The Department of Neurobiology, Care Sciences and Society
Karolinska Institutet, Stockholm, Sweden

THE N-METHYL-D-ASPARTATE RECEPTOR SUBUNIT NR3A: CLONING, EXPRESSION AND INTERACTING PROTEINS

Maria Eriksson

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Sluta stressa, du dör ändå.
Ovanligt klok kvinna
ABSTRACT

This thesis focuses on the subunit NR3A, which together with subunits NR1, NR2A-NR2D and NR3B builds the excitatory glutamate receptor N-methyl-D-aspartate (NMDA). The receptor is most likely a tetramer, containing at least one NR1, probably two NR2 and sometimes one NR3 subunit. NR3A is highly expressed during development and present at moderate levels in the adult brain. Both NR3A and NR3B have an attenuating effect on NMDA receptor currents, when incorporated into the receptor complex.

In study I we cloned and sequenced the human NR3A subunit, which at the time only had been described in rat. We found the homology between the rat and human sequences to be high, and most potential phosphorylation and glycosylation sites to be conserved. In contrast to rat tissue, we did not detect the longer splice variant of NR3A in the human central nervous system. We found NR3A mRNA to be expressed both in the developing human brain and spinal cord, with prominent staining of the cortical plate, ventricular zone and the dorsal half of the spinal cord as well as the neuroepithelial layer surrounding the central canal. Interestingly, the neuroepithelial layer expressed very low levels of NR1 mRNA, suggesting that NR3A might have replaced one of the NR1 subunits in NMDA receptors in this area.

In study II we investigated the occurrence of NR3A mRNA and protein in adult human brain, its association with the other NMDA receptor subunits and the subunits solubility. We found NR3A mRNA and protein to be expressed in adult human brain, especially in layers II/III and V of cerebral cortex and in thalamus and pons. Expression was low or undetectable in caudate nucleus, claustrum, cerebellum and spinal cord. Further we found NR3A to be associated with NR1, NR2A and NR2B in adult human cortex, and to some extent in the spinal cord. NR3A showed a different solubility profile to the other NMDA receptor subunits, being extracted with milder detergents. Both NR1 and NR3A were found as monomers, dimers and tetramers, as well as in larger protein complexes. In contrast, NR2 subunits were only found in tetramers and in larger protein complexes. This probably reflects the presence of an intracellular pool of unassembled NR1 and NR3A subunits.

To learn more about NR3A and its function we screened a fetal human brain cDNA library for proteins interacting with NR3A. Among a number of potentially interesting candidates we choose MAP1S/C19ORF5 and MAP1B for further analysis. The results are presented in study III and IV respectively. We found MAP1S to be highly expressed throughout brain and spinal cord, predominantly in neurons. MAP1S-EGFP over-expressed in cultured hippocampal cells, localized both to dendritic shafts and filopodia. Colocalization of MAP1S-EGFP with β-tubulin III immunoreactivity was prominent, with synapsin and PSD95 immunoreactivity occasional and with NR3A immunoreactivity frequent in dendritic shafts and sparse in filopodia. Judging from the subcellular distribution of MAP1S and NR3A their interaction might be important for transport through shafts or regulation of NR3A-containing receptors in spines.

The distribution of MAP1B immunoreactivity resembled that of MAP1S-EGFP, with prominent overlap with β-tubulin III immunoreactivity, sparse colocalization with synapsin and SAP102 immunoreactivity, and frequent colocalization with NR3A immunoreactivity in dendritic shafts and infrequent colocalization in spines. To address the function of the NR3A-MAP1B interaction, we investigated the expression and distribution of NR3A in MAP1B deficient mice. These mice expressed increased NR3A and decreased NR1 levels compared to wild type, suggesting that NMDA receptors in the MAP1B deficient mice might have an altered subunit composition. NR3A was equally distributed to filopodia in neurons from MAP1B deficient and wild type mice, indicating that MAP1B is not essential for transport of NR3A-containing NMDA receptors to synaptic sites. Instead the interaction might involve regulating the distribution of NR3A-containing receptors between intracellular pools and the cell surface, as well as the distribution between synaptic and extrasynaptic sites.
LIST OF PUBLICATIONS

The thesis is based on the following articles, which will be referred to in the text by their roman numerals.


*These authors have contributed equally to the work.*
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<td>5-hydroxy tryptamine</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring protein</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
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<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor</td>
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<td>Knock out</td>
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<td>LRPPRC</td>
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<td>mGluR</td>
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<td>NMDA</td>
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<td>Shank</td>
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<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
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<td>Variable charge Y chromosome 2 interacting protein-1</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<td>ZO-1</td>
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SAMMANFATTNING PÅ SVENSKA

NERVSYSTEMET OCH NERVCELLEN

Vårt nervsystem delas upp i en central del, bestående av hjärna och ryggmärg, och en perifer del, bestående av nervvävnad belägen utanför de centrala delarna. Den mänskliga hjärnan består av uppskattningsvis 10 miljarder nervceller och ännu fler gliaceller. Gliacellerna bildar till exempel blod-hjärn-barriären och de fettrika myelinskidor som möjliggör att våra elektriska nervimpulser kan vidarebefordras i hög hastighet längs nervcellens utskott.


Figur 2. En synaptisk kontaktzon mellan två nervceller. Överst i bilden syns nervcell 1 som avger molekyler från sitt axon. Molekylerna rör sig genom synapsklyftan och binder till mottagarproteiner, så kallade receptorer som sitter i cellmembranet på nervcell 2. Då molekylerna binder till receptoreerna aktiveras dessa och en rad signaler befordras till nervcell 2.

N-METHYL-D-ASPARTAT (NMDA) RECEPTORN

Den här avhandlingen beskriver proteinet NR3A som ingår som en enhet i receptor NMDA (figur 3). NMDA-receptorn är utbredd i centrala nerverystemet hos däggdjur och är bland annat inblandad i minnesfunktioner och vissa typer av cellldöd. NMDA-receptorn aktiveras av att molekylerna glutamat och glycin binder till den del av receptorn som sticker ut utanför cellen. Receptorn anpassar sig till bindningen genom att ändra form, vilket leder till att en kanal öppnas genom receptor och jonerna Na⁺, K⁺ och Ca²⁺ kan passera från ena sidan av cellmembranet till den andra. Förändringen av receptorns form samt jonströmmarna över cellmembranet ger en rad effekter på nervcellen. Dels kan proteinerna som binder till receptorns inre delar påverkas, vilket kan leda till en kaskad av händelser inuti cellen. Dels kan cellens benägenhet att skicka vidare nervimpulser till omkringliggande celler påverkas. Mitt arbete har främst berört de delar av NR3A som finns inuti cellen och de proteiner dessa delar kan interagera med.

Figur 3. NMDA-receptorn och dess enheter NR1, NR2 och NR3A. Enheter bind till varandra i grupper om fyra och bilder på så sätt en funktionell NMDA-receptor. Receptorn aktiveras av att molekylerna glutamat och glycin binder till den och aktiveringen kan i vissa fall leda till att nya minnen bildas.
AVHANDLINGENS DELARBETEN


I arbete II undersökte vi i vilka delar av människans centrala nervsystem som NR3A förekommer och fann att de högsta nivåerna av proteinet finns i hjärnbarken, thalamus och pons, eller bryggan (figur 4). Hjärnbarken (cortex cerebri) är inblandad i högre kognitiva funktioner och thalamus vidarebefordrar signaler från kroppen till hjärnan och tillbaka igen. Pons förbinder bland annat lillhjärnan med rester av nervsystemet. Vi hittade bevis för att NR3A sitter ihop med de andra komponenterna i NMDA-receptorn, NR1, NR2A och NR2B. Hos råttor har det visat sig att NR3A förekommer i höga nivåer under tidig utveckling. Här såg vi att NR3A förekommer i betydande nivåer även i den vuxna hjärnan hos råttor, apa och människa. Den vuxna råttans tätbundna nivåer av NR3A i sin ryggmärg men vi fann att vuxna människor har mycket lite kvar av proteinet i ryggmärgen. Slutligen undersökte vi hur hårt NR3A sitter förankrad i nervecellens membran och tolka de protein som fäster sig till andra i membranen. Vi fann att NR3A var mer lättlösigt än NR1 och NR2A och NR2B. Det gjorde vi genom att använda oss av diskmedelliknande substanser, detergenter, som löser upp feta cellmembran och i viss mån bindningarna mellan proteiner. Vi fann att NR3A var mer lättlösigt än NR1 och NR2A och NR2B. NR3A förekom också i större utsträckning i en form där det inte var bundet till de andra enheterna. Det indikerar att förutom att ingå i NMDA-receptorkomplexet vid nervcellens yta, kan obundet NR3A finnas i en reservpool inuti cellen.


Syftet med arbete III och IV var att undersöka vilka proteiner som binder till NR3A inuti nervcellen. Kartläggning av proteininteraktionerna ger en ökad förståelse av de signaler NR3A och NMDA-receptorn skickar till mottagarcellen. Kunskapen kan eventuellt användas för att ta fram läkemedel mot sjukdomar där NR3A och NMDA-
receptorn är inblandad. Genom att använda NR3A som bete har vi ”fiskat” i ett bibliotek som innehåller en stor mängd proteiner från den mänskliga hjärnan. Vår fängst bestod av både bottennapp och riktigt intressanta proteiner. Av de intressanta proteinerna valde vi ut två besläktade proteiner att arbeta vidare med, ”microtubule-associated protein” (MAP) 1S, som beskrivs i arbete III och MAP1B som beskrivs i arbete IV. Dessa två proteiner har fått sitt namn på grund av att de kan binda till mikrotubuli-cellskeletten. Efter att vi med metoden ”GST pull-down” verifierat att proteinerna binder till NR3A i provröret, undersökte vi om proteinerna också binder till varandra i hjärnan. Med hjälp av en antikroppsbaserrad metod, immunoprecipitering, kunde vi se att både MAP1S och MAP1B binder till NR3A i hjärnan, vilket indikerar att interaktionen har en funktion! I och med att få studier tidigare publicerats om proteinet MAP1S undersökte vi i vilka organ och i vilka regioner av hjärnan som proteinet finns. Vi kunde se att det finns stora mängder MAP1S i hjärnan men att proteinet även förekommer i andra organ. Arbete III avslutades med att vi med hjälp av så kallad immunocytokemi undersökte i vilka delar av nervcellen MAP1S-proteinet finns. I denna metod tillsätts färgade antikroppar till nervceller odlade på glasplattor. Genom mikroskopering kan man se var de färgade antikropparna har fastnat, det vill säga den plats i nervcellen där det proteinet som antikroppen specifikt känner igen finns. Vi använde antikroppar mot både NR3A och MAP1S och kunde se att de till viss del förekommer i samma del av nervcellen. Genom att använda antikroppar mot två proteiner (synapsin och PSD95) som finns i nervcellernas synapser, kunde vi se att MAP1S till viss del förekommer i synapserna. I synapserna finns även NMDA-receptorn och NR3A, vilket kan betyda att MAP1S är ett av proteinerna som förmedlar signaler i mottagarcellen efter att NMDA-receptorn aktiverats.

I arbete IV undersökte vi interaktionen mellan proteinerna NR3A och MAP1B. Med hjälp av immunocytokemi såg vi att både NR3A och MAP1B förekommer i stor utsträckning i nervcellens dendriter men även i synapserna (figur 5). För att ta reda på funktionen av interaktionen mellan de båda proteinerna använde vi oss av möss som saknar MAP1B-proteinet. Vi fann att dessa möss hade mer NR3A och mindre NR1 än kontrollmössen. Det tyder på att dessa möss skulle kunna ha en annan sammansättning av receptorenhetera i sina NMDA-receptorer än kontrollmössen. I nervceller odlade från dessa möss och från kontrollmössen såg vi att NR3A förekom i lika stor utsträckning i synaptiska strukturer hos båda djuren. Det tyder på att MAP1B inte krävs för att transportera NR3A till synaptiska strukturer. Istället är det mer troligt att interaktionen mellan MAP1B och NR3A är viktig för hur NR3A rör sig i de synaptiska strukturerna.

