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Função essencial da subunidade GluR7 nas sinapses das fibras musgosas

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Presynaptic kainate receptors in the hippocampus: A critical role for GluR7 at the mossy fiber synapse

Paulo César da Silva Pinheiro

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“The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries […] of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day.”

Albert Einstein.

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Publications

The present study was mostly based on work that has been published or submitted for publication in international peer-reviewed journals:

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Pinheiro, P.S., Rodrigues, R.J., Rebola, N., Xapelli, S., Oliveira, C.R. & Malva, J.O. (2005) Presynaptic kainate receptors are localized close to release sites in rat hippocampal synapses. *Neurochemistry International, in press.*

Pinheiro, P.S., Perrais, D., Coussen, F., Barhanin, J., Bettler, B., Mann, J., Malva, J.O., Heinemann, S., & Mulle, C. (2005) GluR7 is a presynaptic kainate receptor subunit involved in facilitation of synaptic transmission. *Neuron, in revision.*

The present work also contributed to the success of other publications under the co-authorship of Paulo Pinheiro, as follows:

Silva, A.P., Xapelli, S., Pinheiro, P.S., Ferreira, R., Lourenco, J., Cristovao, A., Grouzmann, E., Cavadas, C., Oliveira, C.R. & Malva, J.O. (2005) Up-regulation of neuropeptide Y levels and modulation of glutamate release through neuropeptide Y receptors in the hippocampus of kainate-induced epileptic rats. J*ournal of Neurochemistry 93(1), 163-70.*

Araujo, I.M., Xapelli, S., Gil, J.M., Mohapel, P., Petersen, A., Pinheiro, P.S., Malva, J.O., Bahr, B.A., Brundin, P., & Carvalho, C.M. (2005) Proteolysis of NR2B by calpain in the hippocampus of epileptic rats. *Neuroreport 16(4), 393-6.*

Lopes, L.V., Rebola, N., Pinheiro, P.C., Richardson, P.J., Oliveira, C.R., & Cunha, R.A. (2003) Adenosine A3 receptors are located in neurons of the rat hippocampus. *Neuroreport 14, 1645-8*.

Rebola, N., Pinheiro, P.S., Oliveira, C.R., Malva, J.O., & Cunha, R.A. (2003) Subcellular localization of adenosine A1 receptors in nerve terminals and synapses of the rat hippocampus. *Brain Research, 987, 49-58.*

Silva, A.P., Pinheiro, P.S., Carvalho, A., Carvalho, C. & Malva, J.O. (2003) Neuroprotective effects of neuropeptide Y1, Y2 and Y5 receptors in organotypic hippocampal slice cultures. *FASEB Journal 17, 1118-20.*

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ABBREVIATIONS

AC	adenylyl cyclase
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATPA	5-*tert*-butyl-4-isoxazolepropionic acid
BCA	bicinchoninic acid
BSA	bovine serum albumin
CaMKII	Ca^{2+}-, calmodulin-dependent protein kinase II
CAPS	3-(Cyclohexylamino)propanesulfonic acid
cDNA	complementary DNA
CNQX	6-cyano-7-nitroquinoxaline
CNS	central nervous system
D-AP5	D(-)-2-Amino-5-phosphonopentanoic acid
DCG-IV	(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine
EC_{50}	concentration that causes half of the maximal response
DTT	dithiothreitol
EDTA	ethylenediaminetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EPSC	excitatory postsynaptic current
Fura2/AM	acetoxymethyl ester of Fura2
GABA	γ-aminobutyric acid

Abbreviations

GBP	glutamate binding protein
GlyBP	glycine binding protein
G-proteins	guanine nucleotide binding proteins
GYKI 53655	(±)-1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine
HBS	HEPES buffered solution
HEK	human embryonic kidney
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
iGluR	ionotropic glutamate receptor
IPSC	inhibitory postsynaptic current
KABP	kainate binding protein
kainate	2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine
L-AP4	L(+)-2-Amino-4-phosphonobutyric acid
L-CCG-I	(2S,1'S,2'S)-2-Carboxycyclopropylglycine
LTD	long-term depression
LTP	long-term potentiation
LY293558	(3*S*,4*aR*,6*R*,8*aR*)-6-(2-(tatrazol-5-yl)ethyl) decahydroxisoquinoline-3-carboxilate
LY303070	(-)-1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine
LY339434	(2*S*,4*R*,6*E*)-2-amino-4-carboxy-7-(2-naphtyl)hept-6-enoic acid

LY354740	(1*S*,2*S*,5*R*,6*S*)-2-Aminobi-cyclo[3.1.0]hexane-2,6-dicarboxylic acid
LY379268	(1*R*,4*R*,5*S*,6*R*)-4-amino-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY382884	(3*S*,4*aR*,6*S*,8*aR*)-6-(4-carboxyphnyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroxyisoquinoline-3-carboxilate
MAPK	mitogen activated protein kinase
mEPSC	miniature EPSC
mGluR	metabotropic glutamate receptor
MGS0028	(1R,2S,5S,6S)-2-amino-6-fluoro-4-oxobicyclo[3.1.0] hexane-2,6-dicarboxylic acid
mRNA	messenger RNA
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline
NCAM	neuronal cell adhesion molecule
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NS3763	5-carboxyl-2,4-di-benzamidobenzoic acid
PDZ	postsynaptic density 95, discs large
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride

Abbreviations

PSD	postsynaptic density
PSD-95	95 kDa postsynaptic density protein
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SNAP-25	25 kDa synaptosomal associated protein
SYM2081	(2*S*,4*R*)-4-methyl glutamic acid
TBS	tris-buffered saline
UBP296	(*R,S*)-3-3(2-carboxybenzyl) willardiine
UBP302	(*S*)-3-3(2-carboxybenzyl) willardiine

RESUMO

Os receptores do glutamato são os principais mediadores da neurotransmissão excitatória no cérebro e também intervêm na sua modulação. Enquanto que a localização e mecanismos de acção de receptores pós-sinápticos do tipo AMPA e NMDA, que suportam a neurotransmissão, são bem conhecidos muito resta a saber acerca da existência, função e mecanismos de acção de receptores que actuam a nível pré-sináptico. A este respeito, muito resta a saber acerca da localização dos receptores de cainato e o seu papel na neurotransmissão.

Com o presente trabalho procurámos responder a algumas questões relacionadas com a localização sináptica e função de receptores do glutamato. Na primeira parte do trabalho descrevemos a optimização de uma metodologia bioquímica que permite a obtenção de preparações purificadas de proteínas da zona activa pré-sináptica e da densidade pós-sináptica. O processo consiste na solubilização sequencial das proteínas não sinápticas em 1% de Triton X-100 a pH 6.0, seguida da solubilização das proteínas pré-sinápticas e sua separação das densidades pós-sinápticas por aumento do pH para 8.0. Experiências de Western blot usando anticorpos contra proteínas tipicamente pré-sinápticas (SNAP-25 e sintaxina), pós-sinápticas (PSD-95) e não sinápticas (sinaptofisina e NCAM) permitiram verificar a eficiência da separação de proteínas destes compartimentos celulares.

De seguida, investigámos a localização subsináptica de diversas subunidades de receptores ionotrópicos e metabotrópicos do glutamato. Observámos que, no caso dos receptores metabotrópicos do glutamato, a subunidade mGluR7 estava localizada maioritariamente na fracção de proteínas da zona activa pré-sináptica. A distribuição subsináptica das outras subunidades estudadas, mGluR1, mGluR2, mGluR4a e mGluR5 foi mais difícil de reconciliar com os resultados de microscopia electrónica

existentes na literatura revelando, provavelmente, a limitação do uso da técnica no estudo da localização de receptores que apresentam distribuições particulares, como é o caso de receptores perisinápticos, que não estão localizados nem na zona activa pré-sináptica, nem na densidade pós-sináptica.

No caso dos receptores do tipo AMPA, observámos que estes apresentavam uma distribuição subsináptica peculiar, com elevados níveis de imunoreactividade para os anticorpos dirigidos contra as subunidades GluR1, GluR2 e GluR2/3 nas fracções de proteínas da zona activa pré-sináptica, da densidade pós-sináptica e de proteínas não sinápticas. A subunidade GluR4 foi detectada em níveis muito mais modestos e parece predominar pós-sinapticamente.

Quanto aos receptores do tipo NMDA, apesar dos vários estudos relatando acções destes receptores ao nível pré-sináptico, detectámos apenas marcação residual para as subunidades NR1, e NR2A-C na zona activa pré-sináptica. A imunoreactividade para todas as subunidades estudadas estava concentrada essencialmente nas densidades pós-sinápicas e ausente da fracção de proteínas não sinápticas.

A pequena amplitude e cinética lenta das correntes sinápticas mediadas por receptores de cainato parecem sugerir uma localização extrasináptica destes receptores, que seriam activados por glutamato difundido para fora da fenda sináptica. No entanto, a manipulação da concentração extracelular de glutamato não altera estas propriedades. Procurámos, portanto, contribuir para o esclarecimento desta aparente discrepância, estudando a localização subsináptica destes receptores. Em estudos funcionais, utilizando sinaptossomas, observámos que a activação de receptores de cainato com baixas concentrações de agonistas aumenta a libertação exocitótica de glutamato tritiado, num processo dependente de Ca^{2+}. Este efeito foi insensível ao antagonista dos receptores AMPA,

LY303070 (10 μM), mas foi prevenido pelo antagonista misto para receptores do tipo AMPA e cainato, CNQX (30 μM). Verificámos ainda que a eficiência de modulação da libertação de glutamato por receptores de cainato é superior à conseguida pela simples despolarização da membrana através da elevação da concentração extracelular de KCl apesar do último fenómeno ser mais eficiente em aumentar a $[Ca^{2+}]_i$. Por outro lado, verificámos que o aumento da $[Ca^{2+}]_i$ induzido por activação de receptores de cainato (cainato 100 μM) foi só parcialmente inibido pela exposição a bloqueadores de canais de Ca^{2+} sensíveis à voltagem. Este resultados sugerem fortemente que os receptores pré-sinápticos de cainato estão localizados dentro da zona activa, próximo dos locais de libertação de glutamato sendo, provavelmente, directamente permeáveis a Ca^{2+}. Para comprovar os resultados dos estudos funcionais investigámos a distribuição subsináptica das várias subunidades de receptores de cainato. Estas experiências mostraram que todas as subunidades de receptores de cainato estão localizadas na zona activa pré-sináptica e na densidade pós-sináptica. A subunidade KA1 mostrou uma localização preferencialmente pós-sináptica.

A subunidade GluR7 é uma subunidade dos receptores de cainato cuja função no cérebro é essencialmente desconhecida. A distribuição de mRNA para esta subunidade permite antever uma possível participação em receptores pré-sinápticos nas sinapses das fibras musgosas no hipocampo, pelo que decidimos estudar um possível papel fisiológico de GluR7 ao nível destas sinapses. Através do registo de correntes excitatórias pós-sinápticas, no modo de voltagem imposta, em células piramidais da área CA3 em fatias de cérebro de animais de fenótipo selvagem e animais deficientes para a subunidade GluR7 ($GluR7^{-/-}$) estudámos uma possível participação desta subunidade em receptores pós-sinápticos de cainato. Observámos que nem a amplitude da resposta

dos receptores de cainato nos potenciais excitatórios pós-sinápticos nem a sua cinética estavam alterados em animais GluR7$^{-/-}$. Assim, sugerimos que esta subunidade não contribui para receptores de cainato a nível pós-sináptico nas sinapses das fibras musgosas com as células piramidais da área CA3.

De seguida, estudámos fenómenos de modulação pré-sináptica através de protocolos de plasticidade de curta e longa duração. Em animais GluR7$^{-/-}$ observámos que a facilitação sináptica devida à aplicação seguida de dois pulsos de estimulação estava significativamente reduzida para intervalos de 10-40 ms entre os pulsos de estimulação, mas apresentava-se normal para intervalos de 100 ms ou superiores, sugerindo uma acção rápida dos receptores de apenas alguns milisegundos. A elevada facilitação observada normalmente nesta sinapse em resposta a um conjunto de 5 estimulações com uma frequência de 20 Hz estava também fortemente reduzida, mostrando que receptores contendo a subunidade GluR7 contribuem para a facilitação sináptica em resposta a estímulos repetidos. Uma outra forma de plasticidade, a facilitação em frequência, que se desenvolve mais lentamente na gama de frequências baixas com estimulação repetitiva, embora não estivesse alterada para frequências mais baixas (0.2 Hz), apresentava-se significativamente reduzida para frequências de estimulação de 0.5 Hz e superiores.

A potenciação de longa duração (LTP) observada nas sinapses das fibras musgosas é induzida e expressa a nível pré-sináptico e os receptores pré-sinápticos de cainato, embora inicialmente considerados essenciais para este tipo de plasticidade, desempenham um papel permissivo reduzindo o limiar para a sua indução. Investigámos, por isso, se a subunidade GluR7 teria também um papel preponderante neste tipo de plasticidade sináptica. En animais GluR7$^{-/-}$ a LTP das fibras musgosas estava consideravelmente reduzida, mas não completamente ausente.

Adicionalmente, a potenciação pós-tetânica (PTP) estava também severamente reduzida em animais GluR7$^{-/-}$ sem que, no entanto, nenhuma diferença tenha sido observada entre os dois genótipos na potenciação das respostas sinápticas por aplicação de forscolina, indicando que o mecanismo de expressão deste tipo de plasticidade estava intacto. Quer a LTP quer a PTP foram, no entanto, recuperadas para níveis semelhantes aos níveis controlo após elevação da concentração de KCl no meio extracelular ou fornecendo estímulos eléctricos adicionais durante a fase de indução.

Embora não tenhamos observado uma facilitação das respostas das sinapses das fibras musgosas pela aplicação de baixas concentrações de cainato (50 nM) a sua inibição foi consistentemente observada em animais de ambos os genótipos pela aplicação de concentrações de cainato superiores a 100 nM. Esta experiência mostrou que a facilitação e inibição das respostas sinápticas pelos receptores de cainato provavelmente não são mediadas pelos mesmos receptores. Mostrámos ainda que não só a subunidade GluR7 tem uma localização sináptica na ausência da subunidade GluR6, e vice versa, mas também que estas duas entidades co-imunoprecipitam em lisados de cérebro, sugerindo a existência de receptores heteroméricos contendo GluR6 e GluR7.

Estudos em células HEK transfectadas com GluR6 e GluR7 mostraram que estes receptores heteroméricos são bloqueados pelo antagonista misto de receptores AMPA/cainato, CNQX, e, surpreendentemente, também pelo GYKI 53655, um antagonista considerado selectivo para receptores AMPA. Estabelecemos que estes compostos reduzem a facilitação em frequência em animais controlo mas não em animais GluR7$^{-/-}$. Adicionalmente, os níveis de facilitação em animais GluR7$^{-/-}$ eram os mesmos observados em animais controlo na presença dos antagonistas, dando um suporte farmacológico aos dados obtidos com a estratégia de delecção genética.

Resumo

Os nossos resultados reforçam o papel dos receptores de cainato como entidades fundamentais no controlo das sinapses glutamatérgicas. A nível pré-sináptico, verificámos que a subunidade GluR7 desempenha um papel fulcral em fenómenos de plasticidade sináptica de curta e longa duração no hipocampo, levantando importantes questões acerca do possível papel deste receptor em outras zonas cerebrais onde a plasticidade sináptica é semelhante à observada nas sinapses das fibras musgosas.

SUMMARY

Glutamate receptors play a central role in excitatory neurotransmission in the brain and also in synaptic modulation. Whereas the localization and mechanisms of action of postsynaptic AMPA and NMDA receptors, that support neurotransmission, are more or less well understood, much remains to be studied regarding the existence, function and mechanisms of action of receptors that act at the presynaptic level. With this regard, the synaptic localization of kainate receptors and their role in neurotransmission is one of the most poorly comprehended.

With the present effort we sought to answer some of the unresolved issues regarding glutamate receptor localization and function. In the first part of this work we used a new biochemical technique to allow us to obtain purified preparations of proteins from the presynaptic active zone, the postsynaptic density and from non-synaptic pools. This was achieved by the sequential solubilization of non-synaptic proteins in 1% Triton X-100 at pH 6.0, followed by solubilization of presynaptic proteins from the postsynaptic densities by increasing the pH to 8.0. Antibodies directed against typically presynaptic (SNAP-25 and syntaxin), postsynaptic (PSD95) and non-synaptic (synaptophysin and NCAM) proteins allowed us to verify that the methodology yielded preparations of these protein pools with high purity.

We next investigated the subsynaptic localization of several subunits of ionotropic and metabotropic glutamate receptors. We found that, for metabotropic glutamate receptors, the mGluR7 subunit was found mainly on the presynaptic active zone, as previously described. The subsynaptic distribution of the other subunits studied, mGluR1, mGluR2, mGluR4a and mGluR5 was more difficult to reconcile with the results from previous immunogold electron microscopy studies, revealing a possible limitation of the solubilization technique in resolving receptors that present

particular distributions, such as perisynaptic receptors, that are neither localized in the presynaptic active zone nor in the postsynaptic density.

AMPA receptors were found to have a striking subsynaptic distribution, with high amounts of immunoreactivity for GluR1, GluR2 and GluR2/3 in the presynaptic active zone fraction of proteins, in the postsynaptic density and in the non-synaptic pool of proteins. Although there is some evidence that these receptors may be differentially attached to the postsynaptic density, they should not be behaving differently to the solubilization procedure and contribute significantly for the observed presynaptic labbelling. Furthermore, proof for their existence at presynaptic sites is increasingly growing.

Despite numerous evidences for actions of NMDA receptors at the presynaptic level, we found only residual labelling for the NR1 and NR2A-C subunits in the pool of proteins from the presynaptic active zone, with the majority of immunoreactivity concentrated at postsynaptic densities. The small labelling of this fraction of proteins for PSD-95 may indicate that labelling at such sites may, in fact, result from slight contamination of the presynaptic active zone faction with proteins from the postsynaptic density.

Electrophysiological responses mediated by kainate receptors show small amplitude and slow kinetics that may suggest an extrasynaptic localization and activation by low concentrations of glutamate spilling over from the synaptic cleft. However, manipulating the extracellular glutamate concentration does not change these parameters. Therefore, we sought to add some clarity to this question by investigating the subsynaptic localization of these receptors. In functional studies, using synaptosomes, we observed that activation of kainate receptors with low concentrations of agonists increased the exocytotic release of [^{3}H]glutamate in a Ca^{2+}-dependent manner. This effect was insensitive to the AMPA receptor antagonist, LY303070 (10 μM), but was blocked by the general

AMPA/kainate receptor antagonist, CNQX (30 μM). Furthermore, we also observed that kainate (1 μM), although inducing a much more modest increase in the intracellular Ca^{2+} concentration, was able to significantly modify the release of [^{3}H]glutamate, contrarily to what was observed in a situation of elevated extracellular KCl. These results, together with the fact that the Ca^{2+} signal was only partially reduced by blockers of voltage-sensitive Ca^{2+} channels, at the supramaximal concentration of 100 μM kainate, suggest that presynaptic kainate receptors are localized close to glutamate release sites, within the active zone, and are probably directly permeable to Ca^{2+}. To look further into the synaptic localization of kainate receptors we performed Western blot experiments in the subsynaptic fractions. This showed that, not only all kainate receptor subunits are localized both in the presynaptic active zone and postsynaptic density but also that they appear to be restricted to these sites of synaptic contact, as shown by the very faint labelling in the non-synaptic pool of proteins. The KA1 subunit revealed to be preferentially localized at the postsynaptic level.

Although we showed the subsynaptic localization of kainate receptors and a functional role at the presynaptic level, it is important to understand these parameters at individual synapses and the subunits that are important for synaptic modulation in a more intact system. GluR7 is one subunit of kainate receptors whose function in the brain is unknown. The distribution of mRNA predicts the possibility of its participation to presynaptic kainate receptors at hippocampal mossy fiber synapses and, therefore, we decided to study its possible role at this synapse. By performing whole-cell voltage-clamp recordings from CA3 pyramidal cells in brain slices from wildtype mice and mice lacking GluR7 (GluR7$^{-/-}$) we first studied the possible contribution of this subunit for postsynaptic receptors. We found that neither receptor kinetics nor the percent contribution of pure kainate receptor-mediated responses to mossy fiber EPSCs were changed

in GluR7$^{-/-}$ mice suggesting that, in consistency with anatomical data, GluR7 does not contribute to postsynaptic receptors at mossy fiber-CA3 pyramidal cell synapses.

We then turned to presynaptic modulation by using protocols that lead to presynaptic forms of short- and long-term plasticity, which have been shown to be dependent on or modulated by kainate receptors. In animals lacking GluR7 we showed that paired pulse facilitation was significantly impaired at short intervals between stimuli, but normal for intervals of 100 ms or greater, suggesting a fast action of these receptors of only a few milliseconds. The prominent facilitation of mossy fiber responses to a train of 5 stimuli, delivered at a frequency of 20 Hz, was also greatly reduced in GluR7$^{-/-}$ animals, showing that kainate receptors containing this subunit contribute to the facilitation of responses to repetitive stimuli. Frequency facilitation, another form of presynaptic plasticity that develops over a slower time scale with repetitive stimulation in the low frequency range, although not altered at low (0.2 Hz) rates of stimulation, was significantly reduced for stimulation frequencies of 0.5 Hz and higher in the absence of GluR7.

Mossy fiber LTP is both induced and expressed presynaptically and presynaptic kainate receptors, although initially thought to be crucial for this process, are now known to have a permissive role by lowering the induction threshold. Therefore, we investigated whether GluR7 had any participation in this form of long-term synaptic plasticity. In animals lacking the GluR7 subunit mossy fiber LTP was strikingly reduced, but not completely absent, when compared to wildtype animals. Furthermore, PTP was also severely impaired in GluR7$^{-/-}$ mice but no difference was found in the forskolin-induced potentiation of mossy fiber responses, indicating an intact expression mechanism. Mossy fiber LTP and PTP could, however, be rescued to control levels by either slightly increasing the extracellular KCl concentration or by supplying additional stimuli during induction.

Although we were not able to record a facilitation of mossy fiber synaptic responses by the application of low (50 nM) concentrations of kainate, inhibition was always observed in both genotypes for concentrations of kainate above 100 nM, in parallel with the activation of an inward current in the postsynaptic neurons. This shows that facilitation and inhibition are probably not mediated by the same receptors. We further show, using isolated synaptic junctions, that not only GluR7 is localized within synapses in the absence of GluR6, and *vice versa*, but also that GluR6 and GluR7 co-immunoprecipitate from lysates of mouse brain, suggesting the existence of heteromeric kainate receptors containing both subunits.

Studies in transfected HEK cells established that CNQX and, surprisingly, the AMPA receptor selective antagonist, GYKI 53655, acted as antagonists on GluR6/GluR7 heteromeric receptors. These compounds were shown to reduce mossy fiber low frequency facilitation in wildtype but not GluR7$^{-/-}$ mice. Furthermore, the levels of facilitation in GluR7$^{-/-}$ animals were the same as the ones observed in wildtype animals in the presence of the antagonists, lending pharmacological support to the data obtained with the genetic deletion approach.

Our results not only show the subsynaptic localization of glutamate receptor subunits in the hippocampus, but also that kainate receptors are localized within the active zone of synapses, and almost completely absent from extrasynaptic locations. More importantly, they allow establishing, for the first time, a physiological role for the GluR7 subunit of kainate receptors in the brain, and pose pertinent questions about the possible role of these receptors in other brain areas expressing forms of presynaptic plasticity similar to those of the hippocampal mossy fiber synapses.

Chapter **1**

General Introduction

1.1. The hippocampus

The hippocampus is perhaps the most intensively studied structure in the brain, forming, together with the amygdala, the central axis of the limbic system. It is made up by two interlocking sheets of cortex and, in cross-section, has a well defined laminar structure with layers visible where rows of pyramidal cells are arranged. Within the hippocampus, several regions can be identified, namely the subiculum, the dentate gyrus and the *cornu Ammonis* (CA1, CA2 and CA3 subregions; Figure 1.1). The connections within the hippocampal formation generally follow the laminar arrangement and are, until present, thought to be unidirectional, forming the so-called tri-synaptic circuit. The connection of the entorhinal cortex to the hippocampus is made through the perforant path that crosses the subiculum to make synapses with dentate gyrus granule cells. The perforant path is the major input pathway to the hippocampus and its fibers originate mostly from layers II and III of the entorhinal cortex, with minor contributions from the deeper layers IV and V. Axons from cortical layers II/IV project to granule cells of the dentate gyrus but also to CA3 pyramidal cells, while those arising from layers III/V project to pyramidal cells of the CA1 subregion and to the subiculum. The major input to CA3 pyramidal cells comes from the mossy fibers, the axons of dentate gyrus granule cells. Mossy fiber synapses are made *en passant* onto the proximal dendrites of CA3 pyramidal neurons and are formed by large, complex termini containing multiple neurotransmitter release sites and postsynaptic densities. CA3 pyramidal cells, in turn, are connected to CA1 pyramidal cells through the Schaffer Collateral/Associational Commissural pathway. Axons can either originate from CA3 neurons in the same (ipsilateral) hippocampus or from CA3 neurons of the contralateral hippocampus (commissural fibers). CA1 pyramidal neurons then project back to the subiculum and on to the entorhinal cortex. This connection is, however, not

a straightforward unidirectional pathway, forming two closed loop networks (Figure 1.1).

