Proteomic analysis of NMDA receptor–adhesion protein signaling complexes

Holger Husi[1], Malcolm A. Ward[2], Jyoti S. Choudhary[2], Walter P. Blackstock[2] and Seth G. N. Grant[1]

[1] Centre for Genome Research, Centre for Neuroscience, University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ, UK

[2] Glaxo Wellcome Research and Development Ltd, Gunnels Wood Road, Stevenage SG1 2NY, UK

Correspondence should be addressed to S.G.N.G. (seth.grant@ed.ac.uk)

N-methyl-D-aspartate receptors (NMDAR) mediate long-lasting changes in synapse strength via downstream signaling pathways. We report proteomic characterization with mass spectrometry and immunoblotting of NMDAR multiprotein complexes (NRC) isolated from mouse brain. The NRC comprised 77 proteins organized into receptor, adaptor, signaling, cytoskeletal and novel proteins, of which 30 are implicated from binding studies and another 19 participate in NMDAR signaling. NMDAR and metabotropic glutamate receptor subtypes were linked to cadherins and L1 cell-adhesion molecules in complexes lacking AMPA receptors. These neurotransmitter–adhesion receptor complexes were bound to kinases, phosphatases, GTPase-activating proteins and Ras with effectors including MAPK pathway components. Several proteins were encoded by activity-dependent genes. Genetic or pharmacological interference with 15 NRC proteins impairs learning and with 22 proteins alters synaptic plasticity in rodents. Mutations in three human genes (NF1, Rsk-2, L1) are associated with learning impairments, indicating the NRC also participates in human cognition.

A prevailing cellular model of learning involves modification of synapse strength, induced by patterns of neuronal activity during training, which encodes information in neural networks[1,2]. Understanding the molecular mechanisms used by synapses to detect altered patterns of firing, and how signaling cascades then change the strength of synaptic transmission, may provide basic insights into this model and illuminate pathological mechanisms that occur in human learning impairments and other psychiatric disorders[3]. Considerable attention has focused on the NMDA subtype of glutamate receptor[4] because its blockade in the hippocampus impairs both synaptic plasticity and learning[1]. This receptor channel, which allows calcium influx into the postsynaptic spine, regulates kinases, phosphatases and other enzymes, which then regulate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxasole-4-propionic acid) glutamate receptors (AMPARs), spine cytoskeletal changes, translation, transcription and other events. These diverse actions indicate the NMDAR couples to multiple intracellular signaling pathways. Moreover, in a physiological setting, these signals must be subtly integrated, as shown by electrophysiological studies in CA3–CA1 synapses of the hippocampus, where low-frequency synaptic stimulation results in NMDAR-dependent long-term depression (LTD) and higher frequencies in long-term potentiation (LTP) of synaptic strength[2].

LTP comprises a series of temporally distinct processes that can broadly be separated into 'induction' and 'maintenance' phases. The induction phase includes the train of stimuli that activate the NMDA receptor, which lasts for seconds or less, and a period of minutes during which second messenger pathways act, leading to a new stable level of synapse strength in the maintenance phase. Unlike the induction phase, the maintenance phase is resistant to inhibitors of second messengers, such as kinases and phosphatases, although it is sensitive to inhibitors of RNA and protein synthesis for the first two to three hours, indicating a role for new gene expression[1,2]. In parallel with this requirement for gene expression in the late phases of LTP, the NMDAR is required for transcriptional activation and dendritic trafficking of mRNAs and proteins such as Arg3.1/Arc and Homer/Vesl. A recent review[5] of the molecular mechanisms of LTP highlighted the large number of implicated molecules and the lack of a satisfying model. In addition to neurotransmitter receptors and multiple signaling proteins, this list of molecules included adhesion and structural proteins. An approach to the problem of understanding the molecular basis of LTP and its signaling pathways is to combine a functional genetic and pharmacological dissection with structural analysis of the organization of synaptic molecules.

Assembly of receptors with signal transduction proteins into large multiprotein complexes has emerged as a general mechanism of cellular signaling[6]. The NMDAR binds postsynaptic proteins[7–9], including postsynaptic density 95 (PSD-95), which regulates synaptic plasticity in mutant mice[10], consistent with the hypothesis that a signaling complex regulates synaptic plasticity and learning. To further explore this model, we have isolated NMDAR–PSD-95 complexes from mouse brain and analyzed their properties using proteomic techniques, which are powerful tools for analysis of large protein complexes[11,12]. This approach allows the detection of protein interactions that require post-translational modifications such as phosphorylation (for example, SH2 binding of PI3-kinase to NMDAR subunits[13]) and lipid modification (for instance, in PSD-95; ref. 14) and ternary or

