (Fig. 2A). However, duration of the formalin-induced thermal hyperalgesia was prolonged in the MYCBP2-deficient animals (Fig. 2B). Next, we studied the effect of the MYCBP2-deficiency on protein expression in DRGs, to determine the mechanistic basis for the increased duration of hyperalgesia in MYCBP2-deficient cells. Protein expression was determined by hybridizing DRG protein extracts from Cre-positive SNS-MYCBP2 knock-out mice and Cre-negative littermate control mice to an antibody microarray covering 750 proteins. 28 proteins showed a change of expression with a net log of 0.5 or more. Out of these, 11 proteins were involved in cytoskeletal-dependent processes including receptor trafficking, vesicle transport or growth regulation (Table 1). Another 10 proteins were involved in the regulation of gene expression, and 7 proteins...
were related to general signaling events or ubiquitylation. Because MYCBP2 has recently been described to regulate receptor trafficking and the antibody array suggests changes in the trafficking machinery, we studied whether this property of MYCBP2 underlies the prolonged hyperalgesia. The antibody array suggests increased protein levels of myosin Va and myosin VI in MYCBP2-deficient DRGs, which are both known to be involved in receptor internalization and recycling (30–33).

A significant up-regulation of both myosin isoforms in DRGs of SNS-MYCBP2-knock-out mice was detected in the array by two different antibodies against each protein and was confirmed by Western blot analyses (Fig. 3, B and C). Thus, so far the data show that the presence of MYCBP2 is necessary for activity-induced TRPV1 internalization.

**p38 MAPK Activation Prevents TRPV1 Internalization**—Loss of MYCBP2 causes a constitutive p38 MAPK activation in MYCBP2-deficient sensory neurons (11). However, p38 MAPK activation has been demonstrated to promote internalization of µ-opioid- and AMPA receptors which would be in contrast to our findings (14, 18, 20, 34). Therefore, we tested in the next step whether or not p38 MAPK-activation prevents TRPV1 internalization in MYCBP2-deficient neurons. In accordance with a previous report (11), we found that phosphorylation of the α and γ isoforms of p38 MAPK on threonine 180 and tyrosine 182 were increased in DRGs from SNS-MYCBP2 knock-out mice suggesting an augmented activation (Fig. 6A). Then, we investigated if p38 MAPK modulation of TRPV1 internalization by determining the effect of the specific p38MAPK inhibitor SB203580 on desensitization of capsaicin-induced intracellular calcium increases. Preincubation of neurons with SB203580 did not influence desensitization of capsaicin-induced calcium increases in DRG neurons from Cre-negative control mice (Fig. 6, B and C). However, preincubation with SB203580 restored the ability of the

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**Table 1**

| Protein expression changes in PAM-deficient DRGs |
|-------------------------------------------------|
| Only proteins with log2 ratios above 0.5 or below -0.5 are shown. |

| Protein            | Log2 (ratio Cre+/Cre-) |
|--------------------|------------------------|
| Cytoskeleton associated |
| Cadherin           | 1.26                   |
| ROCK2              | 1.22                   |
| PKCB1              | 0.92                   |
| p130BBS            | 0.74                   |
| Tenacin            | 0.63                   |
| Myosin VI          | 0.6                    |
| Myosin Va          | 0.54                   |
| Reelin             | 0.5                    |
| GRP1               | -0.53                  |
| Neurofilament 200  | -0.53                  |
| MAPT               | -0.58                  |
| Signaling/ubiquitylation |
| SUMO1              | 1.14                   |
| Oaz2               | 1.06                   |
| Adap               | 0.94                   |
| Siah2              | 0.72                   |
| Calretinin         | 0.6                    |
| Caspase 4          | 0.5                    |
| Psen2              | -0.51                  |
| Transcription      |
| WSTF               | 0.86                   |
| SP1                | 0.62                   |
| AP endonuclease    | 0.57                   |
| Y14                | 0.57                   |
| Tal                | 0.56                   |
| HDAC5              | 0.54                   |
| Aly                | -0.54                  |
| c-Myc              | -0.56                  |
| ATM                | -0.67                  |
| Sin3A              | -0.83                  |

0.996 ± 0.0007) were significantly increased p < 0.02 and p < 0.001, respectively.

To determine whether TRPV1 desensitization and/or internalization were altered in SNS-MYCBP2 knock-out mice, we determined in a first approach the desensitization of transient calcium increases in cultured adult DRG neurons in response to the TRPV1-agonist capsaicin. No significant differences in the amplitudes of capsaicin-induced intracellular calcium increases were seen between DRG neurons from MYCBP2-deficient mice and control mice using 0.1, 0.2, or 0.5 µM capsaicin (Fig. 4, A and B) showing that TRPV1 activation are unaltered in absence of MYCBP2. Next, we tested whether or not desensitization of capsaicin responses are modulated by MYCBP2. Therefore, cultured DRG neurons were stimulated consecutively with capsaicin to induce activity-dependent desensitization. Four stimulations with 0.2 µM as well as two stimulations with 0.5 µM or 1 µM capsaicin caused significant reductions in the amplitude of the capsaicin-induced calcium increase in DRG neurons from Cre-negative SNS-MYCBP2 knock-out mice (Fig. 4, A–C). In contrast, DRG neurons from Cre-positive SNS-MYCBP2-knock-out mice showed no significant reduction in the capsaicin-induced calcium increases in either protocol (Fig. 4, D, G, H).