![Figur 5. Immunocytokemisk infärgning av en dendrit i en odlad, human nervcell med antikropp som känner igen MAP1B, till vänster och NR3A till höger. Färgningen visar att båda proteinerna finns i dendriterna men att NR3A förekommer i större utsträckning i de tunna utskott där synaptiska kontakter bildas.](image-url)
SLUTSATS

Avhandlingen visar att proteinet NR3A i människa liknar NR3A i råtta, både avseende aminosyrasammansättning och förekomst i hjärnans olika delar. Däremot skiljer sig förekomsten av NR3A i ryggmärg hos råtta och människa, då den vuxna människans ryggmärg nästan helt saknar NR3A. NR3A förekommer i betydande nivåer i vuxen hjärna hos råtta, apa och människa, vilket tyder på att proteinet spelar en viktigare roll i den vuxna hjärnan än tidigare trott. Vi visar också att NR3A är associerad med de andra NMDA-receptorenheterna NR1, NR2A och NR2B i den vuxna hjärnan men även förekommer obundet. Slutligen har vi identifierat två proteiner som interagerar med NR3A i hjärnan, MAP1S och MAP1B. Båda proteinerna är vanligt förekommande i hjärnan och binder till nervcellernas cellskelett. Våra fynd tyder på att interaktionerna mellan NR3A och MAP1S respektive MAP1B är inblandade i regleringen av NR3A i nervcellens synapser. Reglering av NMDA receptorn och dess enheter är viktig för normal mognad av nervceller under hjärnans utveckling och för minnesfunktioner under hela livet.
INTRODUCTION

Our nervous system contains a complex network of neurons and glial cells that communicate with each other in a highly regulated manner. Nerve impulses are electrical signals, propagated through the neuron by ions, and from one neuron to the next usually by chemical substances called neurotransmitters. When the electrical signal reaches the neurons terminal, neurotransmitters are released from the terminal into a narrow, extracellular space, the synaptic cleft, and diffuse to the receptors of the receiving neuron. With respect to their position in the synaptic cleft, neuronal structures are termed pre- and postsynaptic. The functional unit consisting of the presynaptic membrane, the synaptic cleft and the postsynaptic membrane is called the synapse. The amino acid glutamate is the most common excitatory neurotransmitter in the mammalian central nervous system (CNS), affecting the postsynaptic neuron by binding to glutamate receptors situated in the postsynaptic plasma membrane. There are three types of ionotropic glutamate receptors in mammals, \(N\)-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. This thesis focuses on the NMDA receptor subtype.
THE NMDA RECEPTOR

The NMDA receptor is widely distributed throughout the mammalian CNS and has been prescribed a vast array of functions, ranging from involvement in normal brain development and learning to cell death and psychiatric disorders. The receptor is situated at excitatory synapses on dendritic spines, but is also localized to extrasynaptic sites. A large number of proteins in the postsynaptic density (PSD) interact with the NMDA receptor and mediates its signaling upon receptor activation. The receptor is composed of different subunits, which assemble and form an ionotropic receptor permeable to positively charged ions. Each individual NMDA receptor subunit has an extracellular N-terminus, four membrane domains and an intracellular C-terminus (figure 1). Membrane domains three and four are connected by a long extracellular loop, which together with the N-terminus form a ligand-binding pocket. Membrane domains one, three and four span the membrane while domain number two is a re-entrant loop, beginning and ending on the intracellular side of the membrane. The membrane domains are arranged such that the re-entrant loops outline the ion channel pore.

Figure 1. Topology of the NMDA receptor subunits. The long N-terminus, which is situated outside the cell, is followed by four membrane regions connected by intracellular and extracellular loops, and an intracellular C-terminus. The N-terminus forms the ligand-binding pocket together with the extracellular loop between membrane regions III and IV. Abbreviation; Ligand (L).

Identification of the NMDA receptor subunits

There are three types of NMDA receptor subunits, NR1, NR2 and NR3, and they assemble into tetramers to form functional receptors. NR1 was the first identified NMDA receptor subunit, cloned from rat tissue in the early 1990s (Moriyoshi et al., 1991). Cloning the NR1 subunit from mouse tissue in 1992 and from human tissue in 1993 closely followed the initial identification (Yamazaki et al., 1992; Karp et al., 1993). The NR2 subunits, NR2A-NR2D, are encoded by four different genes and have been identified in rat (Monyer et al., 1992), mouse (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992) and human tissue (Foldes et al., 1994; Adams et al., 1995; Lin et al., 1996). As the other NMDA receptor subunits, NR3A was initially cloned and characterized in rat tissue (Ciabarra et al., 1995; Sucher et al., 1995), but was not cloned from human tissue until early this century (Andersson et al., 2001). Around the same time, the final member of the NMDA receptor family, NR3B, was identified and characterized (Nishi et al., 2001; Matsuda et al., 2002; Bendel et al., 2005; Chatterton et al., 2002).
NR1

NR1 is ubiquitously expressed in brain and spinal cord during development and adulthood (Moriyoshi et al., 1991; Brose et al., 1993; Tölle et al., 1993; Monyer et al., 1994). The NR1 transcript contains 22 exons and can be alternatively spliced, resulting in eight different variants of the NR1 subunit (table 1) (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Yamazaki et al., 1992; Hollmann et al., 1993). Exon five encodes a region in the N-terminus of NR1, named the N1 cassette, and can either be included or excluded in the protein. In the 3’ end of the transcript, exons 21 and 22, encoding C-terminal cassettes C1, C2 and C2’, can be alternatively spliced. The NR1 splice variants differ in their anatomical distribution and generate NMDA receptors with different physiological and pharmacological properties (reviewed in Hollmann and Heinemann, 1994; Zukin and Bennett, 1995; Dingledine et al., 1999; Prybylowski and Wenthold, 2004).

Table 1. The NR1 splice variants.

<table>
<thead>
<tr>
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<th>E5/N1</th>
<th>E21/C1</th>
<th>E22/C2</th>
<th>E22/C2’</th>
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<tr>
<td>NR1-1a</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NR1-1b</td>
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<td>NR1-3a</td>
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<td>NR1-4b</td>
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Exons (E) 5, 21 and 22 can be alternatively spliced, resulting in either the presence or absence of N-terminal cassette N1 and C-terminal cassettes C1, C2 and C2’. Nomenclature according to Hollmann and Heinemann, 1994.

NR2

In rat brain NR2B and NR2D mRNAs are expressed prenatally, while NR2A and NR2C mRNAs are detected around birth (Monyer et al., 1994). NR2D mRNA peaks around postnatal day 7 (P7), and is mainly found in midbrain structures, and in the thalamus and brain stem of adult animals. NR2C mRNA and protein is almost exclusively found in cerebellum, and the protein is detected around P5 in rat brain (Wenzel et al., 1997). At birth, NR2B is expressed in most regions of the brain, but during the first postnatal weeks its distribution becomes highly restricted to hippocampus and forebrain. The NR2A protein levels increase after birth and in adult rat NR2A is ubiquitously expressed throughout the brain (Petralia and Wenthold 1994; Wenzel et al., 1995; Wenzel et al., 1997).

The NR2 subunits are also expressed in the spinal cord. Both NR2B and NR2D are expressed in the embryonic spinal cord, while NR2A and NR2C are absent (Monyer et al., 1994). During postnatal development the NR2A and NR2B subunits follow the same distribution pattern as in the brain, with higher NR2B than NR2A levels (Stegenga and Kalb, 2001). The adult rat spinal cord seems to express at least low
levels of all four NR2 subunits, even though the results are slightly contradictory (Tölle et al., 1993; Petralia and Wenthold, 1994; Stegenga and Kalb, 2001). The adult human spinal cord expresses NR2A, NR2C and NR2D (Sundström et al., 1997).

NR3
Two different genes encode the NR3 subunits, NR3A and NR3B, and in some species the NR3A transcript can be spliced into two different variants. The longer splice variant includes 60 base pairs in the 3’ region and has been identified in rat (Sun et al., 1998). Both splice variants have their highest expression during the first postnatal weeks and NR3A-1 seems to be the dominating form in the entorhinal cortex and thalamus. In mouse, NR3B mRNA is mostly found in brain stem and in motor neurons of the spinal cord and at low levels in the cerebellum (Nishi et al., 2001). Similarly, Matsuda and co-workers found NR3B mRNA in mouse midbrain, medulla and spinal cord (Matsuda et al., 2002). In addition, they found that in whole brain the NR3B mRNA levels were constant during development and that NR3B protein could be co-immunoprecipitated with NR1, NR2A and NR2B from transfected human embryonic kidney 293 (HEK293) cells. Investigation of NR3B distribution in adult rat brain confirmed previous studies, and showed both mRNA and protein to be restricted to motor neurons in the ventral horn and to brain stem nuclei (Chatterton et al., 2002). In contrast, one study found NR3B mRNA to be widely distributed throughout adult rat brain (Andersson et al., 2001). So far there is only one study on NR3B expression in adult human CNS, and it shows NR3B mRNA to be expressed in the hippocampus and adjacent cortex (Bendel et al., 2005). We performed immunohistochemistry (IHC) on adult human brain and found expression of NR3B protein in hippocampus (unpublished observation).

NMDA receptor activation

NMDA receptors are most likely tetrameric assemblies of NR1, NR2 and sometimes NR3 subunits (figure 2) (Laube et al., 1998; Rosenmund et al., 1998). NR1 and NR3 bind glycine (Hirai et al., 1996; Yao and Mayer, 2006; Nilsson et al., 2007) and the NR2 subunits bind glutamate (Laube et al., 1997). Recent evidence has shown that D-serine, which binds to the same site as glycine, might be as important for NMDA receptor regulation as glycine (reviewed in Wolosker, 2007). Other endogenous substances, such as L-aspartate, have been suggested to regulate NMDA receptor activity, but the importance of these is still unclear (Collins and Probett, 1981; Lysko et al., 1989). NR1 is present in all known functional NMDA receptors (Forrest et al., 1994) and expression of functional recombinant receptors in mammalian cells require both NR1 and NR2 subunits (McIlhinney et al., 1996). Moreover, NR1 has been shown to be essential for transport of NR3A-containing receptors to the cell surface (Pérez-Otano et al., 2001).
Figure 2. Functional NMDA receptors expressed at the plasma membrane are believed to consist of four assembled subunits, of which at least one is NR1. NR1 and NR3A bind glycine and D-serine and the NR2 subunits bind glutamate.

Activation of endogenous NMDA receptors requires simultaneous ligand-binding to both the glycine and glutamate sites (figure 3) (reviewed in Dingledine et al., 1999; Paoletti and Neyton, 2007). In addition to ligand binding, receptor activation requires depolarization of the plasma membrane, which allows the Mg$^{2+}$ ion situated in the channel pore to be released. This depolarization can be mediated by the glutamate receptor α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), situated in the same postsynaptic membrane. When activated, the NMDA allows influx of Na$^+$ and Ca$^{2+}$ and out flux of K$^+$ through the channel pore.

Figure 3. Activation of the NMDAR requires binding of the ligands glycine (or D-serine) and glutamate as well as depolarization of the plasma membrane. The depolarization can be mediated by the AMPAR, and results in release of the Mg$^{2+}$ ion situated in the channel pore at resting membrane potentials. The channel pore is permeable to positively charged ions, of which K$^+$, Na$^+$ and Ca$^{2+}$ endogenously are the most important. Abbreviations; N-methyl-D-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA).
THE NR3A SUBUNIT

Spatial and temporal distribution of NR3A

The early studies on NR3A performed in rat, showed NR3A mRNA to be elevated just prior to birth and during the first postnatal week. The levels begin to decline between P7 and P14 and remained at a lower, but constant level into adulthood (Ciabarra et al., 1995; Sucher et al., 1995). The highest levels of the NR3A transcript are expressed in spinal cord, brain stem, thalamus, hypothalamus, hippocampus, amygdala and in cortical areas. It was also detected in hindbrain but not in cerebellum. In 2002 Wong and co-workers published an extensive study on the temporal and spatial distribution of NR3A protein in rat CNS (Wong et al., 2002). They found NR3A to be highly expressed at birth, to further increase and peak at P8 and then decline to lower levels in adult rat brain. They detected NR3A in spinal cord, brain stem, thalamus, hypothalamus, hippocampus and amygdala, and in cerebral and cerebellar cortices. Investigation of cerebral cortex at P16 showed NR3A immunoreactivity mainly in layer V, and to some extent in layers II/III and VI. Further, electron microscopy studies showed NR3A to be localized to asymmetric synapses, but not always to be directly associated with the PSD (Wong et al., 2002). A similar temporal and spatial expression pattern of NR3A protein was described by Al-Hallaq and co-workers, who also investigated the association of NR3A with the other NMDA receptor subunits in rat brain (Al-Hallaq et al., 2002). They found that approximately 80% of expressed NR3A subunits were associated with NR1 in P10 cortex, but only 40% in adult rat cortex. However, only about 10% of NR1 and NR2A and 5% of NR2B were associated with NR3A at P10, decreasing to about half in adult rat cortex. This shows that in both postnatal and adult rat cortex the majority of NMDA receptors do not contain NR3A. Moreover, NR3A mRNA and protein has been shown to be expressed in rat and mouse retina (Sucher et al., 2003).

An extensive study investigating the NR3A mRNA levels in adult macaque brain revealed that the distribution in monkey brain slightly differed from that in rat brain (Mueller and Meador-Woodruff, 2005). Just as rat brain, monkey brain expressed NR3A mRNA in cerebral cortex, hypothalamus, thalamus, hippocampus and amygdala, but in contrast to rat brain, high expression was also found in substantia nigra and cerebellum. Investigation of NR3A in human brain was first performed on embryonic tissue, and showed NR3A to be expressed in the developing cortex and in the spinal cord (Mueller and Meador-Woodruff, 2003). Soon after, investigation of adult human prefrontal and temporal cortices, showed NR3A mRNA also in adult human brain (Mueller and Meador-Woodruff, 2004). A recent study showed the developmental profile of NR3A protein in human brain to be similar to what has previously been shown in rat (Henson et al., 2008).