1.2. Glutamatergic neurotransmission in the hippocampus

The hippocampal formation plays an important role in learning and processing of memory in primates (Zola-Moran and Squire, 1990). The repeated electrical stimulation of several pathways within the hippocampus can lead to a long-term enhancement of synaptic responses (LTP) or to a long-term depression of these responses (LTD) which may be brought

Figure 1.1 – The hippocampal network. The hippocampus forms a mainly uni-directional network, with the input from the entorhinal cortex (EC) connecting to the dentate gyrus (DG) granule cells and CA3 pyramidal cells through the perforant path (PP; split into lateral, LPP and medial, MPP). CA3 pyramidal neurons receive massive input from the DG via the mossy fibers (MF) which in turn project to CA1 pyramidal cells via the Schaffer Collateral Pathway (SC) and to CA1 pyramidal cells of the contralateral hippocampus via the Associational Commissural pathway (AC). CA1 neurons also receive direct input from the PP and send axons to the subiculum. Subicular neurons send the major hippocampal output back to the EC, forming a loop. LEC and MEC, lateral and medial entorhinal cortex, respectively. Adapted from http://www.bris.ac.uk/Depts/Synaptic/info/pathway/hippocampal.htm

about by different mechanisms. These phenomena are thought to be the cellular correlates of learning and memory formation (Baudry and Massicotte, 1992; Bliss and Collingridge, 1993) and critically involve the neurotransmitter L-glutamate. In addition to its well recognized role in synaptic plasticity processes (Bliss and Collingridge, 1993), neurodevelopment and synaptogenesis (McDonald and Johnston, 1990), glutamate is also known to potentially act as a potent endogenous neurotoxic agent, playing a critical role in the development and/or progression of diverse neurological disorders (Meldrum and Garthwait, 1990; Beal, 1992).

1.3. Glutamate receptors

Historically, the notion that L-glutamate functions as a neurotransmitter came from the observation that at low concentrations it excites virtually every neuron in the CNS (Curtis et al., 1959; Curtis and Watson; 1960). Early studies also demonstrated that glutamate depolarized membranes as a result of increased conductance to Na^+ ions (Curtis et al., 1972; Zieglgansberger and Puil, 1973). This neurotransmitter is released in a Ca^{2+}-dependent manner involving N- and P/Q-type voltage-sensitive calcium channels (Birnbaumer et al., 1994) and has powerful excitatory actions on neurons when iontophoresed *in vivo*. Glutamate may also be "released" by the reverse operation of glutamate transporters. This situation may occur when the electrochemical gradient of Na^+ and K^+ is strongly reduced by energy depletion during cerebral ischemia (Levy et al., 1998; Obrenovitch and Urenjak, 1997).

It is now known that L-glutamate is the major excitatory neurotransmitter in the mammalian brain and exerts its functions through a multitude of receptors. Glutamate receptors are distinguished on the basis

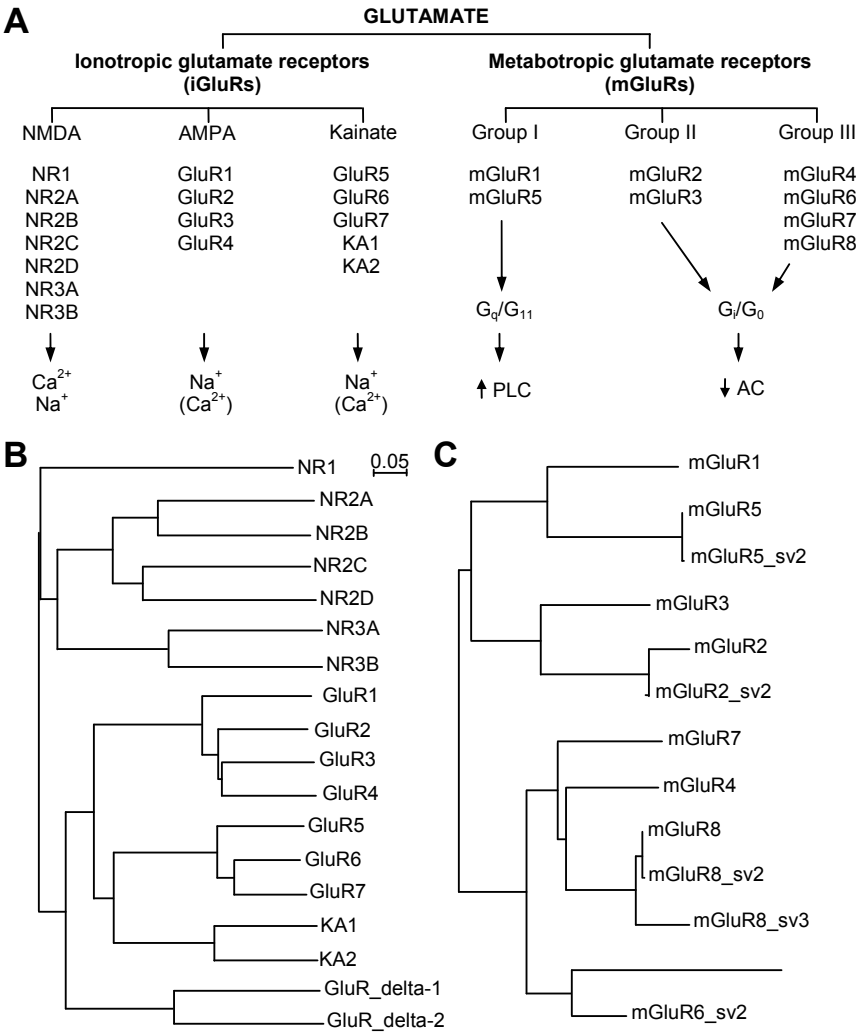

Figure 1.2 – Overview of the glutamate receptor family. **(A)** Illustrates the various groups and subunits that compose both ionotropic and metabotropic glutamate receptors and their main signal transduction mechanisms. Glutamate gates cation-permeable ionotropic receptors and can also activate metabotropic receptors coupled via G-proteins to the activation of phospholipase C (PLC) or the inhibition of adenylate cyclase (AC). **(B and C)** Sequence similarity of ionotropic and metabotropic glutamate receptor subunits. The pharmacological characterization of ionotropic glutamate receptors into AMPA, NMDA and kainate is well reflected in the similarity of their sequences **(B)**. The same is also apparent for the various groups of metabotropic glutamate receptors **(C)**. The bar indicates the normalized distance score derived from the pairwise sequence similarity scores (Feng and Doolittle, 1987). A distance of 0 means identical sequences and a distance of 1 means infinite distance between sequences. Recreated from Kew and Kemp, 2005.

of their signal transduction mechanisms into ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Moreover, several pharmacological differences allow the distinction of different families of receptors within each group (Figure 1.2). The two classes of excitatory amino acid receptors interact in the fine-tuning of neuronal responses under different conditions. The first subunit of ionotropic glutamate receptors was cloned in 1989 (Hollmann et al., 1989) and cloning and identification of other subunits continued until 1992 (reviewed by Hollmann and Heinemann, 1994), when the first subunit of metabotropic glutamate receptors was also cloned (Nakanishi, 1992). Glutamate receptors are expressed mainly in the central nervous system but they can also be found in the periphery. Noteworthy examples are their localization in pancreatic islet cells (Inagaki et al., 1995; Weaver et al., 1996, 1998) and in osteoclasts and osteoblasts (Chenu et al., 1998; Patton et al., 1998). Glutamate signalling in non-neuronal tissue is out of the scope of this work and has been reviewed elsewhere (Skerry and Genever, 2001). The present studies focus more on iGluRs and this chapter will be oriented accordingly.

1.3.1. Metabotropic glutamate receptors

Metabotropic glutamate receptors are transmembrane receptors coupled to heterotrimeric G-proteins. Structurally, mGluRs are formed by a large extracellular bi-lobed N-terminal domain that contains the ligand-binding site and that is connected to the transmembrane domain, which is formed by seven α-helices, by a cysteine-rich domain (Figure 1.3). They also possess an intracellular C-terminal domain whose size is variable depending on alternative splicing (De Blasi et al., 2001. The most conserved domains between the different mGluRs are the sites involved in

Figure 1.3 – Structure of metabotropic glutamate receptors. mGluRs are characterized by their association to G-proteins and the presence of seven transmembrane segments. The extracellular N-terminus is much larger than that of other G-protein coupled receptors and contains the ligand binding domain. The intracellular C-terminus can undergo extensive alternative splicing. Adapted from: http://www.bris.ac.uk/Depts/Synaptic/info/glutamate.html

coupling to G-proteins and the glutamate binding site (Pin and Duvoisin, 1995). Functional mGluRs are thought to comprise homodimers stabilized by both an inter-subunit disulphide bond and hydrophobic interactions (Romano et al., 1996; Kunishima et al., 2000; Tsuji et al., 2000; Tsuchiya et al., 2002).

Up to date, eight different receptors were cloned (mGluR1-8) that are classified into three groups according to their sequence homology, pharmacological properties and intracellular signal transduction pathways used (Figure 1.2A, C). Group I mGluRs include mGluR1 and mGluR5 receptors that are positively coupled to phosphoinositide-specific phospholipase C through G-proteins of the G_q/G_{11} type. Activation of these receptors leads to activation of phospholipase C, production of IP_3, release of Ca^{2+} from intracellular stores and also production of diacylglycerol, which in turn activates PKC, and are activated by quisqualate as their most potent agonist (Masu et al., 1991; Abe et al., 1992; Aramori and Nakanishi, 1992; reviewed by Kew and Kemp, 2005). Group II mGluRs include mGluR2 and mGluR3 and are coupled to the inhibition of adenylyl cyclase activity

through G-proteins of the G_i/G_0 type. A number of potent Group II agonists have been described. These include DCG-IV, LY354740, LY379268 and MGS0028. Finally, group III mGluRs are composed of mGluR4, mGluR6, mGluR7 and mGluR8 and have the same mode of signalling as group II mGluRs. Group III mGluRs are potently activated by (*S*)-AP4 (for a review of mGluR pharmacology see Kew and Kemp, 2005). Although members of the mGluR family can mediate synaptic transmission via activation of slow postsynaptic potentials (Glaum and Miller, 1992) they generally exert a more modulatory role. This is achieved through the activation of intracellular second messenger pathways as already mentioned, but also through the direct action of the βγ subunits of the heterotrimeric G-proteins in modulating, for example, the activity of ion channels (reviewed by Anwyl, 1999). The members of each group of mGluRs share ~70% sequence homology and about 45% homology between classes. Alternatively spliced variants have been described for mGluR1, mGluR4, mGluR5 and mGluR7. Figure 1.2 summarizes the organization and sequence similarity of glutamate receptors.

1.3.2. Ionotropic glutamate receptors

Glutamate is thought to be an ancestral signalling molecule. The proof for this comes from the cloning of ionotropic glutamate receptors in organisms such as *Caenorhabditis elegans* (Hart et al., 1995), the plant *Arabidopsis thaliana* (Lam et al., 1998) and even in the bacteria *Cyanobacterium cynechocystis* (Chen et al., 1999). iGluRs are subdivided, based on pharmacological dissimilarities, into three distinct groups: NMDA, AMPA and kainate receptors (Figure 1.2A, B). All ionotropic glutamate receptors share common structural features, being characterized by the presence of a large extracellular N-terminal domain (S1 domain; that

Figure 1.4 – Structure of the ionotropic glutamate receptors. Shown is a representation of the structure of the GluR5 subunit of kainate receptors to illustrate the general receptor topology. Like the other ionotropic glutamate receptors the N-terminus is extracellular and the C-terminus is intracellular. The C-terminus undergoes extensive splice variation and there are also editing sites at the first and, additionally, at the second transmembrane domains (see text for more details). Adapted from: *http://www.bris.ac.uk/Depts/Synaptic/info/glutamate.html*

exhibits homology with the mGluRs' agonist binding domain) followed by a first transmembrane domain and a pore forming, membrane-residing domain that does not cross the membrane but forms a reentrant loop entering from and exiting to the cytoplasm. The third and fourth transmembrane domains are linked by a large extracellular loop (S2 domain) and the fourth transmembrane domain is followed by an intracellular C-terminus (Dingledine et al., 1999; Mayer and Armstrong, 2004) (Figure 1.4). Native iGluRs are likely organized as tetrameric assemblies, comprising more than one type of subunit, where each subunit contributes with an agonist binding site, a part of the ion permeable pore and also with a cytosolic C terminus that can interact with intracellular proteins and thus modulate receptor properties. Subunits from the different classes of iGluRs do not associate to form receptors.

1.3.2.1. NMDA receptors

NMDA was the first synthetic agonist for iGluRs (Watkins, 1962) and allowed to establish glutamate as an excitatory neurotransmitter. The NMDA receptor family is composed of seven subunits: NR1, NR2A-D and NR3A and B (Figure 1.2) that are all products of separate genes and have the typical topology of iGluRs (Figure 1.4). The NR1 subunit is critical for NMDA receptor function, with receptors being comprised of NR1 and at least one NR2 subunit (Cull-Candy et al., 2001). NR3 subunits can assemble with NR1-NR2 complexes to depress NMDA receptor responses (Ciabarra et al., 1995; Das et al., 1998), and can also assemble with the NR1 subunit alone to form a glycine receptor, that is unaffected by glutamate or NMDA, and whose role in the central nervous system is still not clear (Chatterton et al., 2002). The NR1 subunit has eight different functional variants and one truncated, nonfunctional variant generated by alternative splicing at three sites within the protein, one in the N-terminus and two in the C-terminus (Carrol and Zukin, 2002; see also Dingledine et al., 1999) (Figure 1.5). In the N-terminus splicing occurs in exon 5 (also called the N1 cassette) and in the C-terminus in exons 21 and 22 (also called the C1 and C2 cassettes). Exon 22 (C2) contains an alternate acceptor splice site that, when used, splices out part of exon 22 including the stop codon and engages a new reading frame that encodes an alternative cassette C2' (Figure 1.5). Alternatively spliced forms of NR1 subunits have different regional and developmental expression profiles (Zukin and Bennett, 1995) and are regulated by neuronal activity (Mu et al., 2003), generating channel diversity. The various splice variants also differ considerably in their properties and are differentially localized in the developing and adult animal (e.g., Laurie and Seeburg, 1994; Laurie et al., 1995; Nash et al., 1997; Paupard et al., 1997; Weiss et al., 1998). Recombinant NR2 and NR3 subunits do not form functional homomeric

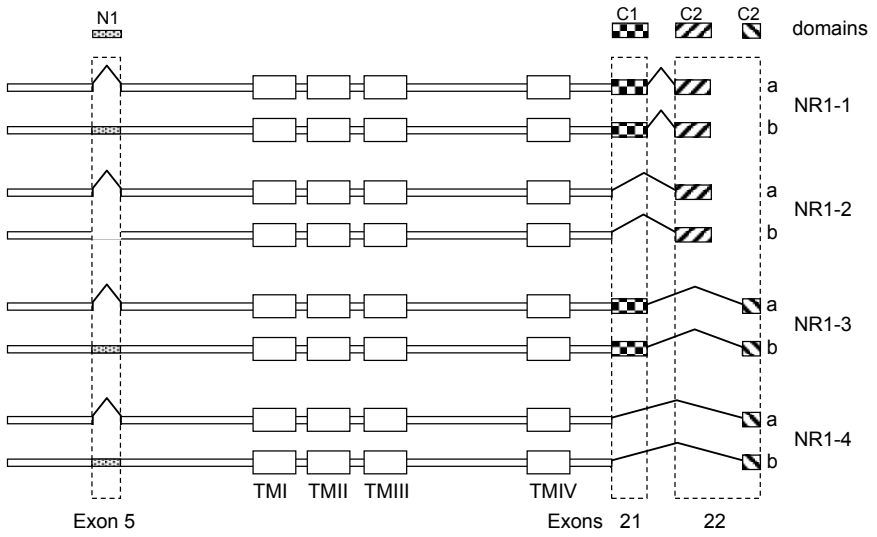

Figure 1.5 – Alternative splicing of NMDA receptor subunits. The different splice variants of the NR1 subunit arise from alternative splicing of exons 5, 21 and 22, giving rise to the cassettes N1, C1, C2 and C2'. TMI to TMIV represent the transmembrane regions of the proteins. Recreated from Dingledine et al., 1999.

receptors (Cull-Candy et al., 2001; Carroll and Zukin, 2002). The NR2 subunits share 38-53% amino acid sequence identity amongst themselves and about 27% homology with NR1 (Monyer et al., 1992; Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Ishii et al., 1993). The NR3 subunits share about 50% sequence homology and about 27% with NR1 and NR2 subunits (Ciabarra et al., 1995).

The NMDA receptor is unique amongst ligand-gated ion channels in its requirement for two obligatory co-agonists; functional NMDA receptors contain at least two glutamate-binding sites and two glycine-binding sites, implying a minimum of four subunits within the functional channel (Cull-Candy et al., 2001). At the neuron's resting membrane potential, binding of glutamate to the NMDA receptor complex is not sufficient for its gating and subsequent passage of ions through the receptor channel; previous depolarization of the membrane above -40 mV is a requisite to remove the

channel block by magnesium ions (Nowak et al., 1984, Mayer et al., 1984). This property renders Ca^{2+} flux through NMDA receptors a coincidence detector for the release of glutamate and activation of AMPA receptors in the postsynaptic membrane; NMDA receptors are functionally active only when sufficient depolarization through activation of AMPA receptors is achieved.

1.3.2.2. AMPA receptors

AMPA receptors are the major mediators of fast glutamatergic neurotransmission. They were originally named quisqualate receptors but renamed AMPA receptors since quisqualate acted also on mGluRs while AMPA acted more selectively on this group of proteins. The family comprises four subunits (GluR1-4) that are products of distinct genes (Figure 1.2B). The various subunits were cloned and expressed in recombinant systems in the late 1980's and were later shown to share 70% of mutual sequence homology (see Nakanishi and Masu, 1994). They were initially though to form pentameric heteromeric assemblies, like nicotinic acetylcholine receptors (Ferrer-Montiel and Montal, 1996), but are now believed to form functional tetramers (Mano and Teichberg, 1998; Rosenmund et al., 1998). Like NMDA receptors, they are likely of heteromeric composition, although homomeric channels are also formed in recombinant expression systems (Boulter et al., 1990; Keinänen et al., 1990). All mRNAs encoding AMPA receptor subunits may suffer alternative splicing in the region coding for the extracellular S2 loop (Flip-Flop variants) but only GluR2 and GluR4 are alternatively spliced in the C-terminal domain, giving rise to a short and a long form (Figures 1.6).

The GluR2 subunit plays a critical role in determining the permeability of heteromeric AMPA receptors to Ca^{2+} and receptors that do

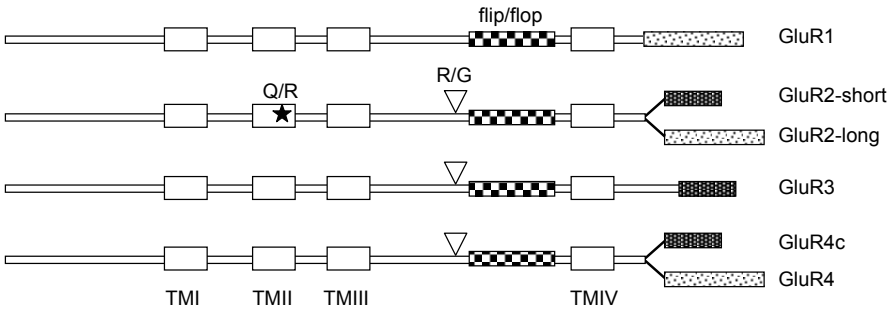

Figure 1.6 – Alternative splicing and editing of AMPA receptors (see text for details). Homologous C-termini are represented by a similar shading pattern and TMI to TMIV represent the transmembrane segments of the proteins. Recreated from Dingledine et al., 1999.

not contain GluR2 are highly Ca^{2+}-permeable, showing a marked inward rectification as the result of a voltage-dependent block of the outward channel conduction by intracellular polyamines (Bowie and Mayer, 1995). In the presence of GluR2, AMPA receptors are rather impermeable to Ca^{2+} and display linear or outwardly rectifying current-voltage relationships. The molecular determinant conferring Ca^{2+} impermeability to GluR2-containing receptors has been identified as an arginine (R) in a critical position in the pore loop (TMII domain), which is a glutamine (Q) at the corresponding site in the other subunits (see Dingledine et al., 1999; Seeburg and Hartner, 2003). This Q/R site is submitted to RNA editing and the edited form of GluR2 has low Ca^{2+} permeability (Hume et al., 1991), low single-channel conductance (Swanson et al., 1996) and an almost linear current-voltage relationship, even in heteromeric receptors (Verdoorn et al., 1991; Hume et al., 1991). The vast majority of GluR2 in the adult is edited to encode an arginine and this is even required for survival (Brusa et al., 1995). GluR2-4 subunits contain an additional editing site in the extracellular region, between the third and fourth membrane domains, where an arginine is edited to a glycine (R/G site), and editing at this site can modulate receptor desensitization kinetics (Lomeli et al., 1994).

1.3.2.3. Glutamate receptors with unknown function

Besides the cDNAs cloned by functional expression of their transcripts, other CNS proteins with some characteristics of glutamate receptors have been cloned following their biochemical isolation and characterization. Delta (δ) receptors are a class of ionotropic glutamate receptors often referred to as “orphan” glutamate receptors because they do not form functional assemblies. They are composed of GluRδ1 and GluRδ2 subunits with very local distribution in the cerebellar Purkinje cells and in the ear, respectively. The role of these receptors in synaptic transmission is unknown but they may function as accessory subunits of other iGluRs or bind some neurotransmitter or modulator other than glutamate (Yuzaki, 2003).

Another glutamate receptor, a kainate-binding protein (KABP), with homology to iGluRs but lacking approximately the first 400 residues, was discovered in the avian and frog brain (Gregor et al., 1989; Wada et al., 1989; Hollmann and Heinemann, 1994). KABPs have four putative transmembrane domains but do not appear to form homomeric or heteromeric assemblies, although they seem to be associated to G-proteins (Willard et al., 1991; Ziegra et al., 1992).

A glutamate-binding protein (GBP) and a glycine-binding protein (GlyBP), that are part of a complex of four proteins, were purified from rat brain synaptic membranes (Ly and Michaelis, 1991; Kumar et al., 1994). Their cDNAs do not have significant homology to the other cloned glutamate receptors (Kumar et al., 1991, 1995), although short stretches of amino acids show high homology to the glycine-binding domains of the NR1 subunit of NMDA receptors (Kumar et al., 1995). Reconstitution of the proteins that form this complex into planar bilayer lipid membranes leads to the formation of glutamate and NMDA activated ion channels whose activity is dependent on the presence of glycine (Aistrup et al., 1996) and is

blocked by MK-801 and ketamine, indicating that they may form NMDA receptor-like channels with the same characteristics as neuronal NMDA receptors.

A 51.6 kDa protein that has high affinity binding sites for both [^{3}H]kainate and [^{3}H]AMPA was also cloned from *Xenopus laevis* (Ishimaru et al., 1996) and denominated XenU1. Upon reconstitution, the protein forms channels that are activated by AMPA, kainate and NMDA (Kerry et al., 1993). The XenU1 protein has four hydrophobic domains that may form transmembrane domains but shares only 36-40% homology with rat non-NMDA receptors.

1.3.2.4. Kainate receptors

Among iGluRs, kainate receptors constitute the main focus of the present work and, hence, this section will be given more attention.

Discovery, cloning and structure

Kainic acid was first isolated from seaweed more than 50 years ago and was known, together with domoic acid, for causing amnesic shellfish poisoning. By the mid-1970s the excitatory and neurotoxic actions of kainate were well known and the hypothesis that this compound acted on a specific subset of receptors was formulated (Davies et al., 1979; Watkins and Evans, 1981). This was supported by the demonstration of high-affinity binding sites for [^{3}H]kainate in the rat brain (London and Coyle, 1979) and that kainate produced distinct depolarizing and desensitizing responses in C-fibers in the dorsal root ganglia (Agrawal and Evans, 1986; Huettner, 1990).

The cloning of cDNAs coding for the various subunits of kainate receptors started in the early 1990s. Two proteins that show high-affinity [^{3}H]kainate binding, with dissociation constants in the range of 5-15 nM (KA1 and KA2 subunits), were cloned (Werner et al., 1991; Herb et al., 1992; Kamboj et al., 1994). Three other kainate receptor subtypes (GluR5, GluR6 and GluR7) were also cloned and expressed as a result of screening with low stringency hybridization probes to GluR1-4 AMPA receptors (Bettler et al., 1990; Egebjerg et al., 1991; Lomeli et al., 1992; Sommer et al., 1992). These receptor subunits show a lower affinity for [^{3}H]kainate than KA1 or KA2, with dissociation constants in the range of 50-100 nM, but comparable to the affinity of a population of binding sites in the rat brain (London and Coyle, 1979; Unnerstall and Wamsley, 1983; Hampson et al., 1987).

