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Neurochondrin is an atypical RII α -specific A-kinase anchoring protein

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Abstract

Protein kinase activity is regulated not only by direct strategies affecting activity but also by spatial and temporal regulatory mechanisms. Kinase signaling pathways are coordinated by scaffolding proteins that orchestrate the assembly of multi-protein complexes. One family of such scaffolding proteins are the A-kinase anchoring proteins (AKAPs). AKAPs share the commonality of binding cAMP-dependent protein kinase (PKA). In addition, they bind further signaling proteins and kinase substrates and tether such multi-protein complexes to subcellular locations. The A-kinase binding (AKB) domain of AKAPs typically contains a conserved helical motif that interacts directly with the dimerization/docking (D/D) domain of the regulatory subunits of PKA. Based on a pull-down proteomics approach, we identified neurochondrin (neurite-outgrowth promoting protein) as a previously unidentified AKAP. Here, we show that neurochondrin interacts directly with PKA through a novel mechanism that involves two distinct binding regions. In addition, we demonstrate that neurochondrin has strong isoform selectivity towards the RII α subunit of PKA with nanomolar affinity.

Keywords

Neurochondrin; A-kinase anchoring proteins; Protein Kinase; A RII-specific

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1. Introduction

Protein kinases are critical mediators of intracellular signaling. However, many kinases exhibit very broad substrate specificity and therefore need to be tightly regulated both spatially and temporally. Specificity and efficiency in cell signaling can be achieved by targeting signaling molecules to subcellular compartments. A-kinase anchoring proteins (AKAPs) are a family of scaffolding proteins (approximately 50 known AKAPs in humans [1–3]) that limit the access of cAMP-dependent protein kinase (PKA) activity in cells to defined pools of its substrates. In addition, AKAPs bind other components of the cAMP signaling cascade including G protein-coupled receptors (GPCRs), adenylyl cyclases (ACs) and phosphodiesterases (PDEs) [4–8]. Furthermore, AKAPs can also interact with other kinases, phosphatases and substrate proteins [1–9].

In its inactive state, the PKA holoenzyme consists of a dimer of regulatory (R) subunits (RI or RII) and two catalytic (C) subunits. Upon cooperative binding of four molecules of the second messenger cAMP to the R-subunits, the C-subunits dissociate and thereby are activated to phosphorylate substrates within close proximity [10, 11]. In general, AKAPs bind PKA through an amphipathic helix that mediates the interaction with the dimerization and docking domain (D/D) of the PKA R-subunit dimer [12–15]. However, so-called non-canonical AKAPs have also been identified that contain divergent A-kinase binding (AKB) regions (reviewed in [3]). For instance, the interaction between pericentrin and RII is mediated by a long 100 amino acid non-helical, leucine-rich region [16]. Another example is the interaction between ribosomal S6 kinase 1 (RSK1) and RI α , which is insensitive to the AKAP disruptor peptide Ht31 [17, 18]. Ht31 binds the canonical AKAP docking site on the R-subunits and thereby globally uncouples PKA from AKAPs [19, 20]. Furthermore, α - and β -tubulin are AKAPs for type I PKA in neurons, however the molecular determinants for PKA binding are still unknown [21].

Neurochondrin is a neuronal cytoplasmic protein that was originally described in the context of chemically induced long-term potentiation (LTP) in rat hippocampus [22]. Overexpression of neurochondrin in Neuro2a cells is associated with neurite outgrowth. Therefore, neurochondrin has an important role in synaptic plasticity [22]. A forebrain-specific neurochondrin knockout revealed dramatic changes in synaptic transmission in the hippocampus [23]. Due to its prominent roles in potentiation and plasticity, neurochondrin is viewed as a regulator for the central nervous system [24, 25]. Structural data and molecular details underlying the function of neurochondrin are unknown.

Here we report a direct interaction between neurochondrin and the RII α subunit of PKA and identify a non-canonical RII-binding domain that consists of two distinct, non-sequential segments. Surface plasmon resonance (SPR) measurements revealed equilibrium binding constants in the low nanomolar range. Since neurochondrin does not bind the other R-subunit isoforms of PKA, we show that neurochondrin acts as a high-affinity, RII α -specific AKAP.