NR3A knock out mice

NR3A is enriched in PSD preparations and co-immunoprecipitates with NR1, NR2A and NR2B but not with glutamate receptor subunits (GluR) GluR2 or GluR6/7, in brain extracts and in over-expressing heterologous cells (Das et al., 1998; Pérez-Otano et al., 2001; Al-Hallaq et al., 2002; Sasaki et al., 2002). Mice lacking NR3A reach adulthood,
are fertile and do not display obvious behavioral abnormalities (Das et al., 1998). NR3A knock out (KO) mice expressed normal NR1, NR2A and NR2B protein levels and displayed increased NMDA-induced currents compared to wild type (WT) mice. The increased currents observed in the KO mice are consistent with earlier studies showing NR3A to have attenuating effects on NMDA receptor currents in *Xenopus laevis* oocytes injected with NR1, NR2 and NR3A (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). The presence of NR3A in the NMDA receptor complex also reduces Ca$^{2+}$ permeability and sensitivity to Mg$^{2+}$ block (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Pérez-Otano et al., 2001; Chatterton et al., 2002; Sasaki et al., 2002). In addition to electrophysiological alterations, neurons in the NR3A KO mice had an increased density of dendritic spines, which displayed enlarged heads and elongated necks (Das et al., 1998). The effect on dendritic spines was more clearly seen in postnatal than adult mice, and preferentially in brain regions normally expressing NR3A. Male NR3A KO mice also show enhanced prepulse inhibition of the startle response (Brody et al., 2005). This is a measure of sensorimotor gating, which is impaired in schizophrenic hyperactivity disorders.

The atypical NR1/NR3 glycine receptor

NR3A has been reported to form an atypical excitatory glycine receptor together with NR1 when injected into *Xenopus laevis* oocytes (figure 4). This novel receptor subtype, which possesses low Ca$^{2+}$ permeability and low sensitivity to Mg$^{2+}$ block, lacks the NR2 subunit, and accordingly is not affected by NR2-binding substances such as glutamate and NMDA (Chatterton et al., 2002; Awobuluyi et al., 2007; Madry et al., 2007). When measuring whole-cell currents from cultured cerebrocortical neurons, Chatterton and co-workers recorded glycine-evoked currents with a similar pharmacological profile as those measured from the NR1/NR3A receptors in oocytes. This result suggests that the atypical glycine receptor is expressed endogenously in neurons (Chatterton et al., 2002). Moreover, over-expression of NR1 together with NR3A in HEK293 cells was reported to result in functional NR1/NR3A glycine receptors (Pina-Crespo and Heinemann, *Soc. Neurosci. Abstr.* 957.1, 2004). These results were presented at the Neuroscience meeting 2004 but have not yet been published.

**Figure 4.** The atypical glycine receptor is composed of NR1 and NR3A subunits and lacks NR2 subunits. The receptor is activated by glycine in the absence of glutamate, possesses low Ca$^{2+}$ permeability and low sensitivity to Mg$^{2+}$ block. The endogenous expression of this atypical receptor is debated.
Earlier studies on HEK293 cells showed NR1-1a/NR3A complexes to be transported to the cell surface and properly inserted into the plasma membrane, but not to form functional receptors in the absence of NR2 (Pérez-Otano et al., 2001). However, Smothers and Woodward recently showed that co-expression of NR1, NR3A and NR3B in HEK293 cells produced glycine-activated currents (Smoth and Woodward, 2007). In contrast to the studies performed in oocytes and by Pina-Crespo and Heinemann, expression of both NR3A and NR3B together with NR1, was required for functional receptors to be formed in HEK293 cells. Analysis of NMDA receptor pharmacology in Xenopus laevis oocytes is complicated by the fact that oocytes express a glutamate binding subunit XenU1 (Soloviev and Barnard, 1997). In addition, a recent study found oocytes to express mRNA encoding orthologs of all four NR2 subunits, XenNR2A-XenNR2D, as well as XenNR2B protein (Schmidt and Hollmann, 2008). The presence of these endogenously expressed glutamate-binding subunits needs to be taken into account when interpreting data from the Xenopus system.

Another recent study investigated the presence of the NR1/NR3A glycine receptor in both WT and hippocampal neurons from transgenic mice over-expressing the NR3A subunit (Tong et al., 2008). They could not detect any glycine-evoked currents in either WT or NR3A over-expressing neurons, suggesting that the atypical NR1/NR3A receptor is not expressed endogenously. This conclusion is contradictory to the previous report by the same group, mentioned above, which conclude that the atypical NR1/NR3A receptor is present in neurons (Chatterton et al., 2002). In Tong’s study the measurements were performed on cultured neurons derived from hippocampus, while cerbrocortical neurons were used in Chatterton’s study. Thus, one possibility for the contradicting results could be that hippocampal and cerbrocortical neurons differ in their subunit composition. Cerbrocortical neurons might express functional NR1/NR3A receptors, while hippocampal neurons do not, despite over-expression of NR3A.

NR3A in glial cells

One of the first studies reporting NMDA receptors in glial cells was performed on Schwann cells (Evans et al., 1992) and was followed by a study illustrating the occurrence of NMDA receptors in cortical astrocytes (Conti et al., 1996). NMDA receptors on glial cells are thought to mediate some of the white matter pathology associated with disorders such as stroke, vascular dementia, multiple sclerosis and brain and spinal cord trauma (reviewed in Lipton 2006; Sty and Lipton, 2007; Verkhratsky and Kirchhoff, 2007).

There are a number of recent studies illustrating the existence of NMDA receptors and NR3A in oligodendrocytes (Káradóttir et al., 2005; Salter et al., 2005; Micu et al., 2006). NMDA receptors are present both in precursors, immature and mature oligodendrocytes in cerebellum and corpus callosum (Káradóttir et al., 2005) and in the optic nerve NR1, NR2A, NR2B, NR2C, NR2D and NR3A, but not NR3B protein was detected (Salter et al., 2005; Micu et al., 2006). Double-labeling revealed co-localization of NR1 and NR2C immunoreactivity and NR2C and NR3A immunoreactivity, suggesting that NR1/NR2C/NR3A complexes might be formed (Káradóttir et al., 2005). The NMDA receptors were relatively insensitive to Mg$^{2+}$.
block, indicating that the receptor complex contains NR3A. Moreover, NMDA receptors were mainly localized to oligodendrocytes processes, and injury to the processes could be prevented by blocking NMDA receptors (Salter et al., 2005). These findings imply that drugs targeting NMDA receptors, and maybe specifically NR3A, might prove useful in preventing and treating white matter disorders.

NR3A and schizophrenia

Dysregulation of the NMDA receptor has been implicated in schizophrenia (reviewed in Olney et al., 1999) and a few studies have addressed the possible involvement of NR3A in the disorder. Mueller and Woodruff found the expression of NR3A mRNA to be elevated in the dorsolateral prefrontal cortex of postmortem brains from individuals with diagnosed schizophrenia (Mueller and Meador-Woodruff, 2004). Moreover, they found NR3A mRNA levels to be decreased in the dorsolateral prefrontal cortex of brains from people with bipolar disorder. They did not detect any change in NR3A mRNA levels in the inferior temporal cortex for either schizophrenia or bipolar disorders. Male NR3A KO mice exhibit an increased inhibition of the startle response (Brody et al., 2005). This response is altered in some schizophrenic patients (reviewed in Braff et al., 1992) and has been linked to the NMDA receptor (reviewed in Geyer et al., 2001). Another study addressed whether single nucleotide polymorphism in the NR3A gene were more frequent in schizophrenia patients compared to matched controls, but did not find any significant correlations (Gulli et al., 2007). Finally, a recent study investigating the NR1 and NR3A protein levels in dorsolateral prefrontal cortex from schizophrenic patients, reported the NR1 and NR3A levels to be normal (Henson et al., 2008). This suggests that the elevated NR3A mRNA levels reported by Mueller and Meador-Woodruff do not reflect an increase of NR3A protein levels, and accordingly is unlikely to affect NMDA receptor composition and signaling.
THE POSTSYNAPTIC DENSITY

The PSD was first described in an electron-microscopy study as an electron-dense area facing the presynaptic active zone (Palay, 1958). Studies of PSD morphology in 1959, led Gray to distinguish synapses with a prominent PSD from those with a less prominent PSD, and classify them into type 1 and type 2 synapses. (Gray, 1959). The type 1 synapses were later shown to be glutamatergic, excitatory synapses, which usually are localized to dendritic spine heads (Peters et al., 1991) (figure 5). The PSD contains membrane-bound proteins such as neurotransmitter receptors and cell adhesion molecules, signaling molecules including kinases and phosphatases, scaffolding proteins such as Shank and the postsynaptic density protein 95 (PSD95) family-members and cytoskeletal components, especially actin and its associated proteins (figure 6) (reviewed in Scannevin and Huganir, 2000; Okabe, 2007; Sheng and Hoogenraad, 2007). This broad array of proteins work in concert to regulate complex mechanisms, for instance receptor trafficking, synaptic strength and the stability of dendritic spines.

The dendritic spine head provides a micro-compartment allowing local changes of ion concentrations and signal transductions (figure 5). The spines contain ER, polyribosomes (PR) and molecules required for protein synthesis. Local protein synthesis is believed to be regulated by synaptic activity and to be involved in synapse-specific forms of long-term synaptic plasticity (reviewed in Martin et al., 2000). Proteins are mainly inserted and removed from the plasma membrane at the extrasynaptic endocytic zones (EZ), situated on each side of the synaptic membrane, which faces the presynaptic terminal. There is a continuous, activity-dependent exchange of proteins between extrasynaptic and synaptic sites (reviewed in Chouquet and Triller, 2003; Sheng and Hoogenraad, 2007).

**Figure 5.** A dendritic spine forming the postsynaptic half of an excitatory synapse. The proteins of the PSD, colored in blue, are continuous with the postsynaptic membrane through the interactions with transmembrane proteins. Actin, indicated by brown lines, is abundant in dendritic spines and smooth endoplasmic reticulum (SER) and polyribosomes (PR) are present. The endocytic zone (EZ) is located in the extrasynaptic region and clathrin-coated vesicles (CV) are indicated. Adapted from Sheng and Hoogenraad, 2007.
The NMDA receptor complex

The NMDA receptor complex is a major constituent of the PSD at glutamatergic synapses, with a large number of proteins directly and indirectly associated with the complex (reviewed in Grant and Husi, 2001; Boeckers, 2006). High throughput studies, examining the PSD composition have estimated the NMDA receptor complex to contain at least 70 different proteins (Husi et al., 2000; Walikonis et al., 2000; Jordan et al., 2004; Colllins et al., 2005; Cheng et al., 2006), but as new protein-interactions are identified the number grows. Below are a few examples of proteins associated with the NMDA receptor complex.

Figure 6. Schematic picture illustrating some of the components of the NMDA receptor complex described in the text. Abbreviations; NMDA receptor subunits NR1, NR2 and NR3A (1, 2 and 3), Ca\(^{2+}\) calmodulin-dependent protein kinase II (CaMKII), protein phosphatase 2A (PP2A), postsynaptic density protein 95 (PSD95), guanylate kinase-associated protein (GKAP), Src homology 3 (SH3) and ankyrin repeat-containing protein (Shank), neuronal nitric oxide synthase (nNOS), inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R), smooth endoplasmic reticulum (SER) and metabotropic glutamate receptor (mGluR).

**Scaffolding proteins**

The membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins comprises PSD95 (Cho et al., 1992; Kistner et al., 1993), synapse-associated protein (SAP) SAP97/human discs large (hDlg) (Lue et al., 1994; Muller et al., 1995), PSD93/Chapsyn110 (Brenman et al., 1996a; Kim et al., 1996) and SAP102 (Muller et al., 1996) (figure 7). All MAGUK proteins are abundant in the PSD, except SAP97, which is mainly found at presynaptic sites and in axons. The MAGUK members contain the protein-protein interaction domains PSD-95/Dlg/zona occludens-1 (ZO-1) (PDZ), Src homology 3 (SH3) and guanylate kinase (GK) through which they connect proteins with each other (reviewed in Sheng and Pak, 2000; Kim and Sheng, 2004).

The NR2 subunits bind to PDZ domains through a conserved amino acid motif in their outermost C-terminal tails, -ESXV (figure 6). A similar PDZ binding motif, -STVV, is present in NR1 splice variants containing the C2’ cassette (Kornau et al., 1995). PSD95
was the first MAGUK member shown to bind to the NMDA receptor and is also the most studied (Kornau et al., 1995; Niethammer et al., 1996). The interactions between NMDA receptors and MAGUK proteins have been implicated in several functions, for instance assembly of signaling complexes, localization and clustering of NMDA receptors at postsynaptic sites and anchoring of NMDA receptors to the cytoskeleton (reviewed in Sheng and Pak, 2000). However PSD95, which is the most abundant MAGUK protein at the PSD, outnumbers NMDA receptors by approximately 20-fold and binds to several proteins in the PSD in addition to the NMDA receptor subunits (Cheng et al., 2006).

**Figure 7.** Schematic picture of a membrane-associated guanylate kinase (MAGUK) protein with its protein-protein interaction domains indicated. Abbreviations; PSD-95/Dlg/ZO-1 (PDZ), Src homology 3 (SH3) and guanylate kinase (GK). The picture is not drawn to scale.

