Collapsin Response Mediator Protein 2 (CRMP2) Interacts with N-Methyl-D-aspartate (NMDA) Receptor and Na\(^+\)/Ca\(^{2+}\) Exchanger and Regulates Their Functional Activity*

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Collapsin response mediator protein 2 (CRMP2) is traditionally viewed as an axonal growth protein involved in axon/dendrite specification. Here, we describe novel functions of CRMP2. A 15-amino acid peptide from CRMP2, fused to the TAT cell-penetrating motif of the HIV-1 protein, TAT-CBD3, but not CBD3 without TAT, attenuated N-methyl-D-aspartate receptor (NMDAR) activity and protected neurons against glutamate-induced Ca\(^{2+}\) dysregulation, suggesting the key contribution of CRMP2 in these processes. In addition, TAT-CBD3, but not CBD3 without TAT or TAT-scramble peptide, inhibited increases in cytosolic Ca\(^{2+}\) mediated by the plasmalemmal Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) operating in the reverse mode. coinmunoprecipitation experiments revealed an interaction between CRMP2 and NMDAR as well as NCX3 but not NCX1. TAT-CBD3 disrupted CRMP2-NMDAR interaction without change in NMDAR localization. In contrast, TAT-CBD3 augmented the CRMP2-NCX3 co-immunoprecipitation, indicating increased interaction or stabilization of a complex between these proteins. Immunostaining with an anti-NCX3 antibody revealed that TAT-CBD3 induced NCX3 internalization, suggesting that both reverse and forward modes of NCX might be affected. Indeed, the forward mode of NCX, evaluated in experiments with ionomycin-induced Ca\(^{2+}\) influx into neurons, was strongly suppressed by TAT-CBD3. Knockdown of CRMP2 with short interfering RNA (siRNA) prevented NCX3 internalization in response to TAT-CBD3 exposure. Moreover, CRMP2 down-regulation strongly attenuated TAT-CBD3-induced inhibition of reverse NCX. Overall, our results demonstrate that CRMP2 interacts with NCX and NMDAR and that TAT-CBD3 protects against glutamate-induced Ca\(^{2+}\) dysregulation most likely via suppression of both NMDAR and NCX activities. Our results further clarify the mechanism of action of TAT-CBD3 and identify a novel regulatory checkpoint for NMDAR and NCX function based on CRMP2 interaction with these proteins.

Glutamate excitotoxicity is a major factor contributing to brain damage in stroke, traumatic brain injury, and various age-related neurodegenerations (1, 2). A prolonged increase in extracellular glutamate concentration that occurs in these neuropathologies results in a sustained elevation in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\))\(^3\), leading to activation of various calcium-dependent degradation enzymes, such as proteases and phospholipases (3–5). Consequently, Ca\(^{2+}\) dysregulation plays a key role in glutamate-induced neuronal injury and cell death (6–8). Ca\(^{2+}\) influx via N-methyl-D-aspartate receptor (NMDAR) is one of the main mechanisms leading to the pathological increase in cytosolic Ca\(^{2+}\) in neurons exposed to glutamate (9). In addition, Ca\(^{2+}\) influx via an Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) operating in the reverse mode (NCX\(_{rev}\)) significantly contributes to glutamate-induced Ca\(^{2+}\) dysregulation (10–12).

The regulation of Ca\(^{2+}\) homeostasis in neurons exposed to glutamate is complex, and all factors involved in this regulation are not yet fully understood. Recently, we found that collapsin response mediator protein 2 (CRMP2), a phosphoprotein traditionally regarded as a regulator of axon guidance and neurite outgrowth (13–15), is also involved in regulation of cytosolic Ca\(^{2+}\) in neurons exposed to glutamate (16). In this study, we demonstrated that CBD3 peptide, a 15-amino acid fragment of CRMP2, fused to the TAT cell-penetrating motif of the

\(\text{NMDG, N-methyl-D-aspartate; NMDAR, NMDA receptor; NCX, Na}^+/Ca^{2+}\) exchanger; NMDG, N-methyl-D-glucamine; NCX\(_{rev}\), NCX operating in the forward mode; NCX\(_{rev}\), NCX operating in the reverse mode; AP-S, d-(–)-2-amino-5-phosphonopentanoic acid; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; AUC, area under the curve.

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HIV-1 protein (TAT-CBD3), decreased surface expression of NMDAR in dendritic spines, antagonized glutamate-induced Ca\(^{2+}\) dysregulation, and protected brain tissue against ischemic damage (16). We hypothesized that TAT-CBD3 alters CRMP2 interaction with NMDAR, attenuates NMDAR activity, and thus exerts its neuroprotective action. However, whether TAT-CBD3 alters CRMP2-NMDAR interaction has not yet been experimentally proven. In addition, suppression of NMDAR activity as a sole mechanism of TAT-CBD3-evoked neuroprotection appears to be inconsistent with our finding that both NMDAR and NCX\(_{\text{rev}}\) are important for glutamate-neuroprotection (12). To resolve this issue and clarify the mechanism of the neuroprotective effect of TAT-CBD3, in the present study, we tested whether CRMP2 interacts with NMDAR and NCX and whether TAT-CBD3 alters these interactions and inhibits both NMDAR and NCX\(_{\text{rev}}\).