2. Material and Methods

2.1 Material

All structural figures were prepared using PyMol (<http://www.pymol.org>).

2.2 Chemicals

Unless mentioned elsewhere, chemicals were either obtained from Sigma-Aldrich (Steinheim, Germany), Carl Roth GmbH & Co KG (Karlsruhe, Germany) or AppliChem GmbH (Darmstadt, Germany).

2.3 Cloning

A human cDNA clone (ImaGenes GmbH, Berlin, Germany (now Source BioScience, Nottingham, England) was used to clone full-length neurochondrin into the bacterial expression vector pPSG IBA 167 (IBA GmbH, Göttingen, Germany). The vector contains the coding sequence for an N-terminal FLAG-One-STrEP fusion peptide. For cloning, the StarGate® cloning system (IBA GmbH, Göttingen, Germany) was used.

The full-length expression clone pRSETb human RII α , a generous gift from Prof. Susan S. Taylor, was used to clone the truncated version comprising the amino acids 1-44 into the bacterial expression vector pGEX-KG [26, 27].

The oligonucleotides for PCR (neurochondrin forward primer: 5' P-AATGTCGTGTTGTGACCTGGCTG-3'; neurochondrin reverse primer: 5' P-TCCCGGGCTCTGACAGGCACTG-3'; hRII α 1-44 forward primer: 5' *Nde*I-GAAGAGCATATGAGCCACATCCAGATC-3'; hRII α 1-44 reverse primer: 5' *Hind*III-GTAAGCTTCTAGGCGCGGGCCTCGCG-3') were from Eurofins Genomics (Ebersberg, Germany).

2.4 Protein expression and purification

FLAG-One-STrEP-neurochondrin was expressed in *E. coli* strain BL21 DE3 RIL. The cells were grown at 37 °C until the A_{600nm} reached 0.6 and then recombinant protein production was initiated by adding 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The induction was carried out overnight at room temperature and the cells were harvested by centrifugation for 20 min at 4,500 $\times$ g and 4 °C. Cell pellets were stored at -80 °C. After thawing, the cells were resuspended in cell lysis buffer (100 mM Tris, 200 mM NaCl, 10% (v/v) glycerol, 1 mM DTT (dithiothreitol), pH 8.0) supplemented with protease inhibitor cocktail (Complete EDTA free, Roche, Mannheim, Germany) and 0.1 mg/mL lysozyme. After incubation on ice for 30 min, cells were lysed using a French Pressure Cell (Fisher Scientific GmbH, Schwerte, Germany). After incubation with 80 U/mL DNase I for 30 min on ice the crude lysate was centrifuged at 15,000 $\times$ g for 30 min at 4 °C. The cleared supernatant was loaded onto a Strep-Tactin® Superflow® cartridge H-PR column (IBA GmbH, Göttingen, Germany) containing a bed volume of 1 mL (preequilibrated with cell lysis buffer). Subsequently, the column was washed with 20 column volumes at a flow rate of 1 mL/min (buffer A: 100 mM Tris, 200 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0). The protein was eluted with 10 column volumes

of elution buffer (buffer A + 2.5 mM d-desthiobiotin (IBA GmbH, Göttingen, Germany)) at a flow rate of 1 mL/min. The purified protein was analyzed by SDS–polyacrylamide gel electrophoresis and subjected to gel filtration analyses.

Human RI or RII subunits were purified using cAMP affinity chromatography as previously described [28]. The truncated version of human RII α (aa 1-44) was purified according to a published protocol [29].

2.5 Gel filtration

Purified neurochondrin (200 μ L) was separated on a Superdex™ 200 10/300 column (GE Healthcare, Freiburg, Germany) in buffer A at a flow rate of 0.5 mL/min. The Superdex™ 200 10/300 column was calibrated using the gel filtration calibration kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's protocol.