Another family of scaffolding proteins, comprising proline-rich synapse-associated protein/SH3 and ankyrin repeat-containing protein 1-3 (ProSAP)/(Shank1-3), contains the protein-protein interaction domains PDZ, SH3, an ankyrin repeat and a sterile alpha motif (SAM). The Shank family is involved in morphological and functional maturation of dendritic spines (Naisbitt et al., 1999; Boeckers et al., 1999; Sala et al., 2001). The Shank proteins bind both to the PSD95 interacting protein guanylate kinase-associated protein (GKAP) and to the metabotropic glutamate receptor (mGluR) binding protein Homer, thus providing a link between ionotropic and metabotropic glutamate receptors at the PSD (Xiao et al., 1998; Tu et al., 1999). Moreover, Homer binds to the inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R) situated in the membrane of the SER, linking the receptor complex to the intracellular Ca$^{2+}$ stores the SER provides (Tu et al., 1998).

**Regulatory proteins**

A large number of kinases and phosphatases reside in the PSD. One of the NMDA receptor-binding kinases found to be present in very high amounts in the PSD is Ca$^{2+}$ calmodulin-dependent protein kinase II (CaMKII) (Kennedy et al., 1983; Cheng et al., 2006). When Ca$^{2+}$ ions flow into the postsynaptic cell through activated NMDA receptors, they bind to calmodulin and thereby regulate CaMKII activity. This regulation has been shown to be involved in certain types of NMDA receptor mediated plasticity (reviewed in Malenka and Bear, 2004; Colbran and Brown, 2004). Based on the abundance of CaMKII at the PSD, CaMKII has been suggested to, besides its enzymatic role, play a structural role at the synapse. CaMKII is linked to the actin cytoskeleton through an interaction with α-actinin (Walikonis et al., 2001) and also interacts with the actin-associated motor protein Myosin Va (Costa et al., 1999).

NMDA receptor signaling also activates the mitogen-activated kinase (MAPK) pathway (Bading and Greenberg, 1991), which just like CaMKII signaling, is involved
in synaptic plasticity (reviewed in Adams and Sweatt, 2002; Miyamoto, 2006). Other kinases and phosphatases present in the PSD include casein kinase 2 (CK2), cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), protein kinase C (PKC), protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP1 and PKA are linked to NR1 through their interactions with the A kinase anchoring protein (AKAP) Yotiao (Lin et al., 1998; Westphal et al., 1999). The physical link provided by Yotiao brings PP1 and PKA in close proximity to the NMDA receptor and both enzymes regulate NMDA receptor activity (Westphal et al., 1999).

The enzyme neuronal nitric oxide synthase (nNOS) is coupled to the NMDA receptor through its interaction with PSD95 and PSD93 (figure 6) (Brenman et al., 1996b; Sattler et al., 1999). When the NMDA receptor is to highly activated, for instance during stroke-induced ischemia, excessive Ca\(^{2+}\) influx leads to excitotoxicity and neuronal death (reviewed in Dirnagl et al, 1999; Hardingham and Bading, 2003). The excitotoxic cell damage can be mediated by signaling molecule nitric oxide (NO), which is synthesized by nNOS following NMDA receptor activation (Dawson et al., 1991). Blocking the interaction between the NMDA receptor and PSD95 prevents the nNOS-mediated excitotoxic cell death both in neurons and in vivo models of focal ischemia (Aarts et al., 2002). Disrupting the interaction between the NMDA receptor and PSD95, with a peptide, has been suggested as a possible strategy for treating stroke (Aarts et al., 2002; Cui et al., 2007).

**Cytoskeletal proteins**

Dendritic spines are actin-rich structures, which contain intermediate filaments but little or no microtubules (reviewed in Sekino et al., 2007). A number of actin-associated proteins, including α-actinin, neurabin, drebrin, spectrin and cortactin (Gray et al., 2005), have been identified in the PSD and so has tubulin, microtubule-associated protein (MAP) MAP1A, MAP1B and MAP2. In addition, the actin-based motor protein myosin and the microtubule-associated motor protein dynein, are present in the PSD. While the kinesin superfamily (KIF) of microtubule-based motor proteins have not been detected (reviewed in Sheng and Hoogenraad, 2007).

The dynamic actin network is connected to several proteins in the PSD and is involved in the regulation of spine morphology (reviewed in Sekino et al., 2007). α-actinin 2 binds both to NR1 and to NR2B, and competes for the same binding site as calmodulin on NR1 (Ehlers et al., 1996; Wyszynski et al., 1997). Calmodulin and α-actinin 2 are involved in the regulation of the interaction between NR1 and the actin cytoskeleton (Zhang et al., 1998). An additional link between NMDA receptors and the cytoskeleton is provided by the interaction between NR1 and neurofilaments. This interaction is splice variant-specific and requires the C1 cassette in the NR1 C-terminus (Ehlers et al., 1998). Moreover, NMDA receptors are linked to the microtubule cytoskeleton through the interactions between MAP1A and PSD93 (Brenman et al., 1998) and PSD95 (Reese et al., 2007). And MAP1A is involved in activity-dependent remodeling of dendritic spines (Szabonyi et al., 2005).
Proteins interacting with NR3A

A number of proteins have been shown to interact with NR3A, and these are summarized in Table 2.

<table>
<thead>
<tr>
<th>Interaction-partner</th>
<th>Protein-type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein phosphatase 2A</td>
<td>Enzyme</td>
<td>Chan and Sucher, 2001</td>
</tr>
<tr>
<td>PACSIN1/Syndapin1</td>
<td>Accessory *</td>
<td>Pérez-Otano et al., 2006</td>
</tr>
<tr>
<td>Plectin</td>
<td>Cytoskeletal</td>
<td>Eriksson et al., 2007</td>
</tr>
<tr>
<td>CARP-1</td>
<td>Signaling</td>
<td>Eriksson et al., 2007</td>
</tr>
<tr>
<td>GPS2</td>
<td>Signaling</td>
<td>Eriksson et al., 2007</td>
</tr>
</tbody>
</table>

*PACSIN1 has also been called a scaffolding and adaptor protein (reviewed in Kessels et al., 2004). Abbreviations: cell cycle and apoptosis regulatory protein-1 (CARP-1) and G-protein pathway suppressor 2 (GPS2).

PP2A

The first NR3A-interacting protein to be identified was the catalytic subunit of PP2A (Chan and Sucher, 2001). The interaction was found through a yeast two-hybrid screen of a human fetal brain cDNA library, using the intracellular C-terminus of NR3A as bait. By truncating the bait, the membrane proximal 37 amino acids of NR3A were found to be required for the interaction with PP2A. Further analysis of these 37 amino acids by alanine-mutagenesis, revealed one histidine and two leucine residues to be highly involved in the interaction between NR3A and PP2A (Ma and Sucher, 2004).

Chan and Sucher found the interaction between NR3A and PP2A to be activity-dependent. Activation by NMDA of NR1/NR2B/NR3A receptors expressed in HEK293 cells resulted in the loss of binding between NR3A and PP2A. Further, the binding of PP2A to NR3A regulated the phosphorylation of Ser897 in the NR1 C-terminus, and breaking the interaction between PP2A and NR3A resulted in a phosphorylated NR1 subunit (Chan and Sucher, 2001).

PACSIN1/Syndapin1

Another NR3A-interacting protein recently found through a yeast two-hybrid screen is PACSIN1 (Pérez-Otano et al., 2006). PACSIN1 is a neuron-specific adaptor protein involved in membrane fission during endocytosis (Kessels and Qualmann, 2002; reviewed in Kessels and Qualmann, 2004). It is localized to synapses, interacts with dynamin and is highly expressed during neuronal development (Plomann et al., 1998; Qualmann et al., 1999).

NR3A was found to be part of a protein complex containing PACSIN1, dynamin and clathrin in rat brain, and PACSIN1 regulated the surface expression of NR3A in neurons in an activity-dependent manner (Pérez-Otano et al., 2006). Blocking synaptic transmission or NMDA receptor signaling, inhibited the PACSIN1-mediated removal of NR3A from the cell surface. The interaction of PACSIN1 is specific for NR3A, as PACSIN1 did not bind to NR1, NR2A or NR2B. Accordingly, PACSIN1 can
specifically remove NR3A-containing receptors from the plasma membrane and this mechanism might be involved in the maturation of synapses during development (Pérez-Otano et al., 2006).

*Plectin, CARP-1 and GPS2*

Plectin, cell cycle and apoptosis regulatory protein-1 (CARP-1) and G-protein pathway suppressor 2 (GPS2) were also identified as NR3A-binding proteins through a yeast two-hybrid screen (Eriksson et al., 2007). The interactions with NR3A have been verified *in vitro* but not *in vivo*. Plectin is a large scaffolding protein, containing several conserved protein domains and is expressed as different isoforms (Wiche et al., 1982; reviewed in Allen and Shah, 1999). CARP-1 is involved in apoptosis through its interaction with protein 14-3-3 and alters the expression of cell cycle regulatory genes (Rishi et al., 2003). GPS2 interferes with G-protein signaling and has been shown to be involved in cell survival and to modulate p53 activity (Spain et al., 1996; Peng et al., 2001)
SUBUNIT ASSEMBLY AND INTRACELLULAR TRANSPORT

As mentioned above, motor proteins can move either along actin filaments or microtubules in order to transport cargo to the correct subcellular compartment. The myosin family of motor proteins move along actin filaments and is most likely important in compartments devoid of microtubules, such as the neck and head of dendritic spines. The dynein and kinesin superfamilies (KIF) of motor proteins are both microtubule-based and transport cargo through axons and dendrites. Axonal transport comprises transport of membrane-bound vesicles, containing for instance components of synaptic vesicles, ion channels and adhesion molecules from, the cell body to synaptic terminals. Molecules transported through dendrites include components of the PSD, neurotransmitter receptors and mRNA (reviewed in Hirokawa and Takemura, 2005; Caviston and Holzbaur, 2006).

Assembly of the NMDA receptor subunits

The NMDA receptor subunits assemble in the ER and unassembled subunits do not exit from the ER. For instance, both NR1 and NR2 are required to be expressed simultaneously in order to be transported to the plasma membrane, and NR1 is required for NR3 to exit the ER (McIlhinney et al., 1996; Pérez-Otano 2001). The retention of unassembled subunits in the ER is believed to be mediated by an ER retention motif, RXR (Zerangue et al., 1999). This motif is found in the NR1 C1 cassette, in NR3A, NR3B and in NR2B. Subunit assembly most likely masks the ER retention signal and thereby allows the assembled subunits to exit the ER (McIlhinney et al., 1996; reviewed in Pérez-Otano and Ehlers 2003; Wenthold et al., 2003). However, NR1 splice variants expressing the C2’cassette, which contains the PDZ-binding motif -STVV, overcome the ER retention mediated by the C1 cassette and exit the ER (Standley et al., 2000; reviewed in Prybylowski and Wenthold, 2004).

A recent publication showed that NR1 and NR2, and NR1 and NR3A form heterodimers that can assemble two and two into tetramers (Schuler et al., 2008). The authors suggest the formation of heterodimers to be the initial step in NMDA receptor assembly. In addition they find that NR2 and NR3A do not form heterodimers, implying that receptors containing NR3A also contain one NR2 and two NR1 subunits. This study was performed in *Xenopus* oocytes, thus it is not yet clear if endogenous NMDA receptor subunits follow the same pattern of assembly and display the same stoichiometry.

The exocyst complex

The exocyst, or Sec6/8, complex is an assembly of eight proteins, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, and was originally identified in yeast (Bowser et al., 1992; Hsu et al., 1996). The exocyst is associated with ER and Golgi and directs intracellular membranes to sites where they fuse with the plasma membrane (Hsu et al., 1999; Yeaman et al., 2001). A novel function of the exocyst was reported by Sans and co-workers, who found the complex to be involved in NMDA receptor trafficking (Sans et al., 2003). Through a yeast two-hybrid screen they found SAP102 to bind to
the exocyst component Sec8. Co-immunoprecipitation showed that SAP102 and Sec8 binds to NR1 and NR2B in rat brain, and disrupting the interaction between SAP102 and Sec8 blocked the delivery of NMDA receptors to the cell surface. However, deletion of the PDZ-binding motifs in both NR2B and Sec8 allowed delivery of NMDA receptors to the cell surface. This suggests that there are alternative routes for NMDA receptor delivery to the cell surface, and perhaps different mechanisms for delivery of synaptic and extrasynaptic receptors. In addition, Sans and co-workers determined that the complex including SAP102, Sec8 and the NMDA receptor is formed in the ER.

Transport along microtubules

NMDA receptors are transported along microtubules in transport packages that are rapidly recruited to sites of axodendritic contact (Washbourne et al., 2002). The NMDA receptor clusters were more mobile than clusters containing GluR1 and rarely colocalized with PSD95. This suggests that PSD95 is not transported together with the NMDA receptor, but associates with the receptor at a later stage (Washbourne et al., 2002).

NR2B-containing receptors are transported along microtubules in membrane-bound vesicles, by the dendrite-specific kinesin motor protein KIF17 (Setou et al., 2000). The authors found NR2B to be linked to KIF17 through a protein complex consisting of mLin-7, mLin-2 and mLin-10, also known as Mint1/X11. Moreover, KIF17 colocalized with mLin-10, NR2B and NR1 in dendrites, indicating that NR2B is associated with NR1 during transport by KIF17. A later study from the same group reported transgenic mice over-expressing KIF17 to exhibit enhanced spatial and working memory (Wong et al., 2002).