In this paper, using live cell fluorescence microscopy applied to rat hippocampal neurons in culture, we show that TAT-CBD3, in addition to NMDAR, inhibits NCX\(_{\text{rev}}\). Using co-immunoprecipitation, we demonstrated that CRMP2 physically interacts with the NR2B subunit of NMDAR as well as with NCX3. TAT-CBD3 disrupted CRMP2-NMDAR interaction without changing NMDAR localization but augmented CRMP2-NCX3 interaction and stimulated NCX3 internalization. Knockdown of CRMP2 did not affect NCX3 localization but eliminated TAT-CBD3-induced NCX3 internalization and prevented NCX\(_{\text{rev}}\) inhibition. However, TAT-CBD3 inhibited not only NCX\(_{\text{rev}}\) but also NCX operating in the forward mode, suppressing cytosolic Ca\(^{2+}\) extrusion in exchange for external Na\(^+\). Based on our results, we propose a model that explains the role of CRMP2 in regulation of NMDAR and NCX and elucidates the mechanism of the TAT-CBD3 neuroprotective effect.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutamate, glycine, EGTA, gramicidin, N-methyl-D-aspartate (NMDA), tetrodotoxin, ouabain, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Sigma. Fura-2AM, Fura-2FF-AM, Fluo-4FF-AM, and SBFI-AM were from Teflabs (Austin, TX). d-(-)-2-Amino-5-phosphonopentanoic acid (AP-5) was from Tocris (Ellisville, MO). Ionomycin was from LKT Laboratories (St. Paul, MN). MK801 (5R,10S)-(+) -5-methyl-10,11-dihydro-5H-benzolo[a,d]-cyclohepten-5-10-imine maleate) was purchased from Calbiochem. Anti-NCX1 and anti-NCX3 antibodies were kindly provided by Drs. Kenneth Philipson and Michela Ottolia (UCLA). A monoclonal anti-NR2B antibody was from BD Biosciences. A polyclonal anti-CRMP2 antibody was purchased from Sigma. TAT (YGRKKRRQRRR), TAT-scramble (YGRK-KRRQRRWRWEAKEMLYFELAVIE (TAT sequence underlined), a random sequence with no homology to any known sequence), TAT-CBD3 (YGRKKRRQRRRARSRLAEIVGPRGL), and TAT-CBD3A6K (YGRKKRRQRRRARSRLKELRVPGRGL) were synthesized (>95% purity) by Genscript Inc. (Piscataway, NJ). All peptides were verified in house by mass spectrometry (Department of Chemistry, Indiana University School of Medicine).

**Cell Culturing**—Primary cultures of hippocampal neurons were prepared from postnatal day 1 rat pups according to institutional animal care and use committee-approved protocols and procedures previously published for hippocampus (17). For fluorescence measurements, neurons were plated on glass bottom Petri dishes without preplated glia as described previously (17). Twenty-four hours after plating, 35 µg/ml uridine plus 15 µg/ml 5-fluoro-2′-deoxyuridine were added to inhibit proliferation of non-neuronal cells. Cultures were maintained in a 5% CO\(_2\) atmosphere at 37 °C in minimum essential medium supplemented with 10% NuSerum (BD Biosciences) and 27 mM glucose.

**Calcium and Sodium Imaging**—We used rat hippocampal neurons cultured for 12–14 days in vitro (12–14 days in vitro). The bath solution contained 139 mM NaCl, 3 mM KCl, 0.8 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 10 mM HEPES, pH 7.4, 5 mM glucose, and 65 mM sucrose. Sucrose was used to maintain osmolality similar to that in the growth medium (340 mosm) (18, 19). Fluorescence imaging was performed with a Nikon Eclipse TE2000-U inverted microscope using Nikon objective Super Fluor ×20, 0.75 numerical aperture and a Photometrics Cool SNAP HQ camera (Roper Scientific, Tucson, AZ) controlled by MetaFluor software version 6.3 (Molecular Devices, Downingtown, PA). The excitation light was delivered by a Lambda LS system (Sutter Instruments, Novato, CA). The images were acquired every 15 s during the time course of the experiment.

For cytosolic Ca\(^{2+}\) measurements, neurons were loaded with either 2.6 µM Fura-2 AM or 2.6 µM Fura-2FF AM for 60 min at 37 °C in the presence of 0.015% Pluronic F-127. The excitation filters (340 ± 5 and 380 ± 7 nm) were controlled by a Lambda 10-2 optical filter changer (Sutter Instruments, Novato, CA). Fluorescence was measured from individual neuronal somata through a 505-nm dichroic mirror at 535 ± 25 nm. The changes in [Ca\(^{2+}\)]\(_s\) were followed by recording Fura-2 or Fura-2FF F\(_{340}/F_{380}\) ratio. In some experiments, the changes in [Ca\(^{2+}\)]\(_s\) were followed simultaneously with changes in [Na\(^+\)]\(_s\), using a Ca\(^{2+}\)-sensitive fluorescent dye, Fluo-4FF-AM, and a Na\(^+\)-sensitive dye, SBFI-AM. Neurons were co-loaded with 2.5 µM Fluo-4FF AM for 30 min and 5.3 µM SBFI-AM for 1 h at 37 °C. The excitation wavelengths were 340 ± 5 and 380 ± 7 nm for SBFI and 480 ± 20 nm for Fluo-4FF. Fluorescence was recorded from individual neuronal somata through a 505-nm dichroic mirror at 535 ± 25 nm. The changes in [Na\(^+\)]\(_s\) were followed by recording the SBFI F\(_{340}/F_{380}\) ratio. The changes in [Ca\(^{2+}\)]\(_s\) were followed by recording Fluo-4FF F\(_{480}\) and normalized as F/F\(_{0}\).

Quantification of Fura-2, Fura-2FF, and Fluo-4FF signals was carried out per the manufacturer’s instructions. [Ca\(^{2+}\)]\(_s\) and [Na\(^+\)]\(_s\) were calculated using the Grynkiewicz method (20), assuming a K\(_{d}\) for Fura-2 of 0.225 µM, a K\(_{d}\) for Fura-2FF of 5.5 µM, a K\(_{d}\) for Fluo-4FF of 9.7 µM, and a K\(_{d}\) for SBFI of 11.3 mM. In all experiments, the fluorescence background was subtracted from the signals. Because Ca\(^{2+}\) binding and spectroscopic properties of fluorescent dyes can differ significantly in intracellular milieu, the cytosolic Ca\(^{2+}\) concentrations presented in this paper should be deemed estimates as stated previously by other investigators (21, 22).

