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Binding and Activation of LRP1-Dependent Cell Signaling in Schwann Cells Using a Peptide Derived from the Hemopexin Domain of MMP-9

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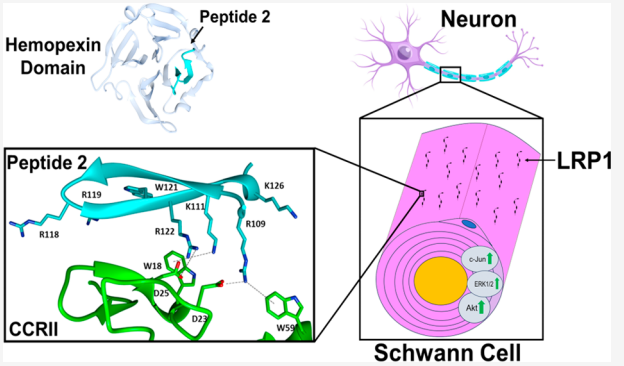
ABSTRACT: Schwann cells (SCs) undergo phenotypic transformation and then orchestrate nerve repair following a peripheral nervous system injury. The low-density lipoprotein receptor-related protein-1 (LRP1) is significantly upregulated in SCs in response to acute injury, activating cJun and promoting SC survival. Matrix-metalloproteinase-9 (MMP-9) is an LRP1 ligand that binds LRP1 through its hemopexin domain (PEX) and activates SC survival signaling and migration. To identify novel peptide mimetics within the hemopexin domain of MMP-9, we examined the crystal structure of PEX, synthesized four peptides, and examined their potential to bind and activate LRP1. We demonstrate that a 22 amino acid peptide, peptide **2**, was the only peptide that activated Akt and ERK1/2 signaling in SCs, similar to a glutathione s-transferase (GST)-fused holoprotein, GST-PEX. Intraneural injection of peptide **2**, but not vehicle, into crush-injured sciatic nerves activated cJun greater than 2.5-fold in wild-type mice, supporting that peptide **2** can activate the SC repair signaling in vivo. Peptide **2** also bound to Fc-fusion proteins containing the ligand-binding motifs of LRP1, clusters of complement-like repeats (CCRII and CCRIV). Pulldown and computational studies of alanine mutants of peptide **2** showed that positively charged lysine and arginine amino acids within the peptide are critical for stability and binding to CCRII. Collectively, these studies demonstrate that a novel peptide derived from PEX can serve as an LRP1 agonist and possesses qualities previously associated with LRP1 binding and SC signaling in vitro and in vivo.



INTRODUCTION

During development and throughout their lifespan, Schwann cells (SCs), the main glia in the peripheral nervous system (PNS), play a key role in maintaining axon integrity in peripheral nerves.[1] After peripheral nerve injury, SCs are the first responders and are essential for promoting functional nerve repair.[2] SCs transdifferentiate into a repair phenotype after injury, playing a pivotal role in clearing out myelin and cellular debris, secreting growth factors and depositing extracellular matrix proteins that are essential for proper axon growth.[2,3] If the repair activities of SCs are impaired, peripheral nerve regeneration may occur incorrectly and result in chronic sensory and motor deficits. The importance of studying SCs is even greater given recent studies, indicating that SCs are nociceptive,[4] and, when SCs are dysfunctional, they contribute to chronic pain states.[5] Targeting SC-dependent signaling pathways would improve strategies for enhancing functional sensory nerve repair.

The endocytic and cell signaling receptor, low-density lipoprotein receptor-related protein (LRP1), is found in many cell types and binds to diverse and numerous ligands.[6,7] After PNS injury, LRP1 is significantly upregulated in SCs and facilitates their survival.[8] Ligand binding to LRP1 activates pro-survival signaling, including activation of extracellular signal-regulated kinase/mitogen-activated protein (ERK1/2/MAP kinase), transcription factor cJun, and the phosphoinositide-3-kinase/protein kinase B (PI3K-Akt) pathway.[8−10] LRP1 also promotes SC survival by antagonizing the unfolded-protein response.[11] Several LRP1 ligands are present in the injured peripheral nerve, including matrix metalloproteinase 9 (MMP-9).[12] MMP-9 is a proteolytic enzyme that binds LRP1 and activates cell signaling and migration in SCs in vitro and in vivo.[9] These activities do not require the MMP-9 proteinase active site but instead are mediated by the MMP-9 hemopexin domain (PEX), which is known to bind and activate LRP1-dependent cell signaling.[13] Thus, designing small peptides that

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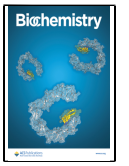

can mimic the activity of PEX on LRP1 and do not exhibit the proteolytic activity of MMP-9 may provide a novel therapeutic potential for an LRP1 agonist that activates the SC repair program.