The KIF17 vesicles move along dendrites and are associated with extrasynaptic NR2B (Guillaud et al., 2003). However, Guillaud and co-workers determined that KIF17 does not move into the synapse, a finding consistent with the fact that KIF17 has not been detected in the PSD (Cheng et al., 2006). Knock-down of KIF17 reduced the amount of NR2B, and increased the amount of NR2A at synapses (Guillaud et al., 2003). This implies that NR2A- and NR2B-containing receptors are delivered to synapses by separate mechanisms, and that the loss of NR2B-containing receptors at synapses is compensated by an increased amount of NR2A-containing NMDA receptors. NR2A replaces NR2B during neuronal development, resulting in mature NMDA receptors with different pharmacological properties from those expressed postnatally (reviewed in Cull-Candy et al., 2001). Regulation of KIF17 activity might be one way to control the NMDA receptor composition at synapse. As expected, depolymerization of microtubules blocks the movement of KIF17 and impairs the delivery of NR2B to the cell surface (Guillaud et al., 2003). Moreover, an intact microtubule cytoskeleton is required for proper NMDA receptor-mediated currents in cortical neurons (Yuen et al., 2005a).

Transport along microtubules is one way for assembled NMDA receptors to reach the correct subcellular compartment. Another possibility is that the receptors are inserted to the plasma membrane soon after the exit from ER and Golgi, and diffuse in the plasma
membrane to the correct location. A study by Washbourne and co-workers show that both ways of trafficking occur in the neuron (Washbourne et al., 2004). Prior to synapse formation, receptor transport packets move along microtubules and are actively exocytosed and endocytosed through the dendritic plasma membrane. The transport packets colocalized with early endosomal antigen 1 and SAP102 (Washbourne et al., 2004) and SAP102 might provide a link to the exocyst complex.

**Receptor trafficking at synapses**

There is a continuous exchange of receptors between synaptic and extrasynaptic sites, as well as between the plasma membrane and intracellular stores. Receptors move by lateral diffusion in the plasma membrane and extrasynaptic receptors are more mobile than synaptic receptors. Insertion and removal of receptors is believed to occur mainly at endocytic zones (EZ) located in the extrasynaptic region (figure 5) (reviewed in Choquet and Triller, 2003; Kennedy and Ehlers, 2006). The endocytotic proteins AP-2, clathrin and dynamin are localized to EZ lateral to the PSD (Petralia et al., 2003; Racz et al., 2004).

The composition of NMDA receptors expressed at extrasynaptic and synaptic sites differ. The subunits NR2B, NR2D and NR3A, which have their expression peaks during development, are preferentially expressed at extrasynaptic sites. While NR2A and NR2C subunits predominate at synaptic sites (reviewed in Pérez-Otano and Ehlers, 2004). This preferential targeting requires subunit-specific regulation and can for instance be mediated by proteins as PACSIN1, which specifically removes NR3A-containing receptors from the plasma membrane (Pérez-Otano et al., 2006).
MICROTUBULE-ASSOCIATED PROTEINS

There are a number of MAPs expressed in neurons (Sloboda et al., 1975) and some of the most studied are MAP1A, MAP1B, MAP2, tau, doublecortin and LIS1. The MAPs bind to microtubules, often in a phosphorylation dependent manner, and regulate their stability. This thesis focuses on the MAP1 family, comprising MAP1A, MAP1B and MAP1S. An ortholog to the mammalian MAP1 proteins, named Futsch, has been identified in *Drosophila* (Hummel et al., 2000).

The MAP1 family

MAP1A, MAP1B and MAP1S are translated as polyproteins, which undergo posttranslational cleavage into a heavy chain (HC) and a light chain (LC) (figure 8). In addition to the heavy chains, the MAP1A polyprotein comprises LC2 and the MAP1B polyprotein comprises LC1. There is also a smaller light chain, LC3, translated independently. The MAP1B-HC can associate both with LC1 and LC3 and the MAP1A-HC can bind all three light chains, LC1, LC2 and LC3 (reviewed in Halpain and Dehmlet, 2006). The HC:LC ratio has been shown to vary between 1:2 and 1:8, depending on cell type and sub-cellular fraction, with a higher proportion of LC in whole brain homogenates than in microtubule preparations (Mei et al., 2000a; Mei et al., 200b).

Both MAP1A and MAP1B are large proteins comprising approximately 3000 and 2500 amino acids respectively, with small variations between species. MAP1S is considerably smaller, the human protein comprises only 1059 amino acids, mainly due to a shorter exon 5 (Orbán-Németh et al., 2005). In addition to its smaller size, the high expression of MAP1S in organs outside the nervous system distinguishes MAP1S from MAP1A and MAP1B. The homology between the three proteins is high predominately in three regions, termed MAP1 homology domains 1-3 (MH1-3) (Orbán-Németh et al., 2005). MAP1A contains three microtubule-binding regions, MAP1B contains two, and only one has been identified in MAP1S. All three proteins contain one actin binding-region situated in their light chains. In addition, one actin-binding region has recently been identified in the MAP1B-HC (Cueille et al., 2007) (figure 8).

![Figure 8. Schematic picture showing the three members of the MAP1 family, MAP1A, MAP1B and MAP1S. The MAP1 family members undergo post-translational proteolytic cleavage into a heavy and a light chain. The shaded boxes MH1, 2 and 3, indicate the MAP1 homology domains, which are regions with highly conserved sequence. The picture is not drawn to scale. Abbreviations; microtubule-binding region (MT) and actin-binding region (A).](image-url)
MAP1S/C19ORF5

Identification
MAP1S is the most recently identified member of the MAP1 family, and its function in the nervous system is still unclear. MAP1S was first identified as an interaction-partner of a fibroblast growth factor-associated protein called leucine-rich PPR-motif containing protein, AAA67549 (LRPPRC), through a yeast two-hybrid screen of a liver cDNA library (Liu and McKeehan, 2002a). The protein was named chromosome 19 open reading frame 5 (C19ORF5), and on basis of its high homology to MAP1A and MAP1B, suggested to be a member of the MAP1 family. The same cDNA was later isolated from testis cDNA libraries, through yeast two-hybrid screening, and given the names variable charge Y chromosome 2 interacting protein-1 (VCY2IP-1) (Wong et al., 2004), and RAS-associated domain family protein 1A-binding protein 1 (RAB1) (Song et al., 2005). Variable charge Y chromosome 2 is a testis-specific protein of unknown function (Stuppia et al., 2001) and RAS-associated domain family protein 1A is a tumor suppressor, implemented in a number of different cancer forms. The names VCY2IP-1 and RAB1, both refer to the proteins interaction-partners, while the name C19ORF5 refers to the chromosomal location of the gene. Despite C19ORF5 being the original name, we have chosen to refer to the protein by its fourth, and most recent name MAP1S (Orbán-Németh et al., 2005). This name indicates the microtubule-binding property of the protein, as well as its homology to MAP1A and MAP1B.

Function
MAP1S was identified in 2002 and there are a limited number of publications concerning the protein, and of these, only the study by Orbán-Németh and co-workers involves nervous tissue (Orbán-Németh et al., 2005). In adult mouse tissue MAP1S is expressed in kidney, lung, spleen, liver and heart, but the highest levels are found in brain and testis. The protein is expressed already at birth, and the levels increase slightly during the first postnatal weeks (Orbán-Németh et al., 2005). Immunoprecipitation from brain tissue shows that the MAP1S heavy and light chains bind to each other in vivo. Deletion of the microtubule-binding domain in the light chain causes MAP1S to lose its microtubule-binding property, indicating that the protein only contains one microtubule-binding region (Orbán-Németh et al., 2005).

A yeast two-hybrid screen of a liver cDNA library, with MAP1S as bait, revealed that MAP1S binds to mitochondria-associated NADH dehydrogenas I, cytochrome c oxidase I and RASSF1 (Liu et al., 2002b). When over-expressed in the cell line HepG2, which is derived from liver, MAP1S causes aggregation of mitochondria and genome destruction (Liu et al., 2005b). The interaction between the microtubule-binding tumor suppressor RASSF1 and MAP1S has been reported by several groups (Liu et al., 2002b; Dallol et al., 2004; Liu et al., 2005a; Song et al., 2005). These studies suggest MAP1S to be involved in apoptosis and cell cycle mechanisms through its interaction with the microtubular cytoskeleton. In addition, there is one study reporting that MAP1S binds to double stranded DNA, but the function of this interaction is not known (Liu et al., 2005c).
MAP1B

MAP1B was identified as a microtubule-binding protein in the 1970s (Sloboda et al., 1975) and is involved in microtubular stabilization, axon generation and growth cone motility (reviewed in González-Billault et al., 2004; Riederer, 2007). MAP1B was cloned in rat in 1989 and its expression was found to be higher during development than in the adult rat brain. During postnatal development MAP1B is replaced by MAP1A, which is highly expressed in the adult nervous system (Riederer and Matus 1985; Safaei and Fischer, 1989). MAP1B expression is maintained in areas undergoing neurogenesis in adult animals such as the olfactory bulb. Moreover, MAP1B expression is induced following injury to the peripheral nervous system (PNS), suggesting a role for MAP1B both in development and regeneration (reviewed in Emery et al., 2003).

Full-length MAP1B is encoded by seven exons but the gene includes two additional exons, 3A and 3U (Lien et al., 1994; Kutschera et al., 1998). If translated, the 3A and 3U gives rise to a MAP1B protein with a truncated N-terminus. Transcripts including either 3A or 3U have been detected both in mouse and rat tissue, and estimated to give rise to 1-10% of total MAP1B transcripts (Lien et al., 1994; Kutschera et al., 1998).

MAP1B knock out mice
In contrast to MAP1S, the function of MAP1B in the nervous system has been extensively investigated, with no less than four different KO mice generated (Edelmann et al., 1996; Takei et al., 1997; González-Billault et al., 2000; Meixner et al., 2000). However, interpreting the function of MAP1B from these studies has not been straightforward since the phenotypes of the KO mice differ significantly. The first KO mouse generated by Edelmann and co-workers displayed a severe phenotype, and died in utero prior to embryonic day 8.5. Therefore, the study describes the heterozygous mice, which have reduced body weight, visual and motor impairments. Morphological examination revealed malformations of cerebellum but unaffected hippocampus, and immunohistochemical analysis showed the Purkinje cells to possess an altered morphology and retinal layers to be disorganized. (Edelmann et al., 1996).

The second KO mouse was generated by Takei and co-workers and displayed a much milder phenotype (Takei et al., 1997). The heterozygous mice did not exhibit either developmental or behavioral abnormalities and could not be distinguished from WT mice. The homozygous mice had slightly decreased body and brain weight and delayed nervous system development. No abnormalities in hippocampus, retina, olfactory bulb or spinal cord were observed by IHC, either at postnatal day 8 or in adult mice. However, myelination of the optic nerve seemed to be delayed, with reduced myelination at postnatal day 8, but normal myelination in the adult. Moreover, they investigated the number and density of microtubules in axons of KO mice but did not find any alterations compared to WT mice.

In the year 2000, studies on two additional MAP1B deficient mice were published (González-Billault et al., 2000; Meixner et al., 2000). Both these strains displayed intermediate, but slightly different phenotypes, compared to the previously generated KO mice. The heterozygous mice generated by Meixner and co-workers were indistinguishable from WT mice, while homozygous mice displayed decreased body
weight as well as a number of developmental abnormalities. The abnormalities included a total lack of corpus callosum in 80% of the homozygous animals, and severely malformed corpus callosum in the remaining. Moreover, the animals demonstrated a reduced thickness of myelin sheaths around axons in the PNS, which resulted in attenuated conduction velocity. To address the visual impairments and disorganized retinal layers reported in heterozygous mice by Edelmann and co-workers, Meixner and co-workers investigated the retina by IHC, but did not detect any abnormalities. Moreover, they did not find any alterations of GABA\textsubscript{C} receptor clustering, which might have been expected since MAP1B has been reported to be involved in GABA\textsubscript{C} receptor clustering in the retina (Hanley et al., 1999). In addition, they claim that the clustering of a number of glutamate receptor subunits, including NR1 and NR2B, as well as PSD95 is unchanged in the homozygous mice.

The phenotype of the fourth MAP1B deficient mouse generated, most closely resembles the mouse generated by Meixner and co-workers. The homozygous mice die at postnatal day 1, exhibit an abnormal limb posture and lack of response to physical stimuli applied to the limbs (González-Billault et al., 2000). Brains from homozygous mice were analyzed by IHC at embryonic day 19. The analysis revealed an increased ventricular volume of 60-70\%, an interrupted corpus callosum and abnormal development of cerebellum. The hippocampus and olfactory bulb displayed abnormal lamination and disorganized cells. Moreover, the thickness of cerebral cortex was reduced and its lamination absent. In contrast, heterozygous mice did not have any alterations of either cerebral cortex or corpus callosum. By the gene-trapping approach used in this study, β-galactosidase was produced under the control of the \textit{Map1b} promoter, allowing the normal distribution of MAP1B expression to be detected. X-gal staining of heterozygous 13 day-old embryos, revealed MAP1B expression to be restricted to the nervous system, with high expression throughout both the CNS and PNS.