Because the up-regulation of myosin Va and VI indicates an altered receptor trafficking in MYCBP2-deficient DRGs, we studied whether a decreased internalization of TRPV1 might be the cause for the apparent lack of desensitization. Therefore, we stimulated, as described above, peripheral sensory neurons from Cre-negative and Cre-positive SNS-MYCBP2 mice twice with 1 µM capsaicin and compared the localization of TRPV1 in stimulated and unstimulated neurons. The percentage of capsaicin-responsive cells was in both genotypes (57.1% for Cre-negative and 55.5% for Cre-positive) comparable. Interestingly, we observed that capsaicin-induced TRPV1-internalization occurred only in Cre-negative but not in Cre-positive SNS-MYCBP2 knock-out mice (Fig. 5, A and B). Thus, the data show that the presence of MYCBP2 is necessary for activity-induced TRPV1 internalization.
MYCBP2-deficient neurons to desensitize capsaicin-induced calcium increases (Fig. 6, D and E). Furthermore, preincubation with SB203580 restored the ability of the MYCBP2-deficient neurons to internalize TRPV1 in response to repeated capsaicin stimulations (Fig. 6, F and G). Next, SB203580 was administered intrathecally (i.th.) 4 and 6 h after formalin injection into SNS-MYCBP2 knock-out mice. While mice receiving saline showed 4 and 6 h after formalin injection significantly decreased paw withdrawal latencies, the paw withdrawal latencies of SB203580-treated animals were not significantly altered as compared with baseline (Fig. 6H), demonstrating the involvement of p38 MAPK in maintenance of thermal hyperalgesia in SNS-MYCBP2 knock-out mice.

Regulation of TRPV1 Internalization by MYCBP2 Is Specific—Then, we examined whether the effect of MYCBP2 on TRPV1 desensitization is specific or presents a general mechanism regulating receptor desensitization. Therefore, cultured DRG neurons were stimulated repeatedly with mustard oil, a selective activator of TRPA1, which is another member of the TRP-family of ion channels. Two consecutive stimulations with 100 μM mustard oil caused comparable reductions of the second mustard oil-induced increase of calcium concentrations in Cre-negative and Cre-positive MYCBP2-knock-out mice (Fig. 7, A–C). Because TRPA1 contributes significantly to the development of mechanical allodynia (35, 36), it would be expected that mechanical allodynia is not altered in Cre-positive MYCBP2-knock-out mice. Accordingly, formalin-induced mechanical allodynia was not significantly changed in SNS-MYCBP2 knock-out mice as compared with the respective control mice (Fig. 7D). These findings suggest that regulation of TRPV1-desensitization by MYCBP2 and p38 MAPK is a receptor-specific mechanism and not caused by a general increase of receptor trafficking.

DISCUSSION

In this report we studied the roles of MYCBP2 in thermoreceptive neurons. In accordance with its known function in synapse formation, synaptogenesis, and neurite growth (4–6, 9, 11) we found a small but significant overgrowth of the central endings of MYCBP2-deficient nociceptive and thermosensitive neurons. The limited extent of the observed overgrowth can be attributed to the fact that in these animals
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SNS/Cre-mediated recombination and, therefore, MYCBP2 deletion, occurs not before day 17 of the embryonal development (22). At this time formation of the synaptic connections in the spinal cord is nearly finalized and leaves only a narrow time window for additional neuronal growth in the SNS-MYCBP2 knock-out mice (37–39). The function of MYCBP2 in the regulation of transcriptional and translational regulation through Myc and mTOR signaling has been demonstrated previously by different cellular pathways which are all activated by increased Ca\(^{2+}\)-sensitive phospholipase C causing depletion of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) during desensitization. In absence of MYCBP2 MAPKKK12 accumulates and causes a constitutive activation of p38 MAPK (9, 11, 43). Also the observed prolonged thermal hyperalgesia was expected, since it was described earlier that activation of p38 MAPK in DRGs increases TRPV1 receptor protein levels at the peripheral endings of sensory neurons and prolongs thermal hyperalgesia (44–46).