In this study, we examined four small peptides derived from PEX and determined whether these peptides replicate LRP1 binding and cell signaling activities observed with recombinant PEX.[9] We show that one peptide binds the ligand-binding domains of LRP1 (Clusters of Complement like repeats II and IV or CCRII and CCRIV) and activates LRP1-dependent cell signaling in human SCs. Intraneural injection of peptide **2** into acutely crush-injured sciatic nerves in wild-type mice resulted in increased activation of cJun, supporting the activation of SC repair signaling in vivo. Our results indicate that SC activation by LRP1 peptides may augment SC repair signaling after injury.

MATERIALS AND METHODS

Human Schwann Cell (SC) Cultures. Human SCs (hSCs) were purchased from ScienCell (Cat. 1700) and grown according to manufacturer's product description. The corresponding SC medium (Cat. 1701), which consisted of 5% fetal bovine serum (FBS), SC growth supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin, was used to culture hSC in humidified incubators at 37 °C and 5% CO_2.

Recombinant MMP-9 Hemopexin Domain Fusion Protein (PEX). Recombinant PEX was produced as a GST-fusion protein using the pGEX-5X-2 plasmid (GE Health Sciences 28954554). Herein, the GST fusion was used due to the poor solubility of recombinant PEX without the GST. The gene sequence for human PEX was synthesized by Genscript with *Eco*RI and XhoI restriction sites, which was spliced into the pGEX plasmid, and the sequence was verified by Eton Bioscience. Recombinant fusion protein GST-PEX was expressed as previously described.[9]

In short, the plasmid was transformed into BL21 (DE3) pLysS Competent Cells (Promega L1195) following the protocol provided by the manufacturer. Bacterial colonies were expanded in 1 L of Luria Broth (LB) medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol in a baffled flask shaken at 150 rpm in a 37 °C incubator. At an OD600 of 0.60, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 μM to induce protein production. The cells were harvested by centrifugation in 2000*g* for 10 min, lysed with a pH 7.4 50 mM tris buffer with 250 mM sodium chloride, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, and 1% Triton X-100, and then snap frozen. The lysate was sonicated and spun at 12,000*g* for 30 min, and the pellet was collected. The pellet was resuspended in a 7.0 M Urea, 7.4 pH 50 mM tris buffer for 1 h at 4 °C with gentle shaking. The suspension was spun for 1 h at 18,500*g*, and the supernatant was collected. The supernatant was dialyzed overnight with 3 changes of pH 7.4 1× phosphate-buffered saline (PBS) buffer to remove urea and refold the protein. The supernatant was gently mixed at 4 °C with glutathione beads overnight. The beads were then washed with pH 7.4 1× PBS buffer with 150 mM sodium chloride. Protein was eluted using a pH 8.0 50 mM Tris buffer with 20 mM reduced glutathione, 0.2 M sodium chloride, and 0.2% SDS. The eluent was then concentrated and dialyzed at room temperature overnight with 3 changes of pH 7.4 1× PBS to remove excess glutathione and sodium dodecyl sulfate (SDS). The protein concentration was calculated using a bicinchoninic acid (BCA) assay (Thermo Fisher 23225).

Recombinant Receptor-Associated Protein (RAP). Recombinant RAP was produced as a GST-fusion protein using a pGEX plasmid as previously reported[9] and described above.

LRP1 Ligand-Binding Domains CCRII and CCRIV Fusion Protein. Cryopreserved Chinese hamster ovary (CHO) cells containing LRP1 CCRII and CCRIV domain sequences spliced into a pFUSE vector containing rabbit Fc antibody domain were previously reported.[14] Vector sequences were isolated and verified that they contained the sequences of the respective fusion proteins. CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM) low glucose with 1× nonessential amino acids, 2 mM glutamine, 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were selected for vector using zeocin at a final concentration of 300 μg/mL. Once cells reached 70% confluency, cells were changed to expression medium Power-CHO 2 (Lonza 12-771Q) containing 4 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 300 μg/mL zeocin. Medium was collected at 24 and 48 h. The collected medium was filtered using a 0.22 μm vacuum filter and concentrated to 50 mL using a spin column. Concentrated medium was incubated with protein A beads overnight with gentle shaking at 4 °C. Beads were then washed with 2 bed volumes of 1× PBS and 1 bed volume of PBS with 5 mM ethylenediaminetetraacetic acid (EDTA) and PBS with 500 mM sodium chloride. Protein was eluted with a pH 3.0 0.1 M citric acid buffer. Eluted proteins were dialyzed overnight in pH 8.0 100 mM Tris at 4 °C with 3 changes of buffer, and protein concentration was determined by BCA.