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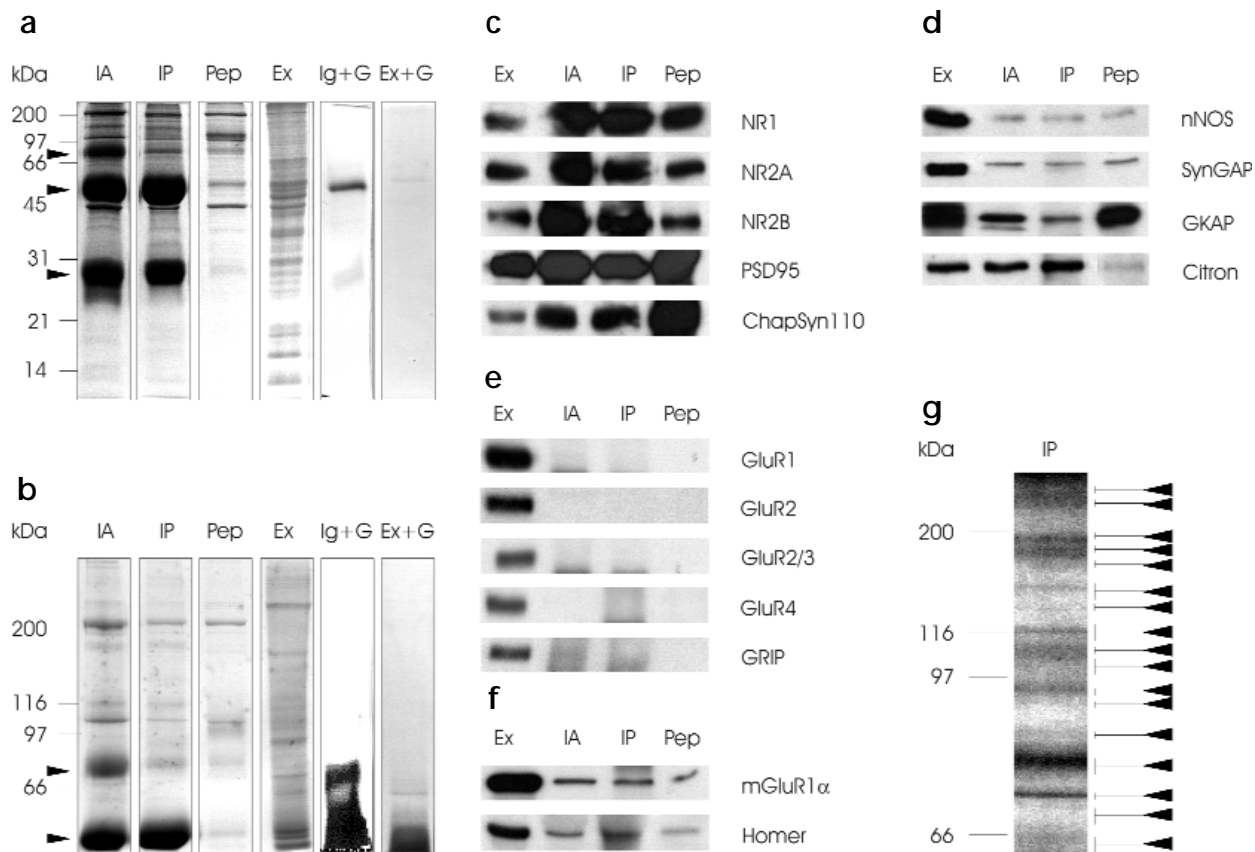
Fig. 1. Basic composition of NRC isolated from mouse brain. (**a**, **b**) Protein staining of SDS-PAGE (**a**, 12%; **b**, 6%) from immunoaffinity (IA), immuno-precipitation (IP) and NR2B peptide affinity (Pep) isolation of NMDAR from mouse brain extracts (Ex). Negative controls are MAP-NR1 Ig bound to protein G-sepharose (Ig+G) and extracts bound to protein G-sepharose (Ex+G). Arrowheads indicate position of Ig species. A complex mixture of specific binding proteins were observed, mainly in the range above 70 kD, and were analyzed by mass spectrometry (**g**; **Table 2**). (**c**) Immunoblot analysis of NMDAR subunits and MAGUK proteins in NRC. NR1, NR2A, NR2B, PSD-95 and Chapsyn-110 were detected. The NRC from 5 mg of starting material, 50 µg of protein loaded in total extract (Ex), was isolated and loaded per lane (IA, IP, Pep). Yield and enrichment cannot be calcu-lated because the IA, IP and Pep samples contained interfering Ig and peptide, and thus did not permit protein quantitation. (**d**) Immunoblot analysis of PSD-95 binding proteins in NRC. We detected nNOS, SynGAP, GKAP and Citron. (**e**) Immunoblot analysis of AMPA receptor subunits and GRIP in NRC. GluR1, GluR2, GluR2/3, GluR4 and GRIP were readily detected in the starting material (Ex) but not in the three NRC preparations. (**f**) Immunoblot analysis of mGluR1 receptor and Homer proteins in NRC. We detected mGluR1α and Homer, indicating that the NRC includes metabotropic glutamate receptors but not AMPA receptors. (**g**) Protein staining of NRC (6% SDS–PAGE) showing position (arrowheads) of bands excised for mass spectrometry.

weak interactions undetected by 2-hybrid screens[11], which have been extensively used to identify NMDAR and PSD-95 binding partners[7–9]. Moreover, biochemical characterization of NRC from brain is required for analysis of mice carrying mutations affecting the complexes as well as other *in vivo* analyses.

Developments in high-sensitivity mass spectrometry, particu-larly nano-electrospray mass spectrometry coupled with search engines to access the huge amount of data produced by the genome sequencing projects, have fueled the growth of proteomics[15]. Ini-tially defined as the protein complement of a genome, the term proteome has been generalized to encompass any large-scale approach to protein characterization. Proteins may exist as dis-crete known proteins in databases (such as SwissProt), as complete or partial sequences in EST databases (for example, dbEST), or as putative open reading frames in genome databases. All three are accessible to searching by combinations of mass and partial sequence information generated by mass spectrometry, searching the protein databases directly and translating the DNA sequences

in six frames. The required numerical search attributes are gener-ated by specific proteolytic digestion of the protein, and subse-quent analysis of the resulting peptide pool by mass spectrometry. Two measurements are usually made; the first is the mass of as many peptides as possible. This peptide mass fingerprint (PMF) alone may be sufficient to identify the original protein, particu-larly if the protein was isolated in a relatively pure state by, for example, two-dimensional gel electrophoresis. More often though, proteins are available only as mixtures, and here PMFs are less use-ful. In this case, mass and partial sequence information are gener-ated by tandem mass spectrometry (MS/MS) and combined to provide powerful discriminating input for mass spectrometry search engines. This latter approach is sufficiently robust to deal with simple mixtures, and if combined with on-line separation techniques can deal with very complex protein mixtures, for exam-ple, from bands from one-dimensional gels, or even total protein complexes. This is a more appropriate approach for searching EST databases, which rarely contain full-length protein sequences.