Despite the varying phenotypes the different KO mice display, these studies give valuable insight into the functions of MAP1B and its involvement in CNS and PNS development. The reason for the discrepancies between mice has been discussed and one contributing factor could be that the mice have different genetic background. Mice with the more severe phenotype are derived from strain 129 (Edelmann et al., 1996; González-Billault et al., 2000) and mice with milder phenotype are derived from strain C57 (Takei et al., 1997; Meixner et al., 200). However, even though genetic background might contribute, it is unlikely that the large variations between the mice generated by Edelmann and Takei are generated solely by that parameter. The KO mice generated by Edelmann and co-workers express a N-terminal fragment of MAP1B, which has been suggested to work in a dominant-negative manner and explain the severe abnormalities observed even in heterozygous mice (Meixner et al., 2000; Tögel et al., 1998). The KO mice generated by Takei and co-workers, on the other hand, express residual levels of a slightly truncated MAP1B protein (Takei et al., 1997). This might compensate for the loss of full-length MAP1B and explain the very mild effect they observe.

In study IV, we used brains and hippocampal neurons from the MAP1B deficient mice generated by González-Billault and co-workers (González-Billault et al., 2000).
Hippocampal neurons from these mice have been reported to display delayed axonal outgrowth and abnormal growth cones, when examined after 2 days in vitro (DIV) (González-Billault et al., 2001). Moreover the axons from cells lacking MAP1B had increased MAP2 immunoreactivity in their axons, compared to cells derived from WT mice, suggesting a certain redundancy between MAP1B and MAP2. Tau immunoreactivity, on the other hand, was similar in neurons lacking MAP1B and WT (González-Billault et al., 2001). The neurons used in study IV where analyzed after 21 DIV and at this time point the neurites morphological difference are much less pronounced. Instead there is a difference in the morphology of filopodia, with longer and thinner filopodia in neurons from MAP1B deficient animals than in WT neurons (E. Tortosa, unpublished observation).

**MAP1B phosphorylation mode I and mode II**

MAP1B is a large protein under the regulation of numerous kinases and phosphatases, with as many as 33 sites found to be phosphorylated in vivo (Collins et al., 2005). Two different modes of MAP1B phosphorylation have been described, mode I and mode II (Ulloa et al., 1993, Ulloa et al., 1994; Garcia-Perez et al., 1998). Mode I is directed by proline-dependent kinases and is present in growing axons during early development. Mode II is mediated by casein kinase II and is present both in axons and dendrites and persist throughout postnatal development.

**MAP1B in disease**

MAP1B has been implicated in a number of pathological conditions of the nervous system, including fragile X mental retardation syndrome, giant axonal neuropathy and cancer. There is also one study describing MAP1B protein levels in post mortem brains from patients with schizophrenia, bipolar disorder and major depression. The authors found decreased MAP1B levels in anterior cingulate cortex of brains from patients with bipolar disorder, but unaltered MAP1B levels in brains from patients with schizophrenia and major depression (Bouras et al., 2001). Even though this is just one single study, parallels can be drawn to the report showing NR3A mRNA levels to be elevated in dorsolateral prefrontal cortex, of brains from patients with schizophrenia (Mueller and Meador-Woodruff, 2004). However, to determine the significance and possible relationship between these altered MAP1B and NR3A levels additional studies need to be performed.

Of the pathological conditions mentioned above, the involvement of MAP1B is probably most studied in the fragile X mental retardation syndrome. The syndrome, which is inherited, involves cognitive, behavioral and emotional dysfunction, in many patients resembling autistic symptoms (reviewed in Bernardet and Crusio, 2006; Koukoui and Chaudhuria, 2007). The syndrome is caused by lack of expression of the fragile X mental retardation protein (FMRP). FMRP is localized to axons, dendrites growth cones, and dendritic spines (Antar et al., 2006). FMRP binds to different mRNAs and regulate their translation, and lack of FMRP results in misregulated MAP1B translation and increased microtubule stability (Lu et al., 2004). Moreover, FMRP KO mice exhibit an increased number of filopodia and a reduced number of mature dendritic spines (Antar et al., 2006).
Giant axonal neuropathy (GAN) is a disorder causing axonal degeneration and aggregation of cytoskeletal components in sensory and motor neurons. The GAN gene encodes gigaxonin, which is a ubiquitously expressed protein that binds to the MAP1B-LC1 (Ding et al., 2002), and over-expression of gigaxonin causes degradation of LC1 (Allen et al., 2005).

One of the groups identifying MAP1S as a binding partner of the tumor suppressor RASSF1A, also found MAP1B to bind to RASSF1A through the same yeast two-hybrid screen (Dallol et al., 2004). The authors showed that RASSF1A is involved in regulating the stability of microtubules, and thereby cell cycle dynamics. However, the possible connection between MAP1B and cancer is still far from clear.
AIMS OF THE THESIS

GENERAL AIM

The aim of this thesis was to determine the function of the human NMDA receptor subunit NR3A, since NMDA receptor signaling is involved in normal brain development as well as number of pathological conditions. Further knowledge about the receptor and its subunits gives insight into developmental processes, and may also open for new treatment-strategies in disorders where NMDA receptor signaling is affected.

SPECIFIC AIMS

- To clone and sequence the human NR3A subunit.
- To investigate the occurrence of NR3A splice variants in human and rat CNS tissue.
- To determine the expression and distribution of NR3A mRNA and protein in embryonic and adult human CNS tissue.
- To determine the solubility of NR3A compared to the other NMDA receptor subunits.
- To establish whether NR3A is associated with the other NMDA receptor subunits in adult human brain and spinal cord.
- To identify and analyze novel NR3A-binding proteins.
RESULTS AND DISCUSSION

STUDY I, CLONING

When this project was started NR3A had only been cloned from and described in rat. Therefore we set out to clone the human NR3A, and this is described in study I. We found that human NR3A comprises 1115 amino acids and has a calculated molecular weight of 122 kD after removal of the first 33 amino acids, which are predicted to be a signal peptide for intracellular targeting. The amino acid sequence of the human subunit was 93% homologous to that of the rat, and most of the potential glycosylation and phosphorylation sites are conserved. So far NR3A has not been shown to be either glycosylated or phosphorylated, therefore it is not clear if the variations in such sites between species are of any significance. Sites that are essential for protein function and/or regulation are likely to be conserved between species.

The hydrophobicity profile of NR3A is similar to the other NMDA receptor subunits, predicting four membrane regions, an extracellular N-terminus and an intracellular C-terminus. The highest homology between the NMDA receptor subunits was observed in the membrane regions and in the extracellular loop between membrane regions three and four. This extracellular loop forms the ligand-binding pocket together with the N-terminus.

The long splice variant of NR3A (NR3A-l) described in rat (Sun et al., 1998), was not identified in either embryonic or adult human CNS tissue. Sun and co-workers report that mRNAs encoding NR3A and NR3A-l have slightly different distribution patterns in adult rat brain, with NR3A-l being the predominant form in the entorhinal cortex and thalamus. Both splice variants present similar developmental regulation, with the highest mRNA levels during the first two postnatal weeks. NR3A-l contains a 20 amino acid insert in the intracellular C-terminus and this can potentially affect both interactions with intracellular proteins and signaling at the PSD, as well as trafficking through the endocytic machinery. Both NR3A splice variants contain the ER retention motif RRR, which is also found in the C1 cassette of the NR1 C-terminus. The retention motif in NR1 has been shown to prevent unassembled NR1 subunits to exit the ER (Prybylowski and Wenthold, 2004). The retention can be overcome in NR1 splice variants that express the C2’ cassette, which contains the PDZ-binding motif – STVV. Assembly with PDZ-containing proteins facilitates the exit of NMDA receptor from the ER. NR3A does not contain a PDZ-binding motif corresponding to those found in the NR1 C2’ cassette and NR2 subunits, and no PDZ-containing proteins have been shown to bind directly to NR3A.

In situ hybridization performed on fetal human CNS tissue revealed that NR3A mRNA is expressed both in the ventricular zone and cortical plate of the developing cerebral cortex and in the fetal spinal cord, where the expression was especially prominent in the dorsal half and around the central canal. Interestingly, in the fetal spinal cord, NR3A mRNA levels were relatively low in regions with high NR1 expression. This suggests that NR3A might replace one of the NR1 subunits in immature NMDA
receptor complexes during brain development, resulting in receptors less permeable to Ca\textsuperscript{2+} and less sensitive to Mg\textsuperscript{2+} block.
STUDY II, MAPPING

Study II describes the expression of NR3A mRNA and protein in adult human CNS tissue. In addition to studying the spatial distribution of NR3A, we investigated the association of NR3A with the other NMDA receptor subunits, and examined the solubility of the subunits. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot showed that NR3A mRNA and protein is expressed in cortices from all four cerebral lobes as well as in thalamus and pons of the adult human brain. In the caudate nucleus, claustrum and cerebellum the levels of NR3A mRNA and protein were low or undetectable. The spatial distribution of NR3A was confirmed by in situ hybridization performed on adult human CNS tissue, which together with IHC on macaque brain revealed that NR3A was mainly present in cortical layers II/III and V. No NR3A-containing cells could be detected in layer I or in subcortical white matter, and few NR3A-containing cells were seen in the caudate nucleus. We saw very low levels of NR3A mRNA and protein in the adult human spinal cord, and in situ hybridization demonstrated that NR3A mRNA was highly confined to motor neurons in the ventral horn. RT-PCR and western blot showed that both postnatal and adult rat spinal cord expressed significant levels of NR3A, and so did embryonic human spinal cord. Except for the different occurrence of NR3A in adult human and rodent spinal cord, the spatial distribution of NR3A in adult human CNS resembled what has previously been published for NR3A in rodent CNS tissue (Al-Hallaq et al., 2002; Wong et al., 2002).

Adult human brain expresses NR3A mRNA (Mueller and Meador-Woodruff, 2004; Bendel et al., 2005) but the presence of NR3A protein had not been reported prior to study II. Recently, a study describing the developmental expression of NR3A mRNA and protein in human prefrontal cortex was published (Henson et al., 2008). The authors found the levels of both NR3A mRNA and protein to peak during the first year of life, decline gradually during the following years and at 25 years of age the levels were comparatively low, but still higher than during gestation. This implies that the temporal expression of NR3A in humans is similar to what has previously been suggested for rodents, with exception for the spinal cord. However, our study revealed that both adult human and rodent brain express significant levels of NR3A protein, the levels corresponding to 20-30% of the levels seen during early postnatal life in both rodents and humans (Al-Hallaq et al., 2002; Wong et al., 2002; Henson et al., 2008). Accordingly, we conclude that in addition to the developmental processes NR3A has been implicated in, the subunit has a significant function in adult brain.

Furthermore, we investigated the association of NR3A with the other NMDA receptor subunits in adult human brain and spinal cord by co-immunoprecipitation. NR3A was associated with NR1, NR2A and NR2B in brain and to some extent in spinal cord. The weaker immunoreactive bands seen in western blots of spinal cord samples, most likely reflect the very low NR3A levels in the tissue. We did not detect any association between NR3A and GluR2, demonstrating that the interaction between NR3A and the other NMDA receptor subunits is specific. The incorporation of NR3A in NMDA receptor complexes in adult CNS further illustrates that NR3A has a functional role in the adult.
We also examined the solubility of NR3A compared to the other NMDA receptor subunits in both human and rat brain. By centrifugation, we prepared a P2-pellet containing plasma membranes and membrane-bound proteins, from which we extracted proteins with different detergents. NR3A showed a remarkably different profile from the other subunits, being readily extracted by the milder detergents Triton-X 100, nonidet P-40 (NP-40) and 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS). These milder detergents did not extract detectable levels of either NR1 or NR2A, which both required sodiumdeoxycholate (DOC) for effective solubilization. Despite the differences regarding milder detergents, the largest amounts of NR3A were extracted with DOC treatment. NR3A has been reported to be more easily extracted from plasma membranes than the other NMDA receptor subunits (Pérez-Otano et al., 2006). Their study was performed on rat brain, and is in accordance with what we see for both human and rat preparations. The unusually high solubility of NR3A indicates that NR3A-containing receptors are less firmly associated with the PSD, and are probably localized to extrasynaptic sites to a higher degree than NMDA receptor complexes lacking NR3A. This is interesting since synaptic and extrasynaptic NMDA receptors are involved in different intracellular protein-interactions and signaling-pathways. For instance, extrasynaptic NMDA receptors have been shown to mediate excitotoxic cell death to a higher degree than synaptic NMDA receptors (reviewed in Hardingham and Bading, 2003).