Peptide Synthesis and Purity. Peptides **1**−**4** were synthesized by PL Laboratories or Anaspec at >95% purity and verified by HPLC. Peptides were all soluble in water up to 10 mg/mL, except for peptide **3**, where we used 10% dimethyl sulfoxide (DMSO).

In Vitro SC Signaling Assays. SCs (50,000 cells) were plated on poly-D-lysine-coated 6-well plates and grown in complete media until 80% confluent. The cells were then washed with serum free medium and incubated for 1 h before treatments. Peptides or GST-PEX was added to SCs for 15 min after serum deprivation for 1 h. Medium was aspirated, and cells were washed with cold PBS before adding cold radioimmunoprecipitation (RIPA) buffer (1% Trion-X, % deoxycholate, 1% SDS, 20 mM vanadate, and EDTA-free protease inhibitor) to lyse the cells. Cell lysates were centrifuged at 20 K × *g* for 15 min at 4 °C, and the supernatant was collected.

In Vivo Cell Signaling Assays. C57BL/6 mice were subjected to nerve crush injury. Briefly, mice were anesthetized initially with 4% isoflurane (IsoSol; VedCo, St. Joseph, MO) and maintained with 2% isoflurane. An incision was made along the long axis of the femur. The sciatic nerve was exposed at the midthigh level by separating the biceps femoris and the gluteus superficialis and then carefully cleared of surrounding connective tissue. Nerves were crushed twice for 15 s with blunt forceps in the same location at the sciatic nerve notch. The muscle and skin layers were closed, and the wound was closed with surgical staples. The crush location was marked by a suture in the muscle. 24 h following crush injury and under isofluorane anesthesia, sciatic nerves were exposed and peptide **2** (1 μM) or vehicle (PBS) was injected intraneurally, distal to

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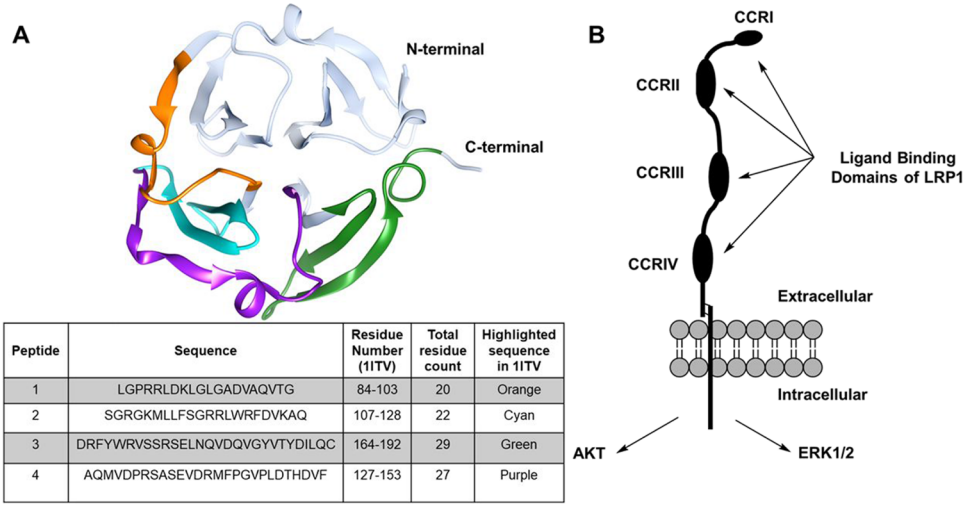

Peptide	Sequence	Residue Number (1ITV)	Total residue count	Highlighted sequence in 1ITV
1	LGPRRLDKLGLGADVAQVTG	84-103	20	Orange
2	SGRGKMLLFSGRRLWRFDVKAQ	107-128	22	Cyan
3	DRFYWRVSSRSELNQVDQVGYVTYDILQC	164-192	29	Green
4	AQMVDPRSASEVDRMFPGVPLDTHDVF	127-153	27	Purple

Figure 1. Design of peptides derived from the hemopexin domain of MMP-9 (PEX). (A) Structural representation of PEX (PDB: 1ITV) is shown along with the selected peptides derived from this protein. (B) Complement repeat domains (CCRs) in LRP1 are labeled I–IV and contain motifs that are known to bind to a diverse group of ligands. CCRII and CCRIV domains constitute the well-described LRP1 binding domains that have been used to show in vitro binding to LRP1.

the crush injury in the sciatic nerve, using a 5 μL Hamilton syringe with a 32-gauge needle. After 15 min, nerve tissue was collected and lysed in RIPA buffer for immunoblot analyses.