**Electrophysiology**—Whole-cell voltage clamp recordings were performed as described previously (23). Briefly, patch
clamp experiments were conducted at room temperature using a HEKA EPC-10 amplifier. Data were collected using the Pulse program (HEKA Elektronik, Lambrecht/Pfalz, Germany). The electrode solution used for recording voltage ramp currents mediated by NCXrev contained 20 mM KCl, 100 mM potassium aspartate, 20 mM tetraethylammonium-Cl, 10 mM HEPES, 0.01 mM K-EGTA, 4.5 mM MgCl₂, and 4 mM Na-ATP, pH 7.3, adjusted with KOH (24). The external solution used for recording currents contained 129 mM NaCl, 10 mM CsCl (to block K⁺ channels), 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM glucose, 10 mM Na-HEPES, pH 7.2, 65 mM sucrose, 10 μM nifedipine (to block voltage-gated Ca²⁺ channels), 20 μM ouabain (to inhibit Na⁺/K⁺-ATPase), and 1 μM tetrodotoxin (to block Na⁺ channels). In our experiments, the first voltage ramp generated ion current that was used as an internal control. Ni²⁺ (5 mM), TAT-CBD3 (10 μM), or CBD3 without TAT (10 μM) was applied to neurons 5 min before the second voltage ramp.

Co-immunoprecipitation—Co-immunoprecipitation experiments were performed on freshly prepared cell lysates from rat hippocampal neuronal cultures at 12–14 days in vitro. Lysates were clarified to remove any additional precipitate by incubating with Protein A-agarose beads for experiments with rabbit primary antibodies or Protein G-agarose beads for experiments with mouse primary antibodies (Santa Cruz Biotechnology, Inc.) for 2 h at 4 °C. Then the lysates were incubated overnight with primary anti-NCX1, anti-NCX3, or anti-CRM2 antibodies under gentle agitation at 4 °C followed by incubation with Protein A-agarose beads or Protein G-agarose beads for 2 h at 4 °C. The immunocomplexed complexes were then washed three times with lysis buffer before being heated at 70 °C in equal volumes of SDS loading dye (Innogen). Samples were then processed by immunoblotting as described previously (5, 16). Blots were probed with anti-NCX1 (NCX1 mAb R3F1), anti-NCX3 (NCX3 polyclonal antibody 95209), anti-NR2B (BD Biosciences), or anti-CRM-2 (Sigma-Aldrich) (all 1:1000). All blots are representative of at least three experiments.

NCX3, NR2B-NMDAR, and CRM2 Immunocytochemistry—Cultured neurons were treated with vehicle (0.1% DMSO), TAT-CBD3, or TAT-scramble peptide for 10 min at room temperature. Then neurons were fixed with 4% paraformaldehyde for 15 min and then washed with PBS. Cells were incubated with a blocking solution containing 2.5% IgG- and protease-free BSA (Jackson ImmunoResearch Laboratories, West Grove, PA), 2.5% goat serum, and 0.1% Triton X-100 in PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with the primary rabbit polyclonal anti-NCX3 (NCX3 polyclonal antibody 95209, 1:500), mouse monoclonal anti-NR2B (1:500; BD Biosciences), or rabbit polyclonal anti-CRM2 (1:500; Sigma). Then cells were incubated with a secondary donkey anti-rabbit antibody or goat anti-mouse antibody conjugated with AlexaFluor 488 for anti-NCX3 and anti-NR2B or AlexaFluor 568 for anti-CRM2 (1:1000) (Invitrogen). Cells were incubated for 1 h at room temperature. The images were taken using a laser, spinning-disk confocal microscope based on a Nikon Eclipse TE2000-U equipped with a Yokogawa spinning disk confocal unit CSU-10, a back-thinned EM-CCD camera Andor iXon EM- DU-897 (Andor Technology, South Windsor, CT), and a motorized flat top stage Prior H-117 (Prior Scientific, Rockland, MA). This setup was controlled by Andor iQ version 1.4 software (Andor Technology, South Windsor, CT). To visualize immunostaining, neurons were illuminated at 488 nm using an air-cooled krypton/argon laser T643-RYB-A02 (Melles Griot, Carlsbad, CA). Fluorescence was collected through a 505-nm dichroic mirror and a 535 ± 25-nm emission filter using an objective Nikon CFI Plan Apo ×100, 1.4 numerical aperture. The fluorescence intensity profiles were generated through randomly chosen cross-sections using AutoQuant software (MediaCybernetics, Silver Spring, MD).

Transfection—To down-regulate CRMP2, hippocampal neurons in culture were transfected in suspension during plating using an electroporator BTX 630 ECM (Harvard Apparatus, Holliston, MA) with a plasmid encoding GFP (4 μg/100 μl of cell suspension, 5 × 10⁶ cells; Clontech) and siRNA (250 nm; Sigma) against CRMP2. The transfected neurons were imaged 7–8 days after transfection.

Statistics—Statistical analysis of the experimental results consisted of one-way analysis of variance followed by Bonferroni’s post hoc test (GraphPad Prism® version 4.0, GraphPad Software Inc., San Diego, CA). The data represent mean ± S.E. of at least three separate, independent experiments.