2.6 Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jasco J815 spectropolarimeter in 25 mM Tris, 50 mM NaCl, pH 8.0 at 20 °C, at protein concentrations of 0.5 to 1 mg/mL in 0.05 mm cuvettes. Six scans were accumulated from 190 to 260 nm with a digital integration time of 1 s, a bandwidth of 1 nm and a scan speed of 50 nm/min. Data were collected in 0.5 nm steps. Spectra determined by taking buffer effects into account. Ellipticities were calculated according to Schmid [30] and the data were analyzed using DICROWEB [31].

2.7 Pull-down approach

Pull-downs of PKA R-subunit and holoenzyme complexes with the respective agonist (Sp-2-AEA-cAMPS) and antagonist (Rp-8-AHA-cAMPS) agaroses (BIOLOG Life Science Institute, Bremen, Germany) from pig brain lysates and subsequent mass spectrometry analyses were performed as described previously [28, 32].

2.8 Surface Plasmon Resonance measurements

Interaction studies were performed using Biacore 3000 instrument (GE Healthcare, Freiburg, Germany) and data were evaluated with the BIAevaluation software version 4.1.1

The R-subunits were captured as previously described [29]. Measurements were performed in running buffer containing 100 mM Tris, 150 mM NaCl, 0.005% (v/v) Tween 20, pH 7.4, at 25 °C instrument temperature. In brief, 8-AHA-cAMP (BIOLOG Life Science Institute, Bremen, Germany) was covalently coupled to CM5 sensor chips (research grade) using standard NHS/EDC chemistry as described previously [28]. Purified RI or RII subunits were injected in running buffer containing 1 mg/mL bovine serum albumin for reversibly immobilization on an 8-AHA-cAMP surface (surface density of 70–600 resonance units). A surface containing 8-AHA-cAMP without captured R-subunit served as reference in order to test for unspecific binding. Blank runs (buffer only) were performed and subtracted.

The neurochondrin peptides (peptide I: YAGDIDAKTRRRIFDAVGFTF, peptide II: KLSSWQRNPALKLAARLAHA) were covalently coupled to CM5 sensor chips (research grade, GE Healthcare). The peptides were dissolved in DMSO. CM5 sensor chip surfaces

were activated for 10 min with NHS/EDC according to the manufacturer's instructions (amine coupling kit, GE Healthcare). Subsequently, each peptide was injected until around 1 resonance unit (RU) was immobilized on the surface. Deactivation of the surface was performed with 1 M ethanolamine-HCl, pH 8.5. Each flow cell was activated, coupled and deactivated individually with a flow rate of 5 μ L/min at 25 °C. One flow cell, activated and deactivated accordingly, was used as reference.

Kinetics of neurochondrin—Neurochondrin was injected at different concentrations (7.8–250 nM, serial twofold dilution) at a flow rate of 30 μ L/min. Both association and dissociation phases were recorded for 300 s. Rate constants (k_a and k_d) and equilibrium binding constants (K_D) were calculated based on nonlinear regression analysis assuming a 1:1 Langmuir binding model using the BIAevaluation software, version 4.1.1 (GE Healthcare, Freiburg, Germany).

Surface competition experiments with peptides Ht31 and Ht31P—

Neurochondrin, in a concentration of 300 nM was injected into the Biacore device over RII α -coated chips at a flow rate of 30 μ L/min in the absence or presence of 5 μ M peptide Ht31, Ht31P (Biaffin GmbH & Co KG, Kassel, Germany) or AKAP18 δ L314E. Association and dissociation phases were recorded for 300 s, respectively. As control, experiments were performed using 5 μ M of each of the peptides Ht31, AKAP18 δ L314E or Ht31P without neurochondrin present and these values were subtracted.

Regeneration—Complete removal of bound material from 8-AHA-cAMP chip surfaces or from neurochondrin peptide containing surfaces was accomplished by three short injections (15 s each) of 0.1% (w/v) SDS, followed by a single injection of 4 M guanidinium hydrochloride (30 s) and an injection of running buffer (including 1 M NaCl, 30 s).