The GluR5-7 subunits are capable of forming functional homomeric receptors when recombinantly expressed (Egebjerg et al., 1991; Bettler et al., 1992; Sommer et al., 1992; Schiffer et al., 1997). GluR5 and GluR6 were also found to associate *in vivo* (Paternain et al., 2000; Christensen et al., 2004). The high-affinity subunits KA1 and KA2 do not form functional homomeric receptors but do originate high-affinity kainate binding sites and can combine with GluR5-7 subunits to form functional receptors with modified pharmacological properties (Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992; Schiffer et al., 1997; Cui and Mayer, 1999). Evidence also suggests that in the absence of GluR5-7 subunits KA2 cannot achieve cell surface expression, being retained in the endoplasmic reticulum (Gallyas et al., 2003). The GluR5-7 receptors share 75-80% homology and the KA1 and KA2 receptors are 68% homologous (but less than 40% homologous with the AMPA receptor subunits GluR1-4), whereas homology between the two subclasses of kainate receptors is just 45%.

Kainate receptor subunits have a similar structure to the other iGluRs (Figure 1.4). They are transmembrane proteins with an extracellular

N-terminal domain followed by a first transmembrane domain (TMI) and a "p-loop" that dips into the lipid bilayer and forms the pore (TMII). Two successive transmembrane domains (TMIII and TMIV) are connected by an extracellular loop and are followed by an intracellular sequence containing the C-terminus. Recent crystallographic data has also revealed the ligand bind cores of the GluR5 and GluR6 subunits in conjugation with several agonists (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). The study by Mayer (2005) also led to the conclusion that the GluR7 subunit contains a mixture of the side chains that differ between GluR5 and GluR6, suggesting the possibility of developing pharmacological agents that bind selectively to this receptor.

Generation of diversity

Like in AMPA receptors, the mRNAs encoding for kainate receptors also suffer post-translational modifications of editing or alternative splicing (Bettler and Mulle, 1995; Dingledine et al., 1999; Lerma et al., 2001) (Figure 1.7). All splice variants of kainate receptors, with the exception of an alternative 15 amino acid exon in the N-terminal domain of GluR5, differ in their C-terminal domain (Figure 1.7). The GluR5 subunit presents four alternative splicing variants, GluR5a-d, but the last one was only found in humans (Gregor et al., 1993). The insertion of the 15 amino acid cassette gives rise to GluR5-1 while the originally discovered subunit is termed GluR5-2. Alternative splicing of the originally cloned GluR5 gene (GluR5-2b) in the C-terminus gives origin to a longer variant by insertion of new amino acids (GluR5-2c) or a shorter variant by insertion of a stop codon (GluR5-2a).

For GluR6, there are two described splice variants, GluR6a and GluR6b. A third variant, GluR6c contains an insertion of the 15ter exon and has only been described in humans. As for the GluR7 subunit, two splice

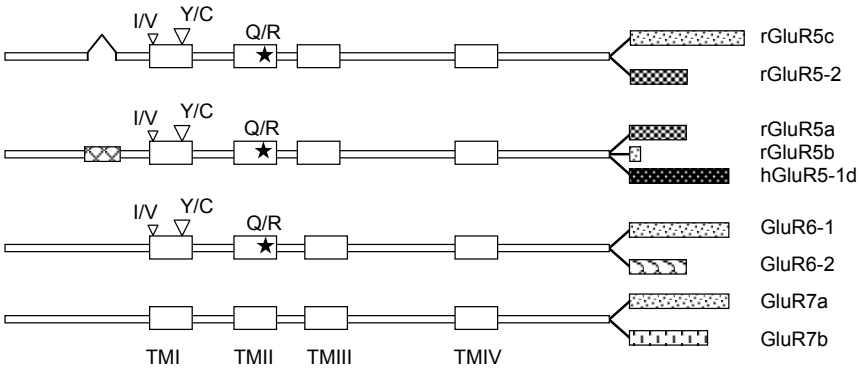

Figure 1.7 – Alternative splicing and editing of kainate receptor subunits. The diagram shows the basic structures of rat (r) and human (h) subunits with the alternative splice cassettes and editing sites (see text for more details). Homologous C-termini are represented by a similar shading pattern and TMI to TMIV represent the transmembrane portions of the proteins. Recreated from Dingledine et al., 1999.

variants were cloned; the originally discovered GluR7a and GluR7b, where almost the entire C-terminus is replaced by an unrelated 55 amino acid-long sequence (Bettler et al., 1992; Schiffer et al., 1997). The physiological relevance of alternative splicing of kainate receptors remains somewhat obscure. Certain modifications to the C-terminus can impact the trafficking processes of the receptors through the cell and also change or abolish sites of interaction with intracellular partner or regulatory proteins (Jaskolski et al., 2005).

The GluR5 and GluR6 subunits of kainate receptors can also be edited at a Q/R site in the second transmembrane domain that also determines the ability of the receptors to permeate Ca^{2+} ions. In analogy with the GluR2 AMPA receptor, the presence of an arginine residue results in a subunit that has low permeability to Ca^{2+} whereas the presence of a glutamine residue leads to subunits with higher Ca^{2+} permeability (Egebjerg and Heinemann, 1993; Burnashev et al., 1992, 1996), with a concomitant increase in the permeability to chloride ions. Like in AMPA receptors, this is also reflected in the electrophysiological properties of kainate receptors;

mature receptors comprised of non-edited subunits display inwardly rectifying current-voltage relationships owing to the block of outward current by intracellular polyamines, whereas receptors containing edited subunits resist polyamine block and have linear current-voltage relations (Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Isa et al., 1995; Kamboj et al., 1995; Koh et al., 1995; Bähring et al., 1997). In the GluR6 subunit, in addition to the Q/R site, Ca^{2+} permeability is also dependent on the edited state of two other codons in the first transmembrane domain: the I/V (isoleucine/valine) and the Y/C (tyrosine/cysteine) sites (Köhler et al., 1993). Fully edited GluR6 subunits at both TMI and TMII are essentially impermeable to Ca^{2+}. Fully unedited receptors also exhibit a higher unitary conductance as compared to receptors that include one or more edited subunits (Howe, 1996; Swanson et al., 1996). RNA editing in kainate receptors is developmentally regulated and, in the adult, up to 95% of GluR6 may exist in the edited form, against only 50-60% for GluR5.

The cytoplasmic C-terminus can be a target for phosphorylation (Dingledine et al., 1999) that may serve to regulate channel function (Raymond et al., 1993; Wang et al., 1993; Ghetti and Heinemann, 2000). GluR6 can also be palmitoylated in the C-terminus (Pickering et al., 1995) and, as well as other subunits, can interact with other proteins that contain specific PDZ domains (Garcia et al., 1998; Hirbec et al., 2003).

Kainate receptor pharmacology

In a recent past the study of kainate receptor physiology has been hampered by the lack of selective ligands and also because both AMPA and kainate receptors display a similar affinity for glutamate (EC_{50} ~300 μM; Paternain et al., 1996). In addition to glutamate, both native and recombinant kainate receptors are also activated by exogenous agonists that include kainate and domoate. However, these compounds also

activate AMPA receptors inducing large sustained currents and the selectivity towards kainate receptors is rather modest (Kiskin et al., 1986; Keinänen et al., 1990; Patneau and Mayer, 1991). In this respect exceptions exist; the GluR7 subunit not only displays a low affinity for glutamate and kainate, as compared to GluR5 or GluR6, but is also insensitive to domoate, which in fact appears to act as a high-affinity antagonist (Schiffer et al., 1997). The potential heterogeneity of kainate receptors, given the innumerous possible assemblies between the various subunits and their variants, complicates the assessment of pharmacological properties within the family.

Several kainate receptor-selective agonists have now been identified (Figure 1.8A). These include ATPA, (*S*)-5-iodowillardiine, SYM2081 and LY339434. ATPA, which is a substituted analog of AMPA, and (*S*)-5-iodowillardiine are potent, selective GluR5 agonists and display low affinity for AMPA and GluR6 or GluR7-containing receptors (Clarke et al., 1997; Swanson et al., 1998; Alt el al., 2004). GluR5 can also be activated by AMPA but ATPA and AMPA can, however, also activate heteromeric receptors composed of GluR6 and KA2 subunits. The gamma substituted glutamate analogs SYM2081 and LY339434 are also selective for kainate over AMPA receptors (Figure 1.8A). While the first displays selectivity for GluR5 and GluR6-containing kainate receptors, the later seems more selective for GluR5 over GluR6 and GluR7 (Small et al., 1998; Pedregal et al., 2000; Alt et al., 2004). Another recently isolated, naturally occurring marine toxin, dysiherbaine, also acts as a potent kainate receptor agonist (Sakai et al., 2001; Swanson et al., 2002) and has allowed to demonstrate that activation of the GluR5 subunit within a GluR5/KA2 heteromer suffices to allow the opening of the receptor channel (Swanson et al., 2002).

Compounds of the quinoxalinedione family, including CNQX and NBQX, act as competitive antagonists at native and recombinant kainate

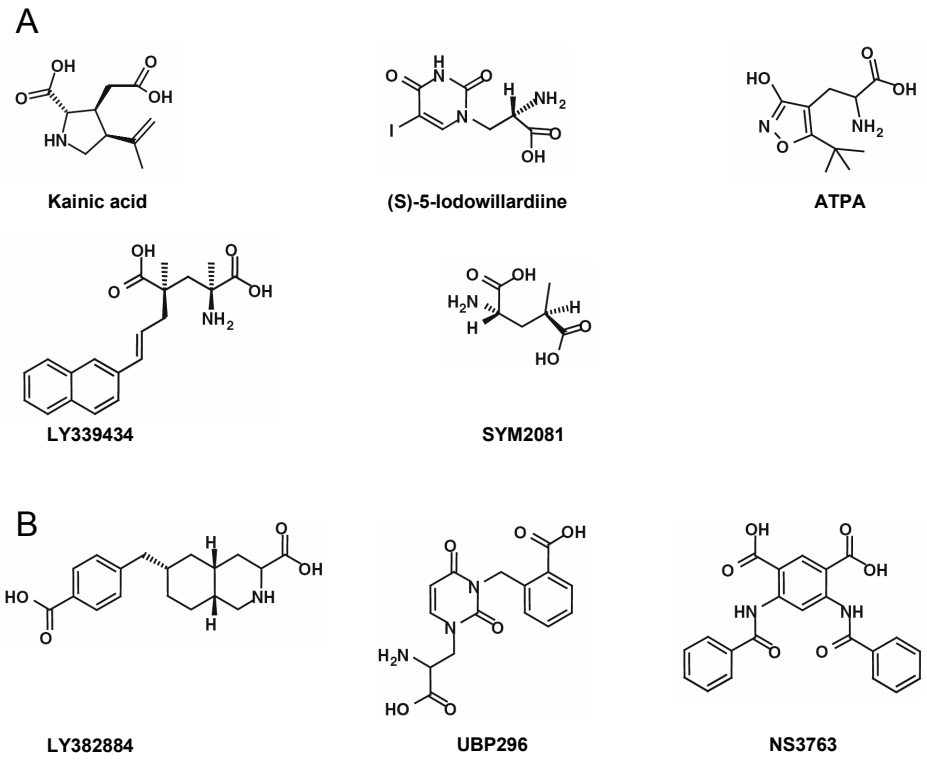

Figure 1.8 – Chemical structures of a few selective kainate receptor agonists **(A)** and antagonists **(B)**. See text for more details. Adapted from Kew and Kemp, 2005.

receptors (Figure 1.8B). However, they exhibit little selectivity for kainate over AMPA receptors. New compounds were synthesized that display more selectivity towards kainate receptors and act as potent antagonists at certain subunits. This is the case for LY382884, a compound derived from the AMPA receptor non-competitive antagonist, LY293558 that displays selectivity towards the GluR5 subunit (Bortolotto et al., 1999; Bleakman et al., 2002; Alt et al., 2004). Recently, the willardiine derivative UBP296 has been reported as the most potent and selective antagonist at GluR5-containing kainate receptors, with activity residing in the *S* enantiomer, UBP302 (More et al., 2004). Another compound, NS3763, was also

recently shown to be a non-competitive antagonist that exhibits selectivity for GluR5-containing receptors (Christensen et al., 2004).

Kainate receptor physiology

Kainate receptors can play diverse roles in glutamatergic synaptic transmission. The actions of kainate receptors at the postsynaptic level have only recently begun to be unraveled due to the synthesis of GYKI 53655 that blocks AMPA receptor mediated responses. Kainate receptor-mediated EPSCs were first described at the synapses between mossy fibers and CA3 pyramidal cells in the hippocampus (Castillo et al., 1997; Vignes and Collingridge, 1997; Mulle et al., 1998) and at excitatory synapses onto interneurons (Cossart et al., 1998; Frerking et al., 1998), and were later described at other synapses of the nervous system (reviewed by Huettner, 2003). Kainate receptor-mediated responses are much smaller and slower than AMPA receptor-mediated responses, allowing for their summation upon repetitive stimulation. These characteristics have led to the hypothesis that kainate receptors have an extrasynaptic localization, where they sense low concentrations of glutamate spilling over from the synaptic cleft (Lerma, 1997). However, the demonstration that these receptors can be activated by single quanta of glutamate (Cossart et al., 2002) and that manipulating the extracellular concentration of glutamate does not change the properties of kainate receptor-mediated responses (Bureau et al., 2000; Kidd and Isaac, 2001) have argued against this idea.

Kainate receptors can also be found at the presynaptic level where they can be activated by endogenous glutamate. They were first described in synaptosomal preparations (Malva et al., 1995, 1996; Chittajallu et al., 1996; Malva et al., 1998; Perkinton and Sihra, 1999) and found to modulate the intraterminal calcium concentration ($[Ca^{2+}]_i$) (Malva et al., 1995), to

regulate glutamate release (Chittajallu et al., 1996; Malva et al., 1996; Perkinton and Sihra, 1999), and synaptic activity at both excitatory and inhibitory synapses in the hippocampus (Clarke et al., 1997; Rodriguez-Moreno et al., 1997) and at inhibitory synapses in the hypothalamus (Liu et al., 1999).

Several plasticity phenomena at the hippocampal mossy fiber-CA3 pyramidal cell synapses seem to be due to the activation of presynaptic kainate receptors. The activation of these receptors by synaptically released glutamate, in response to successive stimuli, contributes to the unusually large frequency facilitation and paired pulse facilitation observed at this synapse (Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001). Presynaptic kainate receptors also have a permissive role in the induction of a presynaptically expressed, PKA-dependent form of LTP found at the mossy fiber synapse (Contractor et al., 2001; Schmitz et al., 2003; present work) and also contribute to a pronounced post-depolarization and Ca^{2+} rise in the presynaptic bouton (Kamiya et al., 2002). Kainate receptors also seem to increase axonal excitability through a direct depolarization of the membrane (Kamiya and Ozawa, 2000; Contractor et al., 2001) and are involved in the modulation of GABA release form interneurons in the CA1 subregion of the hippocampus (Mulle et al., 2000; Cossart et al., 2001).

The notion that kainate receptors are ionotropic glutamate receptors cannot be taken in the strict sense of an ionotropic action. It was suggested that kainate receptors on presynaptic GABAergic terminals reduce neurotransmitter release by a G-protein-mediated activation of phospholipase C and PKC (Rodriguez-Moreno et al., 1997) and it has been later shown that these receptors can be directly coupled to G-proteins (Cunha et al., 1999). In synaptosomes, kainate receptors modulate glutamate release in a PKA-dependent manner (Rodriguez-Moreno and Sihra, 2004) and modulate increases in intracellular Ca^{2+} in a G-protein-

dependent manner in dorsal root ganglion neurons (Rozas et al., 2003). Kainate receptors also regulate a slow after-hyperpolarization of CA1 pyramidal neurons through a pertussis toxin-sensitive metabotropic action (Melyan et al., 2002).

Distribution of transcripts in the hippocampus

In the hippocampal formation mRNA encoding for all the subunits of kainate receptors can be found in the main cell types, whereas the expression in interneurons is somewhat less known (see Figure 1.9). The expression of the KA1 subunit mRNA is restricted to the CA3 pyramidal cells and dentate granule cells, with virtually no expression in CA1 pyramidal cells (Werner et al., 1991; Hikiji et al., 1993; Wisden and Seeburg, 1993; Bahn et al., 1994). Although this distribution overlaps more or less with [^{3}H]kainate binding sites (Monaghan and Cotman, 1982) the distribution of transcripts for kainate receptors is more widespread. The KA2 subunit mRNA is abundant in all main cell types, whereas the GluR5 subunit appears to be virtually absent from the hippocampus, with only some punctate labelling in interneurons (Bettler et al., 1990; Wisden and Seeburg, 1993; Bahn et al., 1994). Labelling of transcripts for the GluR6 subunit are found in all main cells from CA1, CA3 and the dentate gyrus, being higher in the later and lower in the first (Egebjerg et al., 1991; Wisden and Seeburg, 1993), and can also be found in interneurons. mRNA for the GluR7 subunit is found in dentate gyrus granule cells but appears to be absent from pyramidal cells (Bettler et al., 1992; Lomeli et al., 1992; Wisden and Seeburg, 1993; Bureau et al., 1999). This subunit also appears to be expressed in some interneurons of areas CA1 and CA3. This pattern of expression of the different subunits leads to the prediction that CA1 pyramidal cells may have GluR6/KA2 receptors; CA3 pyramidal cells may have GluR6/KA1, GluR6/KA2 or GluR6/KA1/KA2 receptors; dentate

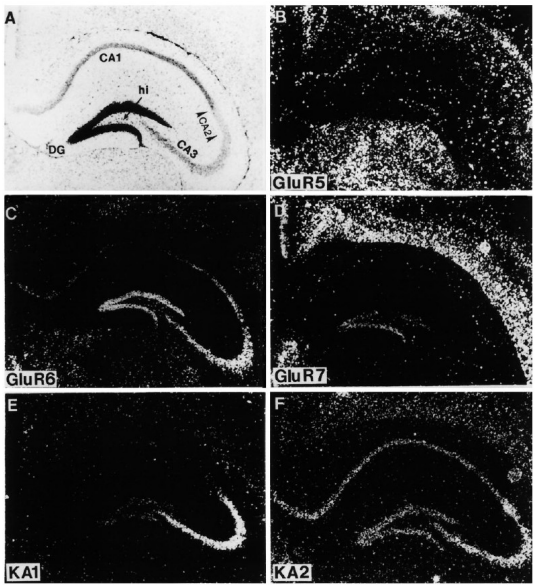

Figure 1.9 – Distribution of mRNA encoding for the five different kainate receptor subunits in the mouse hippocampus using 35^{S}-labelled riboprobes. The first panel **(A)** represents a Nissl-stained section. Adapted from: Bureau et al., 1999

granule cells may, in turn, have any number of receptors derived of various combinations of KA1, KA2, GluR6 and GluR7.

Synaptic localization and function: lessons from mutant mice

Mutant mice technology has allowed for substantial developments in the knowledge of the subunit composition and function of native kainate receptors at certain synapses. To date, global mutants for the GluR5 (Mulle et al., 2000), GluR6 (Mulle et al., 1998) and KA2 (Contractor et al., 2003) subunits have been generated. Mutant mice lacking both the GluR5 and GluR6 subunits, mutants for the GluR5 and GluR6 Q/R editing site and a myc-GluR6 knock-in are also available.

Mice lacking the GluR5 subunit were generated by homologous recombination is ES cells and display normal overall anatomy and health status. In the CA1 subregion of the hippocampus there are no changes in kainate receptor-mediated increase in IPSC frequency, no change in kainate receptor-mediated whole-cell currents from interneurons in the

stratum radiatum and no change in kainate receptor-mediated reduction of IPSC amplitude but these are all abolished if the GluR6 subunit is also removed (Mulle et al., 2000). In the CA3 subregion these mice present no changes in kainate receptor-mediated depression of evoked excitatory synaptic transmission of mossy fiber and associational commissural inputs to CA3 pyramidal cells. They do present, however, loss of kainate receptor-mediated potentiation of evoked excitatory synaptic transmission in perforant path inputs to CA3 neurons and loss of kainate-induced enhancement of mEPSC frequency in mossy fiber synapses (Contractor et al., 2000; Mulle et al., 2000; Contractor et al., 2001). These changes probably reflect expression of GluR5 in interneurons since plasticity phenomena that depend upon the direct activation of mossy fibers seem intact, although pharmacological data point to GluR5 as a presynaptic subunit at the mossy fiber synapse (Bortolotto e al., 1999; More et al., 2004; but see Breustedt and Schmitz, 2004).

Removal of the GluR6 subunit has more dramatic effects in the hippocampal formation and, although overall neuroanatomy is normal, these animals are less active than wildtypes. They don't show any changes in motor learning or special learning but are much more resistant to kainate excitotoxicity. At the cellular level there is a complete loss of kainate receptor-mediated depression of synaptic transmission both in mossy fiber and in associational-commissural inputs to CA3 neurons. In addition, kainate receptor-mediated potentiation of evoked excitatory synaptic transmission in perforant path inputs to CA3 neurons is also lost. At the mossy fiber synapse striking changes are observed; short-term synaptic plasticity is greatly reduced and LTP is nearly abolished, although PKA-dependent potentiation of mossy fiber synaptic transmission is intact (Mulle et al., 1998; Bureau et al., 1999; Contractor et al., 2000; Contractor et al., 2001). Additionally, there are no postsynaptic kainate receptor-mediated currents at this synapse (Mulle et al., 1998) and this is also observed in

cerebellar Golgi cells (Bureau et al., 2000). Therefore, at the mossy fiber synapse GluR6 appears to be a critical subunit at both pre- and postsynaptic kainate receptors.

Mice lacking the KA2 subunit do not differ from their littermates in general health status, breeding or behavior and display more subtle changes at the mossy fiber-CA3 synapse. The facilitatory effect of low concentrations of kainate is lost and the inhibition is observed at lower concentrations of agonist. The contribution of kainate receptors to the postsynaptic current is not changed, although receptor kinetics are faster. A loss of heterosynaptic facilitation is also observed, although this interpretation of the data is not very clear. Presynaptic forms of short- and long-term synaptic plasticity were not changed neither was the overall immunoreactivity for GluR5 and GluR6 subunits of kainate receptors (Contractor et al., 2003). These results reveal that KA2 seems to be part of kainate receptors on both sides of the mossy fiber synapse.

Besides the changes already referred when describing GluR5$^{-/-}$ mice, data from cultured transfected neurons of GluR5$^{-/-}$/GluR6$^{-/-}$ double mutants indicates that surface expression of GluR6b and all GluR5 splice variants is reduced but can be rescued by co-transfection with GluR6a (Jaskolski et al, 2004). Regarding the remaining kainate receptors subunits, whereas no mutant mouse lacking the KA1 subunit has been reported yet, a mouse lacking the GluR7 subunit has been generated and is subject for chapter 5 of the present report.

1.4. Objectives

The role and mechanisms of action of certain glutamate receptors in synaptic transmission are still a matter of much debate. In this respect, it is also important to understand their synaptic localizations. In the present

report we sought to answer several questions specifically regarding the synaptic localization of glutamate receptors. At present, the existence of presynaptic ionotropic and metabotropic glutamate receptors is well established, but knowledge about the subunit composition of such receptors is, in some cases, still lacking. Furthermore, an in the specific case of kainate receptors, their role in modulating presynaptic phenomena at some synapses has been documented, but the molecular identity and modes of action of such receptors are not known.

In the present work, in order to look at the subsynaptic distribution of the various glutamate receptors, in Chapter 3 we first sought to optimize a biochemical technique that allows for the selective solubilization and separation of proteins form the presynaptic active zone, postsynaptic density and of non-synaptic localizations.

We then tried to study the distribution of several metabotropic, AMPA and NMDA receptor subunits. Regarding kainate receptors, we were particularly interested in investigating their role in modifying the release of glutamate and in the correlation of such properties with their localization at the presynaptic level. For this, in Chapter 4, we investigated the modulation of [^{3}H]glutamate release and of changes in the $[Ca^{2+}]_i$ in nerve terminals from the hippocampal CA3 subregion, coupled to the use of Ca^{2+} channel blockers. We also correlated the distribution of the various kainate receptor subunits in the subsynaptic protein fractions with the data from the functional studies.

In order to be more specific in addressing the issue of kainate receptor-dependent modulation of synaptic phenomena we went on to analyze, on Chapter 5, the possible role of a kainate receptor subunit with unknown function in the brain: the GluR7 subunit. For this, we used GluR7$^{-/-}$ mice and investigated a possible participation of GluR7-containing kainate receptors in short- and long-term plasticity phenomena. We also sought to find ways to pharmacologically interfere with presynaptic kainate receptors

in order to show their presynaptic function, as an alternative to the genetic deletion model.