RESULTS

TAT-CBD3 Inhibits NMDAR and Attenuates Glutamate-induced Ca²⁺ Dysregulation—In experiments with Fur-2FF-loaded rat hippocampal neurons (12–14 days in vitro), compared with vehicle control (Fig. 1A), TAT-CBD3 (10 μM) significantly attenuated glutamate-induced Ca²⁺ dysregulation (Fig. 1B), whereas CBD3 without TAT (10 μM) that cannot cross the membrane (Fig. 1C) and TAT alone were without effect (Fig. 1D). In this and subsequent figures, thin, gray traces represent fluorescence signals from somata of individual cells, whereas thick, red traces represent average signals ± S.E. The change in cytosolic Ca²⁺ in neurons exposed to TAT-CBD3, expressed as average area under the curve, showed a decline by 70% compared with vehicle-, TAT-, or CBD3-treated neurons (Fig. 1E). Similarly, TAT-CBD3 strongly attenuated NMDA-evoked increases in cytosolic Ca²⁺, whereas CBD3 without TAT was ineffective (Fig. 2, A, C, and D); from a peak of 1.03 ± 0.05 μM to 0.17 ± 0.05 μM cytosolic Ca²⁺ with TAT-CBD3 (p < 0.01, n = 5 individual experiments with a total of 107 analyzed neurons) and from a peak of 1.15 ± 0.05 μM to 1.10 ± 0.07 μM cytosolic Ca²⁺ with CBD3 without TAT (p > 0.05, n = 5 individual experiments with a total of 98 analyzed neurons). Previously, we established an IC₅₀ for the neuroprotective action of TAT-CBD3 to be about 2 μM and found that 10 μM TAT-CBD3 provided maximal protection against glutamate excitotoxicity (16). Therefore, in the present study, we used 10 μM TAT-CBD3. As a positive control, we used AP-5 (20 μM), a potent and efficacious NMDAR antagonist (25). AP-5 completely blocked NMDA-induced increase in cytosolic Ca²⁺ (Fig. 2B). Collectively, the results revealed that TAT-CBD3-mediated protection against glutamate-induced Ca²⁺ dysregulation and NMDA-evoked increases in cytosolic Ca²⁺ required CBD3 translocation into the cell and most likely occurred due to its interaction with intracellular molecular targets.
CRMP2 Directly Interacts with NR2B-NMDAR and TAT-CBD3 Disrupts This Interaction and Decreases NMDAR Activity—The inhibition of NMDAR activity by TAT-CBD3, a membrane-permeant CRMP2 fragment, suggested a direct interaction between CRMP2 and NMDAR. In the present study, co-IP experiments revealed an interaction between CRMP2 and NR2B-containing NMDARs (Fig. 3A). Similar findings were reported previously (26). Our experiments were focused on CRMP2 interaction with NR2B-NMDAR because this type of NMDAR is predominantly involved in excitotoxicity (27, 28). TAT-CBD3 disrupted the CRMP2-NMDAR complex (Fig. 3A). It is possible that CRMP2 up-regulates NMDAR activity via binding to the NR2B subunit and that TAT-CBD3-mediated dissociation of CRMP2 from NMDAR leads to diminution of NMDAR activity (see Fig. 2C). It is also conceivable that CRMP2 serves as an anchor protein, retaining NMDAR in

**FIGURE 1.** TAT-CBD3, but not CBD3 without TAT or TAT alone, strongly inhibits glutamate-induced Ca$^{2+}$ dysregulation. Unless stated otherwise, in this and other similar figures, rat hippocampal neurons (12–14 days in vitro) were loaded with Ca$^{2+}$-sensitive dye Fura-2FF-AM to monitor changes in $[\text{Ca}^{2+}]_c$. Here and in other figures, the thin, gray traces show fluorescent signals from individual neurons, whereas thick, red traces represent mean ± S.E. (error bars) from individual experiments ($n = 20–25$ neurons/experiment). Here and in all other figures, where indicated, neurons were treated either with vehicle (A) (DMSO, 0.1%) or 10 μM TAT-CBD3 (B) for 10 min prior to glutamate exposure. C, neurons were treated with 10 μM CBD3. The peptide remained in the bath solution throughout the experiment. D, neurons were treated with 10 μM TAT for 10 min prior to glutamate exposure. Where indicated, 25 μM glutamate plus 10 μM glycine (Glu) were applied. E, the summary graph shows the average area under the curve (AUC) (a.u., arbitrary units), which represents a measure of $[\text{Ca}^{2+}]_c$ increase over time. A representative AUC is shown in A as a gray area under the mean ± S.E. trace. Under each experimental condition, the AUC was calculated for the same time (1080 s) following glutamate application. *, $p < 0.01$ comparing vehicle- and TAT-CBD3-treated neurons. $n = 5$ separate, individual experiments; the total number of analyzed neurons is 383.
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FIGURE 2. TAT-CBD3, but not CBD3 without TAT, strongly inhibits NMDA-induced increases in cytosolic Ca\textsuperscript{2+}. Rat hippocampal neurons (12–14 days in vitro) were loaded with Ca\textsuperscript{2+}-sensitive dye Fura-2AM to monitor changes in \([\text{Ca}^{2+}]_c\). In these experiments, where indicated, short pulses of 30 \(\mu\text{M}\) NMDA (plus 10 \(\mu\text{M}\) glycine) were applied to neurons. Following recovery of \([\text{Ca}^{2+}]_c\), after the first NMDA pulse, neurons were treated with either vehicle (A) (DMSO, 0.1%), 10 \(\mu\text{M}\) TAT-CBD3 (C), or 10 \(\mu\text{M}\) CBD3 (D), as indicated. Then one or two more NMDA pulses were applied. B, before the second NMDA pulse, neurons were treated with 20 \(\mu\text{M}\) AP-S as a positive control. E, the summary graph shows changes in average peak cytosolic Ca\textsuperscript{2+} concentration under different conditions. #, \(p < 0.01\) compared with \([\text{Ca}^{2+}]_c\) before treatment. NT, non-treated cells. \(n = 5\) separate, individual experiments; the total number of analyzed neurons is 205. Error bars, S.E.