a

b

c

d
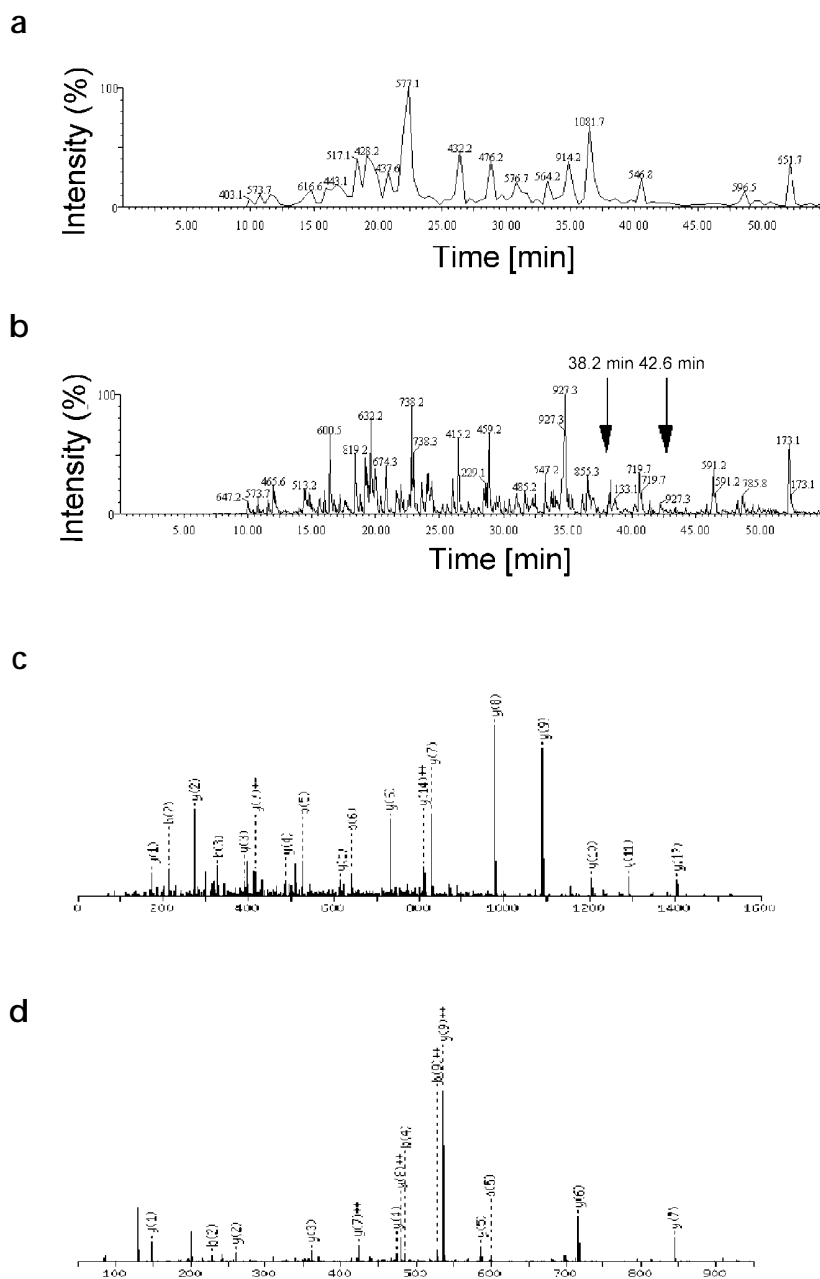
Fig. 2. Mass spectrometry analysis of the NRC. (**a**) Base peak chromatograms generated in MS survey mode (example from trypsin digestion products of a 90 kD protein band) The two most intense ions in each MS scan are automatically selected for collision-induced fragmentation and analysis in MS/MS mode (LC-MS/MS; **b**). (**b**) Base peak chromatograms of one of the two MS/MS channels. The labels at 38.2 min and 42.6 min correlate with two peptides that were selected to generate peptide sequence information. (**c**, **d**) Corresponding MS/MS spectra obtained from the precursors eluting at these times. (**c**) Averaged MS/MS spectrum obtained from peak eluting at 42.6 min in (**b**). A doubly charged ion at mass to charge ratio (m/z) 809.72, corresponding to a peptide of 1617.44 D, was selected for fragmentation by collision-induced dissociation. The fragment ions that originate either from the N terminus (b type ions) or the C terminus (y″ type ions) correspond to an amino-acid sequence that was identified using the Mascot database search program. In this instance, the MS/MS spectrum matched the peptide sequence VNDSILFVNEVDVR from PSD-95 residues 113–126 (accession number Q62108). (**d**) Example of a novel NRC protein identified from the averaged MS/MS spectrum obtained from the peak eluting at 38.2 min in (**b**). The observed doubly charged precursor ion at m/z 538.68, relating to a 1071.65 D peptide, was identified by Mascot search program as the sequence DLKEILTLK, which matches APPL, an adaptor protein for PI3 kinase and AKT/PKB kinase[29].

RESULTS

We began by comparing methods for NRC purification from mouse brain, including standard FPLC chromatography, affinity chromatography with NMDAR ligands, immunoaffinity chromatography with purified antibodies, large-scale immunoprecipitation and peptide affinity (H.H. & S.G.N.G., unpublished data). The clearest results were produced by three methods: immunoaffinity chromatography, immunoprecipitation with an antibody directed against the NMDA R1 subunit, and peptide affinity based on the structure of the NMDA R2B subunit C terminus that binds to the NMDAR-binding protein PSD-95 (**Fig. 1**). Analysis of individual fractions on sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) showed that the purified material was very complex (**Fig. 1a** and **b**, lanes IA, IP, Pep) compared to unspecific interaction of extracted proteins with protein G-sepharose used in immunoprecipitations (lane Ex+G) or

blocked affigel-10 used in antibody and peptide affinity purification (**Fig. 1**), as well as other peptides and antibodies (data not shown). The integrity of the complexes and specificity of protein interactions were examined. NMDAR subunits (NR1, NR2A, NR2B) and their reported interacting proteins[7–9] (PSD-95, Chapsyn-110/PSD-93, calmodulin, α-actinin 2, calcium/calmodulin kinase II (CaMKII), phospholipase Cγ) were readily detected (**Fig. 1c** and **Table 1**), and PSD-95 binding proteins including nNOS, SynGAP, SAPAP/GKAP and Citron were also found (**Fig. 1d**). We next examined AMPA subunits (GluR1–4) and their cognate adapter protein GRIP[16], which were undetected in the NRC despite their abundance in the extract (**Fig. 1e**). Similarly, we did not detect NMDAR complex proteins in immunoprecipitations with AMPAR-specific antibodies (data not shown). Kainate receptor subunits (GluR6/7) were detected, consistent with reports that PSD-95 binds these subunits[17]. The metabotropic (mGluR1α) receptors and their cognate binding partner Homer/Vesl, were found in the NRC (**Fig. 1f**), consistent with reports from 2-hybrid screening that Homer binds Shank, which can bind GKAP to PSD-95 (refs. 18, 19). Therefore the NMDAR and mGluR receptors are associated in distinct complexes from AMPA receptors.