2.9 Far Western blot

For Far Western analysis, equimolar amounts of RI α , RII α and GST-1-44 RII α as well as 125 ng neurochondrin (used as control) were separated by SDS-PAGE and transferred onto PVDF membranes. Renaturation of proteins was carried out overnight at 4 °C in 20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5 (TBS-T buffer). The membrane was blocked in 2% (w/v) bovine serum albumin in TBS-T at room temperature for 1 h. Neurochondrin (0.1 μ M) was incubated with the membrane at room temperature for 1 h followed by washing in TBS-T and incubation with Strep-Tactin® HRP conjugate (IBA GmbH, Göttingen, Germany) at a dilution of 1/100,000 for 1 h at room temperature in TBS-T. To confirm equal loading the membrane-bound proteins were stained with amido black (0.01% (w/v) amido black, 40% (v/v) methanol, 10% (v/v) acetic acid).

2.10 Peptide Array and RII overlay

Peptide spots were generated by automatic SPOT synthesis using the Intavis ResPep-SL (Intavis, Koeln, Germany) as described previously [33–36]. The interaction of spot-synthesized peptides with RII subunits of PKA was investigated by RII overlay assay using radioactively labeled RII subunits [34, 37, 38]. Competition experiments were performed in the presence of 10 μ M of the peptide AKAP18 δ L314E

(PEDAELVRLSKRLVENAVEKAVQQY) or its control AKAP18 δ -PP (PEDAELVRLSKRLPNAPLKAVQQY).

2.11 Peptide synthesis

Peptides were synthesized on solid support using Fmoc chemistry. Rink amide MBHA resin (25 μ mol) was deprotected using 25% piperidine in 1-methyl-2-pyrrolidinone (NMP) for 25 min. All subsequent deprotections were performed under the same conditions. Couplings were performed for 45 min using 0.5 M Fmoc-protected amino acid (0.5 mL, 250 μ mol, 10 equiv.), 0.5 M HCTU (0.495 mL, 247.5 μ mol, 9.9 equiv.), and diisopropylethylamine (87 μ L, 500 μ mol, 20 equiv.) in NMP. After the final deprotection the peptides were then cleaved from the resin in a solution containing 95% TFA, 2.5% triisopropylsilane, and 2.5% water for 4 hours. The resin was removed by filtration through glass wool, and the peptides were precipitated in ice cold *tert*-butyl methyl ether. The precipitate was pelleted by centrifugation and the supernatant was discarded. The dry peptide was dissolved in methanol and characterized by LC/MS using a Zorbax SB-C18 5 μ m column with an Agilent 1200 series HPLC coupled to an Agilent 6120 quadrupole LC/MS. Peptides were purified by reverse-phase HPLC using a gradient of 100% acetonitrile in water containing 0.1% TFA. Neurochondrin peptide I molecular weight = 2418.0 (expected = 2418.7); neurochondrin peptide II = 2229.9 (expected = 2230.6).

3. Results

3.1 Pull down approach to identify new AKAPs

As a non-biased strategy to identify novel AKAPs, we performed pull-down experiments targeting the R-subunits of PKA in pig brain lysates. As previously described [28, 32], lysates were incubated with Rp-8AHA-cAMPS- (antagonist) or Sp-2-AEA-cAMPS- (agonist) agarose. After an extensive wash, bound proteins were eluted with 20 mM cAMP, separated by SDS-PAGE and analyzed by mass spectrometry [28, 32]. In addition to previously described cAMP-binding proteins, neurochondrin was also identified.

3.2 Neurochondrin preferentially binds PKA-RII α through contacts with the D/D domain

In order to verify that neurochondrin was bound to the cAMP agarose through interactions with the R-subunits of PKA, the interaction between neurochondrin and the R-subunits of PKA was examined by SPR. Full-length human RI α , RI β , RII α and RII β were captured on high-density 8-AHA-cAMP sensor surfaces yielding average surface concentrations of 160–600 resonance units (RU). Neurochondrin was injected as an analyte over these surfaces.