Immunoblotting. Protein in nerve and cell extracts was determined by the BCA assay. Protein extracts (10 or 20 μg/lane) were subjected to SDS-PAGE on 8–12% gradient gels and then electrotransferred to nitrocellulose or poly(vinylidene fluoride) (PVDF membranes using a BioRad Trans-Blot Turbo Transfer System (Hercules, CA). Membranes were blocked with a blotting grade blocker (5% nonfat dry milk) (#170-6404, Bio Rad, Irvine, USA) or bovine serum albumin (BSA) (#97061-420, VWR, Swedesboro, USA) in 10 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Primary antibodies (phospho-ERK1/2 (Cell Signaling Cat. #9101; phospho-Akt Cat. #9271; phospho-cJun, Cat. 2130162; total-cJun; 165, and total-ERK1/2, Cat. #9102) diluted in 5% BSA/TBS-T or MILK/TBS-T were incubated with the membranes overnight at 4 °C. Membranes were washed with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antirabbit IgG (#7074, Cell signaling, Danvers, USA) in TBS-T with nonfat dry milk for 1 h at 22 °C, followed by enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Waltham, USA). Blots were imaged using a BioRad ChemiDoc Imaging System (Hercules, CA) or a blue film.

Pulldown Assays. *N*-Hydroxy succinimide (NHS)-activated Sepharose beads from Cytiva (Cat #1709061) were used to immobilize peptides or GST-PEX. Beads were washed and prepared according to the manufacturer's protocols. GST-PEX or peptides were introduced to the washed beads with a 0.02 M pH 8.0 phosphate buffer and allowed to react for 2 h at room temperature with top-down agitation. The remaining NHS-beads were quenched with 0.1 M ethanolamine at pH 8.5 for another hour. Beads were then blocked for nonspecific binding using 0.5% BSA in pH 8.0 phosphate buffer for 30 min at room temperature. CCRII or CCRIV was added and allowed to incubate for 1 h at room temperature with top-down agitation. In competitive ligand-binding assays, GST-RAP (5 molar excess of RAP) was mixed with CCRII or CCRIV prior to being introduced to the beads. The beads were stringently washed with pH 8.0 phosphate buffer in 0.1% Tween 20. Sample buffer was added to the beads, boiled, and analyzed by immunoblot.

Structure Prediction with AlphaFold2. Protein structure prediction was completed with AlphaFold2[15] version 2.3.0 using the multimer[16] functionality for the protein and peptide complex. We downloaded the source code from the AlphaFold2 Github page (https://github.com/deepmind/alphafold). Protein structure prediction was for proteins found in *Homo sapiens*. We predicted the complex of CCRII of LRP1 (UniProt ID: Q07954) with a peptide from PEX) of MMP9 (UniProt ID: P14780). We also predicted the uncomplexed structures of CCRII and PEX using the same UniProt ID numbers. The maximum template release date that we used was from 14 May 14, 2020. We used the full genetic database configuration and included a final relaxation step on the predicted model. Five predictions were generated for the protein–peptide complex, each starting with a random seed. Apart from the maximum template release date, which must be set manually, all of these are the default settings from AlphaFold2. This structure prediction workflow outputs five structures ranked by their predicted template-modeling (pTM) score; we selected the top-ranked structure in each case, even if this structure had a slightly lower predicted local difference distance test (pLDDT) score than a model that ranked lower in the pTM ranking. We used the pLDDT to predict which regions of the protein complex are disordered and used that predicted aligned error to measure which regions of the protein were predicted with high confidence.

Prediction of Relative Stability of Peptide–Protein Complexes. The top predicted complex from Alphafold2, as described above, was used for predicting mutations that would interfere with the protein–peptide interface. First, the interface residues were determined with Molecular Operating Environment (MOE) 2022.[17] Then, the predicted complex structure was put through a residue scan on the interface residues using the Amber10:EHT force field[18,19] in MOE to determine which point mutations would most interfere with the protein–peptide interface. We selected the top 8 mutations and put these through the MOE LowMode MD scan functionality with