We next identified novel components of the NRC using two strategies: a western blotting screen for candidate proteins involved

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Table 1. Summary of molecular composition of the NRC.

	Protein	Mr (kD)	Ex	IA	IP	Pep	Ab	Binding partner
Glutamate receptors								
1	NR1	120	+++	+++	+++	+++	a	2, 3, 18, 35, 68, 95, 96, 101
2	NR2A	180	+++	+++	+++	+++	b	1, 3, 10, 11, 12, 35, 73, 96
3	NR2B	180	+++	+++	+++	+++	c	1, 2, 10, 11, 12, 35, 43, 95, 71, 73, 96, 101
4	GluR1	108	+++	–	–	–	d	
5	GluR2	102	+++	–	–	–	d	
6	GluR2/3	102	+++	–	–	–	e	
7	GluR4	108	+++	–	–	–	d	
8	GluR6 + 7	117	+++	+	+	+	b	10, 12
9	mGluR1α	200	+++	+++	+++	++	d	15, 68, 73, 101
Scaffolding and adaptors								
10	PSD-95	95	+++	+++	+++	+++	f	2, 3, 8, 10, 11, 12, 13, 65, 69, 75
11	ChapSyn110 / PSD-93	110	+++	+++	+++	+++	g	2, 3, 10, 69
12	Sap102	115	+++	+	+	+++	h	2, 3, 8, 10, 13, 65, 68
13	GKAP / SAPAP	95–140	+++	+++	++	+++	e	10, 12, 14
14	Shank	200	+++	+++	+++	+++	i	13, 15, 102
15	Homer	28/45	+++	++	++	++	j	9, 15, 14
16	GRIP	120	+++	–	–	–	e	
17	ABP (GRIP2)	95–130	+++	–	–	–	k	
18	Yotiao	200	+++	+++	+++	+++	l	1, 25, 37
19	AKAP150	150	+++	+++	+++	+++	l	25, 26–34, 39, 68
20	NSF	83	+++	++	++	+++	m	5, 20
PKA								18, 19, 92
21	PKA catalytic subunit	40	+++	++	++	++	f	
22	PKA-R1α and β	48	+++	ch	ch	–	f	
23	PKA-R1a-α	49	+++	ch	ch	–	f	
24	PKA-R2α	51	+++	ch	ch	–	f	
25	PKA-R2β	53	+++	++	++	++	f	
PKC								19, 105
26	PKC α	82	+++	–	–	–	f	
27	PKC β	80	+++	++	++	++	f	
28	PKC γ	80	+++	+++	+++	+++	f	
29	PKC δ	78	+++	–	–	–	f	
30	PKC ε	90	+++	++	++	++	f	
31	PKC η	82	+++	–	–	–	f	
32	PKC θ	79	+++	–	–	–	f	
33	PKC ι	74	+++	–	–	–	f	
34	PKC λ	74	+++	–	–	–	f	
CaM kinase								1, 2, 3, 65, 68, 69
35	CaM kinase II β	60	+++	+++	+++	+++	f	
36	phospho-CaM kinase II	60	+++	++	++	++	n	
Phosphatases								
37	PP1	36	+++	+++	+++	+++	f	18
38	PP2A	36	+++	+++	+++	+	f	
39	PP2B (calcineurin)	61	+++	+	+	+	f	19
40	PP5	50	+++	++	++	++	o	
41	PTP1B	50	+++	ch	ch	–	b	
42	PTP1C	68	+++	–	–	–	b	
43	PTP1D/SHP2	72	+++	++	++	++	f	3
Tyrosine kinases								
44	Src	60	+++	+	+	+	p	47, 59, 60, 90, 101
45	Fyn	59	+++	–	–	–	f	
46	FAK	125	+++	–	–	–	f	
47	PYK2	116	+++	+	+	+	f	44
MAP kinase pathway								
48	ERK (pan ERK)	42/44	+++	++	++	++	f	
49	ERK1	42/44	+++	++	++	++	f	
50	ERK2	42	+++	++	++	++	f	57
51	ERK3	62	+++	–	–	–	f	
52	phospho-ERK1/2	42/44	+++	–	–	–	q	

#	Name	Mr	Ex	IA	IP	Pep	Ab	Binding partner
53	MEK1	45	+++	++	++	+	f	59
54	MEK2	46	+++	++	++	+	f	59
55	MKP2	43	+++	+++	+++	++	f	
56	JNKK1 / MKK4	44	+++	–	–	–	f	
57	Rsk	90	+++	++	++	++	f	50
58	Rsk-2	90	+++	++	+++	+	r	50
59	c-Raf1	74	+++	++	++	+	f	44, 53–54, 60

Small G-proteins and modulators

#	Name	Mr	Ex	IA	IP	Pep	Ab	Binding partner
60	H-Ras	21	+++	cl	cl	+	f	44, 59, 65, 66, 71
61	Rac1	21	+++	++	+	+	f	75, 101, 102
62	Rap1	21	+++	–	–	–	f	
63	Rap2	21	+++	++	++	+	f	
64	RalA	24	+++	cl	cl	+	f	
65	SynGAP	135	+++	++	++	++	e	10, 12, 35, 60
66	NF1	250	+++	+	+	+	r	60, 101
67	p120GAP	120	+++	–	–	–	f	