As shown in Figure 1a, neurochondrin robustly binds the RII α subunit, but only weakly interacts with the RII β or RI-isoforms even though higher immobilization levels were used as compared to RII α . Using a Far Western blot approach [39, 40], this isoform selectivity was confirmed (Figure 1b). Incubation with neurochondrin (+) yielded a strong signal for RII α , whereas no signal was detected for RI α . Moreover, we found that neurochondrin binds to a construct containing the first 44 residues of RII α that includes the dimerization/docking (D/D) domain (Figure 1b). To further characterize this interaction, the RII α subunits were immobilized at surface concentrations of 330 RU on an 8-AHA-cAMP sensor surface.

Neurochondrin was injected in the presence or absence of AKAP disruptor peptides Ht31 [19], derived from AKAP-Lbc; and AKAP188L314E [34], derived from AKAP188. Both peptides target the D/D domain of the R-subunits and thereby inhibit the classical PKA-AKAP interface. Ht31P was used as a control. This peptide contains proline substitutions that prevent α -helical folding of the peptide, thereby preventing interactions with the R-subunits [19, 20]. Figure 2 demonstrates that both Ht31 and AKAP188L314E efficiently block binding between neurochondrin and RII α . As expected, the interaction between neurochondrin and RII α was not affected by the negative control Ht31P.

3.3 Neurochondrin binds RII α with nanomolar affinity

To quantify the interaction of neurochondrin with RII α , interaction studies were performed where RII α subunits were immobilized on an 8-AHA-cAMP surface and neurochondrin was injected as analyte (Figure 3). Association and dissociation rate constants were determined, and an equilibrium binding constant was measured ($K_D = 7.8 \pm 0.1$ nM).

3.4 Neurochondrin contains a unique RII-binding domain comprised of two different regions

The data described above suggest a canonical interaction between neurochondrin and the RII isoform [3]. Since the majority of AKAPs interact with PKA through an amphipathic helix formed by their AKB sequence, the secondary structure of neurochondrin was investigated. Indeed, a secondary structure prediction using PredictProtein [41] (Appendix A, Supporting Table A1) revealed a large α -helical structure (65% of total protein) and helicity was verified by circular dichroism (CD; Appendix A, Supporting Fig. A1). For mapping the RII-binding (RIIB) domain/s of neurochondrin, we employed a combination of peptide spot technology and overlays using recombinant protein [34, 36, 42, 43]. Arrays of overlapping peptides spanning the entire sequence of neurochondrin (729 amino acids) were spot-synthesized (25-mers, 20 amino acid residues overlap) and subjected to RII overlay assays. The RII subunits bound to two series of overlapping peptides (Figure 4a, A11–A15, D6–D11), indicating two potential interacting sites. Since a decreased apparent affinity is expected with increasing distance from the key binding residues, the core regions of the RIIB regions were identified as KTRRR (peptides A11–A15 in Figure 4c) and WQRNP (peptides D6–D11 in Figure 4d). By using peptide substitution arrays and RII overlays these core regions were confirmed (Appendix A, Supporting Fig. A4). Surprisingly however, the AKAP disruptor peptide, AKAP188L314E that binds the D/D domain of the R-subunits with higher affinity than Ht31 [34] could not prevent RII binding by these key neurochondrin peptides (Figure 4a). Thus, it appears that neurochondrin binds the RII subunit through two distinct regions (Figure 4); since the binding of the full-length construct of neurochondrin to RII α is affected by Ht31, this suggests that only under this condition the D/D domain of the R-subunit is involved (Figures 1–3).

To quantify the interaction of RII α with the individual neurochondrin binding regions, interaction studies were performed using neurochondrin peptides that corresponded to the identified regions. The synthetic neurochondrin peptides (Table 1) were designed according to the peptide array studies (Figure 4) so that the core region for each sequence was flanked by 4 to 11 amino acids (Table 1). For binding studies, the synthetic peptides were covalently

immobilized on a CM5 sensor chip surface and RII α was injected as analyte at concentrations ranging from 7.8 to 250 nM (Figure 5).

Association and dissociation rate constants were determined, and equilibrium binding constants were calculated for both peptides. Both peptides bound the RII α subunit with low nanomolar affinities (Table 2).