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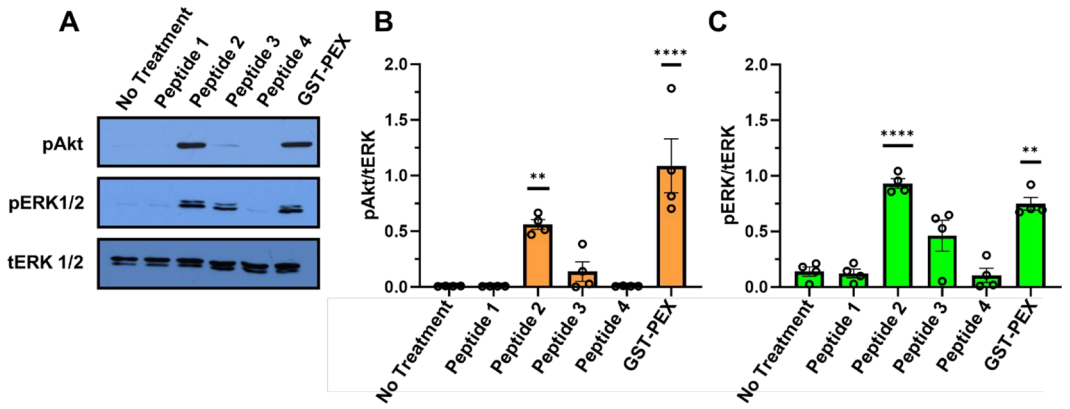
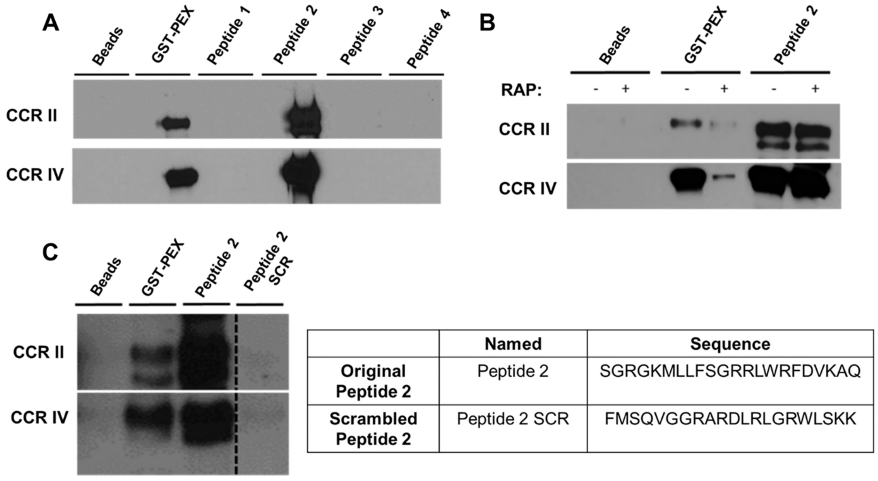


Figure 2. Activation of the cell signaling by peptides derived from PEX in hSCs. (A) Representative immunoblots of hSCs stimulated by GST-PEX (100 nM) or by PEX-derived peptides **1**−**4** (10 μM) for 15 min. Densitometry of immunoblots for (B) pAkt/tERK or (C) pERK/tERK. The data are presented as normalized to tERK1/2 ($N = 4$). Statistical analysis relative to the blot intensity for no treatment was performed using Brown−Forsythe and Welch ANOVA tests (**$p < 0.01$, ****$p < 0.0001$).



	Named	Sequence
Original Peptide 2	Peptide 2	SGRGKMLLFSGRRLWRFDVKAQ
Scrambled Peptide 2	Peptide 2 SCR	FMSQVGGRARDLRLGRWLSKK

Figure 3. Pulldown studies of recombinant CCRII and CCRIV by peptides **1**−**4** and GST-PEX. (A) Peptides **1**−**4** and GST-PEX were used to pull down the LRP1-binding domains CCR II and IV. (B) Pulldown of CCRII and CCRIV by peptide **2** or GST-PEX in the presence or absence of GST-RAP, a known binder of LRP1 CCR II and IV. (C) Pulldown studies examining the binding of CCR II and CCR IV by a scramble of peptide **2** compared to GST-PEX or peptide **2**.

default parameters to estimate changes in the stability of the complex caused by the mutations.

RESULTS AND DISCUSSION

PEX is a 23 kDa protein that consists of 4 propeller-like domains.[13,20,21] Peptide candidates were designed using the crystal structures of PEX (PDB entry 1ITV) and were determined to be the peptide sequences that were in the most hydrophilic regions along the surface and contained limited or defined secondary structure. The propeller region adjacent to the N-terminus is the region hindered by the interaction with the rest of the MMP-9 protein and was avoided in peptide selection. With this information and criteria in mind, four peptides were designed (Figure 1A) and commercially synthesized at 98% purity. Peptides **1**, **2**, and **4** were found to be soluble at concentrations of 10 mg/mL in pure water, while peptide **3** required 10% DMSO to completely dissolve in water at this concentration.