the plasma membrane. In this case, disruption of CRMP2-NMDAR interaction should lead to NMDAR internalization that could explain TAT-CBD3-evoked suppression of NMDAR activity. To test this possibility, we used immunocytochemistry in conjunction with confocal fluorescent microscopy. In these experiments, neurons were treated for 10 min with a vehicle (0.1% DMSO) or 10 \(\mu\text{M}\) TAT-CBD3 and then fixed, permeabilized with 0.1% Triton X-100, stained, and analyzed using confocal microscopy. Fig. 3, B–E, shows representative inverted fluorescent images and fluorescence intensity profiles for the respective images. The experiments were repeated three times under each condition. The total number of analyzed neurons was 58 (vehicle-treated) and 67 (TAT-CBD3-treated). Under each condition, all analyzed cells had similar patterns of NR2B localization, as illustrated in Fig. 3, B and D. The NMDAR appeared to be localized in both the plasma membrane and cytoplasm, the latter probably reflecting internalized NMDAR (Fig. 3, B–E). A similar NMDAR distribution pattern has been reported previously by others (29). Application of secondary antibody alone failed to produce staining (data not shown), indicating the lack of nonspecific interactions. The monoclonal anti-NR2B antibody (BD Biosciences) used in these experiments demonstrated excellent specificity, detecting only a single band in immunoblotting experiments (Fig. 3B, inset). This ensures that immunostaining shown in Fig. 3, B and D, is exclusively due to NR2B-NMDARs. Contrary to our expectations, immunocytochemistry experiments failed to provide evidence for TAT-CBD3-induced NMDAR relocalization (Fig. 3, D and E). This suggested that disruption of the CRMP2-NR2B complex might affect NMDAR activity without its translocation from the plasma membrane.

CRMP2 Directly Interacts with NCX3 and TAT-CBD3 Strengthens This Interaction but Leads to NCX3 Internalization and Decreases Its Activity—Recently, we showed that inhibition of both NMDAR and NCX\textsubscript{rev} is necessary for robust attenua-
tion of glutamate-induced Ca\(^{2+}\) dysregulation (12). Based on these findings, we hypothesized that TAT-CBD3, in addition to inhibiting NMDAR, should also inhibit NCX\(_{\text{rev}}\). To test the effect of TAT-CBD3 on NCX\(_{\text{rev}}\) activity, we induced NCX reversal by replacing external Na\(^{+}\) for equimolar N-methyl-D-glucamine (NMDG). Prior to Na\(^{+}\)/NMDG replacement, neurons were preincubated with ouabain (1 mM), an inhibitor of Na\(^{+}\)/K\(^{+}\)-ATPase, for 10 min to increase cytosolic Na\(^{+}\) and thus facilitate NCX reversal. The Na\(^{+}\)/NMDG replacement produced a robust increase in cytosolic Ca\(^{2+}\) and a decrease in cytosolic Na\(^{+}\) (Fig. 4, A and B). Because Na\(^{+}\)/NMDG replacement could evoke release of endogenous glutamate (30), these experiments were performed in the presence of AP-5 (20 \(\mu\)M) to prevent activation of NMDAR and exclude Ca\(^{2+}\) influx via this mechanism. CNQX (20 \(\mu\)M), an antagonist of AMPA/kainate receptor, did not influence Na\(^{+}\)/NMDG-induced increase in cytosolic Ca\(^{2+}\) (data not shown), indicating that AMPA/kainate receptor is not involved. In our previous paper (12), we demonstrated that MK801, an NMDAR antagonist, also appeared to be a potent inhibitor of NCX\(_{\text{rev}}\). In the present study, MK801 completely inhibited Na\(^{+}\)/NMDG-induced increase in cytosolic Ca\(^{2+}\) and slowed down a decrease in cytosolic Na\(^{+}\) (Fig. 4, C and D), implicating NCX\(_{\text{rev}}\) in these ion alterations. Thus, in our experiments, Na\(^{+}\)/NMDG replacement caused an increase in cytosolic Ca\(^{2+}\) due to a reversal of NCX.

TAT-CBD3 (10 \(\mu\)M) strongly inhibited the Na\(^{+}\)/NMDG-induced increase in cytosolic Ca\(^{2+}\) (Fig. 5, A and B), suggesting that TAT-CBD3 inhibits NCX\(_{\text{rev}}\). TAT-CBD3 A6K, a mutated version of CBD3 that demonstrated increased conformational stability and greater anti-nociceptive activity in several rodent models of neuropathic pain compared with the parent TAT-CBD3 peptide (31), also significantly inhibited Na\(^{+}\)/NMDG-induced increase in cytosolic Ca\(^{2+}\) (Fig. 5C), whereas TAT-scramble peptide and CBD3 without TAT were without effect (Fig. 5, D and E). Similar results were obtained with these peptides when NCX reversal was induced by application of 5 \(\mu\)M gramicidin (data not shown). Fig. 5F shows a summary of the data, expressed as average area under the curve, obtained with Na\(^{+}\)/NMDG-induced increases in cytosolic Ca\(^{2+}\) and the effects of various peptides.

Na\(^{+}\)/Ca\(^{2+}\) exchange is electrogenic; therefore, it is possible to record ion currents mediated by NCX (32, 33). Previously, this approach was used in experiments with cardiomyocytes (24, 34). We successfully adapted this technique to neurons in culture (12, 23) and have previously provided a detailed explanation as to why NCX\(_{\text{rev}}\) activity, but not voltage-gated Ca\(^{2+}\) channel activity, is the main contributor in generating these currents (30). In these experiments, we employed a voltage ramp protocol with a change in voltage from −120 mV to +80 mV. Because of favorable Na\(^{+}\) and Ca\(^{2+}\) concentrations across the plasma membrane, NCX operates in the reverse mode under positive voltages, extruding 3 Na\(^{+}\) out of the cell for every Ca\(^{2+}\) transported in. This results in a net outward current, the peak of which when recorded at +80 mV can be used as a measure of NCX\(_{\text{rev}}\) activity (12). Consequently, in addition to Ca\(^{2+}\) imaging, we used an electrophysiological, whole-cell, voltage ramp protocol to demonstrate NCX\(_{\text{rev}}\) inhibition by TAT-CBD3. Ni\(^{2+}\), an NCX inhibitor (24, 34), was used as a positive control. Ni\(^{2+}\) (5 \(\mu\)M) significantly decreased (by 62 ± 7%, \(n = 5\)) the peak ion current generated by the voltage ramp, indicating that this current is predominantly mediated by NCX\(_{\text{rev}}\) (Fig. 6A). TAT-CBD3 (10 \(\mu\)M), but not TAT-scramble...
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![Diagram](image)