Though, it was unexpected that constitutive p38 MAPK activation prevented capsaicin-induced internalization of TRPV1 receptors, because p38 MAPK activation was not known to regulate TRPV1 internalization and was previously demonstrated to promote the internalization of various receptors. Desensitization of TRP channels is mediated by different cellular pathways which are all activated by increased Ca\(^{2+}\) level. In sensory neurons and heterologous expression systems TRPV1 desensitization is due to activity-evoked activation of the calcium-dependent phosphatase PP2B, leading to dephosphorylation and desensitization of the TRPV1 channel (47, 48). It has also been reported that Ca\(^{2+}\)/calmodulin (Ca\(^{2+}\)/CaM), an ubiquitous calcium sensor, may play a role in TRPV1 Ca\(^{2+}\)-dependent desensitization by binding to the N-terminal ankyrin repeat domain (49, 50). In addition it has been shown that Ca\(^{2+}\) influx through TRPV1 activates a Ca\(^{2+}\)-sensitive phospholipase C causing depletion of phosphatidylinositol 4,5-bisphosphate (PIP\(_3\)) during desensitization, and that the recovery of the channel from desensitization requires resynthesis of PIP\(_3\) (51–54).

Regarding the role of p38 MAPK in receptor desensitization it was shown that p38 MAPK activation was necessary.
for μ-opioid receptor endocytosis and is even sufficient to trigger constitutive internalization of μ-opioid receptors in the absence of agonists (20). Similarly, p38 MAPK activation induces serotonin and metabotropic glutamate receptors (mGluR)-dependent long-term depression by activating receptor trafficking machineries to facilitate AMPA receptor internalization (18, 34). In accordance with these reports the MYCBP2 ortholog in C. elegans, rpm-1, prevented in central neurons activity-dependent internalization of AMPA receptors by degrading DLK and inhibiting p38 MAPK signaling (14). In contrast to these reports, internalization of TRPV1 was absent in sensory neurons of SNS-MYCBP2 knock-out mice and was reconstituted by p38 MAPK inhibition.

Thus, our data suggest that p38 MAPK fulfills versatile roles in the modulation of activity-dependent receptor and ion-channel internalization which seem to depend on the specific receptor or ion channel. This notion that p38 MAPK fulfills different roles in receptor trafficking is further supported by the finding that regulation of receptor trafficking by p38 MAPK can occur at different stages. In this regard, it has been reported that phosphorylation of epidermal growth factor (EGF)-receptor between amino acids 1002–1022 mediates stress-induced internalization (19) while EGF receptor phosphorylation at serine 1046 and 1047 by p38 MAPK mediates ubiquitination and degradation of already internalized EGF receptor but not internalization itself (55, 56).

The direct ubiquitination of TRPV1 by PAM, as described for the heme receptor Rev-erbα (57), can be ruled out, because p38 MAPK mediates the MYCBP2 effects. The mechanisms underlying the regulation of TRPV1 internalization by p38 MAPK have been partly described in other studies. One of the important downstream targets of p38 MAPK that is involved in μ-opioid receptor and AMPA receptor internalization is Rab5 (14, 18, 58), a member of the Rab family of small GTPases that function as specific regulators of vesicle transport between organelles (40, 59). After its activation Rab5 triggers endocytosis by facilitating the formation of clathrin-coated pits and sorting of membrane proteins into

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**FIGURE 6.** The p38MAPK signaling pathway is activated in DRGs from Cre-positive SNS-MYCBP2lox/lox mice. A, Western blot analysis of DRGs from naive Cre-negative and Cre-positive MYCBP2lox/lox mice for phospho-p38MAPK as well as α- and γ-p38MAPK expression. The right panel shows the densitometric analysis of three Western blots. Student’s t test, *, p < 0.05. B–E, delta ratio mean of capsaicin-induced maximal calcium increases in cultured DRG neurons from adult Cre-negative (panels B and C) and Cre-positive MYCBP2lox/lox mice (panels D and E) of 29–35 cells. Neurons were stimulated twice with 0.5 μM capsaicin with a recovery time of 15 min between both stimulations. In panels C and E cells were preincubated with 10 μM SB203580 for 30 min. Student’s t test, *, p < 0.05. F, representative DRG neurons from Cre-positive SNS MYCBP2lox/lox mice were stained for TRPV1 expression. Cells were either unstimulated or stimulated twice with 1 μM capsaicin. Where indicated the cells were preincubated with 10 μM SB203580 for 30 min. G, percentage of Cre-negative and Cre-positive MYCBP2lox/lox cells showing TRPV1 internalization is shown. Data are presented as average of four independent experiments. Two-tailed Student’s t test, *, p < 0.05. H, Cre-positive SNS- MYCBP2lox/lox mice were given intrathecally 10 μg SB203580 or saline 4 and 6 h after formalin injection (BL: baseline). The withdrawal latencies are shown. The data are expressed as the mean ± S.E. of 5–6 animals. Student’s t test, *, p < 0.05; **, p < 0.01.
endosomes. Although Rab5-mediated endocytosis is able to explain facilitation of μ-opioid and AMPA receptor internalization by p38 MAPK, its involvement in the inhibition of TRPV1 internalization by p38 MAPK is unlikely. Taken together, we show in this report that MYCBP2 modulates TRPV1 desensitization through p38 MAPK signaling which presents not only a novel model for the modulation of TRPV1-mediated signals but also expands the role of p38 MAPK in the regulation of activity-induced receptor internalization.

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