To determine whether peptides **1**−**4** activated SCs signaling in vitro, we utilized a commercially available human SC line as we described previously.[9,10] The holoprotein, MMP-9, and the hemopexin domain, PEX, are known to robustly activate ERK1/2 and Akt in an LRP1-dependent manner in primary rat SC cultures.[9] In human SCs, other LRP1 ligands such as tissue plasminogen activator robustly activate ERK1/2, Akt, and cJun.[10] Based on these published studies, peptides **1**−**4** were first tested for their ability to activate (e.g., phosphorylate) Akt and ERK1/2 in human SC cultures. A glutathione s-transferase (GST)-fused holoprotein, GST-PEX, was used as a control. Of the four peptides, only peptide **2** consistently and significantly activated Akt and ERK1/2 signaling (e.g., increases pAkt and pERK1/2) compared to no treatment, similar to GST-PEX (Figure 2A−C).

To test if the peptides directly bind LRP1, pulldown assays with recombinant Fc fusion proteins of human CCR domains II and IV were used. Both CCR II and CCR IV are domains in LRP1 that have been shown to bind to metalloproteases.[22] Peptides or GST-PEX was immobilized on NHS-ester beads, and Fc fusion proteins for CCR domains II and IV were added. Next, we immunoblotted Fc to determine the presence of the LRP1 CCR fusion proteins. Only GST-PEX and peptide **2** pulled down the LRP1 CCR II and IV domains (Figure 3A). When we added RAP, a competitive antagonist to LRP1 routinely used to block binding of ligands to LRP1,[9,23] binding

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of GST-PEX was substantially inhibited (Figure 3B) but not peptide **2**. We attribute this finding to the small size of peptide **2** compared to GST-PEX, which may not be inhibited sterically by RAP for binding to LRP1 as previously observed with other protein LRP1 ligands. Moreover, a previous study showed that RAP predominantly interacts with the CR4−7 repeats within the CCR domains,[24] whereas our computational modeling predicts that peptide **2** predominantly interacts with the CR1 repeat within the CCR domains of LRP1 (Figure S1). Similarly, when we added free GST-PEX in these pulldown studies, free GST-PEX was able to substantially inhibit the binding of immobilized GST-PEX to CCRII, but like RAP, free GST-PEX was not able to inhibit the binding of immobilized peptide **2** to CCRII (Figure S3). Analogously, free peptide **2** was able to inhibit the binding of immobilized peptide **2** to CCRII but not the binding of immobilized GST-PEX to CCRII. Computational modeling using AlphaFold2 for the complex between PEX and CCRII suggests that PEX predominantly interacts with the CR2−5 repeats within the CCR domains (unpublished results), which supports the observation that PEX and RAP can compete with each other for binding to LRP1, but neither of these proteins compete for binding of peptide **2** to LRP1. To ensure that the specificity of peptide **2** to LRP1 was sequence-dependent, a scramble of the peptide was also tested in the pulldown assay. The scrambled peptide **2** (peptide **2** SCR) was unable to pull down the CCR domains (Figure 3C).

Based on the results of the *in vitro* cell signaling and pulldown studies, we investigated whether peptide **2** could induce cJun signaling *in vivo*. cJun is a key cell signaling pathway activated in the SC Repair program.[25] Peptide **2** (1 μM) or vehicle was injected intraneural into crush injured nerves (after 24 h, a time when LRP1 is upregulated in SCs[8]) in wild-type mice. Nerves were collected after 15 min, lysed, and immunoblotted for phospho-cJun and total cJun (Figure 4). Densitometric analyses of immunoblots from three independent studies revealed that peptide **2** increased phospho-cJun by greater than fivefold compared to naïve nerve and by greater than 2.5-fold compared to vehicle-treated injured nerves ($p < 0.05$). Because LRP1 is upregulated in SCs after injury and that peptide **2** did not activate cJun signaling in naïve nerves, our results showing that peptide **2** activated cJun after injury are consistent with LRP1-dependent cell signaling.

In order to provide a molecular interpretation of the binding of peptide **2** to the CCR domains of LRP1, we next turned to in silico modeling and stability studies. Initial AlphaFold2 modeling predicted that the charged residues on peptide **2** R109, K111, and R122 cluster at the interface with peptide **2** and CCRII (Figures 5A and S1). Here, we kept the PEX amino acid numbering from the PDB: 1ITV (residues 107−128) for the numbering of the sequence of peptide **2** (Figure 1A). Such electrostatic interactions between LRP1 and LRP1 ligands have precedence in literature, where previous studies found that positively charged residues on protein ligands play a large role in binding to LRP1.[26] To further examine which of these positively charged residues on peptide **2** might be crucial for binding to LRP1, possible amino acid point mutations to alanine that may impair the interaction of CCRII-peptide **2** were computationally examined (Figure 5B). Apart from the six positively charged lysine or arginine residues in peptide **2**, we also examined mutation of W121 to an alanine as tryptophan is found to have specific roles in stabilizing proteins and beta structures.[27,28] In all mutations, the