FIGURE 5. Na\(^+\)/Ca\(^{2+}\) exchanger reversal induced by Na\(^+\)/NMDG replacement is inhibited by TAT-CBD3. Neurons were loaded with Fura-2FF-AM. Where indicated, neurons were treated either with vehicle (A), 10 μM TAT-CBD3 (B), 10 μM TAT-CBD3 A6K (C), or 10 μM TAT-scramble peptide (D) for 10 min prior to NCX reversal induced by Na\(^+\)/NMDG replacement. E, neurons were treated with 10 μM CBD3, and the peptide remained in the bath solution throughout the experiment. The bath solution was supplemented with 5 μM nifedipine, 1 μM tetrodotoxin, and 20 μM AP-5. In addition, neurons were preincubated for 10 min with 1 mM ouabain (Ouab). Ouabain remained in the bath solution throughout the experiment. F, the summary graph shows the average AUC (a.u., arbitrary units), which represents a measure of [Ca\(^{2+}\)] increase over time. Under each experimental condition, the AUC was calculated for the same time (1080 s) following Na\(^+\)/NMDG replacement. Data are mean ± S.E. (error bars). *, \(p < 0.01\) comparing TAT-CBD3-, TAT-CBD3 A6K-, and vehicle-treated neurons. n = 4 separate, individual experiments with each condition; the total number of analyzed neurons is 404.

(10 μM) applied to neurons for 5 min also significantly attenuated the peak ion current (by 53 ± 6%, \(n = 5\)), suggesting that indeed TAT-CBD3 decreases NCXrev activity (Fig. 6, B and C). Fig. 6D shows a summary graph, analyzing changes in the peak ion current under different conditions.

Previously, we showed that CRMP2 directly interacts with CaV2.2 (35) and NMDAR (Fig. 3). Here, we hypothesized that CRMP2 could also physically interact with NCX and that TAT-CBD3 inhibition of NCXrev could possibly be due to alterations of a putative CRMP2-NCX interaction. In co-immunoprecipitation experiments, we found no evidence for interaction of NCX1 with CRMP2 (Fig. 7A). However, CRMP2 did co-immunoprecipitate with NCX3 (Fig. 7, B and C), suggesting a physical interaction between these proteins. Surprisingly, TAT-CBD3 notably strengthened the CRMP2-NCX3 interaction, whereas TAT-scramble was without effect (Fig. 7C).

In our previous study, we had also reported that TAT-CBD3, in an activity-dependent manner, down-regulated surface expression of NR2B-containing NMDAR in dendritic spines (16). Consistent with this, in our present study, we found that TAT-CBD3 disrupts the CRMP2-NMDAR interaction (Fig. 3).

In stark contrast, TAT-CBD3 enhanced the CRMP2-NCX3 interaction, and consequently, we expected a commensurate increase in NCX3 surface expression. In immunocytochemistry experiments to test this hypothesis, neurons were treated for 10 min with vehicle, TAT-CBD3, or TAT-scramble (both at 10 μM) and then fixed, permeabilized with 0.1% Triton X-100, stained, and analyzed using confocal microscopy. Fig. 8 shows representative inverted fluorescent images and fluorescence intensity profiles for the respective images. The experiments were repeated three times under each condition. In vehicle-treated neurons, NCX3 was found to be localized in both plasma membrane and, to a lesser extent, the cytoplasm (Fig. 8, A and B). A similar pattern was observed in 51 of 59 analyzed neurons. The cytoplasmic localization of NCX3 most likely reflects a pool of internalized NCX. The anti-NCX3 antibody (provided by Drs. Kenneth Philipson and Michela Ottolia, UCLA) used in these experiments demonstrated superb specificity, detecting only a single band in Western blotting experi-
ments (Fig. 8A, inset). This selectivity ensures that immunostaining shown in Fig. 8 (A, C, and E) is exclusively due to NCX3. In contrast to our expectations regarding an increase in NCX surface expression, after treatment with TAT-CBD3, we found that TAT-CBD3 triggered internalization of NCX3, resulting in a decreased expression in the plasma membrane and an increased pool of internalized NCX3 (Fig. 8, C and D). A similar pattern was observed in 63 of 65 analyzed neurons. TAT-scramble did not change NCX3 localization (Fig. 8, A; a similar pattern was observed in 48 of 51 analyzed neurons).

To establish a link between CRMP2 and TAT-CBD3-triggered NCX3 internalization, we down-regulated CRMP2, using siRNA. We hypothesized that if TAT-CBD3-induced NCX3 internalization depends on CRMP2 association with NCX3, then CRMP2 down-regulation should prevent or attenuate this process. To test this hypothesis, neurons were concomitantly transfected during plating with a GFP construct and siRNA against CRMP2. As illustrated in Fig. 9, A–D, neurons expressing GFP had an almost complete lack of CRMP2 (Fig. 9, A–D). Notably, in non-transfected cells, CRMP2 was found in both processes and neuronal somata. In all analyzed neurons (n = 21), expression of GFP coincided with significant CRMP2 down-regulation. Consequently, in the following experiments, we considered GFP fluorescence as an indicator of neurons with down-regulated versus transfected cells. The NCX3 expression pattern was similar in both types of cells (Fig. 9, E–H and I–L). Importantly, pretreatment with 10 \( \mu \)M TAT-CBD3 for 10 min failed to induce internalization of NCX3 in neurons with down-regulated CRMP2 (Fig. 9, I–L). In addition, CRMP2 down-regulation robustly attenuated TAT-CBD3-induced inhibition of NCX3rev (Fig. 10). The results of these experiments demonstrate that CRMP2 is necessary for the effect of TAT-CBD3. Overall, our data show that the increased CRMP2 association with NCX3 correlates with TAT-CBD3-induced NCX3 internalization and subsequent inhibition of the exchanger.