In line with our peptide array studies, further SPR analyses revealed that Ht31 did not disrupt the binding of RII α to the neurochondrin peptides I or II (Figure 6). Thus, neurochondrin appears to be capable of binding RII α in a non-canonical manner. Of note, a small increase in the binding signal was observed when RII α was injected in the presence of Ht31. However, this signal is likely due to the mass increase of the RII α -Ht31 complex.

4. Discussion

In this study, we identified a previously unknown function of neurochondrin, namely, its ability to bind PKA R and thereby act as an AKAP. SPR and Far Western blot analyses were used to demonstrate that full-length neurochondrin interacts directly with PKA-RII-subunits. Both methods were previously used to demonstrate AKAP / PKA interaction [29, 37, 39, 40]. Neurochondrin preferentially interacts with the RII α isoform with nanomolar affinity ($K_D = 8$ nM). Binding of the other R-subunits was nearly undetectable. Since the interaction with RII α is prevented by the peptides Ht31 and AKAP188L314E, it is highly likely that it involves the D/D domain. A typical AKB sequence forms an amphipathic helix that is 14–18 amino acids in length. The hydrophobic face of the amphipathic helix docks into the hydrophobic pocket formed by the D/D domain. Residues of the hydrophilic face appear to influence the binding affinity [12, 15, 34, 36, 44, 45]. A variety of AKB-targeting peptides such as Ht31, AKAP188L314E or STAD-2 are designed to interfere with the interaction site [45, 46]. Although these findings strongly suggest that full-length neurochondrin binds RII α in a typical AKAP-like fashion, our further observations argue that the binding interface may be more complex and is composed of at least two separate interfaces.

We identified two distinct high-affinity binding sites in neurochondrin that bind RII based on our peptide array studies (Figure 4). The identified peptides diverge from a typical AKB sequence [36, 44]. For example, peptide II contains a central proline that would introduce a kink into the otherwise helical peptide sequence and thereby may induce a conformation that limits only a portion of the helix as being amenable for RII binding. As such, it is unlikely that a short helix such as the sequence immediately flanking the central sequence WQRNPA in peptide II (ALKLAARLAHA) could bind RII with such high affinity. An RRR motif as in peptide I is not present in other AKBs of AKAPs. Triple R motifs seem to be involved in protein trafficking and biosynthesis [47]. Since Ht31 and AKAP188L314E do not interfere with the interaction between RII α and each of the individual peptides, we conclude that the binding mode of the peptides must be distinct and perhaps not target the canonical binding groove formed at the D/D interface. The question remains as to how the two binding regions from full-length neurochondrin engage in an interaction with RII α that can be inhibited with Ht31. This complex binding interface may become clearer once the 3D structure of neurochondrin has been determined.

AKAP-RII binding is influenced by various interactions including H-bonds and salt bridges [34, 48]. In general, charged amino acid residues, putative salt bridges, or H-bond partners affect the isoelectric point (pI) of a peptide. The calculated pI for several peptides derived from AKB domains of AKAPs varies from 3.43 to 6.23 [36]. Hence, these peptides are negatively charged under physiological conditions. In contrast, both RII-binding regions of neurochondrin exhibit pI values of around 9 (Appendix A, Supporting Table A2 and A3). Thus the interaction between neurochondrin and RII α may not be restricted only to the D/D domain but may extend to negatively charged surface regions. Hence, it is possible that charge-dependence may be a critical aspect for mediating interactions between neurochondrin and RII α (Figure 7b, arrows).

Multiple binding regions for PKA are found in several AKAPs including AKAP220, AKAP350/450 and BIG2 [49–51]. In these AKAPs, each of the interactions can be prevented with Ht31 or similar peptides indicating that these AKAPs bind more than one PKA holoenzyme. SKIP, for example, binds two molecules of PKA type I [52]. Yet, the functional significance of binding more than one molecule of PKA is not known [53]. For neurochondrin it remains to be determined whether the two binding sites mediate binding of two molecules of PKA in the full-length protein or whether the two sites join to bind one molecule of PKA.