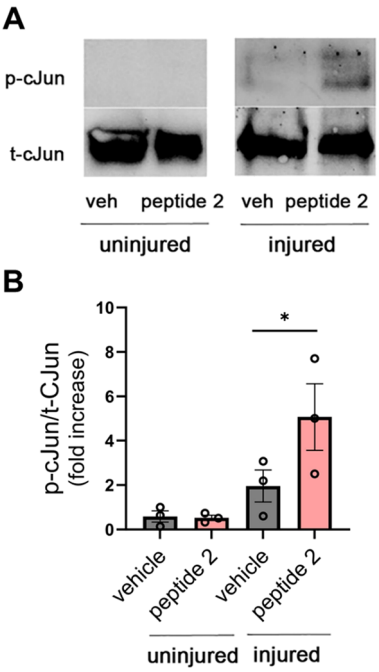

Figure 4. Peptide **2** activates cJun in acutely injured sciatic nerves in vivo. (A) Representative images of immunoblots of nerve extracts from uninjured and injured nerve with intraneural injections of vehicle or peptide **2** (1 μM) for 15 min probed with anti-phospho cJun (p-cJun) and total cJun (t-cJun). (B) Densitometric analyses of p-cJun to t-cJun in vehicle or peptide **2**-injected nerves. Data are fold increase compared to naïve nerve. $N = 3$/group. One-way ANOVA and Neuman−Keuls post hoc test (*$p < 0.05$).

calculations predicted a decreased stability of the peptide−fCCRII complex with the largest effects being found in mutants R119A and R122A.

In order to support the in silico predictions of the stability of peptide−CCRII complexes, we generated seven mutants of peptide **2** shown in Figure 5B and analyzed their capability to bind to CCRII using the same in vitro pulldown study as described for Figure 3. Figure 5C shows immunoblots of the CCRII fusion protein that were pulled down by peptide **2**, the peptide **2** scramble (Figure 3C), and the seven peptide **2** mutants. Densitometry analysis shown in Figure 5D shows that mutations R109A, K111A, R118A, R119A, W121A, and R122A had a significantly negative effect on binding to CCRII compared to unmutated peptide **2**, with R119A and R122A having a similar capability to pull down CCRII as the scramble peptide (peptide **2** SCR). The effect on pulldown of CCRII by these peptide 2 mutants correlated well with the computed stability of the peptide−protein complex (Figure S2).

CONCLUSIONS

Utilizing the crystal structure and sequence of the MMP-9 hemopexin domain (PEX), four peptides were selected as candidates for LRP1 binding and activation based on their location and secondary structure within the intact protein. Of the four selected peptides, only peptide **2** activated Akt and ERK1/2 signaling in primary hSCs and bound to the LRP1 binding domains, CCR II and CCR IV, suggesting that this peptide is a true human LRP1 binder. Among these 4 peptides, only peptide **2** contains two somewhat proximal lysine residues that is consistent with critical lysine residues identified in the

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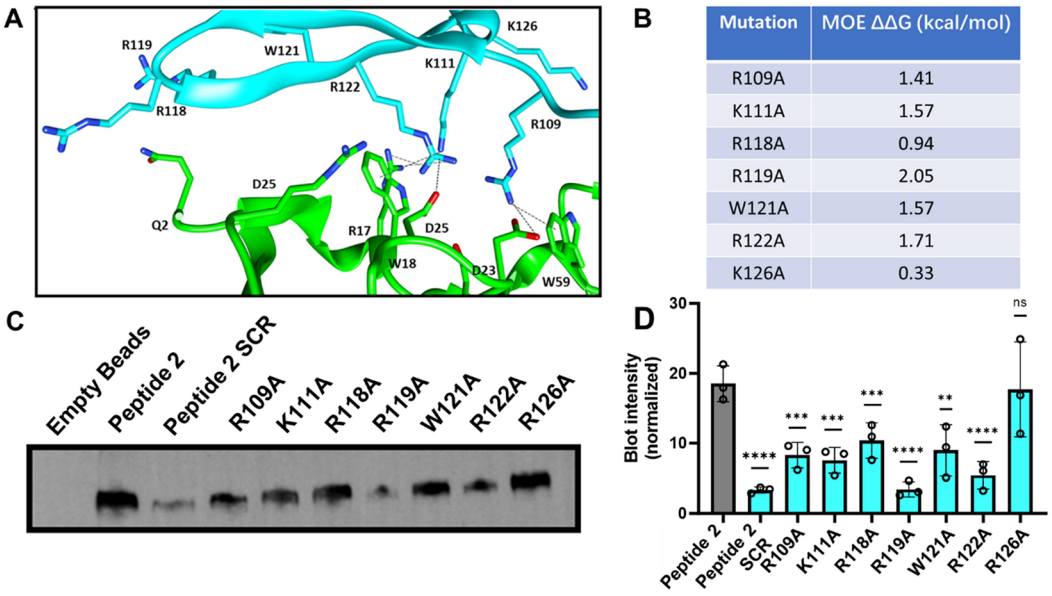