Chapter 2

Materials and methods

2.1. Solubilization of synaptic proteins

2.1.1. Preparation of synaptosomes

Synaptosomes were prepared based on the method described by Phillips and collaborators (2001), with modifications. The hippocampi from 8-12 animals were quickly dissected out and homogenized at 4ºC with a Teflon-glass homogenizer in 15 mL of isolation solution [0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma)]. The concentration of sucrose was raised to 1.25 M by addition of 70 mL of 2 M sucrose and 30 mL of 1 mM $CaCl_2$, and the suspension was divided into ultracentrifuge tubes. The homogenate was overlaid with 8 mL of 1.0 M sucrose, 0.1 mM $CaCl_2$ and with 5 mL of homogenization solution and centrifuged at 100,000 $\times$ g_{max} for 3h at 4ºC. Synaptosomes were gently collected at the 1.25/1.0 M sucrose interface, diluted 1:10 in cold 0.32 M sucrose with 0.1 mM $CaCl_2$ and pelleted by centrifugation at 15,000 $\times$ g_{max} during 30 min at 4ºC. The synaptosome pellets were ressuspended in a small volume of 0.32 M sucrose with 0.1 mM $CaCl_2$ and a small sample was taken for gel electrophoresis. Synaptosomes were either used immediately or stored frozen at -70 ºC.

2.1.2. Solubilization of non-synaptic and presynaptic proteins

The solubilization procedure was modified from the one decribed by Phillips and colleagues (2001). Synaptosomes were diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2$\times$ solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) pelleted by centrifugation at 40,000 $\times$ g_{max} for

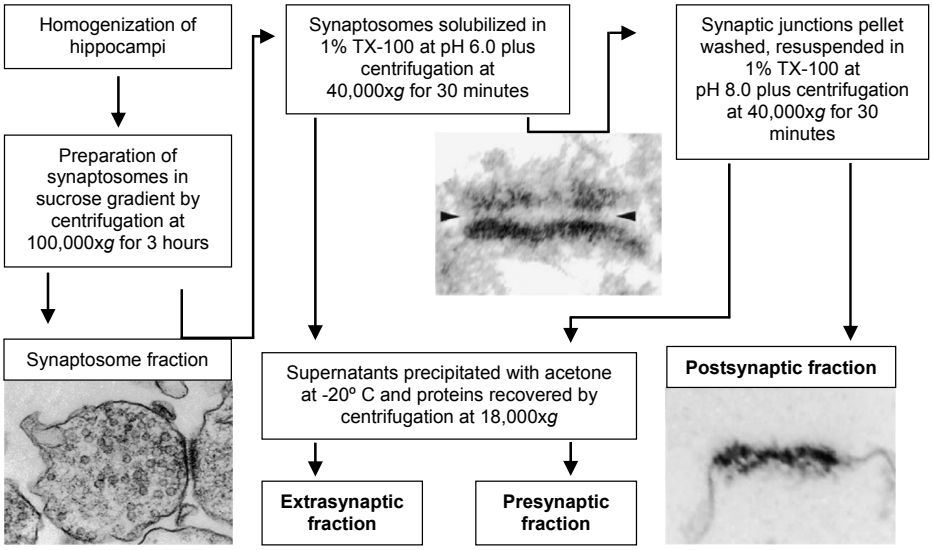

Figure 2.1 – Schematic representation of the major procedures in the method used to isolate presynaptic active zone, postsynaptic density and non-synaptic proteins from nerve terminals. Pictures adapted from Phillips et al., 2001.

30 min at 4ºC. Proteins in the supernatant (non-synaptic fraction) were precipitated with 6 volumes acetone at –20ºC and recovered by centrifugation at 18,000× g_{max} for 30 min and at -10ºC. The synaptic junctions pellet was washed twice in pH 6.0 solubilization buffer and mechanically ressuspended with a small Teflon-glass homogenizer in pH 8.0 solubilization buffer (1% Triton X-100, 20 mM Tris, pH 8.0, using 10 volumes of the initial synaptosome suspension), incubated for 30 min on ice with mild agitation and centrifuged at 40,000 × g_{max} for 30 min and 4ºC (Figure 2.1). The increase in pH from 6.0 to 8.0 disrupts the extracellular matrix that holds the presynaptic active zone tightly bound to the postsynaptic density, which results in the solubilization of presynaptic proteins, leaving the postsynaptic densities essentially preserved (Phillips et al., 2001). For maximum recovery of presynaptic proteins a second or

even third (depending on the amount of material available) solubilization step at pH 8.0 was performed from the pelleted insoluble material. The supernatants were pooled (presynaptic fraction) and processed as above for recovery of the solubilized proteins. PMSF (1 mM) was added to the suspensions in all extraction steps. The proteins recovered from the supernatants and from the final insoluble pellet (postsynaptic fraction) were solubilized in 5% SDS. Samples were further treated with SDS-PAGE sample buffer [6x concentrated; 350 mM Tris, 10% (w/v) SDS, 0.6 M DTT, 30% (v/v) glycerol, 0.06% (w/v) bromophenol blue], boiled (5 min at 98ºC) and stored at -20ºC. For some experiments the procedure was terminated before solubilizing the presynaptic active zone proteins and the synaptic junctions were treated as described for using in Western blotting.

2.1.3. Protein quantification

The protein concentration in the various samples was determined using the bicinchoninic acid (BCA) method (Pierce) and bovine serum albumin as a standard.

2.1.4. Western blot

Extracts from immunoprecipitation experiments (section 2.6) or ten to twenty five micrograms of protein from the different subsynaptic fractions were separated by SDS-PAGE on 7.5% acrylamide/bisacrilamide gels, using a Bicine/SDS-based electrophoresis buffer (pH 8.3), and transferred onto PVDF membranes (750 mA, 50 min at 4ºC in a solution containing 10 mM CAPS and 10% methanol, pH 11.0). Membrane blocking was performed for 1h at room temperature in Tris-buffered saline containing 5%

low-fat milk and 0.1% Tween 20. Primary antibodies raised against typically presynaptic (syntaxin, SNAP-25), postsynaptic (PSD-95, NMDA R1) and non-synaptic (synaptophysin, NCAM) proteins, and glutamate receptor subunits (see Table 1) were applied overnight at 4ºC and were detected using alkaline phosphatase conjugated secondary antibodies. Immunoblots were visualized using the Enhanced ChemiFluorescence detection reagent and a Versa Doc 3000 imaging system (BioRad). Broad range molecular weight standards (BioRad) were always included in each gel to allow estimation of the relative molecular weight of immunoreactive protein bands.

2.2. Measurements of $[Ca^{2+}]_i$ in hippocampal synaptosomes

2.2.1. Preparation of synaptosomes

A partially purified synaptosomal fraction (P_2) was isolated from the rat hippocampus, essentially as described previously (Malva et al., 1996). The CA3 subregion of the hippocampi of each rat was micro-dissected (see Silva et al., 2001 for details), homogenized in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, and centrifuged at 3,000 $\times$ g_{max} for 2 min. The resulting pellet was ressuspended, followed by sedimentation at the same speed. The combined supernatants were centrifuged for 12 min at 14,600 $\times$ g_{max}, and a P_2 pellet was obtained. This pellet was ressuspended in buffered sucrose medium, divided into 4 equal aliquots and the synaptosomes stored as drained pellets (Malva et al., 1996). All procedures were performed at 4ºC.

2.2.2. Ratiometric $[Ca^{2+}]_i$ measurements

The evaluation of changes in the $[Ca^{2+}]_i$ was performed by ratiometric Fura-2 fluorescence analysis. Synaptosomes were loaded with 5 μM Fura-2/AM (Molecular Probes) in a solution containing 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.2 mM H_3PO_4, 10 mM glucose, 10 mM HEPES-Na and 0.02% Pluronic F-127, pH 7.4 with 0.1% fatty acid-free bovine serum albumin for 20 min at 25ºC. After this loading period, synaptosomes were pelleted and the extracellular probe was removed. The synaptosomal pellet was ressuspended in 2 mL of assay medium containing 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES-Na, pH 7.4, incubated for 10 min at 37ºC for complete hydrolysis of Fura-2/AM to Fura-2 and transferred into an acrylic cuvette. The fluorescence of Fura-2-loaded synaptosomes was monitored at 510 nm using a computer-assisted Spex Fluoromax spectrofluorometer, with double-wavelength excitation at 340 nm and 380 nm, using 5 nm slits. The calibration was made in the presence of 2.5 μM ionomycin (R_{max}) followed by 25 mM EGTA (R_{min}). The fluorescence ratios were converted into $[Ca^{2+}]_i$ values by using the calibration equation for measurements with double-wavelength excitation and considering the dissociation constant of the Fura-2/Ca^{2+} complex as 224 nM (Grynkiewicz et al., 1985).

2.3. [^{3}H]Glutamate release from hippocampal synaptosomes

The method to monitor the release of [^{3}H]glutamate was adapted from the methodology already described (Lopes et al., 2002). Briefly, synaptosomes prepared from micro-dissected CA3 subregion slices (P2 pellet, prepared as described for $[Ca^{2+}]_i$ measurements) were loaded with [^{3}H]glutamate (2 μM) during 5 min at 37ºC, layered at the surface of

Whatman GF/C glass microfiber filters and superfused at a flow rate of 0.8 mL/min with a solution containing 120 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose, saturated with a 95% O_2/ 5% CO_2 mixture for 20 min before the beginning of each experiment. Agonists were generally applied after 7 min of sample collection and kept until the end of the experiment (total of 15 min), unless otherwise stated. Whenever receptor antagonists were used they were applied 10 min before starting sample collection and kept during the whole experiment. Higher potassium concentrations used in certain experiments were achieved by isosmotic substitution of NaCl by KCl in the superfusion medium. Sample collection was performed every minute and 0.5 mL of each sample was added to 5 mL of a liquid scintillation cocktail. Radioactivity was measured using a Packard Tri-Carb 2000 CA liquid scintillation counter with quench correction.

2.4. Generation of GluR7 mutant mice

GluR7$^{-/-}$ mice were generated by homologous recombination in ES cells. The targeting construct consisted of a 12 kb BamHI/BamHI fragment from 129Sv mouse gene in which a PGK-*neo*^{R} cassette replaced a 3.9 kb fragment overlapping the second membrane domain of GluR7 (exon 12 in *grik1* or *grik2* genes). Transfection and selection procedures into W9.5 ES cells were as previously described (Vetter et al., 1996). Positive clones (2/360) were identified by Southern blotting and injected into C57BL/6 blastocysts. One chimera transmitted the mutation through the germline and was backcrossed to C57BL/6; the resulting C57BL/6x129Sv mice were intercrossed to produce GluR7$^{-/-}$ mice. The GluR7$^{-/-}$ mice used in the present study were backcrossed with C57BL/6 mice for 5 generations (>90% C57BL/6 background). GluR7$^{-/-}$ mice did not differ from control mice

in breeding or general health status, and did not show any overt behavioral phenotype.

Note: GluR7$^{-/-}$ mice were generated at The Salk Institute, in Dr. Stephen Heinemann's laboratory, by Drs. Christophe Mulle, Bernhard Bettler and Jacques Barhanin and data about the methodologies was included due to the fact that it has not been published before.

2.5. Electrophysiology

2.5.1. Slice preparation

Paraggital hippocampal slices were prepared from postnatal day 14 to 22 C57BL/6 wildtype, or mutant mice lacking specific kainate receptor subunits, using standard techniques (Marchal and Mulle, 2004). The mice were decapitated and the brain rapidly removed under ice-cold artificial cerebrospinal fluid (ACSF) containing 120 mM NaCl, 2.5 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$ and 10-25 mM glucose. Slices were cut at a thickness of 320-350 µm using a Leica VT 1000 S vibroslicer and placed in a submerged chamber containing ACSF at room temperature for at least one hour before starting the recordings. The ACSF was saturated with a mixture of 95% O_2/5%CO_2 at all times.

2.5.2. Electrophysiological recordings in slices

Individual slices were transferred to a recording chamber and bathed continuously with ACSF saturated with a mixture of 95% O_2/5%CO_2 at a flow rate of 1-2 mL/min. Whole-cell voltage-clamp recordings were

made at room temperature (22-25ºC) on pyramidal cells of the hippocampal CA3 field, visualized by infrared video microscopy, under a Zeiss Axioskop upright microscope equipped with a 63x magnification water immersion objective. Patch pipettes were pulled from borosilicate capillaries to have a resistance of 3-4 MΩ when filled with a solution containing 122 mM CsCl, 2 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM HEPES, pH adjusted to 8.0 with CsOH. This solution was added 4 mM of Na_2ATP reconstituted in water before starting the recordings, which would also set the pH of the intracellular medium at 7.3. Cells were voltage-clamped at -70 mV and minimal intensities of stimulation, provided by a constant current isolated stimulator (Digitimer), were used to limit polysynaptic activity and the recruitment of non-mossy fiber circuits. Bicuculline (10 μM) was kept in the bathing solution at all times during recordings to block $GABA_A$ receptors. Excitatory postsynaptic currents were evoked in CA3 pyramidal cells by stimulation of the mossy fibers with a monopolar glass electrode, filled with a HEPES-based extracellular solution, placed in the dentate gyrus or in the *stratum lucidum* in the vicinity of the recorded cell. To confirm that mossy fiber responses were being recorded it was routinely verified after the experimental procedure that the group II mGluR agonist L-CCG-I, applied at a concentration of 10 μM, inhibited the EPSC amplitude by a large extent (>70%). Recordings were made using an EPC9 amplifier (HEKA), at a sample rate of 10 KHz, and analyzed using PulseFit (HEKA) and IGOR Pro programs (WaveMetrics).

Short-term synaptic plasticity

For short-term plasticity experiments, AMPA receptor-mediated EPSCs were recorded in the presence of 10 μM bicuculline and kainate receptor-mediated EPSCs were recorded in the same conditions after the application of the AMPA receptor antagonist, GYKI 53655, at a

concentration of 50 µM. In some experiments mossy fiber EPSCs were recorded in cells held at +40 mV to remove the magnesium block of NMDA receptors and allow for the simultaneous monitoring of the AMPA and the NMDA component of synaptic responses. Several protocols were used to compare short-term plasticity at the mossy fiber synapses between wildtype and GluR7$^{-/-}$ mice. Frequency facilitation was calculated by comparing the mean EPSC peak amplitude of 20 cycles of stimulation at a frequency of 0.1 Hz to that obtained for 30 cycles at 0.2 Hz, 60 cycles at 0.5 Hz, 60 cycles at 1 Hz and 100 cycles at 3 Hz. EPSC amplitude was allowed to return to the initial level between the test frequencies by repeating the basal 0.1 Hz stimulation paradigm. Paired pulse ratio was calculated from the mean peak EPSC amplitude of two pulses of stimulation given at intervals of 10 ms, 20 ms, 40 ms, 100 ms and 200 ms. To study the summation property of mossy fiber synapses a train of 5 stimuli at a frequency of 20 Hz was applied 20 times every 20 seconds at normal stimulation intensity and the mean peak EPSC amplitude obtained at each stimulation was normalized to the amplitude of the first EPSC in the series.

Long-term synaptic plasticity

Long-term synaptic plasticity was studied by following the induction and expression of long-term potentiation (LTP). LTP was induced, unless otherwise stated, by a long high frequency stimulation (L-HFS) protocol, consisting of 100 stimulations at a frequency of 100 Hz, repeated 3 times with a 10 second interval between trains, in the presence of 10 µM bicuculline and 50 µM of the NMDA receptor antagonist D-AP5. Baseline stimulation was done at a frequency of 0.05 Hz and the same intensity of stimulation was used for baseline recording and for the high-frequency train. Mossy fiber responses were monitored for an additional 40-50 min following the induction protocol and L-CCG-I was applied at the end of the

experiment. In some experiments a modified induction protocol or slightly different ionic conditions were used and these are indicated where appropriate.

2.5.3. Electrophysiological recordings of glutamate-evoked currents in HEK 293 cells

HEK 293 cells were co-transfected with GluR1, GluR6a or GluR7a subunits and GFP at a cDNA ratio of 2:1. To study heteromeric receptors, GluR7a and the edited form of GluR6a (GluR6aR) were co-transfected with GFP at a ratio of 1:3:2. Six to eight hours after transfection cells were re-plated on glass coverslips and recorded the following day. For recordings, cells were placed at room temperature in a HEPES buffered solution (HBS) containing 145 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$ 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to 320 mOsm/L, and to pH 7.4 with NaOH. Cells were observed with fluorescent illumination and isolated, brightly fluorescent cells were chosen. Recordings were made with borosilicate glass pipettes filled with a solution containing 122 mM CsCl, 2 mM NaCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 4 mM Na_2ATP and 0.06 mM spermine, adjusted to 310 mOsm/L, and pH 7.2 with CsOH. Pipette resistance was 2-4 MΩ. After the whole-cell configuration was achieved, the cell was gently pulled and lifted off the coverslip, and placed under the flow of a theta tube containing HBS, with the other barrel containing HBS, 30 mM glutamate and 30 mM sucrose to clearly see the interface between the two flows. Fast application was achieved by moving the theta tube laterally with a piezoelectric device (Burleigh) and application of glutamate was made every 20 s. Only minimal rundown of the responses was observed over 30 min, and was not corrected for. Series resistance was less than 20 MΩ, and compensated by 70 % or more. Cells were held

at –80 to –40 mV, except for current-voltage (I-V) curves where membrane voltage was held between -80 and +80 mV in 10 mV steps and voltage stepping was performed 100 ms before the glutamate application. Antagonists were applied by exchanging the flowing solutions with manual 4-way valves for at least 3 minutes and exchange between the solutions took about 1 minute. To measure the effect of antagonists at a given concentration, averages of 5 traces were taken and the ratio of their amplitude to the amplitude of traces in the control situation calculated. 1-3 concentrations of antagonist were applied for each cell. Concentration-response curves were fitted with the Hill equation, i.e. fraction = 1 - 1/(1 + $(IC_{50}/C)^h$), with C being the antagonist concentration. Least square fit was performed with Igor Pro.

2.6. Co-immunoprecipitation experiments

To determine if the GluR6 and GluR7 subunits of kainate receptors were associated *in vivo*, and in the absence of a suitable specific anti-GluR7 antibody, we used the following experimental approach; we immunoprecipitated kainate receptors from transgenic mice expressing myc-GluR6 from brain extracts after crossing of these mice with a $GluR6^{-/-}$ background (myc-GluR6x$GluR6^{-/-}$ mice; Coussen et al., 2002). Myc-GluR6 is detected either with an anti-GluR6/7 antibody or with an anti-myc antibody as a 135 kDa band, whereas the band labeled by the anti-GluR6/7 antibody at 115 kDa corresponded to GluR7. For each immunoprecipitation one brain was homogenized in 6 mL of homogenization solution containing 20 mM HEPES, 0.15 mM EDTA and 10 mM KCl, pH 7.5, supplemented with a cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin-A and pefabloc, 10 µg/mL), and centrifuged for 10 min at 520 x g_{max}. The supernatant was further centrifuged for 30 min at 18,000 x g_{max}, the

resulting pellet homogenized with 20 strokes in 15 mL of the same solution containing 15% sucrose and centrifuged at 520 x g_{max} for 10 min to remove genomic DNA. The supernatant containing the membranes was centrifuged again for 30 min at 18,000 x g_{max}. Brain membranes were solubilized in a solution containing 20 mM HEPES, 1% Triton X-100, 150 mM NaCl and 0.15 mM EDTA, with the same protease inhibitor cocktail and pH 7.5 with 20 strokes, incubated for 1 h, and samples were centrifuged for 45 min at 18,000 x g_{max}. All steps were performed at 4ºC. The solubilized fraction was cleared of non specific binding with protein-G Sepharose for one hour at 4°C. This Triton X-100 supernatant, containing 100% of kainate receptors, was then incubated with 5 µg of anti-myc antibody (Table 1) for one hour at 4°C. Then, 30 µL of protein-G Sepharose (Sigma) were added and incubated over night at 4°C. The resin was washed four times with 1 mL of loading buffer and four times with 1 mL of the same solution containing 500 mM NaCl. Beads were ressuspended in 80 µL of loading buffer and used for Western blot.

2.7. Antibodies

All antibodies used in this work are commercially available and are listed in table 1.

2.8. Animal care and maintenance

Animals used in the present studies were housed in standard conditions with a 12h light/12h dark cycle and food and water were supplied *ad libitum*. Killing of the animals was performed by decapitation and all

efforts were made to reduce animal suffering and the number of animals used to a minimum.

Table 1: Primary and secondary antibodies used for Western blot

Primary antibody	Dilution	Origin	Secondary antibody	Dilution	Origin
Rabbit anti-GluR1	1:400	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-GluR2	1:400	Chemicon	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-GluR2/3	1:400	Pharmingen	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-GluR4	1:400	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-GluR5	1:500	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-GluR6/7	1:600	Upstate	Goat anti-rabbit	1:20,000	Amersham
Goat anti-KA1	1:100	Santa Cruz	Rabbit anti-goat	1:10,000	Zymed
Rabbit anti-KA2	1:600	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-NMDA R1	1:400	Chemicon	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-NMDA R2A	1:800	Chemicon	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-NMDA R2B	1:200	Molecular Probes	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-NMDA R2C	1:800	Molecular Probes	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-mGluR1	1:1000	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-mGluR2	1:1000	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-mGluR4a	1:1000	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-mGluR5	1:1000	Upstate	Goat anti-rabbit	1:20,000	Amersham
Rabbit anti-mGluR7	1:1000	Upstate	Goat anti-rabbit	1:20,000	Amersham
Mouse anti-Syntaxin	1:3000	Sigma	Goat anti-mouse	1:20,000	Amersham
Mouse anti-SNAP-25	1:3000	Sigma	Goat anti-mouse	1:20,000	Amersham
Mouse anti-Synaptophysin	1:3000	Sigma	Goat anti-mouse	1:20,000	Amersham
Rat anti-NCAM	1:250	Pharmingen	Goat anti-rat	1:5,000	Santa Cruz
Mouse anti-PSD-95	1:50,000	Upstate	Goat anti-mouse	1:20,000	Amersham
Mouse anti-myc	IP	Roche	-	-	-
Rabbit anti-mycTag	1:1500	Upstate	Goat anti-rabbit	1:20,000	Amersham

IP: immunoprecipitation

Chapter 3

Subsynaptic localization of glutamate receptor subunits

3.1. Introduction

The study of the localization of synaptic receptors has ever been hampered by technical difficulties, mostly imposed by epitope accessibility due to the complex nature of synaptic structures (Chatha et al., 2000). Even in ultra-thin sections used for immunogold electron microscopy many antibodies prove to be ineffective at labelling proteins that sometimes are present in obvious amounts.

The concept of the synapse was originally defined in functional terms but the need for the existence of a stable physical location was also realized. The synapse, put in simple terms, is the macromolecular structure that connects neurons with each other. More elaborately, we can define it as a highly specialized junction between two neurons, or between a neuron and an effector cell (e.g., muscle or gland cell), at which electrical and/or chemical signals are passed from one cell to another. The majority of synapses in the CNS are chemical synapses; they are formed by a presynaptic element separated from the postsynaptic neuron by the synaptic cleft. In the presynaptic bouton, vesicles filled with neurotransmitters release their contents into the synaptic cleft which will activate receptors in the postsynaptic element. The pre- and postsynaptic elements are strongly bond to each other and, once formed, this connection is very resistant to physical separation. The postsynaptic element exhibits a continuous, electron dense thickening below its membrane, the so-called postsynaptic density or PSD. On the presynaptic side a grid or web of electron dense particles, arranged in a regular network, has been observed (Bloom and Aghajanian, 1968; Pfenninger et al., 1972).

A recent study showed that through selective changes in pH, during a gentle solubilization process, it was possible to more or less selectively solubilize proteins from different synaptic compartments (Phillips et al., 2001). In this chapter we describe the improvement of a simple but

powerful way to separate non-synaptic, presynaptic active zone and postsynaptic density proteins based on the original report, but with some modifications. This methodology yields highly purified fractions of these proteins and was also used in the present chapter to study the subsynaptic localization of AMPA, NMDA and various mGluRs. Kainate receptors are given more attention in the following chapters.

3.2. Results

Distribution of synaptic markers in the protein fractions

As originally described (Phillips et al., 2001), starting with hippocampal synaptosomes, non-synaptic proteins are solubilized in 1% Triton X-100 at pH 6.0, leaving the synaptic junctions (formed by the presynaptic and the postsynaptic elements still attached together) intact.

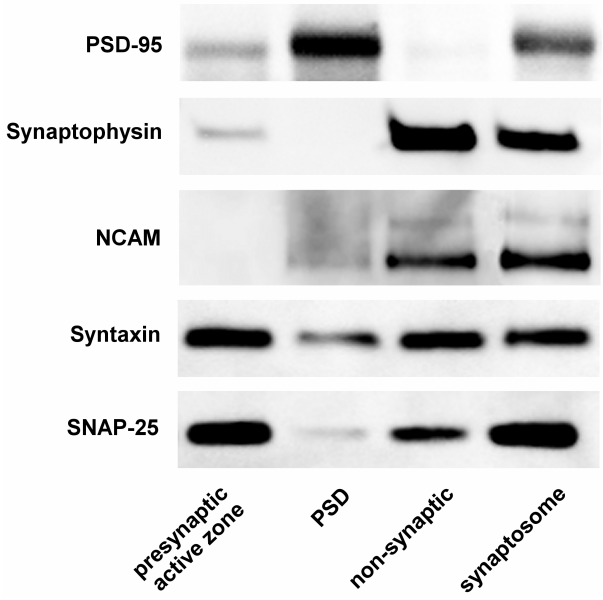

Figure 3.1 – Typical Western blot analysis of the synaptic fractions to verify the efficiency of the solubilization process. Shown are representative Western blots for proteins characteristic of the postsynaptic density (PSD-95), of non-synaptic localization (synaptophysin, NCAM) and of the presynaptic active zone (SNAP-25, Syntaxin) in the fractions derived from rat hippocampal nerve terminals.