The solubility of the NMDA receptor subunits was further investigated by size exclusion chromatography of solubilized plasma membranes. This demonstrated that NR3A, NR1 and NR2A are all present in large protein complexes, containing both the subunits themselves and associated proteins. In addition, all three subunits are found in tetrameric NMDA receptor complexes, but in contrast to NR2A, both NR1 and NR3A are also found as dimers and monomers. The NR1 subunits have previously been shown to be localized both to intracellular membranes, were they reside as monomers, and to the cell surface where they are part of functional NMDA receptor assemblies (Huh and Wenthold, 1999). The intracellular pool of NR1 has been estimated to contain 60% of the total amount of NR1, and over-expression of NR1 did not have any significant effect on the number of NMDA receptors present at the cell surface (Prybylowski et al., 2002). In contrast, only about 10% of NR2A and NR2B subunits are localized to intracellular pools and over-expression of either of these resulted in an increase of NMDA receptor subunits at the cell surface, predominantly at extrasynaptic sites (Prybylowski et al., 2002). Our results suggest that NR3A, just like NR1, is present as monomers in intracellular pools. Thus, the amount of NR3A subunits is probably not limiting for the number of NR3A-containing NMDA receptors at the cell surface.
The cellular response of NMDA receptor activation is mediated by ion fluxes through the channel pore, as well as by protein-protein interactions. Identifying NR3A-binding proteins can give insight into which signaling pathways NR3A-containing receptors are involved in. Apart from mediating cellular responses, protein-protein interactions are essential for transport of NMDA receptors to appropriate subcellular locations, and the local trafficking of receptors between synaptic and extrasynaptic sites of dendritic spines. Both study III and study IV are the result of a yeast two hybrid-screen performed in order to find novel NR3A-binding proteins. We screened a fetal, human brain cDNA library, using the intracellular C-terminus of human NR3A as bait. Among a number of interesting candidates, we chose MAP1S/C19ORF5 and MAP1B for further investigation, and this is described in study III and IV respectively. We found MAP1B interesting since it had been shown to be involved in the regulation of other neurotransmitter receptors (Hanley et al., 1999; Yuen et al., 2005b), and just like NR3A, it is developmentally regulated with its highest expression levels during postnatal development (Riederer and Matus, 1985; Safaei and Fischer, 1989). Since very little is known about the function of MAP1S in the nervous system, we wanted to investigate this protein and its interaction with NR3A further. Based on the homology between MAP1S and MAP1B, it could be envisioned that they are involved in similar mechanisms regarding NR3A.

The 3’ part of MAP1S cDNA, encoding amino acids 705-1059/stop was isolated in the screen, and the interaction between MAP1S and NR3A was verified in vitro by GST pull-down experiments. We determined that the interaction between MAP1S and NR3A also takes place in the brain, by demonstrating co-immunoprecipitation from solubilized rat brain membranes. In addition, we showed that the membrane-proximal part of the NR3A C-terminus is required for the interaction with MAP1S.

There are a limited number of publications on MAP1S and only one of them concerns neuronal tissue (Orbán-Németh et al., 2005). This led us to investigate the occurrence of MAP1S in different organs and brain regions. We found MAP1S to be highly expressed in cerebral cortex and cerebellum of both adult and eight day-old rats. Adult rat also expressed MAP1S in testis, heart and skeletal muscle and postnatal rat expressed MAP1S in heart, muscle and liver. Interestingly, in brain and testis the posttranslationally cleaved heavy chain dominated over the full-length MAP1S, distinguishing them from the other organs. In addition, we saw that the levels of MAP1S are higher in adult than in embryonic human forebrain and higher in postnatal than adult rat forebrain. However, the heavy chain predominated over the full-length MAP1S in all forebrain samples, and the dominance was increased with age. MAP1B is almost exclusively expressed in its cleaved form, and the fact that we detect so much full-length MAP1S suggests that the posttranslational cleavage of MAP1B and MAP1S is regulated differently. The MAP1B heavy chain has been shown to associate with 2 to 8 light chains, which suggests that the MAP1B light chain has a longer half-life than the heavy chain since they are synthesized in a 1:1 ratio. (Mei et al., 2000a; Mei et al., 200b). The ratio between MAP1S heavy and light chain has not been investigated so far.
and it is not clear if the function of full-length, uncleaved protein is different from that of cleaved and re-assembled protein.

Next we compared the levels of MAP1S in human cortical cells cultured under conditions adapted to obtain cultures of mainly neurons, or cultures of mainly glial cells. We found the MAP1S levels to be higher in neurons than in glial cells. We also examined the occurrence of MAP1S in different CNS regions and in dorsal root ganglia of postnatal rat, and found MAP1S to be expressed in all regions examined. Previous publications on MAP1S have focused on its role in regulation of cell cycle events and cell death (Liu et al., 2005b; Liu et al., 2005a; Song et al., 2005). Therefore, it is of significant interest to find that the MAP1S levels are high in post-mitotic neurons, a fact that opens for novel functions of MAP1S, one being regulation of NR3A-containing NMDA receptors.

We studied the subcellular distribution of MAP1S in primary rat hippocampal and human cortical cells cultured for 14 DIV. Due to different fixation requirements, we were not able to successfully perform double-staining with the MAP1S antibody. Therefore, we chose to transfect the neurons at 10 DIV with EGFP-tagged MAP1S, and after fixation at 14 DIV we performed immunocytochemistry (ICC) on the cells. We found MAP1S-EGFP to be localized throughout dendritic shafts, and the distribution resembled that of β-tubulin III immunoreactivity. But, MAP1S-EGFP was also localized to filopodia-like protrusions which lacked β-tubulin III immunoreactivity. Since filopodia and spines contain little or no microtubules, MAP1S in these structures might be associated with actin, NR3A or other so far unidentified binding-partners. Moreover, we found a partial colocalization of MAP1S-EGFP and the presynaptic marker synapsin and postsynaptic marker PSD95, indicating that MAP1S can be localized to synaptic structures. MAP1S-EGFP and NR3A immunoreactivity colocalized in dendritic shafts and occasionally in filopodia-like processes. An interaction between MAP1S and NR3A in dendritic shafts might involve regulation of the transport of NR3A-containing NMDA receptors along microtubules. However, the interaction between the two proteins in filopodia or spines, more likely involves regulation of expression of NR3A-containing NMDA receptors at synaptic and extrasynaptic sites.
STUDY IV, INTERACTION WITH MAP1B

Study IV focuses on the interaction between NR3A and MAP1B. MAP1B was identified as a potential binding-partner of NR3A in the yeast two-hybrid screen described above. The interaction between NR3A and MAP1B was verified in vitro by GST pull-down experiments and in vivo by co-immunoprecipitations from solubilized rat brain membranes. Moreover, the microtubule-binding region of the MAP1B light chain was shown to be required for the interaction with NR3A.

Truncation analysis showed that the microtubule-binding region of the MAP1B light chain is required for the interaction with NR3A.

To examine the subcellular distribution of MAP1B and NR3A we performed ICC on human cortical cells cultured for 21 DIV. This analysis showed that MAP1B was highly colocalized with both β-tubulin III and actin in dendritic shafts and to some extent with actin in filopodia. Colocalization between MAP1B and the synaptic proteins synapsin and SAP102 was sparse. The infrequent occurrence of MAP1B immunoreactivity at synaptic sites of cultured neurons is in accordance with two recently published papers (Davidkova and Carroll, 2007; Kitamura et al., 2007). However, a much higher presence of MAP1B in synapses was suggested in an electron-microscopy study on adult rat brain, estimating MAP1B to be present in as many as 50% of cerebral cortex synapses (Kawakami et al., 2003). NR3A immunoreactivity was frequently seen throughout dendritic shafts where it colocalized with β-tubulin III immunoreactivity, but also in actin-containing filopodia and spines. The distribution of NR3A and MAP1B resembled each other, consequently colocalization between NR3A and MAP1B immunoreactivity was more frequently seen throughout dendritic shafts, than in filopodia-like processes.

Through truncation-analysis of MAP1B we determined the microtubule-binding region in MAP1B-LC1 to be required for the interaction with NR3A. Thus, it is possible that by binding to MAP1B-LC1, NR3A blocks the interaction between MAP1B-LC1 and microtubules. However, since the MAP1B-HC contains an additional microtubule-binding region, MAP1B might be able to bind to microtubules and NR3A simultaneously.

To learn more about the function of the interaction between NR3A and MAP1B, we investigated the protein levels of NR3A and NR1 in MAP1B deficient mice. Compared to WT mice, the levels of NR3A were increased and the levels of NR1 decreased in MAP1B deficient animals, with a 35% increase in the NR3A/NR1 ratio. We did not see any effect on GluR2 protein levels, indicating that the absence of MAP1B does not have a general effect on glutamate receptors but is specifically affecting the NMDA receptor subtype. The fact that NR3A and NR1 seem to be mutually regulated strengthens our hypothesis that NR3A can replace at least one of the NR1 subunits in NMDA receptor assemblies. The elevated NR3A levels in MAP1B deficient mice might result in an increased amount of NR3A-containing NMDA receptors at the cell surface, which would lead to an attenuation of NMDA receptor signaling in these animals.
Next we studied the subcellular distribution of NR3A in 21 DIV cultured hippocampal neurons from MAP1B deficient and WT mice. We visualized filopodia/spines by phalloidin staining of actin filaments and counted the number of NR3A-immunoreactive filopodia/spines. The fraction of filopodia/spines containing NR3A was equal in MAP1B deficient and WT animals, which implies that MAP1B is not required for transport of NR3A-containing NMDA receptors to filopodia/spines. Instead we postulate that MAP1B might be involved in regulating the expression of NR3A-containing receptors at the cell surface. The subcellular distribution of both GABAc and 5-HT1A and 5-HT3 is regulated by MAP1B (Hanley et al., 1999; Yuen et al., 2005b; Sun et al., 2008) and similar mechanisms might underlie the regulation of NR3A-containing NMDA receptors.

Finally, we compared the microtubular-binding properties of MAP1S and MAP1B. We found that both MAP1S and MAP1B bind strongly to paclitaxel-stabilized microtubules, showing that the microtubule-binding properties of MAP1S and MAP1B are shared. Study III and IV suggest that MAP1S and MAP1B have similar functions in neurons, one being involvement in the regulation of NR3A-containing NMDA receptors.
CONCLUSIONS AND FUTURE PERSPECTIVES

The homology between the rat and human NMDA receptor subunit NR3A is high, 93% at the amino acid level. The spatial and temporal distribution of NR3A is similar in mammals, with the exception of the spinal cord and cerebellum. Adult rats express comparable NR3A levels in the spinal cord and cerebral cortex, whereas NR3A levels in the adult human spinal cord have declined markedly, compared to earlier developmental stages and to the levels in the adult cerebral cortex. Moreover, we could not detect NR3A mRNA or protein in adult human cerebellum, in contrast to earlier studies reporting expression of NR3A in rodent cerebellum.

NR3A is less firmly bound to plasma membranes than the other NMDA receptor subunits. Just as NR1, NR3A is incorporated into tetrameric NMDA receptor complexes but also exists as dimers and monomers. Both NR1 and NR3A can bind glycine and it is likely that NR3A replaces one of the NR1 subunits in certain NMDA receptor complexes, especially during development when the NR3A levels are high.

NR3A binds to the C-terminal regions of MAP1S and MAP1B. Both MAPs are highly expressed in neurons, with prominent distribution throughout dendritic shafts and with occasional localization to dendritic filopodia/spines. In addition to the previously reported interaction with actin, we showed that MAP1S and MAP1B both bind to stabilized microtubules. Little is known about the function of MAP1S in the nervous system, whereas MAP1B is known to be essential for normal brain development and is involved in axon guidance and in myelinization, both in the CNS and PNS. Moreover, MAP1B is involved in the regulation of several neurotransmitter receptors, such as GABAc and 5-HT.

Based on our findings in study IV, I find it unlikely that MAP1B is involved in transport of NR3A-containing NMDA receptors along microtubules. I favor the idea that interaction between the MAPs and NR3A is involved in regulation of the local distribution of NR3A-containing NMDA receptors between intracellular pools and the plasma membrane and/or the distribution between synaptic and extrasynaptic sites. To see whether the MAPs have an effect on the number of NR3A-containing receptors at the cell surface, the MAPs could either be over-expressed in heterologous cells, or knocked down by antisense treatment of neurons. The cells could then be analyzed by ICC.

When analyzing the distribution of NR3A in neurons from MAP1B deficient and WT mice, we found that the localization of NR3A to filopodia was not affected by the lack of MAP1B. However, it would be interesting to examine the morphology of the NR3A-containing spines in MAP1B deficient and WT mice. Dendritic spines in the MAP1B deficient mice have a more immature morphology than spines in WT mice (E. Tortosa, unpublished observation). NR3A is associated with development and might not be equally distributed between immature filopodia and more mature, mushroom shaped spines. As a complement to the morphological examination, it would be interesting to stain the cells for synaptic proteins, for example PSD95 and synapsin and compare their localization to the localization of NR3A immunoreactivity.
The MAP1B deficient mice have increased NR3A protein levels. However, at the moment it is not clear if these additional NR3A subunits are part of functional NMDA receptor assemblies expressed at the cell surface. Since the presence of NR3A in the NMDA receptor complex attenuates receptor currents, this might be elucidated by electrophysiological measurements. Decreased NMDA receptor currents would indicate the presence of NR3A in receptor assemblies at the cell surface, while currents similar to those in WT cells would suggest that the excess NR3A seen in MAP1B deficient animals is mainly localized to intracellular pools. More than half of the NR1 subunits expressed in a neuron have been estimated to be localized to intracellular pools, where they have a high turnover rate compared to NR1 subunits incorporated into receptors and expressed at the cell surface. Our data suggests that NR3A has a similar distribution between intracellular membranes and the cell surface as NR1.

Both MAPs bind to actin and to microtubules, and their distribution is dependent on cytoskeletal integrity. If the MAPs are associated with the cytoskeleton when interacting with NR3A, cytoskeletal depolymerization would also affect the distribution of NR3A. This could be investigated by exposing cultured neurons to actin or microtubule-depolymerizing drugs and by ICC determine if the distribution of the proteins is altered.

The interaction between NMDA receptor subunits and intracellular proteins are often activity dependent. For instance, NR3A’s interaction with both PACSIN1 and PP2A has been shown to be regulated in an activity-dependent manner. Whether there is such a relationship between NR3A and its interaction with the MAPs could be tested by treating cultured neurons with NMDA receptor agonists or antagonists. The effect of the treatment could then be evaluated by co-immunoprecipitation and ICC.