If our findings about TAT-CBD3-induced internalization of NCX3 are correct, then we predict that in addition to a decrement in NCX3rev activity, we should also observe a diminution of the activity of NCX operating in the forward mode (NCXfor). To test this hypothesis, we treated neurons with ionomycin (5 \( \mu \)M), a \( Ca^{2+} \) ionophore. Ionomycin caused delayed \( Ca^{2+} \) dysregulation in neurons (Fig. 11A), demonstrating that neurons possess very effective mechanisms antagonizing elevations in cytosolic \( Ca^{2+} \). It is known that NCX3rev cannot function in the absence of...

**FIGURE 7.** CRMP2 co-immunoprecipitates with NCX3, but not with NCX1. TAT-CBD3 strengthens CRMP2-NCX3 interaction. A, immunoprecipitation (IP) was performed with IgG or anti-CRMP2 antibody followed by Western blotting (WB) with anti-NCX3 antibody. B, immunoprecipitation was performed with IgG or anti-NCX3 antibody followed by Western blotting with anti-CRMP2 antibody. C, prior to immunoprecipitation, where indicated, neurons were incubated either with 10 \( \mu \)M TAT-CBD3 or with 10 \( \mu \)M TAT-scramble peptide for 10 min. Each experiment was repeated three times.

**FIGURE 8.** TAT-CBD3 triggers NCX3 internalization. A, C, and E, confocal, inverted fluorescence images of representative neurons stained with anti-NCX3 antibody. B, D, and F, fluorescence intensity profiles for straight lines in A, C, and E, respectively. n = 3, separate, individual experiments. The total numbers of analyzed neurons are 59 (vehicle-treated), 65 (TAT-CBD3-treated), and 51 (TAT-scramble-treated). Inset in A, Western blot produced with cell lysate and anti-NCX3 antibody (provided by Drs. Kenneth Phillips and Michela Ottolia, UCLA) to illustrate antibody specificity. Scale bars, 15 \( \mu \)m.
Consequently, to demonstrate the role of NCX for in ionomycin-induced Ca\textsuperscript{2+} increase, we inhibited NCX for activity by removing external Na\textsuperscript{+} and replacing it with equimolar NMDG. In these experiments, ouabain was omitted from the bath solution, so that cytosolic Na\textsuperscript{+} remained low, and Na\textsuperscript{+}/NMDG replacement did not cause an increase in cytosolic Ca\textsuperscript{2+}. In addition, these experiments were performed in the presence of AP-5 (20 μM), an NMDAR antagonist, to exclude any contribution by NMDARs to the ionomycin-induced Ca\textsuperscript{2+} increase. The removal of external Na\textsuperscript{+} significantly accelerated the onset of ionomycin-induced Ca\textsuperscript{2+} dysregulation, illustrating a key role for NCX for in maintaining low cytosolic Ca\textsuperscript{2+} (Fig. 11B). As we expected, TAT-CBD3 significantly accelerated Ca\textsuperscript{2+} dysregulation in neurons treated with ionomycin (Fig. 11C). To quantify our observations, we measured the time from the beginning of ionomycin application to the onset of Ca\textsuperscript{2+} dysregulation (t\textsubscript{dys}) (Fig. 11D). A similar approach was used recently to provide a statistical analysis of glutamate-induced Ca\textsuperscript{2+} dysregulation (37, 38). The statistical analysis confirmed that suppression of NCX for by removing external Na\textsuperscript{+} or by application of TAT-CBD3, significantly accelerated the onset of Ca\textsuperscript{2+} dysregulation in neurons (Fig. 11D). These observations suggest that TAT-CBD3 decreases the activities of NCX regardless of the mode of operation and supports a model whereby TAT-CBD3-induced NCX internalization is probably responsible for the suppression of NCX activity.

**DISCUSSION**

Sustained elevations in cytosolic Ca\textsuperscript{2+} play a causative role in glutamate-induced neuronal death (6–8). Elevated cytosolic Ca\textsuperscript{2+} activates Ca\textsuperscript{2+}-dependent degradation enzymes, such as proteases and phospholipases, and thus hastens cell death (3–5). Therefore, it is vitally important for neurons to fight against rampant Ca\textsuperscript{2+} elevations and maintain low cytosolic Ca\textsuperscript{2+}. In neurons, Ca\textsuperscript{2+} extrusion by NCX for and Ca\textsuperscript{2+} uptake by mitochondria are major mechanisms that antagonize Ca\textsuperscript{2+} increases in the cytosol. Mitochondria may accumulate substantial amounts of Ca\textsuperscript{2+} before they undergo permeability transition and become damaged (39). Following Ca\textsuperscript{2+}-dependent activation of the permeability transition pore, mitochondria...
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(12). TAT-CBD3 strongly attenuates glutamate-induced $\text{Ca}^{2+}$ dysregulation. Based on these observations, we hypothesized that TAT-CBD3 should inhibit both NMDAR and NCX$_{\text{rev}}$. The results presented in this paper strongly support this hypothesis and link NMDAR and NCX activity to CRMP2 signaling.