Solubilization of the synaptic junctions in 1% Triton X-100 at pH 8.0 selectively strips away the presynaptic web from the insoluble postsynaptic densities by disrupting the extracellular matrix that holds these structures tightly bound. This is shown in figure 3.1 where non-synaptic proteins such as synaptophysin and NCAM are present in the non-synaptic (pH 6.0-soluble) fraction, being completely or almost completely excluded from the postsynaptic density or presynaptic active zone protein fractions. Presynaptic proteins, such as syntaxin and SNAP-25, were, to a large extent, recovered in the presynaptic active zone (pH 8.0-soluble) fraction, with little immunoreactivity in the pH 8.0-insoluble collection of proteins. On the other hand PSD-95, a typically postsynaptic protein, was greatly enriched in the insoluble postsynaptic density fraction and almost completely excluded (less than 5%) from the presynaptic active zone and non-synaptic fractions (Figure 3.1).

Subsynaptic distribution of metabotropic glutamate receptors

Metabotropic glutamate receptors act essentially as modulators of neurotransmission and their localization in both glutamatergic and GABAergic neurons suggests a role in modulating excitatory and inhibitory neurotransmission. The pattern of synaptic distribution of the various mGluRs has been studied using pre- and/or postembedding immunogold electron microscopy and here we studied their localization in the presynaptic, postsynaptic and non-synaptic protein fractions to compare it with the data existing in the literature. Using this methodology, immunoreactivity for mGluR1 was found, to a large extent, to be localized within the synapse, with more prominent labelling in the postsynaptic collection of proteins. Immunoreactivity for this receptor was also important in the pool of non-synaptic proteins (Figure 3.2). A very similar pattern of distribution was observed for the mGluR2 receptor (Figure 3.2). The

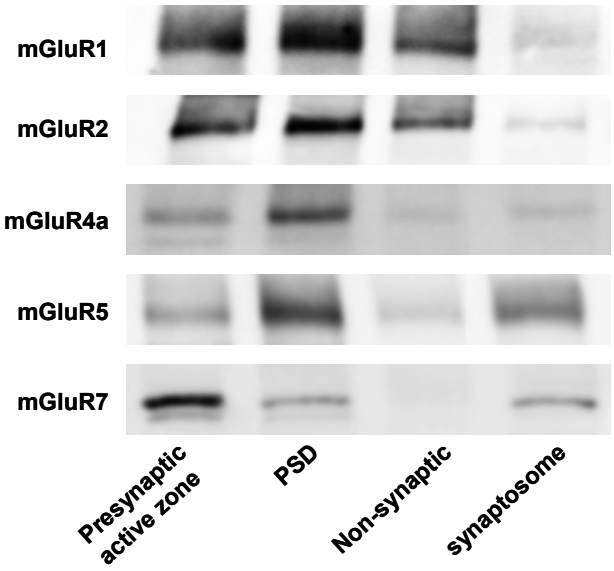

Figure 3.2 – Representative Western blots of immunoreactivity for mGluRs in the subsynaptic fractions. High Immunoreactivity for mGluR1 and mGluR2 was found in the postsynaptic density fraction but important levels were also localized in the presynaptic active zone and non-synaptic pools of proteins. mGluR4a and mGluR5 also presented a preferential postsynaptic localization and less labelling was observed for the other pools of proteins. In contrast, mGluR7 was found mostly in the presynaptic fraction and absent from the non-synaptic sites.

labelling for mGluR4a was more prominent in the postsynaptic fraction with less important labelling at both the presynaptic active zone and non-synaptic collections of proteins, and mGluR5 presented a similar subsynaptic distribution (Figure 3.2). As for the mGluR7 receptor, the only Group III receptor studied in the present work, its subsynaptic distribution was in striking contrast with that seen for the other receptors since immunoreactivity was mostly present in the presynaptic active zone protein fraction. Furthermore, some immunoreactivity was also found at the postsynaptic density but was mostly absent from the non-synaptic pool of proteins. As expected, all subunits were detected in the starting synaptosome fraction.

Subsynaptic distribution of NMDA receptors

NMDA receptors are important modulators of synaptic transmission and were initially believed to be localized exclusively on the postsynaptic side of the synapse. However, initial observations that these receptors were

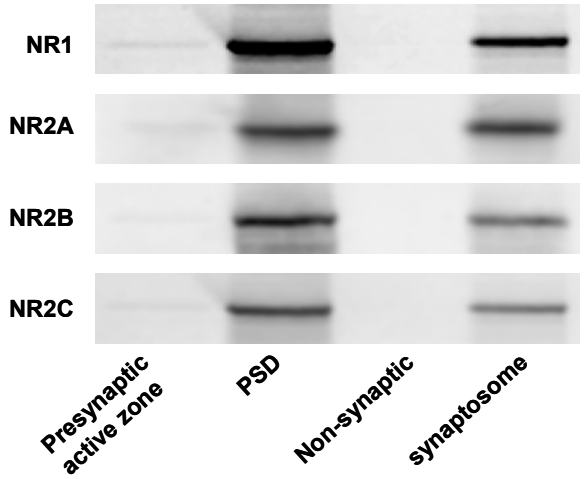

Figure 3.3 – Representative Western blots of immunoreactivity for NR1 and NR2A, B and C subunits of NMDA receptors across the subsynaptic fractions isolated by differential solubilization of synaptic proteins. All subunits are mainly found in the pool of proteins from postsynaptic densities, with very faint immunoreactivity labelling the presynaptic active zone. No immunoreactivity was found in the non-synaptic collection of proteins for any of the antibodies used.

localized presynaptically, for example in the spinal cord dorsal horn (Liu et al., 1994), started changing this view. The ultrastructural localization of NMDA receptors is fairly well documented. Here we also studied the subsynaptic distribution of some of the more well known subunits composing the NMDA family of ionotropic glutamate receptors to assess the usefulness of the technique to investigate the synaptic distribution of ionotropic receptors. The distribution of the NR1, NR2A, NR2B and NR2C subunits of NMDA receptors in the various protein fractions was very similar (Figure 3.3). Some faint labelling was observed, in all cases, in the pool of proteins from the presynaptic active zone and no immunoreactivity was present in the non-synaptic pool of proteins. Most of the immunoreactivity for these receptors (>95%, compared to the other fractionated material) was concentrated in the fraction containing the postsynaptic densities.

Subsynaptic distribution of AMPA receptors

AMPA receptors are the main mediators of glutamatergic synaptic transmission. As such they were, together with the NMDA receptors,

thought to be restricted to the postsynaptic side of the synapse, opposed to neurotransmitter release sites. However, growing evidence suggests that these receptors are also localized presynaptically. A possible role for AMPA receptors in modulating the release of catecholamines (Desce et al., 1991; Malva et al., 1994; Pittaluga and Raiteri, 1992; Wang et al., 1991), glutamate (Barnes et al., 1994; Patel and Croucher, 1997) and GABA (Satake et al., 2000) has been proposed. However, postembedding immunogold electron microscopy studies report that AMPA receptors are located exclusively on the postsynaptic specialization (reviewed by Ottersen and Landsend, 1997). Western blotting analysis of the solubilized fractions using antibodies directed against AMPA receptor subunits revealed considerably high levels of immunoreactivity in the presynaptic fraction of synaptic junctions (Figure 3.4). Immunoreactivity for the GluR1 subunit was very abundant in all three fractions; presynaptic active zone, postsynaptic density and non-synaptic. The GluR2 subunit was detected in high levels in the postsynaptic density fraction and, as occurred for the

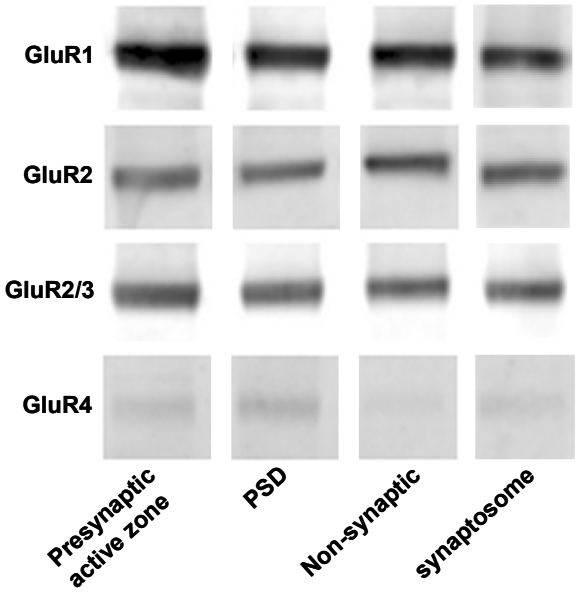

Figure 3.4 – Representative Western blots of the distribution of immunoreactivity for AMPA receptors across the subsynaptic fraction of hippocampal synapses. All subunits were found, as expected, in the postsynaptic fraction of proteins but, additionally, high levels of immunoreactivity were found in the presynaptic active zone and non-synaptic fractions for GluR1, GluR2 and GluR2/3. GluR4 was harder to detect and showed a preferential localization in the fraction of postsynaptic densities.

GluR1 subunit, strong immunoreactivity was also seen in both the presynaptic active zone and non-synaptic collections of proteins. The use of an anti-GluR2/3 antibody revealed a distribution of immunoreactivity similar to that of the GluR2 subunit across all fractions. However, since the GluR3 subunit seems to be present in the hippocampus in very modest levels (Tsuzuki et al., 2001) the strong immunoreactivity we observed may be due to the GluR2 subunit alone, since the antibody detects both. Labelling for the GluR4 subunit, as expected for the hippocampal formation (Boulter et al., 1990; Keinänen et al., 1990), was much less abundant than for the other AMPA receptor subunits, but still immunoreactivity for GluR4 was detected in all three fractions. However, unlike the other subunits, immunoreactivity for GluR4 had a preferential localization in the postsynaptic density fraction.

3.3. Discussion

An alternative and powerful way to study synaptic receptors

Starting with hippocampal nerve terminals we selectively solubilized non-synaptic proteins and presynaptic active zone proteins from the postsynaptic densities. The original method aimed at studying the structure and organization of the presynaptic web (Phillips et al., 2001). We now introduced slight modifications in order to make this method as efficient as possible in producing enriched preparations of synaptic proteins and to optimize the procedure. For this, synaptosomes were concentrated at the end of the isolation step, allowing for smaller reaction volumes to be used. This procedure also constitutes another purification step since non-synaptic membranes, that could contaminate the synaptosome fraction during its collection from the sucrose gradient, are further excluded.

We also realized that a single solubilization step was often not sufficient for maximum recovery of presynaptic proteins and that these frequently contaminated the postsynaptic density proteins in high amounts. Therefore, we began to perform two to three solubilization steps at pH 8.0 and introduced pellet washes. In these conditions we were able to obtain highly purified preparations of proteins from the presynaptic active zone, from the postsynaptic density and also from non-synaptic pools of proteins. Even so, labelling in the postsynaptic fraction for typical presynaptic proteins was evident. This may not result from contamination or incomplete solubilization since it has been previously suggested that typically presynaptic, exocytosis-related proteins may be involved in the active insertion and recycling of AMPA receptors in the postsynaptic specialization (Luscher et al., 1999). A significant proportion of these two proteins was solubilized at pH 6.0, probably reflecting synaptic and non-synaptic pools, as also discussed by Phillips and collaborators (2001).

On the other hand, a faint labelling for PSD-95 in the presynaptic fraction was also perceptible. This is probably due to a slight contamination of the presynaptic fraction, since this protein is characteristically localized within the postsynaptic density. Furthermore, the vesicle protein synaptophysin was found in minor amounts in the presynaptic active zone, likely reflecting a pool of vesicles already docked to the anchoring proteins in the presynaptic active zone. This simple technique, with the limitations in discriminating certain pools of receptors as discussed bellow, may thus prove to be a powerful tool for the research on synaptic receptors. Because the dense synaptic protein matrix has been detergent-disrupted, it circumvents the problems of epitope accessibility that may be imposed in tissue sections.

Diverse localizations of mGluRs at hippocampal synapses

In recent years it has become evident that, besides activating ionotropic receptors that mediate synaptic transmission, glutamate can also activate G-protein linked receptors, suggesting that this neurotransmitter can modulate transmission and neuronal excitability at the same synapses at which it elicits fast excitatory responses. (Pin and Duvoisin, 1995; Conn and Pin, 1997). A great deal of progress was made in defining various presynaptic and postsynaptic effects of mGluR agonists in the hippocampus (reviewed by Conn and Pin, 1997) when little was still known about their synaptic localization. In the rat brain, the activation of mGluRs contributes to both presynaptic and postsynaptic responses (Miles and Poncer, 1993; Gereau and Conn, 1995; Sánchez-Prieto et al., 1996; refer to Anwyl, 1999 for an extensive review on the actions of mGluRs).

Immunocytochemical studies have established that mGluR1 and mGluR5 are exclusively postsynaptic in the hippocampus (Martin et al., 1992; Shigemoto et al., 1993; Lujan et al., 1996). mGluR5 was also found both perisynaptically and extrasynaptically on postsynaptic spines (Lujan et al., 1996, 1997). In our study most of the immunoreactivity for mGluR1 and mGluR5 was also found in the postsynaptic fraction of proteins, with the fraction of proteins from the presynaptic web showing, nevertheless, obvious labelling. This may be due to the inability of the technique in distinguishing receptors with particular localizations, such as perisynaptic receptors. In this context, it is impossible to state if postsynaptic labelling comes mainly from the perisynaptic pool of receptors that may be attached to the structure of the PSD or if these receptors contribute to the labeling observed in the fraction of proteins from the presynaptic active zone. Nevertheless, one study localized important levels of mGluR5 in presynaptic axon terminals (Romano et al., 1995) and this receptor was also found to co-localize with adenosine A2A receptors in more than half of

striatal glutamatergic nerve terminals and to be involved in the modulation of glutamate release (Rodrigues et al., 2005). Also, it is impossible to clearly identify whether labelling in the non-synaptic pool of proteins could be due to perisynaptic receptors that where solubilized at pH 6.0, receptors that have an extrasynaptic localization or receptors contained in vesicles in process of recycling.

In contrast to Group I mGluRs, mGluR2 and mGluR3 are mainly found at presynaptic sites in the hippocampus (Petralia et al., 1996; Shigemoto et al., 1997), although in other brain structures such as the olfactory bulb (Hayashi et al., 1993) and in cerebellar Golgi cells (Ohishi et al., 1994; Neki et al., 1996, 1996b) they are found at both presynaptic and postsynaptic sites. In the present study mGluR2 was found to be present mostly in the collection of proteins containing postsynaptic densities, but important levels of immunoreactivity were also found in the fraction of proteins from the presynaptic active zone and from non-synaptic sites. This, once again, is most probably due to a limitation of the technique in discriminating pools of receptors that have perisynaptic localization and, furthermore, non-synaptic labelling can be contributed from non-synaptic membrane from both the presynaptic and the postsynaptic elements.

In the hippocampus, the selective group III mGluR agonist, L-AP4, reduces synaptic transmission at several excitatory pathways (Koerner and Cotman, 1981; Lanthorn et al., 1984; Cotman et al., 1986). Group III mGluRs were postulated to serve as presynaptic autoreceptors involved in reducing glutamate release from presynaptic terminals (Glaum and Miller, 1993) and these receptors were subsequently found to be mainly or exclusively presynaptic in the hippocampus (Bradley et al., 1996; Shigemoto et al., 1996, 1997; Kinoshita et al., 1998; Dalezios et al., 2002; Somogyi et al., 2003). Furthermore, mGluR7 was shown not only to be localized on the presynaptic membrane specialization (Shigemoto et al., 1996), within the presynaptic active zone (Dalezios et al., 2002; Somogyi et

al., 2003), but also to be mostly restricted to glutamatergic terminals. Consistent with these previous reports, our study shows that mGluR7 is mostly present in the fraction of proteins from the presynaptic active zone, with much less immunoreactivity in postsynaptic densities and no labelling in the non-synaptic pool of proteins. The result obtained for the mGluR7 receptor shows, in a simple way, the usefulness of the technique to investigate the distribution of receptors localized within the synapse, both pre- and postsynaptically.

NMDA receptors are essentially concentrated at postsynaptic densities of hippocampal synapses

Neurotransmission involving NMDA receptors has been implicated in a variety of unique roles: NMDA receptor activation is associated with long-lasting changes in synaptic strength (Ali and Salter, 2001), organization of afferent fibers with respect to target neurons during development (Collingridge and Singer, 1990) and may be a trigger for glutamate neurotoxicity (Choi and Rothman, 1990). Although NMDA receptors were initially described as having an exclusively postsynaptic localization (Petralia et al., 1994, 1994b), some studies showed presynaptic labelling for these receptors (Liu et al., 1994; Paquet and Smith, 2000) and accumulating evidence shows that they exert important modulatory actions at the presynaptic level. They were shown to increase the size of GABAergic terminals and enhance GABA release in cerebellar cultures (Fiszman et al., 2005) and cerebellar interneuron-Purkinje cell synapses (Duguid and Smart, 2004), to facilitate axonal excitability (Suárez et al., 2005), to modulate glutamate release from sensory neurons in the spinal cord dorsal horn (Bardoni et al., 2004), and provide a mechanism for coincidence detection in the induction of timing-dependent LTD (Sjöström et al., 2003), to cite a few examples.

Despite numerous evidences for the presence and function of these receptors at the presynaptic level, we detected only residual (less than 5%) labelling in the fraction of proteins from the presynaptic active zone for all the NMDA receptor subunits studied. This, together with the fact that a more or less similar amount of immunoreactivity for PSD-95 was also found in the same collection of proteins, suggests that even the small presynaptic signal for NMDA receptor subunits might be due to slight contamination of this fraction with material originating from the PSD. One possible explanation is that the presynaptic effects of NMDA receptor activation are mediated by receptors localized at dendritic or axonal compartments outside of and away from the areas of synaptic contact, being excluded from our preparation. Some presynaptic effects of NMDA receptors were also reported to be mediated by nitric oxide (NO; Sandor et al., 1995; Sequeira et al., 2001). NO is a diffusible messenger that can hypothetically be produced at postsynaptic sites and exert its actions presynaptically, which would serve as an alternative explanation to our present results.

A large pool of presynaptic AMPA receptors?

AMPA receptors are considered the most important receptors for fast excitatory neurotransmission and are of paramount importance for the postsynaptic modulation of synaptic strength (Malinow and Malenka, 2002). The existence of presynaptic AMPA receptors was suggested some years ago, but the lack of definite evidence for such receptors contributed to considerable skepticism. In the past few years, a number of reports have supported the notion of their existence, which is now bordering on general acceptance. Initially, AMPA receptors were described on the basis of their role in modulating the release of catecholamines from synaptosomes and brain slices (Desce et al., 1991; Malva et al., 1994; Pittaluga and Raiteri 1992). Also, in the hippocampus, these receptors were proposed to

modulate the exocytotic release of glutamate (Barnes et al., 1994; Patel and Croucher, 1997) and GABA (Satake et al., 2000; Patel et al., 2001).

Postembedding immunogold electron microscopy studies on AMPA receptor expression and localization on hippocampal synapses refer to these receptors as exclusively postsynaptic, with little or no importance being given to some presynaptic labelling (Nusser et al., 1998; Takumi et al., 1999). Nevertheless, in the developing striatum, GluR1 immunoreactivity was observed in presynaptic neurites forming synapses (Martin et al., 1998). GluR2 and GluR2/3 immunoreactive gold particles were also detected at some presynaptic sites in organotypic hippocampal slices (Fabian-Fine et al., 2000). Additionally, in the retrochiasmatic area and bed nucleus of the stria terminalis, GluR3 immunoreactive axon terminals of oxytocin-containing hypothalamic magnocellular neurons were found in synaptic contact with unlabeled dendrites (Ginsberg et al., 1995). Our data clearly support these previous functional and biochemical evidences for the existence of presynaptic AMPA receptors. Moreover, the data in the present study is indicative of considerably high abundance of GluR1 and GluR2/3 subunits at the presynaptic membrane. Although the levels of immunoreactivity for presynaptic AMPA receptors reported in the present work are particularly high, especially when compared to the postsynaptic fraction, these may reflect, in part, an overestimation due to the method used for the separation. Because of differences in protein composition between the presynaptic web and the postsynaptic density, a slightly higher protein yield is obtained for the postsynaptic density fraction. Therefore, the relative levels of AMPA receptor immunoreactivity in the presynaptic fraction are likely being slightly overestimated when the same amount of protein from each fraction is probed by Western blotting. This rationale holds true for every other protein analyzed.

Given the data obtained not only for synaptic marker proteins but also the diverse subsynaptic distributions of the other structurally related

glutamate receptors (see below), it seems unlikely that AMPA receptors could be behaving differently to the solubilization procedure. In fact, AMPA receptors are found anchored to the PSD in association with NMDA receptors (Lisman and Zhabotinsky, 2001). More recently, El-Husseini and colleagues reported that the dispersion of synaptic clusters of PSD-95 by acute blockade of palmitoylation causes a selective loss of synaptic AMPA receptors (El-Husseini et al., 2002), further suggesting a tight association of these receptors to the postsynaptic density. Nevertheless, these receptors were also reported to be more loosely bound to the postsynaptic density (Malenka and Nicoll, 1999) and to be stabilized and solubilized, although in cultured neurons, in a differential way when compared to NMDA receptors (Allison et al., 1998), raising the possibility that part of the high immunoreactivity observed for the presynaptic active zone fraction may indeed result from receptors stripped from the PSD. As expected, AMPA receptor subunits were found to be very abundant in the fraction of postsynaptic densities, since these receptors are the main mediators of fast excitatory synaptic transmission (Collingridge et al., 1983). Standard protocols for electrophysiological recordings are based on postsynaptic AMPA receptor activity, and this may be the reason for the lack of electrophysiological evidences for presynaptic AMPA receptor activity. But recently, electrophysiological recordings of $GABA_A$ receptor-mediated IPSC's, allowed the detection of a presynaptic AMPA receptor-mediated inhibition of GABA release (Satake et al., 2000). These receptors were also recently found, using a variation of the postembedding method, at presynaptic locations in corticostriatal and thalamostriatal synapses (Fujiyama et al., 2004).

Presynaptic activation of AMPA receptors, besides its role in modulating the release of neurotransmitters (Reviewed by Schenk and Matteoli, 2004), has also been implicated in regulating the motility of axonal filopodia (Chang and De Camilli, 2001; Tashiro et al., 2003). These

receptors also display metabotropic activity by coupling to the activation of MAPK independently of ion influx (Schenk et al., 2005). These diverse and widespread actions of presynaptic AMPA receptors further support the results reported in the present study.

We also observed that immunoreactivity for all the AMPA receptor subunits was found in significant levels in the non-synaptic fraction. This pool of proteins is formed by synaptosomal contents that are released after lysis and by vesicle and cellular membranes physically excluded from the synaptic junctions, as shown by the labelling for synaptophysin and NCAM (Figure 3.1). Therefore, intracellular membranes and the receptors associated with them in the normal intracellular traffic and recycling are likely present in this fraction. The high density of labelling in the non-synaptic pool of proteins is, however, not outstanding since it is known that in the hippocampus and cerebral cortex a major pool of these receptors resides on synaptic vesicles, so that functional receptors can be inserted into the synapses in response to neuronal activity. This is also true for presynaptic pools of AMPA receptors (Schenk et al., 2003). Labelling in this protein fraction most probably also includes receptors diffusing laterally in the membrane that can then be inserted and stabilized within synapses (Groc et al., 2004).

In summary, in this chapter we describe a technique which allowed us to show that metabotropic, AMPA and NMDA glutamate receptors have diverse, and sometimes distinct subsynaptic localizations hippocampal synapses. Our data supports some of the early anatomical and functional observations regarding AMPA and NMDA receptors and mGluRs.