SPR measurements with peptides derived from the two distinct RII-binding regions within neurochondrin revealed nanomolar binding affinities for RII α subunits (peptide I: $K_D = 9.3$ nM, peptide II: $K_D = 9.6$ nM). Interestingly, the core amino acid residues of both regions (RII-binding region I: KTRRR; RII-binding region II: WQRNP, Appendix A, Supporting Table A2 and A3) are evolutionary highly conserved; the sequence KTRRR is identical in *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus*, suggesting a conserved function of this site. We identified the AKAP function of neurochondrin in pig brain lysate. Neurochondrin engages in various protein-protein interactions in neurons [23, 54–57] and apparently plays a role in controlling plasticity [22–25]. Where and how neurochondrin introduces PKA into the functioning of neuronal processes remains to be determined.

4.1 Conclusion

We propose a 3D structural model for neurochondrin by using comparative modeling methods based on the fold recognition patterns [58]. Figure 8 shows that the model of neurochondrin is almost entirely comprised of α -helical repeats. The superhelical structure creates a shallow groove that may serve as a protein-protein interaction platform [59, 60]. Non-globular folding was confirmed by gel filtration, which revealed an apparent molecular weight of 125 kDa in lieu of the theoretical weight of 84 kDa (Appendix A, Supporting Fig. A3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Neurochondrin is a new A-kinase anchoring protein.
- Neurochondrin has a strong isoform selectivity towards the RII α subunit.
- Neurochondrin interacts with the RII α isoform with nanomolar affinity.
- Neurochondrin interacts directly with PKA through a novel mechanism.

Figure 1. Neurochondrin selectively binds RIIα through the dimerization/docking (D/D) domain
 (a) Surface plasmon resonance measurements. Human RIIα (~160 RU), RIIβ (~300 RU), RIα (~600 RU) and RIβ (600 RU) subunits were captured in separate flow cells of 8-AHA-cAMP sensor chips. Binding of neurochondrin (500 nM) and dissociation was monitored for 300 s, respectively. As an additional control, binding of neurochondrin to 8-AHA-cAMP without immobilized R-subunit was subtracted. Shown is a representative plot from 5 independent experiments. RU: response units. (b) Far Western blot analysis. Equimolar amounts of RIα, RIIα and GST-1-44 RIIα as well as 125 ng neurochondrin (used as control) were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with (+) and without (–) neurochondrin. Binding of neurochondrin to the R-subunits was detected using Strep-Tactin® HRP conjugates (F: hyperfilm). As loading controls proteins on the membranes were stained with amido black (M: membrane). Neurochondrin interacts with RIIα as well as with the 1-44 RIIα deletion construct, which only contains the D/D domain (aa 1-44).

Figure 2. Inhibition of the interaction between neurochondrin and RII α subunits by blocking the D/D domain with peptides Ht31 or AKAP18 δ L314E

Human RII α (330 RU) was captured on 8-AHA-cAMP surfaces. Neurochondrin (300 nM) was injected alone (black) or in the presence of the inhibitor peptides Ht31 or AKAP18 δ L314E (5 μ M each). Association and dissociation phases were monitored for 300 s each. Ht31 (red) and AKAP18 δ L314E (blue) efficiently prevent association of neurochondrin with RII α . The inactive control peptide Ht31P had no inhibitory effect (5 μ M; gray). Representative plot (n=2). RU: response units.

Figure 3. Neurochondrin binds RIIα with high affinity

SPR studies were performed with 70 RU of immobilized RIIα subunit (8-AHA-cAMP surface). Neurochondrin was injected at concentrations ranging from 7.8 nM to 250 nM for 300 s. After the end of the injection, the dissociation phase lacking neurochondrin was monitored for 300 s. A blank surface was subtracted and a Langmuir 1:1 binding model was applied (red lines). Shown is a representative sensorgram including fits ($n=2$). Association and dissociation rate constants were determined to $5.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $4.5 \times 10^{-4} \text{ s}^{-1}$, respectively, yielding an equilibrium binding constant of 8 nM.