Figure 5. AlphaFold2 prediction of the CCRII−peptide **2** complex and the effect of amino acid mutations on the stability and binding. (A) AlphaFold2-predicted complex of CCRII (green) and peptide **2** (cyan). (B) Effect on free energy of peptide **2**−CCRII interaction derived from mutations to peptide **2** calculated using low mode MD simulations in MOE. All peptide **2** mutants in this study had an overall destabilizing effect on the peptide **2**−CCRII complex compared to unmutated peptide **2.** (C) Immunoblot of pulldowns of the CCRII with alanine mutants of peptide **2**. Pulldowns of the CCRII by unmutated peptide **2**, the peptide **2** scramble (shown in Figure 3C), and the empty beads were included in this blot as controls. (D) Quantification of pulldown studies where each lane was normalized to the control−empty beads. Statistical analysis relative to the blot intensity for Peptide **2** was performed using Brown−Forsythe and Welch ANOVA tests (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = not significant).

binding region of other LRP1 ligands[29,30] such as coagulation factor VIII,[31] PAI-1,[26] and more recently, SP16.[23] When lysine 111 was converted to an alanine, the level of binding to CCRII was reduced. Notably, when arginine residues 119 or 122 were substituted with an alanine, binding to CCRII was substantially reduced, with the predicted stability of the peptide−CCRII complexes also reduced compared to nonmutated peptide **2**. We also employed scrambled peptide **2** that failed to bind CCR domains, suggesting the importance of the specific peptide sequence. Other well-known LRP1 ligands such as alpha-2 macroglobulin encompass putative LRP1 recognition sequences including proximal lysine residues that likely interact with negatively charged amino acids in the LRP1 complement repeats.[32] The role of arginine residues contributing to LRP1 binding is less explored, but due to its positive charge, it may play a role similar to that of lysine residues. Further studies are underway to understand the role of lysine and arginine residues on ligands in the LRP1 bioactivity. Further characterization of this peptide and the properties that drive strong and specific binding and activation of LRP1 in Schwann cells could lead to the generation of new druggable compounds that may aid in sensory nerve recovery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.3c00705.

Additional images of the predicted complex between peptide **2** and the CCRII domain of LRP1 and a correlation analysis of the predicted stability of peptide **2** mutants and CCRII versus the relative capability of these peptides to pull down CCRII (PDF)

Accession Codes

UniProt ID: Q07954, UniProt ID: P14780

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Author Contributions

J.H.K., W.M.C., and J.Y. conceived and designed the research. J.H.K., A.S., M.N., S.C.L., and C.S. executed the experiments. A.S., C.S., and R.E.A. designed and executed the in silico experiments and analyzed the data. S.L.G. helped analyze the in vitro data and guided the design of additional experiments. J.H.K., A.S., W.M.C., and J.Y. wrote the manuscript. All authors reviewed and edited the manuscript. J.H.K. and A.S. contributed equally to this paper.

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The authors declare the following competing financial interest(s): J.Y. is a founder, equity interest holder, and advisor of Amydis, Inc. All other authors declare no competing financial or non-financial interests.

■ ABBREVIATION

SC, Schwann cells; PNS, peripheral nervous system; LRP1, low-density lipoprotein receptor-related protein-1; PEX, hemopexin domain; GST, glutathione S-transferase; CCR, clusters of complement-like repeats; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; Akt, protein kinase B; MMP-9, matrix metallopeptidase 9; hSC, human Schwann Cells; FBS, fetal bovine serum; LB, Luria broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; tris, hydroxymethylaminomethane; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BCA, bicinchoninic acid; RAP, recombinant receptor-associated protein; CHO, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; RIPA, radioimmunoprecipitation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; BSA, bovine serum abumin; TBS, Tris-buffered saline; HRP, horseradish peroxidase; NHS, *N*-hydroxy succinimide; pTM, predicted template-modeling; pLDDT, predicted local difference distance test; MOE, molecular operating environment; MD, molecular dynamics; DMSO, dimethyl sulfoxide; α2M, alpha-2 macroglobulin.

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