Chapter 4

Presynaptic kainate receptors are localized close to release sites in rat hippocampal synapses

4.1. Introduction

Kainate receptors have been shown to be present at both presynaptic and postsynaptic sites. Postsynaptically, the activation of kainate receptors by endogenously released glutamate gives rise to EPSCs of small amplitude and slow kinetics when compared to AMPA receptor-mediated EPSCs (Castillo et al., 1997; Vignes and Collingridge 1997). Accumulating evidence seems to show that these properties of kainate receptors are due to intrinsic receptor properties (Bureau et al., 2000; Castillo et al., 1997; Cossart et al., 2002; Kidd and Isaac, 2001) rather than to an extrasynaptic or non-synaptic localization, as initially suggested (Lerma 1997). Kainate receptors can also be activated presynaptically by endogenous glutamate. The activation of kainate autoreceptors has been shown to modulate the $[Ca^{2+}]_i$ (Malva et al., 1995), to regulate neurotransmitter release at both excitatory and inhibitory synapses in the hippocampus (Malva et al., 1996; Malva et al., 1998; Chittajallu et al., 1996; Clarke et al., 1997; Rodriguez-Moreno et al., 1997) and at inhibitory synapses in the hypothalamus (Liu et al., 1999). Some studies have pointed to GluR5 (Vignes et al., 1998; Bortolotto et al., 1999; More at al., 2004; but see Breustedt and Schmitz., 2004), GluR6 (Mulle et al., 2000; Contractor et al., 2001; Schmitz et al., 2003) and KA2 (Contractor et al., 2003) as presumed presynaptic kainate receptor subunits at certain synapses. At the electron microscopy level KA1 was found to be localized pre- and postsynaptically at mossy fiber synapses in the hippocampus, whereas KA2 was found to be mostly postsynaptic (Darstein et al., 2003). However, no clear consensus seems to exist as to whether all subunits can contribute to presynaptic and postsynaptic receptors and whether or not the subunit composition of kainate receptors can be different at the two sides of the synaptic cleft.

Presynaptic kainate receptors mediate some of the unusual short-term plasticity properties observed at mossy fiber terminals in the hippocampus (Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001) and they are required for the effective establishment of mossy fiber LTP (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001), by lowering the threshold for its induction (Schmitz et al., 2003). The presynaptic modulation of short-term plasticity by kainate receptors is a fast phenomenon; presynaptic kainate receptors can sense glutamate released by a single stimulus and contribute to the large facilitation of release to a subsequent stimulus given in a time-window of a few milliseconds, suggesting that these receptors should be localized very close to release sites. However, the demonstration of their presence at such sites is missing. In the present chapter, using data from functional and molecular studies in isolated hippocampal nerve terminals, we sought to show the subsynaptic localization of the various kainate receptor subunits. We studied the efficiency of modulation of [^{3}H]glutamate release and of $[Ca^{2+}]_i$ by activation of kainate receptors as an indirect approach and we also used purified presynaptic, postsynaptic and non-synaptic protein extracts, obtained from nerve terminals, for the direct immunological identification of the various subunits and their subsynaptic distribution.

4.2. Results

Activation of presynaptic kainate receptors modulates the release of [^{3}H]glutamate

Previous studies on the modulation of glutamate release by presynaptic kainate receptors generally used high concentrations of agonists (e.g., Perkinton and Sihra 1999; Rodriguez-Moreno and Sihra

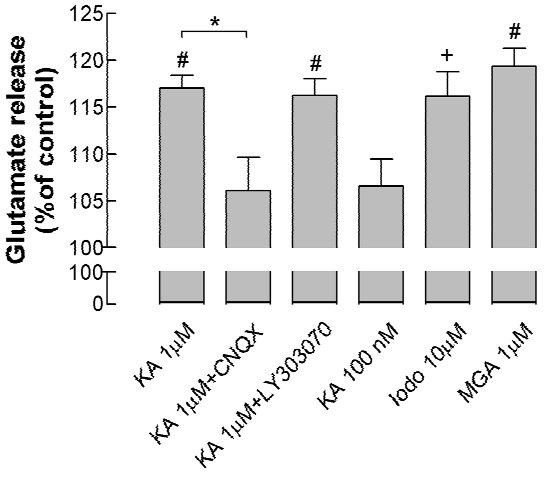

Figure 4.1 – Activation of presynaptic kainate receptors increases the basal release of [^{3}H]glutamate. In synaptosomes isolated from the rat hippocampal CA3 subregion activation of presynaptic kainate receptors with 1 μM kainate (KA), 1 μM MGA or 10 μM iodowilardiine (Iodo) causes a significant increase in the basal release of [^{3}H]glutamate (117.0 ± 1.39%, 119.3 ± 2.0% and 116.1 ± 2.6% of control, respectively). CNQX (30 μM), but not LY303070 (10 μM), significantly reduced the effect of 1 μM kainate (106.1 ± 3.6% and 116.2 ± 1.8% of control, respectively). At a concentration of 100 nM, kainate did not significantly alter the basal release of [^{3}H]glutamate (106.6 ± 2.8% of control). + $P < 0.005$ and # $P < 0.001$, in relation to the respective controls, Mann-Whitney test; * $P < 0.05$, Mann-Whitney test.

2004) that may cause unpredicted or uncontrolled effects. An earlier study in our laboratory (Malva et al., 1995) showed that activation of presynaptic kainate receptors in nerve terminals from the CA3 subregion caused an increase in $[Ca^{2+}]_i$, in a dose-dependent manner, with an EC_{50} of approximately 0.81 μM kainate. We wondered, therefore, whether kainate at concentrations considerably lower than the ones used in other studies would be efficient at modulating the release of glutamate. In synaptosomes prepared from the CA3 subregion (reportedly where the highest density of kainate binding sites resides; Monaghan and Cotman 1982; Represa et al., 1987) 100 nM kainate did not significantly alter the basal release of [^{3}H]glutamate (106.6 ± 2.8% of control; n = 4; $p > 0.05$; unpaired *t* test) (Figure 4.1). However, we observed that 1 μM kainate was the lowest concentration of agonist that caused a significant increase in the basal release of [^{3}H]glutamate in the absence of any additional depolarizing stimulus (117.0 ± 1.4% of control; n = 8; $P < 0.001$; unpaired *t* test) and this concentration was used for further studies (Figure 4.1). The release of [^{3}H]glutamate induced by the application of 1 μM kainate was not significantly different from the respective control in the presence of the

AMPA/kainate receptor antagonist CNQX (30 μM; 106.1 ± 3.6% of control, n = 4, P > 0.05; unpaired *t* test) or when the extracellular medium had virtually no calcium. Contrarily, kainate-induced glutamate release was unchanged in the presence of the AMPA receptor-selective, non-competitive antagonist, LY303070 (10 μM; 116.2 ± 1.8% of control; n = 8; P < 0.001; unpaired *t* test) (Figure 4.1), indicating the selective involvement of kainate receptors in this process. Other agonists, such as (*S*)-5-iodowillardiine (10 μM) and (2*S*,4*R*)-4-methyl glutamic acid (MGA; 1 μM) were also able to significantly modify the basal release of [^{3}H]glutamate (116.1 ± 2.6% of control; n = 8; P < 0.005 and 119.3 ± 2.0% of control; n = 8; P < 0.001, respectively; unpaired *t* test).

Since the increased release of [^{3}H]glutamate could be simply due to a kainate receptor-mediated direct depolarization of nerve terminals (Kamiya and Ozawa 2000; Schmitz et al., 2000) or, alternatively, to direct Ca^{2+} influx through the receptor channel, we compared the efficiency of 1 μM kainate to cause a rise in $[Ca^{2+}]_i$ with that of depolarizing nerve terminals by changing the extracellular potassium concentration. We found that, in synaptosomes isolated from the CA3 subregion, 1 μM kainate caused a batch increase in the $[Ca^{2+}]_i$ of 32.8 ± 4.5 nM (n = 6) whereas increasing by 2 mM the extracellular KCl concentration augmented the batch $[Ca^{2+}]_i$ by 74.7 ± 5.7 nM (n = 5) (Figure 4.2A). Depolarization of the synaptosomes with 20 mM KCl caused a very large increase in the $[Ca^{2+}]_i$ (926.9 ± 25.4 nM; n = 3) (Figure 4.2A). We next tested both situations (1 μM kainate and 2 mM KCl) in parallel in [^{3}H]glutamate release experiments and, interestingly, kainate proved to be more efficient at modifying the release of [^{3}H]glutamate (114.5 ± 2.9% of control; n = 8) than an elevation by 2 mM in the extracellular KCl concentration (96.9 ± 2.4% of control; n=8) which caused a more than two-fold greater elevation in $[Ca^{2+}]_i$ (Figure 4.2B, C). In order to determine if differences in the efficiency of the response

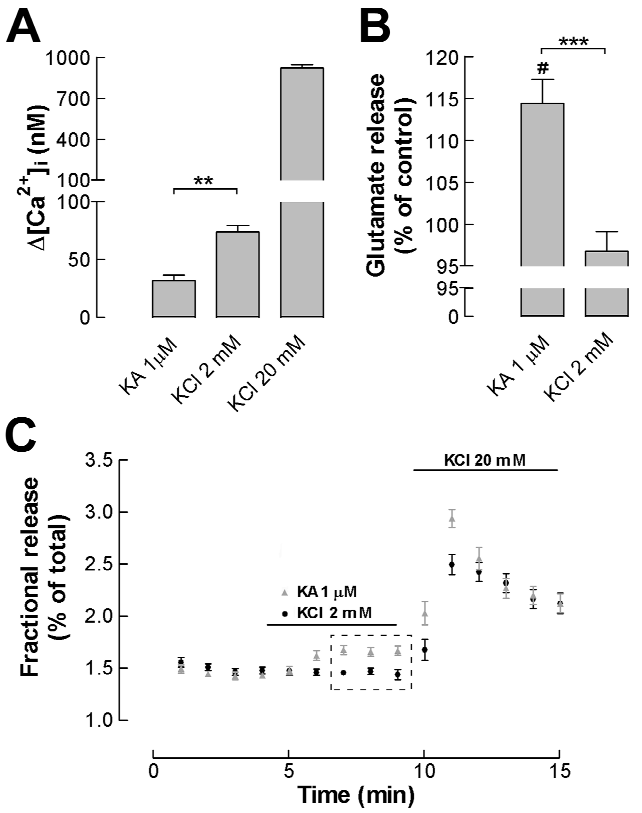

Figure 4.2 – Modulation of [^{3}H]glutamate release by kainate receptors involves a very small increase in the $[Ca^{2+}]_i$. **(A)** Activation of presynaptic kainate receptors present in CA3 subregion nerve terminals with kainate (1 μM) causes an increase in the $[Ca^{2+}]_i$ (32.8 ± 4.5 nM) that is much smaller than that caused by a 2 mM potassium-induced depolarization (74.7 ± 5.7 nM), whereas a 20 mM potassium depolarization causes massive calcium entry into nerve terminals (926.9 ± 25.4 nM). **(B)** Depolarization of nerve terminals with 2 mM potassium fails to change the basal release of [^{3}H]glutamate (96.9 ± 2.4% of control versus 114.5 ± 2.9% of control for 1 μM KA). **(C)** Representative experiment of the data shown in B, averaged from the area defined by the rectangle. # $P < 0.005$ in relation to the respective control, Mann Whitney test; ** $P < 0.005$ and *** $P < 0.001$, Mann-Whitney test.

following kainate receptor activation could depend on their synaptic localization and permeability to calcium we decided to use Ca^{2+} channel blockers as research tools. At the supra maximal concentration of 100 μM kainate, but still kainate receptor-selective in this preparation (Malva et al., 1995; Malva et al., 1996; Rodriguez-Moreno and Sihra, 2004), ω-CgTxGVIA (500 nM) plus ω-CgTxMVIIC (500 nM) reduced the calcium

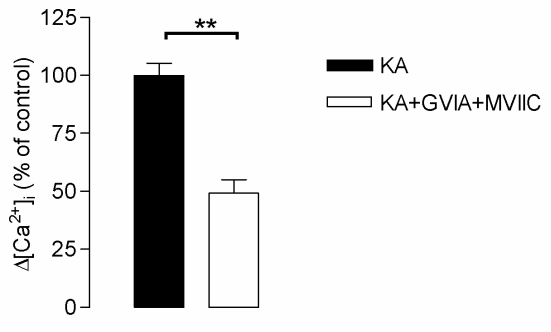

Figure 4.3 – Blocking voltage-gated calcium channels with 500 nM ω-CgTxGVIA + 500 nM ω-CgTxMVIIC reduces calcium influx upon activation of kainate receptors (100 μM KA) to 49.2 ± 5.9%. ** P < 0.005, Mann-Whitney test.

signal to 49.2 ± 5.9% (n = 5; P < 0.005 in relation to kainate alone; unpaired *t* test) (Figure 4.3), indicating that this remaining calcium should be entering directly through the receptor channel. These results strongly suggest that kainate receptors localized very close to release sites, within the presynaptic active zone, are mediating the release of [^{3}H]glutamate probably by direct permeation of Ca^{2+} through the receptor.

Presynaptic kainate receptors are localized within the active zone

It is pertinent the idea that the small calcium rise attained with low kainate concentrations could in fact be a large, localized increase in the concentration of this ion in the vicinity of kainate receptors localized within the presynaptic active zone, and this idea may help to explain its efficiency in triggering the release of glutamate. Therefore, starting with hippocampal nerve terminals we selectively solubilized non-synaptic, presynaptic active zone and postsynaptic density proteins to try to investigate the subsynaptic distribution of kainate receptor subunits, as performed in the previous chapter for other glutamate receptors. After evaluating the purity of the fractions using marker proteins (see chapter 3 for details) we next sought to study the presence of kainate receptor subunits in the presynaptic active zone. For this purpose the various fractions of synaptic material were

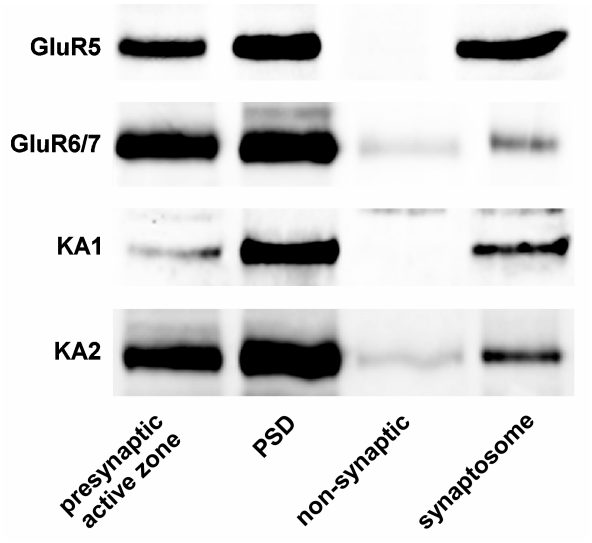

Figure 4.4 – Representative Western blots of GluR5, GluR6/7, KA1 and KA2 immunoreactivity in the subsynaptic fractions isolated by differential solubilization of synaptic proteins. All subunits are present in the collection of proteins from the presynaptic active zone and the postsynaptic density, with very faint or no labelling at all in the collection of non-synaptic proteins. The KA1 subunit predominates in the postsynaptic density when compared with the other subunits.

probed by Western blot with commercially available antibodies against kainate receptor subunits (Table 1).

Strong immunoreactivity was found for all subunits on the postsynaptic density and, with exception of the KA1 subunit, strong immunoreactivity was also found in the collection of proteins from the presynaptic active zone (Figure 4.4). Interestingly, the labelling for kainate receptor subunits in the non-synaptic fraction of proteins was always barely above background staining. These results strongly suggest that, in rat hippocampal nerve terminals, kainate receptors are localized within the sites of synaptic contact (the presynaptic active zone and the postsynaptic density) since they almost completely withstood solubilization at pH 6.0. This distribution is somewhat similar to the distribution of adenosine A_3 receptors in the rat hippocampus (Lopes et al., 2003) but is in striking contrast to what we previously reported for AMPA receptors (Pinheiro et al., 2003) or adenosine A_1 receptors (Rebola et al., 2003).

4.3. Discussion

The subsynaptic localization of neurotransmitter receptors is important in determining their synaptic functions and the outcome of their activation. Electrophysiology gives very precise results regarding response timing, duration and intensity, and data obtained with this technique suggests that presynaptic kainate receptors should be localized close to glutamate release sites, probably within the presynaptic active zone, due to their fast activation following single stimuli, as already suggested (Jaskolski et al., 2005). We show here that the activation of presynaptic kainate receptors, present at nerve terminals of the hippocampal CA3 subregion, with various kainate receptor agonists causes an increase in the basal release of [^{3}H]glutamate, an effect that has been observed previously in hippocampal CA1 subregion synaptosomes challenged with 3 μM domoic acid (Cunha et al., 2004). This effect was shown to be calcium-dependent and sensitive to the AMPA/kainate receptor antagonist, CNQX, while insensitive to the AMPA receptor-selective antagonist, LY303070. In contrast to our data, an effect over the basal release of glutamate from cerebrocortical synaptosomes was not observed in studies performed using a fluorimetric assay (Perkinton and Sihra 1999, Rodriguez-Moreno and Sihra 2004) but was previously reported by our group, using the same technique, in synaptosomes isolated from the hippocampal CA3 subregion (Malva et al., 1996). Additionally, the increase in $[Ca^{2+}]_i$ in synaptosomes isolated from the CA3 subregion of the rat hippocampus, through the activation of kainate receptors, is much larger than that observed in the other subregions (Malva et al., 1995), probably reflecting either a higher density of kainate receptors, or receptors that are more permeable to Ca^{2+} than those found in CA1 or the dentate gyrus.

We also investigated the efficiency of kainate in modulating glutamate release *versus* the rise in calcium needed to do so. The

reasoning behind these experiments was to study if a potassium-induced depolarization would mimic the effects of kainate (Schmitz et al., 2000) in terms of intraterminal calcium elevation and of modification of glutamate release. The stimulation of hippocampal CA3 subregion synaptosomes with 2 mM KCl caused a more than two-fold augmentation in the $[Ca^{2+}]_i$ compared to that achieved with 1 μM kainate; however it did not cause any change in the basal release of [^{3}H]glutamate. Our interpretation of this data is that presynaptic kainate receptors are localized within the presynaptic active zone, very close to release sites. Entry of calcium directly through the receptors, but also in part through voltage sensitive calcium channels (Figure 4.3) that we have previously shown to be localized within the presynaptic active zone and postsynaptic density but absent from non-synaptic sites (Rebola et al., 2003), may cause very confined “calcium hotspots”, just beneath the membrane. This would cause vesicle fusion and glutamate release without the need of a pronounced depolarization of the nerve terminals. In fact, the GluR7 subunit of kainate receptors, that plays a crucial role for the function of presynaptic receptors at mossy fiber synapses onto CA3 pyramidal cells (see chapter 5), exists only in the non-edited form and displays electrophysiological properties compatible with a high permeability to Ca^{2+} ions (Schiffer et al., 1997).

Another hypothesis to explain our results is that there is a massive depolarization of nerve terminals and massive calcium entry upon kainate receptor activation that would trigger the release of large amounts of glutamate. This would only occur at a very restricted population of nerve terminals containing kainate receptors and would explain the low calcium rise in the large volume where the experiment is carried out, but the comparatively high levels of glutamate that is released. This, however, does not seem likely given the very high density of labelling and binding for kainate receptors in this region (Monaghan et al., 1986; Represa et al., 1987; Darstein et al., 2003; Yoneyama et al., 2004). To further support our

conclusions, Pastuszko and colleagues have shown that application of kainate to synaptosomes causes only minor changes in transmembrane electrical potential and only when very high concentrations of this agonist are used (in the millimolar range). They also show that the minor depolarization observed was insensitive to tetradotoxin (Pastuszko et al., 1984).

To further show that kainate receptors are localized within the active zone of hippocampal synapses we used a biochemical procedure for the selective and sequential solubilization of non-synaptic and presynaptic proteins from synaptic junctions, obtaining purified preparations of these proteins, as well as a purified preparation of postsynaptic densities (chapter 3). This simple method is very powerful since it circumvents the problems of epitope accessibility that one often encounters when using other methods, because the synaptic scaffold has been detergent-disrupted, and has allowed the study of the subsynaptic localization of other receptors (Lopes et al., 2003; Pinheiro et al., 2003; Rebola et al., 2003).

An earlier study (Henley, 1995) showed, through sub-cellular fractionation techniques, that in the rat hippocampus almost all [^{3}H]kainate binding sites are found in the synaptosomal fraction with almost no binding to the microsomal fraction, where most [^{3}H]AMPA binding was located, suggesting the localization of kainate receptors within the synapse. The fact that presynaptic kainate receptors are localized within the active zone as we show here is further supported by the labelling for mGluR7 (Chapter 3), that was repeatedly reported as a presynaptic receptor and already shown to be mainly localized within the active zone of GABAergic and non-GABAergic terminals by immunogold labelling (Dalezios et al., 2002; Somogyi et al., 2003; see also chapter 3). Furthermore, in our preparation, it seems that kainate receptors are not only localized within the active zone and postsynaptic density, but they are also mostly restricted to these areas of synaptic contact. This is revealed by the immunoreactivity pattern of

synaptophysin and NCAM (see chapter 3), excluding kainate receptors from non-synaptic sites (either vesicular or non-synaptic membrane). In further support of our data, the KA1 subunit was found, using a selective antibody not available commercially, to be localized mainly in postsynaptic structures (Fogarty et al., 2000).

In conclusion, in this chapter we show two main findings; not only our data demonstrates that presynaptic kainate receptors are localized within the active zone, close to glutamate release sites, where they are in a privileged location for a fast modulation of presynaptic events, but also that all kainate receptor subunits are able to exist pre- and postsynaptically in rat hippocampal nerve terminals, not implying that they do so in the same synapses. It also adds to the notion that kainate receptors are not significantly localized at extrasynaptic sites, at least in cellular membranes included in our preparation. These results may help in understanding some of the apparently complex processes by which kainate receptors arbitrate synaptic modulation, especially at the presynaptic level.

Chapter 5

GluR7 is a presynaptic kainate receptor subunit involved in facilitation of synaptic transmission

5.1. Introduction

It has recently become evident that, besides their role in mediating fast glutamatergic neurotransmission, ionotropic glutamate receptors are also localized in presynaptic/axonal compartments where they can regulate axonal excitability or neurotransmitter release by acting as heteroreceptors or autoreceptors (reviewed by Engelman and MacDermott, 2004). Among these, glutamate receptors of the kainate type play a most prominent role in the regulation of synaptic transmission at the presynaptic level. Pharmacological activation of kainate receptors either facilitates or depresses GABAergic or glutamatergic synaptic transmission in several brain regions (Huettner, 2003; Jaskolski et al., 2005a; Lerma, 2003). A variety of possible mechanisms may account for these pharmacological effects, that can be due to either Ca^{2+} entry through presynaptic kainate receptors, direct depolarization of the nerve terminal or metabotropic regulation of voltage-gated Ca^{2+} channels (Engelman and MacDermott, 2004 ; Huettner, 2003; Lerma, 2003). There are, in comparison, relatively few reports that describe a presynaptic role for kainate receptors activated by endogenous sources of glutamate. The most compelling evidence for a physiological function of presynaptic kainate receptors is found at the synapses between mossy fibers and CA3 pyramidal cells, where they participate in short- and long-term synaptic plasticity (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2000; Schmitz et al., 2001).

To understand the precise molecular and biophysical mechanisms by which kainate receptors facilitate synaptic transmission and plasticity it is crucial to identify the subunit composition of the receptors involved. To date, the molecular identity of presynaptic kainate receptors at the mossy fiber synapse is a matter of much debate where the use of molecular, genetic and pharmacological tools has given conflicting results. Antagonists

for the GluR5 subunit block the presynaptic action of kainate receptors (More et al., 2004; Lauri et al., 2001; but see Breustedt and Schmitz, 2004), leaving postsynaptic kainate receptor-mediated EPSCs unaffected. Although these compounds clearly point to a pharmacological difference between pre and postsynaptic receptors, these data are at odds with the analysis of GluR5$^{-/-}$ and GluR6$^{-/-}$ mice, as only the latter show significant differences when compared with wildtype mice (Contractor et al., 2001). In addition, the expression of GluR5 mRNA cannot be detected by *in situ* hybridization in the rat (Wisden and Seeburg, 1993) or mouse (Bureau et al., 1999) dentate gyrus granule cell layer, where mossy fibers originate. CA3 pyramidal cells express GluR6, KA1 and KA2 subunits whereas dentate granule cells express GluR6, GluR7, KA1 and KA2 subunits. Thus, receptors comprising the GluR7 subunit are possible candidates for presynaptic kainate receptors at the mossy fiber-CA3 pyramidal cell synapse.

The GluR7 subunit of kainate receptors has been cloned more than 10 years ago and shares 75% amino acid identity with GluR5 and GluR6 and about 40% with GluR1-4 and KA1 (Bettler et al., 1992).The isolated GluR7 cDNAs encode for a predicted mature protein of 888 amino acids and a calculated molecular mass of ~100 kDa. GluR7 does not appear to be edited at the Q/R site and a glutamine is always found in this position. The pattern of GluR7 expression in the brain is widespread, being most prominently seen in the deep cortical layers, hippocampal dentate gyrus, reticular thalamic nucleus, mammilary bodies, pons and cerebellum (Bettler et al., 1992; Lomeli et al., 1992). Recombinant GluR7 receptors expressed in HEK 293 cells form functional ion channels that only respond to high concentrations of glutamate and kainate (EC_{50} = 6 mM for glutamate) (Schiffer et al., 1997). Strikingly, not only GluR7 is insensitive to the potent kainate receptor agonist, domoate, but it seems that this compound in fact acts as a high affinity antagonist at these receptors (Schiffer et al., 1997).