Figure 4. RII-binding domains of neurochondrin

Mapping the RII-binding domain of neurochondrin. Panels (a) and (b): The entire sequence of neurochondrin (729 amino acid residues) was spot-synthesized as overlapping peptides (25-mers, 20 amino acid residues overlap) and probed for RII binding using RII overlay assays. Signals were detected by autoradiography. RII overlay assays were performed in the presence of the inactive control peptide AKAP18δ-PP (left) or the peptide AKAP18δL314E, which blocks AKAP-PKA interactions [34]. The binding was not affected by the presence of either peptide. Panels (c) and (d): Two potential RII-binding domains were identified. Peptide sequences binding RII: position A11–A15 and D6–D11. (e) As control, binding of RII to the peptides Ht31 and AKAP18δL314E and the corresponding inactive control peptides Ht31PP and AKAP18δ-PP were monitored.

Figure 5. Determination of association and dissociation rate constants for the binding of the RII α subunit to designed neurochondrin peptide I and II

Surface plasmon resonance studies were performed using covalently immobilized neurochondrin peptides I (a) and II (b). RII α was injected in the indicated concentrations for 300 s. After the injection, the dissociation phase lacking RII α was monitored for additional 300 s. A blank surface was subtracted and a Langmuir 1:1 binding model was applied (red lines). RII α shows high affinity binding with both neurochondrin peptides (see Table 2). Representative plot (n=2). RU: response units.

Figure 6. The neurochondrin-derived peptides individually interact with RIIα in a non-canonical manner

Neurochondrin peptides I (a) and II (b) were immobilized covalently on a Biacore sensor surface. RIIα (500 nM) was either injected alone (black) or in the presence of the peptide Ht31 (5 μM), which inhibits canonical AKAP-PKA interactions. Association and dissociation phases were monitored for 300 s. Ht31P is the corresponding negative control peptide for Ht31. The interactions between RIIα and the neurochondrin peptides were not affected by Ht31 (red) or Ht31P (dark gray).

Figure 7. Surface charges of the neurochondrin RII-binding regions and the D/D domain of RIIα
 (a) Model of neurochondrin. Both RII-binding regions of neurochondrin exhibit pI values of approximately 9. Therefore, they are positively charged under physiological conditions (blue label). (b) D/D domain of RIIα complexed with the D-AKAP2 peptide (PDB ID: 2HWN). Shown is the hydrophobic groove, formed by the D/D domain, where AKAPs usually bind (demonstrated by the D-AKAP2 peptide in yellow). The D/D domain of RIIα contains charged regions (negative charges are labeled in red whereas positive charges are labeled in blue). The arrows label negatively charged regions that may be important for the neurochondrin binding interface.

Figure 8. Phyre2-structure prediction of neurochondrin

The full-length amino acid sequence of neurochondrin was analyzed using Phyre2 [58]. (a) Neurochondrin shows a primarily helical architecture. Neurochondrin contains an N-terminal armadillo (ARM)-domain (aa: 36–468) with characteristic repeats of three helices (blue: helix I, magenta: helix II, yellow: helix III). (b) Different views of Neurochondrin (magenta: loop, cyan: helix). The N terminus is labeled with black dots.

Table 1
Neurochondrin peptides for kinetic binding studies with RII α

The synthetic neurochondrin peptides were designed according to the peptide array studies (Figure 4). For quantification, peptide I contains an additional tyrosine at the N-terminus.

peptide I	YAGDIDAK TRRR IFDAVGFTF
peptide II	KLSS WQ RNPALKLAARLAHA

Table 2
Apparent rate and equilibrium dissociation constants for the interaction between RII α and peptides representing two RII-binding regions of neurochondrin

Interaction studies using Biacore technology were performed as depicted in Figure 5 to determine rate constants (k_a and k_d) and equilibrium binding constants (K_D) for the interaction of neurochondrin peptides with human RII α .

	k_a	k_d	K_D
	$M^{-1}s^{-1}$	s^{-1}	nM
peptide I	$1.2 \pm 0.1 \times 10^5$	$1.1 \pm 0.2 \times 10^{-3}$	9.3 ± 3.1
peptide II	$1.1 \pm 0.5 \times 10^5$	$8.4 \pm 0.4 \times 10^{-4}$	9.6 ± 4.8