Other signaling molecules

#	Name	Mr	Ex	IA	IP	Pep	Ab	Binding partner
68	Calmodulin	15	+++	+++	+++	+++	b	1, 9, 12, 19, 35, 69, 92
69	nNOS	155	+++	++	++	++	f	10, 11, 35, 68
70	eNOS	140	+++	–	–	–	f	
71	PI3 kinase	85	+++	+	+	+	f	3, 60, 101
72	Calpain	30	+++	–	–	–	s	
73	PLC γ	150	+++	++	++	++	r	2, 3, 9, 94, 101
74	cPLA2	110	+++	+++	++	++	r	
75	Citron	183	+++	+++	+++	++	r	10, 61
76	VAV	95	+++	–	–	–	f	
77	Arg3.1	55	+++	+++	+++	+++	t	
78	CREB/CREM	46/26	–	–	–	–	f	
79	NCK	47	+++	–	–	–	f	
80	SHC	52/66	+++	–	–	–	f	

Cell-adhesion and cytoskeletal proteins

#	Name	Mr	Ex	IA	IP	Pep	Ab	Binding partner
81	N-Cadherin	150	+++	++	++	+++	u	81, 87, 88, 90
82	E-Cadherin	120	+++	–	–	–	f	
83	P-Cadherin	120	+++	–	–	–	f	
84	Cadherin-5	130	+++	–	–	–	f	
85	Desmoglein	165	+++	++	++	+	f	85, 88
86	α-Catenin	102	+++	–	–	–	f	87, 88, 95
87	β-Catenin	92	+++	++	++	+	f	81, 86
88	γ-Catenin	82	+++	–	–	–	f	85, 86
89	L1	200	+++	+++	+++	++	f	89
90	pp120cas	120	+++	+++	++	+	f	44, 81
91	Paxillin	68	+++	–	–	–	f	
92	MAP2B	280	+++	++	+	+	f	25, 68, 101
93	Vinculin	117	+++	–	–	–	v	
94	Actin	45	+++	+++	+++	+++	w	73, 95, 96, 97, 102
95	α-actinin 2	110	+++	+++	+++	+++	v	1, 3, 86, 94
96	Spectrin	240/280	+++	++	++	++	v	1, 2, 3, 94, 96
97	Myosin (brain)	205	+++	+++	+++	++	v	94
98	Tau	50–68	+++	–	–	–	f	
99	Tensin	215	+++	–	–	–	f	
100	Ezrin	80	+++	–	–	–	f	
101	Tubulin	50	+++	+++	+++	+++	v	1, 3, 9, 61, 66, 71, 73, 92, 96, 106
102	Cortactin	80/85	+++	+	+	++	x	14, 44, 61, 94, 103
103	CortBP-1	180/200	+++	+	++	+++	x	102
104	Clathrin heavy chain	180	+++	++	++	+++	f	106
105	Dynamin	100	+++	++	++	+++	f	26–34
106	Hsp-70	70	+++	++	++	+++	f	101, 104

Immunoblotting screen of NRC and known binding partners within NMDAR complexes. Classes of proteins are boxed, and specific molecular names are indicated with identifying numbers (1–106). Mr, relative molecular mass; Ex, mouse brain extracts; IA, complexes isolated by MAP–NR1 immunoaffinity; IP, complexes isolated by MAP–NR1 immunoprecipitation; Pep, complexes isolated by NR2B peptide affinity; Ab, antibody source (see Methods); binding partner, identified interactions from published *in vitro* studies. Specific brain proteins were assayed in three preparations of complexes (IA, IP, Pep) using immunoblotting, and signals were scored as strong (+++), medium (++), weak (+) or undetectable (–). Some proteins could not be analyzed in IA or IP because of comigration of Ig (ch, comigration heavy chain Ig; cl, comigation light chain Ig). The scoring is not suitable for comparison of different proteins because it depends on the properties of specific antibodies, but it is suitable to indicate a relative enrichment from extract. For each protein found in the complexes, the reported associated proteins are indicated (binding partners) by reference to the numbering scheme in the first column.

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Table 2. Summary of mass spectrometry analysis of NRC proteins.

Protein name	Mr (kD)	Accession number	Number of peptides
Bassoon	418	O88778	11
Myosin B heavy chain (non muscle type)	229	P35580	27
p53 binding protein-1	214	Q12888	2
Tight junction protein ZO-1	195	P39447	13
Clathrin heavy chain	192	P11442	1
NR2B	166	Q01097	85
NR2A	165	P35436	36
SynGAP	143	AAC08071	9
NR1	105	P35438	208
α-actinin 2	103	Q62744	25
Sap97	100	Q12959	2
Hypothetical 97.8 kD protein	98	CAB43675	3
Dynamin	96	Q61358	4
Chapsyn 110	95	Q63622	108
Sap102	93	P70175	9
Phosphofructokinase	86	P12382	7
NSF vesicular fusion protein	83	P46460	1
PSD-95	80	Q62108	112
APPL adaptor protein	80	AF169797	8
Sarcolemmal associated protein-3	74	Q28623	3
GKAP/SAPAP	74	P97841	4
HSP70-like HS71 protein	71	U73744	24
Kinesin light chain 2	67	O88448	2
PP2A regulatory R1 α chain	65	P30153	1
CaM kinase II β	60	P28652	1
PP2B (Calcineurin) α chain	59	P20652	3
α-Internexin	56	P46660	2
CaM kinase II α	54	P11798	4
RNA binding protein FUS/TLS	53	P35637	1
Tubulin α-4 chain	50	P05215	3
Est736.26		AI047568	2
Est700.75		AI428173	1
Est571.14		AA982950	1
Est762.20		AA592427	1

Visualized protein bands of 60–300 kD were prepared and analyzed as described (Methods and **Fig. 2**). Mr, relative molecular mass; number of peptides, total number of matching peptides sequenced by MS/MS for a specific protein. Note that the number of peptides sequenced from each protein does not directly correlate to quantity of protein. Peptide lengths were 10–20 residues, and the peptides were unique and did not match any other sequences in the databases.

with NMDAR signaling and scaffolding (**Table 1**) and protein identification using mass spectrometry (**Fig. 2** and **Table 2**). The protein bands indicated in Fig. 1g were analyzed by online LC-MS/MS (**Fig. 2**). It is important to note that these strategies do not show absolute levels and stoichiometry of subunits.