GluR7 exists in two different C-terminal splice variants (GluR7a and GluR7b; section 1.3.2.4.) which were shown to exhibit differential trafficking to the plasma membrane (Jaskolsky et al., 2005b). The GluR7 subunit can also co-assemble with GluR5, GluR6, KA1 and KA2 to form heteromeric receptor channels in heterologous expression systems (Schiffer et al., 1997; Cui and Mayer, 1999). The co-expression of GluR7 with GluR6 markedly reduces the responses to agonists (Cui and Mayer, 1999). Although GluR7 is expressed at high levels in various neuronal populations, its physiological role in the brain is unknown. By analyzing GluR7 deficient mice ($GluR7^{-/-}$) we demonstrate that GluR7 is a presynaptic kainate receptor that facilitates synaptic transmission, and plays a permissive role in short- and long-term synaptic plasticity, at the hippocampal mossy fiber synapse. This shows, for the first time, a physiological function for the GluR7 subunit of kainate receptors in the brain.

5.2. Results

Generation of $GluR7^{-/-}$ mice

The GluR7 gene (grik3) was disrupted by insertion of a neoR cassette that replaced genomic sequences including the entire exon coding for membrane domain 2 (TMII) necessary for receptor function. $GluR7^{-/-}$ mice did not differ from their littermates in breeding and general health status, and did not display any overt phenotype. In the absence of a selective anti-GluR7 antibody that would allow to directly test for the loss of the GluR7 protein, we sequenced the RT-PCR product obtained with the $polyA^+$ mRNA from wildtype and $GluR7^{-/-}$ mouse brains amplified with primers in TMI and in TMIV. We verified the loss of the sequence corresponding to TMII and the insertion of a stop codon (see Figure 5.1).

Figure 5.1 – Strategy used in the generation of mutant mice for the GluR7 subunit. **(A)** Top line: genomic map of the GluR7 locus around the exon coding for the TMII domain. Middle line: the targeting vector containing a neomycin resistance marker under the control of a phosphoglycerate kinase promoter (pgkneo). Bottom line: an illustration of the GluR7 locus after homologous recombination. Abbreviations for restriction sites are: E, EcoRI; B, BamHI; N, NotI ; H, HindIII. **(B)** Southern blot hybridization analysis of ES cells genomic DNA using a probe on HindIII digested DNA (left), and a neomycin probe on BamHI digested DNA (right). **(C)** Sequences of the RT-PCR product obtained with the polyA+ mRNA from wildtype (top) and GluR7$^{-/-}$ (bottom) mouse brains amplified with two primers in TMI and in TMIV. In the absence of a selective GluR7 antibody, the sequencing allows to verify the loss of the sequence corresponding to TMII and the insertion of a stop codon.

Note: *Generation of GluR7$^{-/-}$ mice was achieved by Drs. Christophe Mulle, Bernhard Bettler and Jacques Barhanin at Dr. Steve Heinemann's laboratory, but was included here since it was not published before and is part of a scientific report in revision for publication.*

GluR7 does not contribute to postsynaptic kainate receptors in CA3 pyramidal cells

We have used GluR7$^{-/-}$ mice to explore the potential contribution of this subunit at the synapses between mossy fibers and CA3 pyramidal cells in hippocampal slices. Mossy fiber synaptic responses were recorded in the whole-cell voltage-clamp mode in CA3 pyramidal cells, in the presence of bicuculline (10 µM) to block $GABA_A$ receptors. Mossy fiber synaptic responses were evoked at minimal stimulation intensity at a rate of 0.05-0.1 Hz. They were characterized by large paired pulse facilitation, pronounced frequency facilitation when shifting stimulation frequency to 1 Hz, and inhibition by L-CCG-I (10 µM), an agonist of group II mGluRs present on mossy fiber terminals.

To test whether deletion of the GluR7 gene altered the properties of postsynaptic kainate receptors, we isolated kainate receptor-mediated EPSCs by inhibiting AMPA receptors with the non-competitive antagonist GYKI 53655 (50 µM). As previously described, the mossy fiber EPSC mediated by these receptors displayed small amplitude as compared to the mixed AMPA/kainate mediated-EPSC (Figure 5.2A), as well as a slow onset and decay time (Castillo et al., 1997; Vignes and Collingridge, 1997) (Figure 5.2B). The proportion of the mossy-fiber EPSC mediated by kainate receptors was not significantly altered in GluR7$^{-/-}$ mice (5.8 ± 1.3 %; n = 8 for wildtype and 5.6 ± 0.9 %; n = 8 for GluR7$^{-/-}$; P = 0.94, unpaired *t* test) (Figure 5.2A, B). A change in decay time constant, such as that observed in KA2$^{-/-}$ mice (Contractor et al., 2003), was also not found for GluR7$^{-/-}$ mice (48.1 ± 4 .6 ms; n = 7 for wildtype and 44.6 ± 3.4 ms; n = 8 for GluR7$^{-/-}$; P = 0.54, unpaired *t* test) (Figure 5.2C, D). These results indicate that

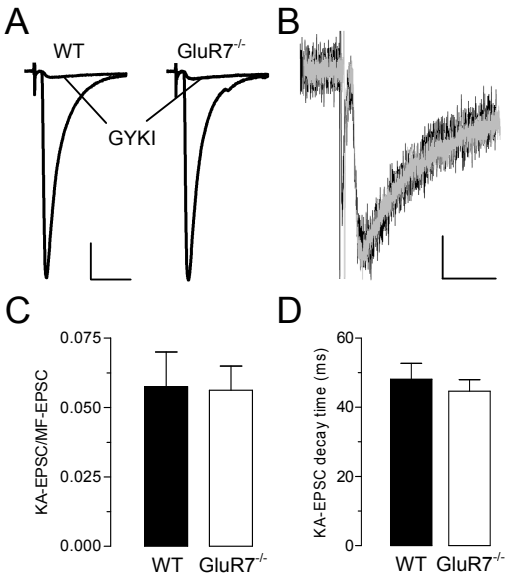

Figure 5.2 – Postsynaptic kainate receptor properties are not changed in GluR7$^{-/-}$ mice. **(A)** Sample traces of mossy fiber EPSCs (MF-EPSCs) showing the mixed AMPA/kainate receptor-mediated component and the isolated kainate receptor-mediated component in the presence of GYKI 53655, recorded from slices of wildtype and GluR7$^{-/-}$ mice and evoked at a stimulation frequency of 1 Hz. Scale bar: y axis, 50 pA for wildtype and 40 pA for GluR7$^{-/-}$; x axis, 25 ms. **(B)** Sample traces of pure kainate receptor-EPSCs from wildtype (grey trace) and GluR7$^{-/-}$ (black trace) mice. Scale bar: y axis, 1.9 pA for wildtype and 2 pA for GluR7$^{-/-}$; x axis, 20 ms. **(C)** The contribution of KAR-mediated responses for MF-EPSCs, expressed as the ratio of kainate receptor-mediated postsynaptic current in the presence of GYKI 53655 to the total AMPA/kainate receptor-mediated current, is not changed in GluR7$^{-/-}$ mice. **(D)** The decay kinetics of pure kainate receptor-EPSCs, obtained by a single exponential fit to the decay phase of the synaptic current, are not different between wildtype and GluR7$^{-/-}$ mice.

postsynaptic kainate receptors at the mossy fiber synapse do not comprise GluR7, which is consistent with the lack of expression of GluR7 mRNA in CA3 pyramidal cells (Bureau et al., 1999; Wisden and Seeburg, 1993).

GluR7 contributes to short-term synaptic plasticity at mossy fiber synapses

Presynaptic kainate receptors are involved in short- and long-term synaptic plasticity at the mossy fiber synapse (Bortolotto et al., 1999; Contractor et al., 2003; Schmitz et al., 2001). Pharmacological studies have pointed to GluR5 as a key presynaptic subunit (Bortolotto et al., 1999; Lauri et al., 2001; More et al., 2004) but, in apparent contradiction with these results, electrophysiological analysis of GluR5, GluR6 and KA2 mutant mice have so far identified kainate receptors containing the GluR6, but not the GluR5 nor the KA2 subunits, as important modulators of mossy fiber synaptic strength during synaptic plasticity (Contractor et al., 2003;

Contractor et al., 2001; Schmitz et al., 2003). GluR6 is also the key subunit for postsynaptic kainate receptors at the mossy fiber synapse (Mulle et al., 1998). The question arises as to whether presynaptic and postsynaptic kainate receptors are composed of different combinations of kainate receptor subunits, with GluR6 being part of pre and postsynaptic kainate receptors. The marked expression of GluR7 mRNA in the dentate gyrus granule cells (Wisden and Seeburg, 1993; Bureau et al., 1999) prompted us to examine the contribution of GluR7 to presynaptic kainate receptors. We thus studied several forms of short-term synaptic plasticity by looking at paired pulse facilitation, high frequency facilitation and low frequency facilitation that were shown to depend on presynaptic kainate receptors containing the GluR6 subunit (Contractor et al., 2001). We first compared paired pulse facilitation in the two genotypes (Figure 5.3A). When paired mossy fiber EPSCs were evoked at intervals ranging from 10 to 40 ms, the paired pulse ratio was markedly smaller in GluR7$^{-/-}$ mice (for 40 ms interval, 4.3 ± 0.3; n = 14 for wildtype and 2.5 ± 0.2; n = 10 for GluR7$^{-/-}$; P = 0.0002, unpaired *t* test). However, at inter-stimulus intervals of 100-200 ms, no significant difference was observed between the two genotypes.

Mossy fiber synaptic transmission displays prominent facilitation in response to a short train of stimuli applied at a high frequency (20 Hz). High frequency facilitation has been shown to be inhibited by the AMPA/kainate antagonist CNQX (Schmitz et al., 2001) and the GluR5 selective antagonist LY382884 (Lauri et al., 2001). We studied whether high frequency facilitation was also impaired in GluR7$^{-/-}$ mice by applying a train of 5 stimuli at 20 Hz every 10 seconds, and normalized the five EPSCs to the amplitude of the first event in the train (Figure 5.3B). In keeping with results described above for paired pulse facilitation, a marked difference in high frequency facilitation was observed between the two genotypes (for the fifth EPSC, 690 ± 106 %; n = 12 for wildtype and 356 ± 52; n = 13 for GluR7$^{-/-}$; P = 0.008, unpaired *t* test). These results suggest that GluR7-containing

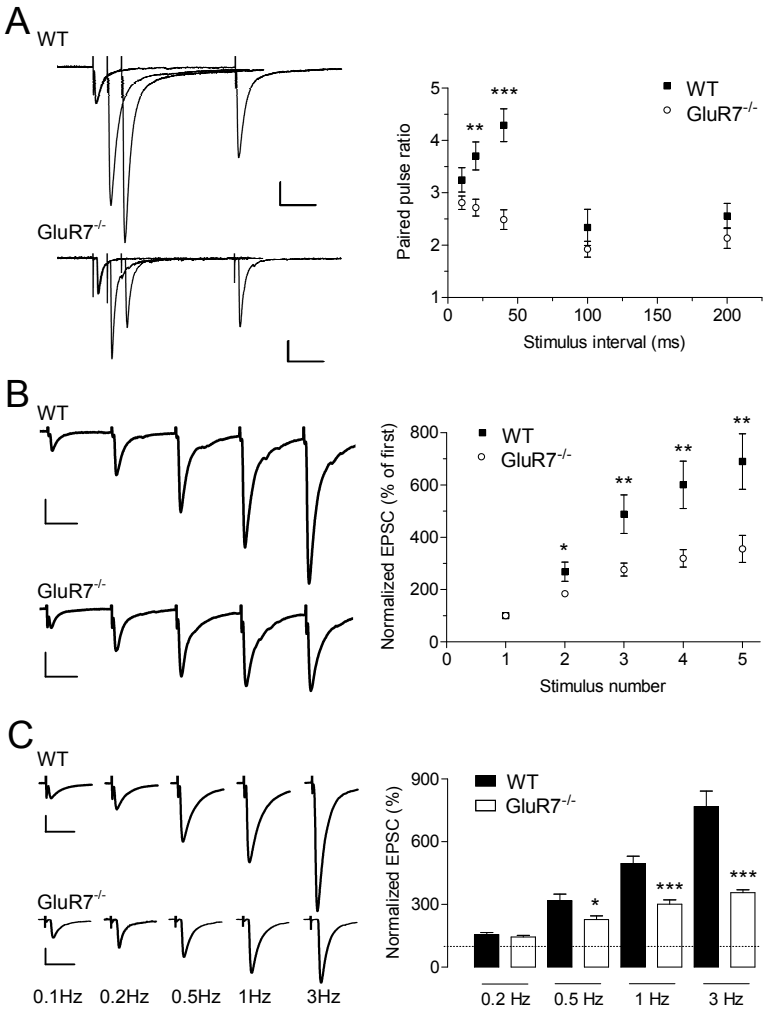

Figure 5.3 – Mossy fiber short-term synaptic plasticity is impaired in GluR7$^{-/-}$ mice. **(A)** Left panel: representative traces of averaged mossy fiber EPSCs evoked by paired stimuli delivered at intervals of 20 ms, 40 ms and 200 ms for wildtype and GluR7$^{-/-}$ mice. Scale bars: y axis, 60 pA for wildtype and 50 pA for GluR7$^{-/-}$; x axis, 50 ms. Right panel: summary graph of paired-pulse ratios at intervals ranging from 10 ms to 200 ms. Significant differences between wildtype and GluR7$^{-/-}$ mice were found for inter-stimulus intervals of 20 and 40 ms. ** P < 0.01; *** P < 0.001. **(B)** Left panel: Representative traces of averaged mossy fiber EPSCs evoked by a train of 5 stimuli delivered at a frequency of 20 Hz in slices from wildtype and GluR7$^{-/-}$ mice. Scale bars: y axis, 100 pA; x axis, 50 ms. Right panel: Summary graph of mossy fiber EPSC amplitudes in response to the 20 Hz train normalized to the amplitude of the first EPSC in the series. Significant differences were found between both genotypes for all the subsequent EPSCs in the series. * P < 0.05; ** P < 0.01. **(C)** Left panel: representative traces of averaged mossy fiber EPSCs at stimulation frequencies of 0.1-3 Hz for wildtype and GluR7$^{-/-}$ mice. Scale bars: 100 pA for wildtype and 50 pA for GluR7$^{-/-}$; x axis, 25 ms. Right panel: Summary graph of mossy fiber EPSC amplitudes for stimulation frequencies of 0.2-3 Hz normalized to the amplitude of the responses at 0.1 Hz. Significant differences between wildtype and GluR7$^{-/-}$ mice were found for stimulation frequencies of 0.5 Hz, 1 Hz and 3 Hz. * P < 0.05; *** P < 0.001.

kainate receptors facilitate mossy fiber synaptic transmission when repetitive stimuli occur within short intervals. These forms of short-term plasticity mainly occur because residual Ca^{2+} in the presynaptic terminal increases the release probability for subsequent EPSCs (Kamiya et al., 2002), suggesting a rapid action of GluR7-containing receptors (probably less than 10 ms) on the entry of Ca^{2+}.

Mossy fiber synaptic transmission displays another form of short-term plasticity, low frequency facilitation, that develops over a slower time scale with repetitive stimulation in the low frequency range (0.05 to 5 Hz), and that requires activation of CaMKII (Salin et al., 1996). We tested mossy fiber low frequency facilitation in GluR7$^{-/-}$ by increasing the stimulation frequency from 0.1 Hz to 4 different rates (0.2 Hz; 0.5 Hz; 1Hz; 3Hz). At low rates (0.2 Hz), low frequency facilitation was not different between the two genotypes. However, it was markedly impaired in GluR7$^{-/-}$ mice for the higher rates of stimulation (767 ± 75 %; n = 8 *vs.* 356 ± 15 %; n = 8 at 3 Hz for wildtype and GluR7$^{-/-}$, respectively; P = 0.0001, unpaired *t* test) (Figure 5.3C), indicating a role for GluR7 in this form of plasticity. The expression of low frequency facilitation does not require the presence of presynaptic kainate receptors, but GluR7-containing receptors markedly facilitate this form of short-term plasticity. Similar data were obtained with GluR6$^{-/-}$ mice but not GluR5$^{-/-}$ mice (Contractor et al., 2001), suggesting that both

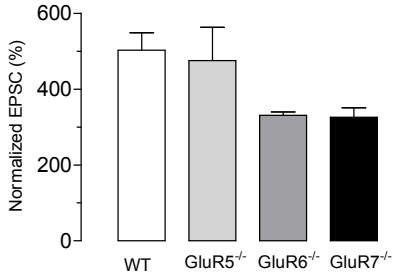

Figure 5.4 – Summary graph of mossy fiber EPSC amplitudes obtained at a frequency of stimulation of 1 Hz and normalized to the amplitude of the responses obtained at 0.1 Hz. Mossy fiber frequency facilitation is markedly reduced in GluR6$^{-/-}$ and GluR7$^{-/-}$ mice as compared to wild-type mice. However, no changes are observed in GluR5$^{-/-}$ mice. Data for wild-type and GluR7$^{-/-}$ mice is the same as in figure 5.3C.

subunits are necessary for the facilitatory effect of presynaptic kainate receptors on mossy fiber synaptic transmission. These results were reproduced in the present study and are shown for comparative purposes (Figure 5.4).

GluR7 contributes to long-term synaptic plasticity at mossy fiber synapses

Kainate receptors play an important role in mossy fiber LTP (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001). Mossy fiber LTP is a cAMP-dependent presynaptic form of synaptic plasticity that does not require activation of NMDA receptors, but depends upon Ca^{2+} entry in the mossy fiber nerve terminal and subsequent activation of a Ca^{2+}-stimulated adenylyl cyclase AC8 (Henze et al., 2000; Wang et al., 2003). As for short-term plasticity, the identity of kainate receptors involved in mossy fiber LTP is controversial, since genetic manipulations point to GluR6 as the major subunit whereas pharmacological studies identify the GluR5 subunit. Here we also examined the potential contribution of GluR7 to mossy fiber LTP. Mossy fiber LTP was induced in wildtype and GluR7$^{-/-}$ mice using a high frequency stimulation (HFS) protocol, consisting of 100 stimulations at a frequency of 100 Hz, repeated 3 times with a 10 second interval between trains, in the presence of bicuculline (10 μM) and the NMDA receptor antagonist D-AP5 (50 μM). In the first minute after the last tetanic burst mossy fiber EPSCs showed a marked post-tetanic potentiation (PTP) (Figure 5.5A). A significant difference in the magnitude of PTP was observed between the two genotypes (941 ± 121 %; n = 8 for wildtype and 534 ± 86 %; n = 9 for GluR7$^{-/-}$; P = 0.014, unpaired *t* test) (Figure 5.5A, B). This sharp increase was transient and was followed by a sustained enhancement of synaptic transmission that lasted over 40 minutes (Figure 5.5A). The percent increase in EPSC amplitude, averaged between 20 and 30 minutes after the tetani, was markedly decreased in GluR7$^{-/-}$ mice (181 ±

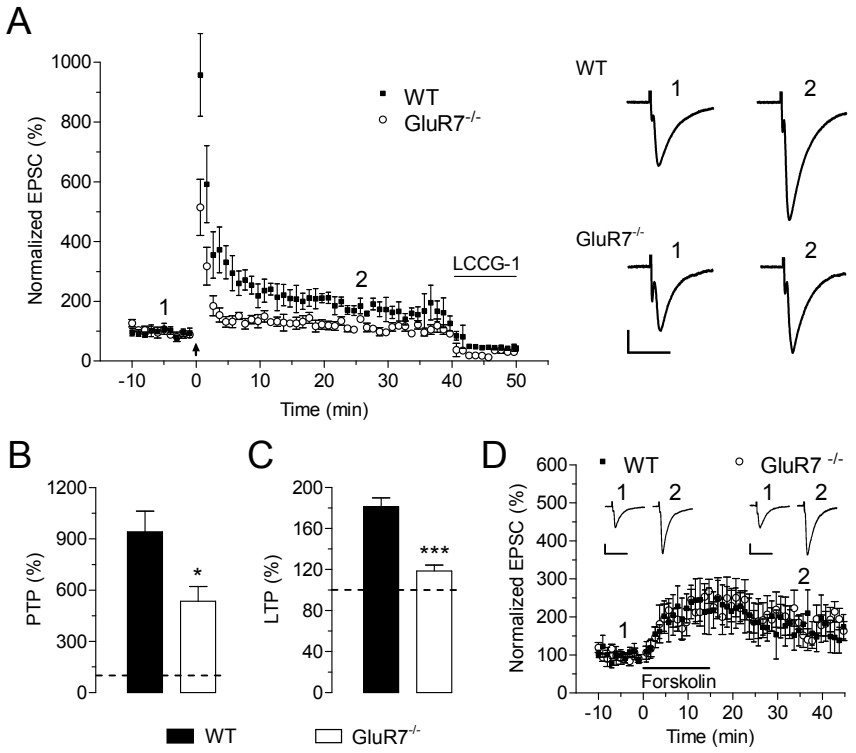

Figure 5.5 – Mossy fiber PTP and LTP are impaired in the absence of the GluR7 subunit. **(A)** Time course of mossy LTP in wildtype and GluR7$^{-/-}$ mice. LTP was induced by a high frequency stimulation protocol consisting of a 1 s duration 100 Hz train repeated 3 times with 10 s intervals in the presence of bicuculline and D-AP5. The trains were delivered at the moment indicated by the arrow and at the end of each experiment the group II mGluR agonist L-CCG-I (10 μM) was applied to confirm that the responses were indeed originating from mossy fibers. Sample traces from recordings in wildtype and GluR7$^{-/-}$ mice are shown to the right for the time points indicated in the graph. Basal stimulation was performed at 0.05 Hz. Scale bars: y axis, 50 pA; x axis, 20 ms. **(B)** Summary graph of mossy fiber PTP, calculated as the percent increase in mean EPSC amplitude averaged in the first minute following the induction protocol. PTP was observed in both genotypes but was significantly reduced in slices from GluR7$^{-/-}$ mice. * $P < 0.05$. **(C)** Summary graph of mossy fiber LTP, calculated as the percent increase in mean EPSC amplitude averaged between 20-30 min after the induction protocol. LTP was significantly reduced, but not completely abolished, in GluR7$^{-/-}$ mice. *** $P < 0.001$. **(D)** Time course showing that PKA-dependent enhancement of mossy fiber synaptic transmission by forskolin is not changed in GluR7-/- mice. Forskolin (10 μM) was applied for 15 min and mossy fibers EPSCs monitored for an additional 30 min. Sample traces for slices from wildtype and GluR7$^{-/-}$ mice are shown for the indicated time points in the time course plot. Scale bars: y axis, 50 pA for wildtype and 25 pA for GluR7$^{-/-}$; x axis, 20 ms.

8 %; n = 8 for wildtype and 119 ± 6; n = 9 for GluR7$^{-/-}$; $P < 0.0001$, unpaired *t* test) (Figure 5.5C). Mossy fiber LTP critically depends on the stimulation of adenylyl cyclase and subsequent activation of a cAMP-dependent

protein kinase. Forskolin, an activator of adenylyl cyclases, induces a long-lasting enhancement of mossy fiber EPSCs and occludes burst induced mossy fiber LTP (reviewed by Malenka and Bear, 2004). Application of forskolin (10 µM, 15 minutes) increased mossy fiber EPSCs to the same extent in both genotypes (177 ± 39 %; n = 5 for wildtype and 184 ± 29 %; n = 8 for GluR7$^{-/-}$; P = 0.89, unpaired *t* test) (Figure 5.5D). These data indicate that the impairment of mossy fiber LTP in GluR7$^{-/-}$ mice occurred upstream of PKA in the signalling cascades, and might therefore be linked to changes in Ca^{2+} entry and subsequent activation of adenylyl cyclase. These results are again closely similar to what was observed with GluR6$^{-/-}$ mice (Contractor et al., 2001).