The studies I have presented are part of the characterization of NR3A and NR3A-interacting proteins, performed both by us as well as other research groups. In this thesis I discuss the role of NR3A-interacting proteins in receptor transport, trafficking and signaling. It is my belief that these efforts will give us a greater understanding of NR3A, and possibly also NR3B, which both have profound effects on NMDA receptors. When such understanding is reached, we will also be able to evaluate NR3A-related processes in the brain and spinal cord, and their influence on health and disease.
MATERIALS AND METHODS

HUMAN TISSUE (PAPERS I, II, III AND IV)

Adult human post mortem CNS tissue, from individuals without any record of a neurological or psychiatric disorder, was provided by Netherlands Brain Bank (papers II and III) and by Huddinge Brain Bank, Sweden (paper II). Adult tissue was used for in situ hybridization, IHC, immunoprecipitation and western blot in paper II and for western blot in paper III. Embryonic human CNS tissue was collected at elective, routine, first trimester abortions and written consent had been given by the pregnant women. The tissue was used for reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization in paper I, for RT-PCR in paper II, for western blot in paper III and culturing cells used for ICC in papers III and IV. The use of human tissue was performed in compliance with Dutch and Swedish law and the procedures were approved by the Regional Ethical Committee in Stockholm.

ANIMAL TISSUE (PAPERS I, II, III AND IV)

Eight-day old Sprague-Dawley rats were decapitated, CNS tissue dissected and used for RT-PCR in papers I and II, for western blot and solubilization-experiments in paper II, for immunoprecipitation and western blot in papers III and IV and for microtubule-binding experiments in paper IV. Adult rats were anestitized, decapitated, CNS tissue dissected and used for RT-PCR in paper II and for western blot in papers II and III. Hippocampi were dissected from embryonic day 18 rats and dissociated cells cultured and used for ICC in papers III and IV. The handling and procedures were approved by the Ethical Committee on Animal Research in Southern Stockholm.

Female macaque monkeys ranging from 6 months to 13 years of age were used for IHC in paper II. The care of the animals and all anesthesia and euthanasia procedures in this study were performed according to the National Institutes for Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at Emory University, Atlanta USA.

Cortices from newborn Swiss-Webster mice were dissected, the cells dissociated and cultured under conditions to form a monolayer of astrocytes. Hippocampi were dissected from 18 day-old WT and MAP1B deficient R1/NMRI mouse fetuses, the dissociated cells plated on cover slips and after adhering combined with the astrocyte monolayer. The hippocampal cells were used for ICC in paper IV. Newborn WT and MAP1B deficient R1/NMRI mice were decapitated, the brains dissected and used for western blot in paper IV. The handling and procedures were approved by the Ministry of Agriculture, Spain.

HUMAN BRAIN cDNA LIBRARIES (PAPER I)

We isolated the first fragment of human NR3A from an adult, human hippocampal cDNA library by PCR-amplification using degenerate primers. The isolated fragment was highly homologous to the rat NR3A sequence and used as a template to synthesize a digoxigenin (DIG)-labeled probe. The probe was then used to screen a fetal, human brain cDNA library, and an additional part of the human NR3A sequence obtained.
However, the full-length human NR3A cDNA was not retrieved from the cDNA libraries.

**REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) (PAPERS I AND II)**

Complementary DNA (cDNA) was reverse transcribed from mRNA prepared from rat or human brain CNS tissue, by gene specific primers. In paper I, the full-length human NR3A cDNA was amplified by PCR and then sequenced. The cDNA obtained was also used to investigate the occurrence of NR3A splice variants in both paper I and II. In paper II we also used this method to investigate in which regions of the adult human brain NR3A mRNA was present.

**SUBCLONING, MUTAGENESIS AND SEQUENCING (PAPERS I, III AND IV)**

In paper I the full-length human NR3A cDNA was obtained by RT-PCR and the nucleotide sequences determined. In papers III and IV different DNA constructs have been prepared by subeloning NR3A, MAP1S and MAP1B cDNA into yeast, bacterial and eukaryotic expression vectors. Some of the DNA constructs were further modified by inserting mutations into their nucleotide sequence by PCR-based mutagenesis. The sequence of all prepared DNA constructs were verified by sequence-analysis.

**IN SITU HYBRIDIZATION (PAPERS I AND II)**

To investigate the expression of NR3A mRNA in embryonic human CNS tissue in paper I, we performed *in situ* hybridization using $[^{35}\text{S}]$-labeled oligonucleotide probes. In paper II we investigated the expression of NR3A mRNA in different regions of the adult human brain using first a full-length rat NR3A (rNR3A) $[^{35}\text{S}]$-riboprobe and then verifying the result using a mixture of two human-specific $[^{35}\text{S}]$-labeled NR3A oligonucleotide probes. The $[^{35}\text{S}]$-labeled sections were exposed to film for 7-50 days, depending on the strength of the signal and then developed. As negative control, sections were hybridized in the presence of a 100-fold excess of unlabeled probe. For visualization of cells, a week counterstaining with cresol violet was used.

**IMMUNOHISTOCHEMISTRY (PAPER II)**

To investigate the expression of NR3A protein in brains from higher mammals we performed IHC on macaque monkeys using a NR3A antibody. NR3A-labeled brain sections were incubated with biotinylated goat anti-rabbit IgG and visualized using 3,3'-diaminobenzidine (DAB) as chromogen. Control sections, processed as above except for the omission of the primary immunoreagent, did not contain DAB label.

**SOLUBILIZATION OF RECEPTOR SUBUNITS (PAPER II)**

We investigated the solubility of the NR3A subunit, by preparing a membrane fraction (P2-pellet) of rat or human brain tissue by centrifugation and then extracting membrane-bound proteins from the fraction. We used different detergents, varying in their strength and a total of six different conditions for the extractions. We loaded equal volumes of solubilized protein on SDS-gels and compared the occurrence of NR3A protein between the different extraction conditions. We also investigated which extraction conditions solubilized the other NMDA receptor subunits. To avoid that
solubility of other cellular proteins would affect our results, the same volume of each supernatant, rather than a fixed amount of total solubilized protein, was loaded in each well. This will more accurately allow a comparison of solubility.

**SIZE EXCLUSION CHROMATOGRAPHY AND SPOT BLOT (PAPER II)**

To analyze the presence of NR3A in protein complexes we separated the membrane fraction (P2-pellet), solubilized with either the mild detergent CHAPSO or with CHAPSO followed by the stronger detergent DOC, on a Suprose 6 column. The solubilization buffer was used to elute protein monomers, dimers, tetramers and larger complexes from the column. High molecular weight complexes passed through the column at higher speed than low molecular weight complexes and were accordingly collected in earlier fractions. The fractions were filtered through a nitrocellulose membrane and the membranes probed for NMDA receptor subunit antibodies to determine which subunits were present in different fractions. The protein spots were detected using a CCD-camera and a standard set of known proteins were used to identify the molecular weights of proteins in the different fractions.

**PREPARATION OF TISSUE AND WESTERN BLOT (PAPERS II, III AND IV)**

Human or animal CNS tissue was homogenized in cold buffer and protein concentrations determined. Samples were separated by SDS-PAGE on Tris-glycine gels, transferred to nitrocellulose membranes, incubated with primary antibody over night and with secondary antibody for 1 hr, both at room temperature, see table 2. In paper II protein bands were detected on film and in papers III and IV with a CCD-camera. In papers III and IV the optical density of protein bands were determined and quantified. In paper IV the relative optical density of protein bands from MAP1B deficient animals, compared to WT animals was calculated and values analyzed with Mann-Whitney U-test. α-Tubulin was used as an internal control to ensure that the same amount of protein was loaded in all wells.

**CO-IMMUNOPRECIPITATION (PAPERS II, III AND IV)**

To determine if two proteins can bind to each other in vivo we performed a number of co-immunoprecipitations, from adult human brain in paper II and from eight-day old rat brain in papers III and IV. In either case, a membrane fraction (P2-pellet) was prepared by centrifugation and then solubilized with DOC at pH 9. To remove excess detergent, solubilized samples were dialyzed over night and also diluted 1:10 before use for immunoprecipitation. After preclearing, the samples were incubated with primary antibody over night, precipitated with Dynabeads and the precipitated and coprecipitated proteins analyzed by western blot.

**YEAST TWO-HYBRID SCREEN (PAPERS III AND IV)**

To find novel proteins interacting with NR3A we performed a yeast two-hybrid screen of a fetal, human brain cDNA library. As bait, we used the full-length intracellular C-terminus of human NR3A, comprising amino acids 952-1115. The initial screen resulted in more than 88 interacting clones. To reduce the number of false positive binding-partners, all clones were exposed to more stringent selection conditions, which resulted in 10 remaining clones. These clones were sequenced and one of them encoded MAP1S and three encoded MAP1B.
GST PULL-DOWN (PAPERS III AND IV)

To determine whether the different clones interacting with NR3A in yeast, also bind to NR3A in a different in vitro system, we performed glutathione-S-transferase (GST) pull-down experiments. In addition, in paper IV we performed GST pull-down experiments with truncated MAP1B fragments, to determine which part of MAP1B is required for binding to NR3A.

CELL CULTURES, TRANSFECTION AND IMMUNOCYTOCHEMISTRY (PAPERS III AND IV)

HEK 293 cells were either transiently or stably transfected with NR3A cDNA. The cells were lysed on ice with Triton X-100 and NP-40, unsolubilized material pelleted and a known amount of total protein used for GST pull-down experiments.

The subcellular distribution of NR3A, MAP1S and MAP1B was investigated by immunocytochemical staining of primary neurons cultured in Neurobasal media supplemented with glutamine, glutamate and B27. In paper III we used rat hippocampal and human cortical cells transfected with MAP1S-EGFP at10 DIV and fixed at 14 DIV. The localization of MAP1S-EGFP was then compared to that of β-tubulin III, the presynaptic marker synapsin, the postsynaptic marker PSD95 and NR3A. In paper IV we investigated the location of MAP1B and NR3A in relation to β-tubulin III, actin, synapsin and the postsynaptic protein SAP102 in human cortical cells cultured for 21 DIV. In paper IV we also examined the distribution of NR3A in hippocampal neurons from MAP1B deficient and WT mice cultured for 21 DIV. The actin cytoskeleton was visualized by Alexa Fluor® 488-conjugated phalloidin, and the number of NR3A immunoreactive filopodia counted.

STABILIZATION OF MICROTUBULES (PAPER IV)

To investigate the microtubule-binding properties of MAP1S, we treated rat brain homogenate with the microtubule-stabilizing drug paclitaxel. The stabilized microtubules were then pelleted by high-speed centrifugation and the distribution of MAP1S between pellet and supernatant analyzed by western blot. As comparison, we looked at the distribution of MAP1B in the different fractions, since the microtubule-binding property of MAP1B is well documented.
Table 2. Primary antibodies employed in the studies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution WB</th>
<th>Dilution ICC</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin, AC-40</td>
<td>Mouse</td>
<td>1:1 000</td>
<td>-</td>
<td>Abcam</td>
</tr>
<tr>
<td>α-tubulin, YL1/2</td>
<td>Rat</td>
<td>1:2 000</td>
<td>-</td>
<td>Abcam</td>
</tr>
<tr>
<td>β-tubulin type III</td>
<td>Rabbit</td>
<td>1:2 000</td>
<td>1:500</td>
<td>Biosite</td>
</tr>
<tr>
<td>β-tubulin type III, SDL.3D10</td>
<td>Mouse</td>
<td>-</td>
<td>1:1 200</td>
<td>Sigma</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>1:1 000</td>
<td>-</td>
<td>Promega</td>
</tr>
<tr>
<td>GluR2, MAB397</td>
<td>Mouse</td>
<td>1:500</td>
<td>-</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GST</td>
<td>Goat</td>
<td>1:10 000</td>
<td>-</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>MAP1B, AA6</td>
<td>Mouse</td>
<td>1:500</td>
<td>1:300</td>
<td>Abcam</td>
</tr>
<tr>
<td>MAP1B</td>
<td>Mouse</td>
<td>1:250</td>
<td>-</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>MAP1S, mAb4G1</td>
<td>Mouse</td>
<td>1:2 000</td>
<td>-</td>
<td>(Liu et al., 2005b)</td>
</tr>
<tr>
<td>NR1, 54.1</td>
<td>Mouse</td>
<td>1:500</td>
<td>1:300</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>NR2A</td>
<td>Rabbit</td>
<td>1:500</td>
<td>-</td>
<td>Merck Biosciences</td>
</tr>
<tr>
<td>NR2B</td>
<td>Rabbit</td>
<td>1:500</td>
<td>-</td>
<td>Invitrogen</td>
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<tr>
<td>NR3A</td>
<td>Rat</td>
<td>1:4 000</td>
<td>1:500</td>
<td>Custom made by Zymed Invitrogen Corporation</td>
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<td>PSD95, K28/43</td>
<td>Mouse</td>
<td>-</td>
<td>1:500</td>
<td>Upstate Cell Signaling Solutions</td>
</tr>
</tbody>
</table>

Abbreviations; Western blot (WB) and Immunocytochemistry (ICC).
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