The NMDA and mGlu receptors have been implicated in the induction of synaptic plasticity through activation of second messenger pathways[1,2]. We therefore examined a wide range of different kinases and phosphatases in the NRC and found several specific serine-threonine kinase and phosphatase family members. We found protein kinase A (PKA) catalytic subunit and regulatory subunit R2β (R1, R1α and R2α were not detected) and protein kinase C (PKC) isoforms β, γ, ε, but not α, δ, η, θ, ι, λ isoforms. That a subset of a protein family associated with the NRC, indicating specific association, was observed repeatedly for multiple classes of proteins in this study. The α and β subunits of CaMKII were associated, as reported[20], and also found to be

in the phosphorylated active form. The presence of these kinases, which are involved in induction of synaptic plasticity, suggests that counterpart phosphatases may also be present in the NRC. We found that PP1 and PP2A were strongly associated and PP2B (calcineurin) and PP5 were also associated. PKA and PP1 are linked to the NR1 subunit via an adapter protein (Yotiao) and can modulate NMDAR currents *in vitro*[21]. We found Yotiao and another PKA adaptor, AKAP150, suggesting that PKA may be linked to the NRC at multiple locations, perhaps in distinct pathways.

Because tyrosine phosphorylation is also involved with NMDAR[22] and synaptic plasticity[23], we examined cytoplasmic tyrosine kinases and found Src (but not Fyn) and the calcium-activated kinase Pyk2 (but not the Pyk2 homologue focal adhesion kinase, FAK) associated with the NRC. Although tyrosine phosphatase inhibitors influence NMDAR channels[22], the identity of relevant tyrosine phosphatases has remained unclear. We

detected PTP1D/SHP2, which associates with NR2B[24], but neither the related PTP1B or PTP1C in the NRC, implicating this tyrosine phosphatase in NRC regulation.

How NMDAR-mediated kinase and phosphatase signal integration occurs is unclear, but the involvement of Ras, which integrates downstream signals in other cellular contexts, is suggested by the altered plasticity and learning in mice with mutations in modulators of Ras (Ras-GRF[25] or NF1; ref. 26). We found both H-Ras and Rap2 small G proteins and their GTPase activating proteins NF1 and SynGAP (which binds PSD-95), but not p120GAP in the NRC. Rap1, which may couple PKA to c-Raf1, was not detectable in the NRC. Ras mediates its effects by differential activation of several downstream effector pathways[27], including the c-Raf1–MEK–ERK/MAPK pathway, PI3 kinase and RalA. The different MAPK pathways are organized into modules composed of the key enzymes producing that cascade, tethered within a cell to produce signal specificity[28]. This was also the case for the NRC, as ERK1 and ERK2 and their upstream activating kinases MEK1, MEK2 and c-Raf1 were found, but not JNKK1/MKK4 and ERK3. Moreover, the ERK phosphatase (MKP2), which inactivates ERK, was also identified. In addition to ERK/MAPK pathway, mass spectrometry identified APPL[29], an adaptor protein linking the p110 subunit of PI3 kinase to AKT/PKB kinase. These observations that ERK/MAPK and the other Ras effectors, PI3 kinase and Ral were detected along with adaptors and regulators indicates that glutamate receptors drive Ras, which is coupled to distinct downstream pathways organized as modules within the NRC.

The role of ERK/MAPK pathways in synaptic plasticity and learning has received considerable attention because phosphorylation of ERK accompanies these processes, which are also disrupted by inhibitors of MEK[30]. Phosphorylation of ERKs has mainly been implicated in regulating transcription, through phosphorylation of Rsk-2, which translocates to the nucleus to phosphorylate transcription factors CREB and CREM. Interestingly, phospho-ERK was not present in the NRC, although it was readily detected in the extract, consistent with its ability to translocate from the NRC on phosphorylation. Moreover, Rsk-2 was found within the NRC, although the transcription factors CREB and CREM were not detectable. We noticed that several of the NRC proteins (Homer, NR1, NR2B, PKCγ, ERK2, c-Raf1, HSP70) are encoded by activity-dependent genes, and we therefore tested Arg3.1/Arc[31,32], a postsynaptic protein of unknown function that is also rapidly regulated by LTP. Arg3.1 was readily detected in the NRC, suggesting it may participate in signaling and dynamic organization of the NRC. Thus the NRC contains signaling mechanisms that could contribute to transcriptional activation following synaptic activation, and the NRC could then itself undergo structural changes secondary to altered gene expression. Another potential ERK effector, cPLA[33], is also regulated by Citron, which binds PSD-95. Mice lacking cPLA2 show resistance to ischemic neuronal damage[34], and cPLA2 generates arachidonic acid, a candidate transynaptic retrograde signaling molecule.

Changes in synapse structure may be fundamental to the storage of long-term memory, and roles for cell-adhesion molecules and cytoskeletal structural proteins in this process are suggested[35]. Many of the signaling proteins described above are known regulators of cytoskeleton and cell adhesion. Actin cytoskeleton is involved in NMDAR channel properties[36], NMDAR-mediated LTP[37] and NMDAR localization[38], and is dynamically regulated in spines by neural activity[39]. We found α-actinin 2 and spectrin (Fodrin), which bind NMDAR subunits[40], and actin–binding proteins, including cortactin, cortactin–binding proteins

(CortBP1 and Shank) and MAP2, but not Ezrin, Tensin or Vinculin, in the NRC. Surprisingly, the cell-adhesion proteins N-Cadherin and Desmoglein, two cadherin family members and their cytoplasmic interacting proteins β-catenin, ZO-1 and p120cas were detected, although E-cadherin, P-cadherin, cadherin-5, α-catenin and γ-catenin were negative. L1 adhesion protein, which is required for learning and synaptic plasticity[41], was also detected in the NRC. These adhesion proteins may be involved in structural organization of the NRC at the synapse; however, the involvement of cadherins[42] and L1 in synaptic plasticity[41] make it tempting to speculate that this glutamate receptor–cell-adhesion protein complex could provide multiple trans-synaptic signaling pathways, whereby adhesion-mediated signaling is coupled to transmitter signaling mechanisms.