CRMP2 is a member of the CRMP protein family originally known as an axon guidance and outgrowth regulator (13–15). CRMP2 is cleaved by calpain following focal ischemia, thereby implicating it in neurotoxicity associated with ischemia (42). It appears that cleaved CRMP2 is neuroprotective, whereas cleavage of CRMP3 and CRMP4 is detrimental for neurons (42–44). CRMP2 physically interacts with numerous proteins in the cell, including NMDAR and CaV2.2 (26, 35, 45–49). It has been shown that TAT-CBD3 disrupts interaction of CRMP2 with CaV2.2, leading to its internalization and a decrease in ion currents mediated by this channel (35). In our previous paper, using a pH-sensitive fluorescent NR2B sensor, we showed that TAT-CBD3 decreases surface expression of NMDAR in dendritic spines (16), but it remained unclear whether TAT-CBD3 disrupts a CRMP2-NMDAR interaction. It was also unknown whether CRMP2 interacts with NCX and whether TAT-CBD3 affects NCX$_{\text{rev}}$ activity. Here, we demonstrate for the first time that CRMP2 physically interacts with NCX3 and that TAT-CBD3 strengthens this interaction, triggers NCX3 internalization, and consequently suppresses NCX activity in both forward and reverse modes. In contrast to strengthening the CRMP2-NCX3 complex, TAT-CBD3 disrupted CRMP2-NMDAR interaction, suggesting different mechanisms by which CRMP2 interacts with NMDAR and NCX3.

Although NCX3 internalization may account for the suppression of NCX activity, the mechanism of NCX3 internalization and the role of TAT-CBD3-augmented CRMP2-NCX3 interaction in this process are not clear. It is conceivable that TAT-CBD3 competes with CRMP2 for binding with other proteins (e.g. CaV2.2 (35, 45) and NMDAR (Fig. 3)) and releases CaV2.2 (35, 45) and NMDAR (Fig. 3). In our previous paper, using a pH-sensitive fluorescent NR2B sensor, we showed that TAT-CBD3 decreases surface expression of NMDAR in dendritic spines (16), but it remained unclear whether TAT-CBD3 disrupts a CRMP2-NMDAR interaction. It was also unknown whether CRMP2 interacts with NCX and whether TAT-CBD3 affects NCX$_{\text{rev}}$ activity. Here, we demonstrate for the first time that CRMP2 physically interacts with NCX3 and that TAT-CBD3 strengthens this interaction, triggers NCX3 internalization, and consequently suppresses NCX activity in both forward and reverse modes. In contrast to strengthening the CRMP2-NCX3 complex, TAT-CBD3 disrupted CRMP2-NMDAR interaction, suggesting different mechanisms by which CRMP2 interacts with NMDAR and NCX3.

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One of the goals of our study was to test the hypothesis that TAT-CBD3 inhibits deleterious NCX$_{\text{rev}}$, which, instead of extruding Ca$^{2+}$ from the cell, causes Ca$^{2+}$ influx (50). Our results strongly support this hypothesis. However, our study also revealed an unexpected consequence of TAT-CBD3 treatment. Because TAT-CBD3 triggers NCX internalization, it suppresses both forward and reverse modes of NCX. However, NCX$_{\text{rev}}$ is critical for mitigating increases in cytosolic Ca$^{2+}$ and, thus, maintaining cytosolic Ca$^{2+}$ at the low, physiological level. Consequently, suppression of NCX$_{\text{rev}}$ should be detrimental for the cell. How then can one reconcile this, given TAT-CBD3-mediated protection against glutamate-induced Ca$^{2+}$ dysregulation? A likely explanation is that TAT-CBD3-induced inhibi-
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**FIGURE 11. TAT-CBD3 inhibits the forward mode of NCX.** Neurons were loaded with Fura-2FF. A, 5 μM ionomycin in combination with 20 μM AP-5 was applied to neurons. Where indicated, neurons were subjected to Na\(^{+}\)/NMDG replacement (B) or treated with 10 μM TAT-CBD3 (C) for 10 min prior to the addition of ionomycin. D, the summary graph shows the time from the beginning of ionomycin application to the onset of Ca\(^{2+}\) dysregulation (\(t_{\text{dys}}\), which reflects NCX internalization activity. Data are mean ± S.E. (error bars), *, \(p < 0.01\) comparing the effect of Na\(^{+}\)/NMDG replacement and TAT-CBD3- and vehicle-treated neurons; \(n = 5\) separate, individual experiments; the total number of analyzed neurons is 101.

**FIGURE 12. Proposed mechanisms of CRMP2- and TAT-CBD3-mediated regulation of Ca\(^{2+}\) signaling in neurons.** CRMP2 binds to NMDAR, NCX, and CaV2.2 and, presumably, determines their localization and functional activity. We speculate that TAT-CBD3 binds to NMDAR and CaV2.2 and triggers CRMP2 dissociation from these proteins, leading to augmentation of CRMP2 binding to NCX. Disruption of the CRMP2-CaV2.2 complex leads to CaV2.2 internalization and a decrease in its activity (35). (Notably, voltage-gated Ca\(^{2+}\) channels do not significantly contribute to glutamate-induced Ca\(^{2+}\) influx via NMDAR.)

In conclusion, in this paper, for the first time we demonstrated a link between CRMP2 signaling, NMDAR and NCX3 activities, and maintenance of Ca\(^{2+}\) homeostasis in neurons exposed to excitotoxic levels of glutamate. Our experiments revealed that CRMP2 physically interacts with both NMDAR and NCX3. Moreover, we showed that TAT-CBD3 strengthens CRMP2-NCX3 interaction and induces NCX3 internalization, leading to suppression of NCX activity. Concomitantly, TAT-CBD3 disrupts the CRMP2-NMDAR interaction without detectable relocation of NMDAR. Nevertheless, this also leads to suppression of NMDAR activity. Thus, our study impli-
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cates CRMP2 signaling in regulation of cytotoxic Ca\(^{2+}\) in neurons exposed to glutamate, suggests different mechanisms of CRMP2 interaction with NMDAR and NCX, and further clarifies the mechanism of TAT-CBD3-mediated defense against glutamate-induced Ca\(^{2+}\) dysregulation. Our experiments with TAT-CBD3 provide further support to our hypothesis that inhibition of both NMDAR and NCX might be crucial for robust attenuation of glutamate-induced Ca\(^{2+}\) dysregulation. Overall, the new knowledge obtained in our study could be instrumental in designing new neuroprotective agents and in a better understanding of the mechanisms of their action.

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