Mass spectrometry of large-scale isolated NMDAR complexes has confirmed and extended the identification of NRC components beyond yeast 2-hybrid assays and immunoblotting (**Tables 1** and **2**). Some NRC components were not detected by MS, which may be due to the low levels of protein in the selected gel fragments. In addition to identification of previously unsuspected known proteins, the MS approach also identified matching peptides that are represented in the dbEST database, indicating novel proteins in the NRC. During the course of this work, as the databases expanded, several EST matches were subsequently found to match known proteins. For example, the peptide sequence DLKEILTLK (**Fig. 2d**) was originally identified as mouse hippocampus EST (AV153731), and later a report of the full-length cDNA sequence allowed us to match with seven other peptides, thus identifying this NRC protein as APPL, a novel adaptor for PI3 kinase and AKT/PKB[29]. Although it is clear that some protein complexes contain dozens of proteins[11,12], the large number of NRC proteins identified in this study does not imply that all proteins exist in a single homogeneous complex. Heterogeneity could result from different complexes in the starting material, as may be found at different synapses. Moreover, the large size of the complex may reflect dynamic assembly, perhaps involving activity-dependent genes, or transient protein–protein interactions of signaling complexes, which is a feature of many signaling pathways involving the proteins in the NRC[6].

DISCUSSION

The proteomic approach complements and extends studies using yeast two-hybrid screens for the identification of proteins in neurotransmitter receptor complexes. Fourteen proteins that interact with NR1 and NR2, as determined by two-hybrid screens, also coimmunoprecipitate with NMDAR from brain (PSD-95, Chapsyn110/PSD-93, SAP102, GKAP/SAPAP, Yotiao, SynGAP, Calmodulin, nNOS, PI3-kinase, Citron, MAP2B, actin, α-actinin2, spectrin). Twelve proteins interact with these 14 proteins, but were not formally shown to exist in NRCs (mGluR, GluR6/7, Homer/Vesl1, Shank, PKA-R2β, PP1, PKA catalytic subunit, PLCγ, Cortactin, CortBP-1, phosphofructokinase, APPL). Four proteins coimmunoprecipitate with NMDARs without defined interaction domains (CaMKII, Src, PTP1D/SHP2, tubulin). All 30 of these proteins were detected in our analysis, including PI3 kinase, which interacts via SH2 phosphorylation with NR2 and therefore escapes detection in two-hybrid screens. We identified a third group of 19 proteins, not previously known to be structurally linked to NRCs, but functionally implicated in NMDAR-dependent synaptic plasticity, including PKC (β, γ, ε), PP2A, PP2AR1α, PP2B, NF1, Ras, Rap2, c-Raf1, MEK1/2, ERK1/2, MKP2, Rsk-2, cPLA2, N-cadherin, L1, Arg3.1/Arc and HSP70. A fourth set of six proteins that were not structurally or

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functionally linked to NMDAR but are known to interact with the above proteins include β-Catenin, Z0-1, pp120[CAS], myosin, Clathrin and Dynamin. There remains a set of 19 proteins, including 5 ESTs, Pyk2, AKAP150, PP5, Rac1, RalA, NSF, SAP97, Desmoglein, Bassoon, P53 binding protein, Sarcolemmal associated protein-3, Kinesin light chain, RNA binding protein FUS/TLS and α-internexin. Interestingly, this latter set includes proteins that might be involved in postsynaptic receptor trafficking and spine translational regulation.

The structure of the NRC isolated from the mouse brain indicates that subsets of neurotransmitter receptors, cell-adhesion proteins, adapters, second messengers and cytoskeletal proteins are organized together into a physical unit comprising signaling pathways. Several features of the NRC provide insights into the specific functions of this complex, particularly in the physiological context of NMDAR-dependent synaptic plasticity. The simplest general function for this NRC may be in the 'induction phase' of synaptic plasticity in contrast to the 'expression phase' as described in electrophysiological experiments. Induction involves activation of the NMDAR and mGluR, Ca^{2+} influx and second messenger signaling and occurs within the first hour after the train of stimuli. Expression or maintenance of synaptic plasticity after one hour is resistant to inhibition of NMDAR or second messengers and is mediated by AMPA receptors. Thus the NRC contains the molecular machinery for the induction of plasticity, and AMPA receptor complexes are separate entities mediating expression. The assembly of specific signaling modules composed of effector pathways (for example, Ras-ERK/MAP or Ras-PI3 kinase) might regulate specific components of LTP or LTD. In addition to trafficking and phosphorylation of AMPA receptors, the expression of plasticity is reported to involve multiple cellular mechanisms, including cytoskeletal changes in spine structure, local translation regulation and gene transcription. These functions could be linked to the NMDA receptor via the NRC proteins.

This model of the NRC as an induction device is supported by targeted mouse mutations, transgenic expression and pharmacological inhibition of NRC proteins that alter the induction of synaptic plasticity. Induction of synaptic plasticity is altered by targeted mouse mutations in NR1, NR2A, mGluR1, PSD-95, PKA catalytic and regulatory subunits, PKCγ, CaMKII, nNOS, NF1 or H-Ras, by transgenic expression of NR2B, CaMKII, PP2B, PKA inhibitors or L1, and by pharmacological inhibition of NMDAR, mGluR, PKA, PKC, CaMKII, MEK, tyrosine kinases, PP1, PP2A, PP2B, Cadherins, L1, actin polymerization, Calmodulin, nNOS, PI3 kinase or cPLA2. A physiological parallel with these disruptions in NRC proteins is observed during postnatal development of the hippocampus and cortex. The threshold for NMDAR-mediated LTP and LTD is age dependent[2], and during this time the levels of expression of many NRC proteins and their association into NMDAR complexes changes[43]. The NRC is also well suited to induce bidirectional synaptic plasticity (LTP and LTD) because many calcium-sensitive proteins with kinases and phosphatases are beneath the NMDAR calcium pore in a microdomain. The composition of this complex goes some way to addressing questions raised[5] regarding the apparent diversity of molecules involved with synaptic plasticity, as a substantial subset of these molecules are components of the NRC and map onto the function of induction.

In addition to a role in synaptic plasticity, the NRC described here is likely to be important for cognitive function and in particular learning and memory. Learning impairments in rodents are associated with genetic and pharmacological disruption of multiple components of the NRC (NR1, NR2A, NR2B, PSD-95,

PKA subunits, PKC isoforms, CaMKII, PP1, PP2A, PP2B, MEK, NF1, nNOS, cPLA2 and L1). Although these data support a role for the NRC in both learning and synaptic plasticity, it may be premature to conclude that synaptic plasticity is itself required for learning, as the NRC seems to comprise multiple effector pathways, which may be differentially required for learning or plasticity or other cellular processes[44]. Because the term NRC was used to describe the channel formed by NMDAR subunits, 'Hebbiasome' or 'potentiosome' are alternative terms describing the function of the described complexes in controlling synaptic potential and Hebbian synaptic properties.

We were surprised to find that three of the proteins (NF1, ref. 45; L1, ref. 46; Rsk-2, ref. 47) detected in this proteomic study were encoded by genes that underlie human mental retardation and learning impairment. This implies that the defect in these patients is in the function of the NRC. Moreover, these findings suggest that pharmacological manipulation of the NRC, as distinct to NMDARs alone, may provide new therapeutic avenues for these patients. Proteomic tools combined with genetic dissection of the NRC signaling pathways in mutant mice should lead to an understanding of the specific contributions of each pathway to the cellular mechanisms of synaptic plasticity and learning, as well as provide a new route toward understanding human neuropsychiatric conditions.

METHODS

Antibodies. Affinity-purified NR1-specific sheep polyclonal antibodies (MAP-NR1) were generated using a multiple-antigen peptide (MAP) of the last 20 amino acids of NR1 ((H-RRAIEREEGQLQLCSRHRES)8-MAP; Diagnostics Scotland, Carluke, UK). Antibody sources (column Ab in **Table 1**): a, J.H. Morrison; b, Upstate Biotech, Waltham, Massachusetts; c, Chemicon, Wealdstone, UK; d, Pharmingen, San Diego, California; e, R. Huganir; f, Transduction Laboratories, Lexington, Kentucky; g, M. Watanabe; h, Alomone Labs, Jerusalem, Israel; i, H. Kreienkamp; j, K. Inokuchi; k, E. Ziff; l, J. Scott; m, J. Henley; n, Promega, Madison, Wisconsin; o, P. Cohen; p, Oncogene, Nottingham, UK; q, New England Biolabs, Hitchin, UK; r, Santa Cruz Biotechnology, Santa Cruz, California; s, Calbiochem, Nottingham, UK; t, D. Kuhl; u, D. Coleman; v, Sigma, Poole, UK; w, Roche Molecular Biochemicals, Lewes, UK; x, J. Parsons.

Purification of the NMDAR complex. The receptor complex was isolated from mouse forebrain extracts using covalently coupled MAP-NR1 immunoaffinity resins, immunoprecipitation with the same antibody, or peptide-affinity chromatography with a hexapeptide of the NMDA-R2B C-terminus (SIESDV; H.H. and S.G.N.G., unpublished data). In brief, samples were homogenized in 1% (w/v) deoxycholate-containing buffer at pH 9.0, spun for 30 minutes at $50.000 \times g$ at 4ºC, followed either by incubation with MAP-NR1 antibody and subsequent protein G-sepharose precipitation, or by immunoaffinity chromatography using MAP-NR1 antibody–substituted affigel-10 (BioRad, Hemel Hempstead, UK; 5 mg antibody per ml resin), or the NR2B peptide resin (5 mg peptide per ml affigel-10 resin). The resins were washed after an overnight incubation at 4ºC with 100 to 1000 column volumes of extraction buffer at 4ºC, and proteins were separated from the resin by boiling in 4% SDS for 30 minutes.

Western blotting. Samples were separated by SDS-PAGE and transferred to PVDF membrane at 4ºC for 90 minutes at 75 V in 10% (v/v) methanol and 10 mM CAPS, pH 11.0. Dilution of primary antibodies was between 1:100 and 1:1000 depending on the quality of the IgG. Signals were detected using peroxidase-linked secondary IgGs and enhanced chemiluminescence.

Mass spectrometry sample preparation. NMDAR complex samples were separated by SDS-PAGE and stained by Coomassie blue or silver staining[48]. Individual protein bands of 60–300 kD were excised, reduced, alkylated and digested with trypsin[49]. The resultant peptide mixtures were

analyzed by on-line liquid chromatography tandem mass spectrometry (LC-MS/MS) to generate peptide sequence information[50].

Online LC-MS/MS analysis. Chromatographic separations of the peptide mixture were done on a 180 μm PepMap column using an Ultimate LC system (LC Packings, Amsterdam, Netherlands) delivering a gradient to formic acid (0.05%) and acetonitrile. The eluting peptides were ionized by electrospray ionization on a Q-TOF hybrid mass spectrometer (Micromass, Wythenshawe, UK) fitted with a Z-spray source. The instrument, in automated switching mode, selects precursor ions based on intensity for peptide sequencing by collision-induced fragmentation tandem MS. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z of the precursor; a total of nine MS/MS scans were acquired per precursor. Several hundred MS/MS spectra were generated per run, allowing the analysis of complex mixtures without any prior interpretation. The mass spectral data was processed into peak lists containing m/z value, charge state of the parent ion, fragment ion masses and intensities, and correlated with proteins and nucleic acid sequence databases using Mascot software (Matrix Science, London, UK). Proteins were identified based on matching the MS/MS data with mass values calculated for selected ion series of a peptide. A non-redundant protein database and a nucleotide database (dbEST) were searched without applying any constraints on molecular weight or species. Most proteins were identified with several peptide matches, although a few were assigned on the basis of a single peptide provided near-complete peptide sequence had been obtained.

Note: A complete list of references for all protein interactions can be found on the Nature Neuroscience web site (http://www.nature.com/neuro/web_specials/).

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