Rescue of mossy fiber LTP in GluR7$^{-/-}$ mice

Mossy fiber LTP was not completely abolished in GluR7$^{-/-}$ mice (119 ± 6 %; n = 9; P = 0.007 in relation to baseline, unpaired *t* test), adding to the notion that kainate receptors play a facilitating or permissive rather than an inducting role in mossy fiber LTP (Schmitz et al., 2003). We thus examined whether LTP could be rescued in GluR7$^{-/-}$ mice with experimental conditions that enhance presynaptic excitability during the induction protocol, and convert a subthreshold LTP tetanus into an effective one (Schmitz et al., 2003). To depolarize the nerve terminal and therefore facilitate LTP induction, we increased the concentration of extracellular KCl to 5 mM during a short period before and during the induction protocol. The KCl concentration was returned to normal just after the induction protocol. The amplitude of mossy fiber EPSCs slightly increased upon application of 5 mM KCl (151 ± 35 %; n = 7) in GluR7$^{-/-}$ mice and the tetanus that only induced limited LTP in these mice in normal (2.5 mM) extracellular KCl concentration, now induced a large and sustained increase in mossy fiber EPSC amplitude (172 ± 39 %; n = 7) (Figure 5.6A, C). LTP was also

rescued in GluR7$^{-/-}$ mice with a more robust tetanus protocol consisting of 9 bursts of 100 Hz trains instead of 3 (Figure 5.6B, C). In these conditions, a sustained enhancement of mossy fiber EPSC amplitude was observed to a

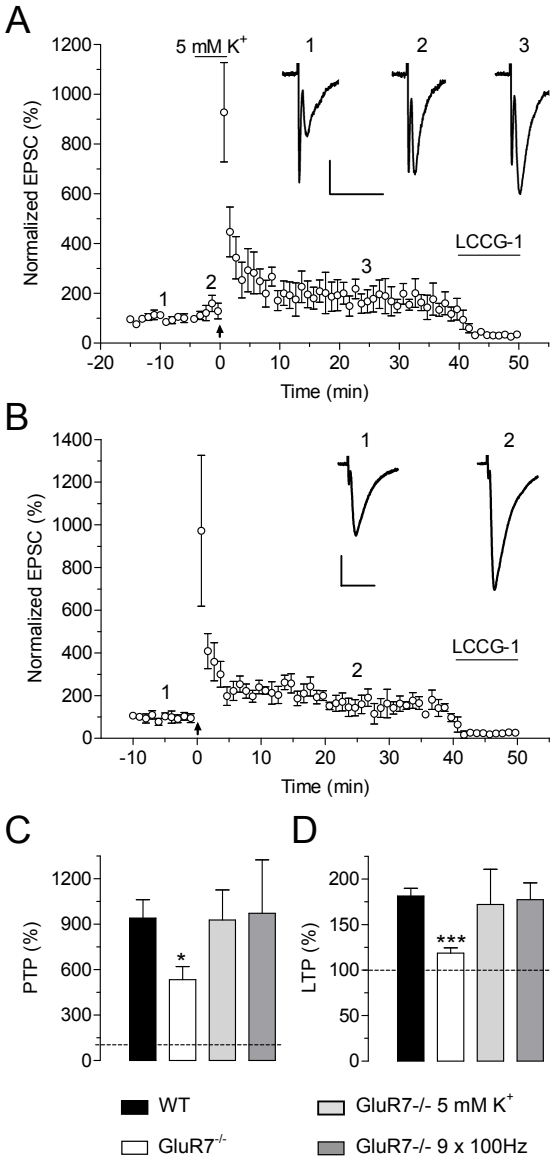

Figure 5.6 – Mossy fiber LPT can be restored by protocols that increase excitability. **(A)** Time course of mossy fiber LTP in slices of GluR7$^{-/-}$ mice where 5 mM K^+ was applied for 5 min before and during the normal induction protocol, that was performed at the time point indicated by the arrow. Mossy fiber responses were monitored for an additional 40 min after the tetanic stimulation, at which point L-CCG-I was applied. In these conditions PTP and LTP were restored. K^+ by itself slightly increased mossy fiber responses. Sample traces for the indicated time points in the graph are also shown. Basal stimulation was performed at 0.05 Hz. Scale bars: y axis, 20 pA; x axis, 20 ms. **(B)** Time course of mossy fiber LTP in slices of GluR7$^{-/-}$ mice where the normal induction protocol was repeated 3 times at 10 s intervals in the presence of bicuculline and D-AP5 at the time point indicated by the arrow. In these conditions PTP and LTP were also restored. Sample traces for the indicated time points are shown. Basal stimulation was performed at 0.05 Hz. Scale bars: y axis, 40 pA; x axis, 20 ms. **(C)** Summary graph of mossy fiber PTP, calculated as the percent increase in mean EPSC amplitude averaged in the first minute after the induction protocol. Impaired PTP observed in GluR7$^{-/-}$ mice can be restored to the levels found in wildtype mice by increasing the extracellular K^+ concentration or by providing additional stimuli for the induction protocol. **(D)** Summary graph of mossy fiber LTP, calculated as the percent increase in mean EPSC amplitude averaged between 20-30 min after the induction protocol. Reduced LTP in slices from GluR7$^{-/-}$ mice can be restored to levels similar to those found in slices from wildtype animals by the same experimental protocols that restore PTP.

magnitude similar to control conditions in wildtype mice (177 ± 19 %; n = 5). In parallel, both protocols also lead to restored PTP in GluR7$^{-/-}$ mice (Figure 5.6D). Similar results were obtained with GluR6$^{-/-}$ mice in a previous study (Schmitz et al., 2003) suggesting that kainate receptors containing both GluR6 and GluR7 facilitate the induction of mossy fiber LTP either by depolarizing the nerve terminal or by directly mediating Ca^{2+} entry into the presynaptic boutons.

GluR7-containing receptors are not involved in the inhibitory action of kainate on mossy fiber EPSCs

At the mossy fiber synapse, activation of kainate receptors by the endogenous release of glutamate is involved in the facilitation of mossy fiber synaptic responses. However, at this synapse, pharmacological activation of kainate receptors by the exogenous agonist kainate modulates the release of glutamate in a bidirectional manner depending on the concentration that is used; low concentrations seem to facilitate release whereas high concentrations cause its inhibition. The most commonly accepted interpretation for this phenomenon is that a small depolarization of the nerve terminal in response to low concentrations of kainate might be enough to facilitate glutamate release, whereas a larger depolarization might lead to inactivation of Na^+ or Ca^{2+} channels and subsequently inhibit neurotransmitter release (see Lerma, 2003). However, this interpretation is not fully satisfactory and does not take into account the possibility that different receptor subtypes might be involved in each action. Analysis of mutant mice revealed that GluR6 containing receptors are involved in both the facilitatory (Contractor et al., 2003) and the inhibitory (Contractor et al., 2000) actions of kainate. We examined whether GluR7 was similarly involved in both actions. In our hands, the application of 50 nM kainate did not cause any change in the amplitude of mossy fiber EPSCs (93 ± 11%; n

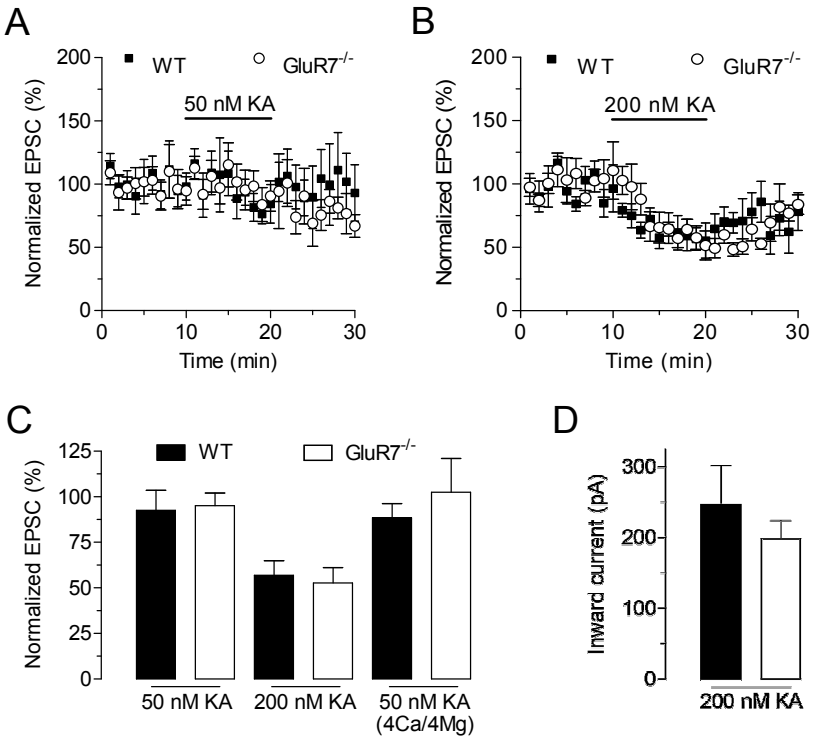

Figure 5.7 – GluR7-containing kainate receptors are not involved in the inhibitory action of kainate on mossy fiber responses. **(A)** Time course of mossy fiber EPSCs during the application of 50 nM kainate in the extracellular medium. At this concentration, and in our experimental conditions, kainate applied for 10 min has no enhancing effect on mossy fiber synaptic transmission in slices from wildtype or GluR7$^{-/-}$ mice. Basal stimulation was performed at 0.05 Hz. **(B)** Time course of mossy fiber EPSCs during the application of 200 nM kainate in the extracellular medium. When kainate is applied for 10 min a marked decrease in mossy fiber EPSC amplitude is observed. The degree of inhibition of synaptic transmission is similar in slices from both wildtype and GluR7$^{-/-}$ mice and responses are recovered upon washout. Basal stimulation was performed at 0.05 Hz. **(C)** Summary graph for the changes in mossy fiber synaptic transmission upon application of low (50 nM) or high (200 nM) concentrations of kainate. In either case no differences between genotypes are observed and the enhancing effect of kainate is not apparent even in the presence of higher concentrations of divalent cations (4 mM Ca^{2+} and 4 mM Mg^{2+}). **(D)** Bath application of 200 nM kainate causes the activation of a postsynaptic inward current, with similar average amplitude, in neurons recorded in slices of wildtype and GluR7$^{-/-}$ mice.

= 6 for wildtype and 95 ± 7%; n = 9 for GluR7$^{-/-}$; basal stimulation rate at 0.05Hz) (Figure 5.7A, C). Since the sensitivity to kainate was reported to be dependent on the extracellular concentration of Ca^{2+} (Lauri et al., 2003), we repeated the experiment with 4 mM Ca^{2+} and 4 mM Mg^{2+} in the extracellular medium. Even under these conditions, we could not find a concentration of kainate that lead to a significant increase in the amplitude of mossy fiber

EPSCs (98 ± 8% for wildtype, n = 10; 114 ± 11% for GluR7$^{-/-}$; n = 8, for 50 nM kainate) (Figure 5.7C). The reason for this is unclear, but it may be in part due to a species or a genetic background difference. In fact, it has been shown that not only different strains of mice have different susceptibility to kainate induced seizures and neuronal damage (Golden et al., 1991; Ferraro et al., 1995; McKhann et al., 2003; Schauwecker, 2003; Schauwecker et al., 2004) but also show marked dissimilarities in calcium signalling at distal dendrites (Shuttleworth and Connor, 2001). Nevertheless, at concentrations higher than 100 nM, kainate consistently induced a similar decrease in synaptic transmission in both wildtype and GluR7$^{-/-}$ mice (inhibition to 57 ± 8 % of control; n = 6 for wildtype and to 53 ± 9% of control; n = 8 for GluR7$^{-/-}$; P = 0.74 between genotypes at 200 nM kainate, unpaired *t* test) (Figure 5.7B, C), in parallel with the activation of an inward current in the postsynaptic neuron (199 ± 25 pA; n = 6 for wildtype and 247 ± 55pA; n = 8 for GluR7$^{-/-}$; P = 0.40 at 200 nM kainate, unpaired *t* test) (Figure 5.7D). In contrast with what was observed with GluR6$^{-/-}$ mice (Contractor et al., 2000), the inhibitory action of high concentrations of kainate on synaptic transmission was thus preserved in GluR7$^{-/-}$ mice. These results indicate that GluR7 contributes to the facilitation of mossy fiber synaptic transmission (in short-term synaptic plasticity protocols) but not its depression, suggesting that two different populations of kainate receptors are involved in the bi-directional regulation of glutamate release.

Co-assembly and sub-cellular localization of GluR6 and GluR7 in vivo

The facilitatory function of presynaptic kainate receptors during short and long-term synaptic plasticity is similarly impaired in both GluR6$^{-/-}$ and GluR7$^{-/-}$ mice. The fact that both subunits seem necessary for the facilitation of synaptic transmission suggests that they might co-assemble to form presynaptic heteromeric kainate receptors at the mossy fiber

synapse. It is already known that GluR6 and GluR7 readily co-assemble to form functional heteromeric receptors in recombinant systems (Cui and Mayer, 1999; Jaskolski et al., 2005b). We examined whether the two subunits also co-assembled *in vivo* using a biochemical assay. In the absence of a suitable anti-GluR7 antibody, we used the following experimental approach. We prepared Western blots from hippocampal extracts of knock-in transgenic mice expressing myc-GluR6a under the control of the α-CAMKII promoter on a GluR6$^{-/-}$ background (myc-GluR6xGluR6$^{-/-}$ mice) (Coussen et al., 2002). In these conditions myc-GluR6 is detected either with an anti-GluR6/7 antibody or with an anti-myc antibody as a 135 kDa band, whereas the band labeled by the anti-GluR6/7 antibody at 115 kDa corresponds to GluR7 alone (Figure 5.8A). Immunoprecipitation with an anti-myc antibody yielded two bands corresponding to myc-GluR6 and GluR7 (Figure 5.8A) indicating that myc-GluR6 and GluR7 were associated within a heteromeric complex in the hippocampus.

The impaired presynaptic function in knock-out mice could be due to miss-targeting of kainate receptor subunits to presynaptic sites if either GluR6 or GluR7 is absent. To test this hypothesis, we used a biochemical procedure similar to the one described in chapter 3 to separate non-synaptic proteins from synaptic junctions. The separation procedure was not carried further due to the scarceness of material and to the fact that preliminary experiments showed a less efficient separation of presynaptic proteins from the postsynaptic densities in material from the mouse brain. We also analyzed the enrichment of synaptic markers in these two different fractions isolated from hippocampi of wildtype, GluR6$^{-/-}$ and GluR7$^{-/-}$ mice (Figure 5.8B). PSD95, a characteristic protein of the postsynaptic density, is present in the synaptic junctions but is excluded from the non-synaptic collection of proteins. The same separation occurs with the metabotropic receptor mGluR7 which is known to be present very close to synaptic

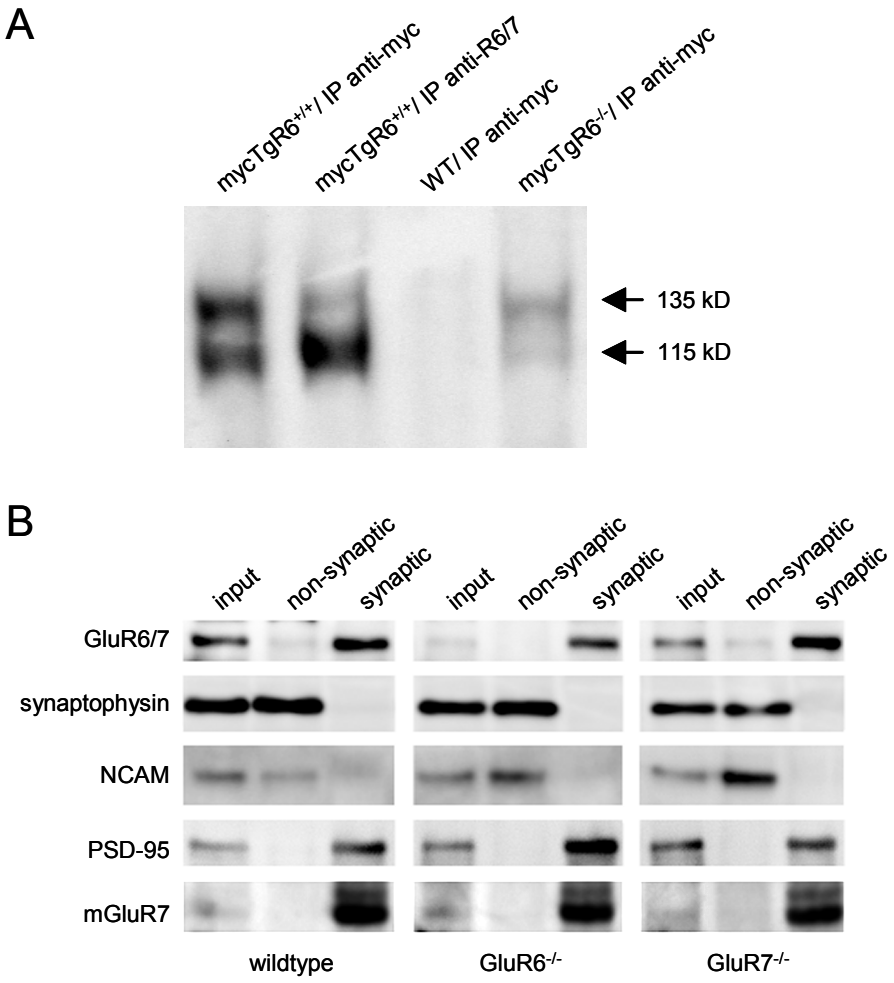

Figure 5.8 – Co-assembly of GluR6 and GluR7 subunits in the brain, and subcellular localization. **(A)** Kainate receptors were purified by immunoprecipitation from the mouse brains of different genotypes (wildtype, mycGluR6 and mycGluR6 x GluR6$^{-/-}$), with an anti-myc antibody. Western blots were probed with an anti-GluR6/7 antibody. In brains from mycGluR6 x GluR6$^{-/-}$ mice, the upper band (135 kD) corresponded to the immunoprecipitated transgene product (myc-GluR6a) and the lower band (115 kD) to GluR7. **(B)** Starting with hippocampal nerve terminals form wildtype, GluR6$^{-/-}$ and GluR7$^{-/-}$ mice we selectively solubilized non-synaptic proteins from synaptic junctions. Proteins typically excluded from the zone of synaptic contact such as NCAM and synaptophysin are exclusively localized in the solubilized non-synaptic fraction. On the other hand, proteins known to be localized at areas of synaptic contact, such as PSD-95 at the postsynaptic level and mGluR7 at the presynaptic level, were found exclusively in the synaptic junctions fraction. Analysis of the protein fractions with an anti-GluR6/7 antibody revealed that, in the absence of the GluR6 subunit, GluR7 is nevertheless found at synapses. Similarly, in the absence the GluR7 subunit GluR6 is also found at synapses.

release sites at the presynaptic level (Somogyi et al., 2003). On the contrary, the adhesion molecule NCAM and the synaptic vesicle protein synaptophysin are only detected in the non-synaptic fraction of proteins (Figure 5.8B). The anti-GluR6/7 antibody labeled the synaptic junctions fraction in wildtype, GluR6$^{-/-}$ (labelling of GluR7) and GluR7$^{-/-}$ (labelling of GluR6) mice. Altogether, these results provide strong evidence that GluR6 and GluR7 can co-assemble in native kainate receptors and are likely localized in the active zone near vesicular release sites.

Pharmacological evidence for presynaptic GluR7-containing kainate receptors

The data using a genetic deletion approach provide powerful arguments in favor of GluR7 as a presynaptic kainate receptor at the mossy fiber synapse. However, potential unknown compensatory mechanisms might also explain the impairment of short and long-term synaptic plasticity at the mossy fiber synapse in GluR7$^{-/-}$ as well as GluR6$^{-/-}$ mice. To directly test for the presence of a presynaptic GluR7 subunit, a selective antagonist would prove very useful. We thus sought to antagonize the facilitatory effects of presynaptic kainate receptors with pharmacological agents in wildtype mice, and to further check that the antagonists were not operant in GluR7$^{-/-}$ mice. There is yet no report of a selective GluR7 (or GluR6/GluR7) antagonist. We thus performed pharmacological experiments on recombinant kainate receptors comprising the GluR7 subunit. We expressed the non-edited variants (Q forms) of GluR1, GluR6a and GluR7a individually in HEK 293 cells. Since the genetic and biochemical data point to a heteromeric GluR6/GluR7 kainate receptor responsible for presynaptic facilitation we also co-transfected GluR6a and GluR7a which readily co-assemble as heteromers in heterologous cells (Cui and Mayer, 1999; Jaskolski et al., 2005b). To ascertain that a heteromeric receptor was

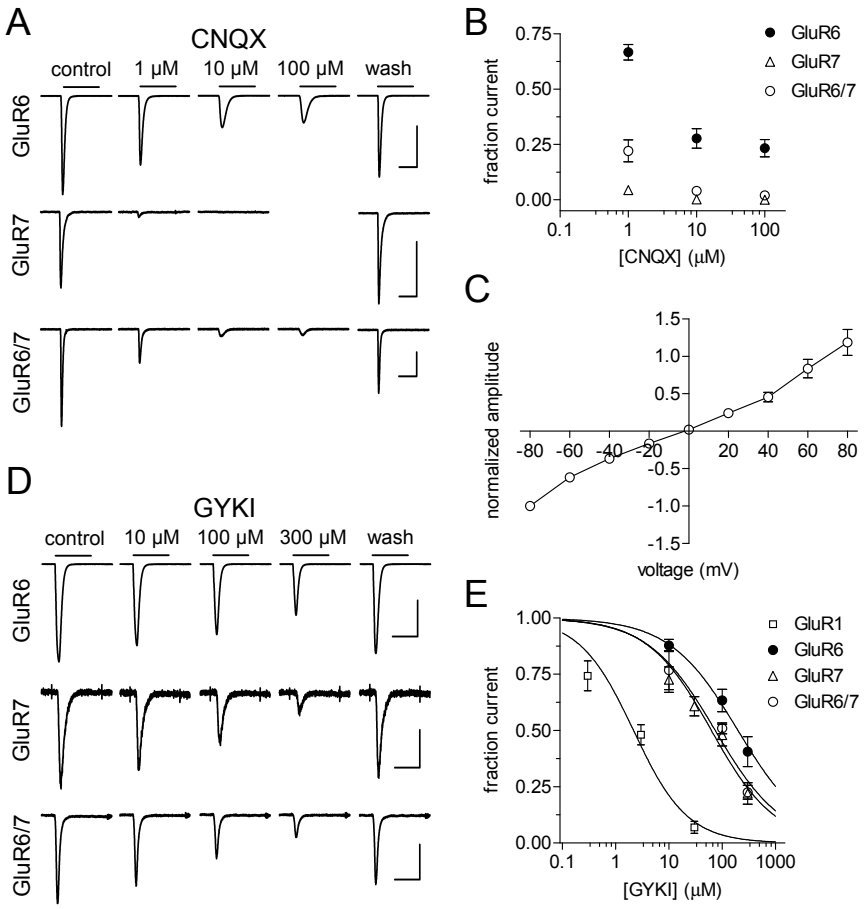

Figure 5.9 – Effect of AMPA/kainate receptor antagonists on recombinant GluR6/GluR7 receptors. **(A)** Currents evoked by applications of 30 mM glutamate for 100 ms on lifted HEK 293 cells expressing GluR6, GluR7a, alone or in combination. Black lines indicate the time of glutamate application. Varying concentrations of CNQX reversibly decreased the amplitude of the evoked currents. Scale bars: x axis, 50 ms; y axis, 2nA (GluR6), 400 pA (GluR7), 200 pA (GluR6/7). **(B)** Concentration-dependent effect of CNQX on current amplitude for GluR6, GluR7 and GluR6/GluR7 receptors. **(C)** Currents evoked at different potentials in cells expressing GluR6 and GluR7 were normalized on their amplitude at –80 mV, and averaged (n = 8). The IV curve is linear, showing that GluR6R is incorporated into receptors. **(D)** Effect of GYKI 53655 on currents evoked by 30 mM glutamate, presented as in A. Scale bars: x axis, 50 ms; y axis, 2 nA (GluR6), 50 pA (GluR7), 200 pA (GluR6/7). **(E)** Concentration-dependent effect of GYKI 53655 on current amplitude. Data points were fit with the Hill equation to calculate IC_{50} values for each GluR subunit combination (see text).

indeed formed, we used an excess of GluR7a cDNA (3 times) and the edited (R form) of GluR6a that, once incorporated into a receptor, yields a

non rectifying I-V curve (Cui and Mayer, 1999) (Figure 5.9C). Since GluR7 is activated only by high concentrations of glutamate (Schiffer et al., 1997) we applied 30 mM glutamate for 100 ms to activate kainate and AMPA receptors using a fast piezoelectric application device on lifted transfected cells (Figure 5.9A). We first tested the effects of CNQX, which binds to GluR7 with a slightly better affinity than to GluR6 on recombinant kainate receptors (Bettler et al., 1992). CNQX, at a concentration used to inhibit short-term plasticity at the mossy fiber synapse (10 µM; Schmitz et al., 2001), completely blocked GluR7 and GluR6/GluR7 containing kainate receptors activated by 30 mM glutamate (Figure 5.9A, B). Currents mediated by homomeric GluR6 receptors were not completely abolished even at the highest CNQX concentration tested (100 µM), probably because of the high glutamate concentration used here (30 mM), that will compete with CNQX for the binding site. This competition is reflected by a change in current kinetics observed for higher concentrations of CNQX (Figure 5.9A).

A recent study in the mouse hippocampus intriguingly revealed that the extent of synaptic plasticity of kainate receptor-mediated EPSCs at mossy fiber synapses was attenuated as compared to AMPA receptor mediated EPSCs (Ito et al., 2004). We reasoned that this difference might lie in the use of GYKI 53655 to unmask postsynaptic kainate receptor-mediated EPSCs that might influence facilitatory kainate receptors at the presynaptic level. Therefore, we also examined whether GYKI 53655 antagonized recombinant GluR6 and GluR7 receptors expressed in HEK 293 cells. As expected, GluR1 was very sensitive to GYKI 53655 (IC_{50} = 2.2 ± 0.4 µM; n = 3). Both GluR6 and GluR7 homomeric receptors appeared sensitive to GYKI 53655 with IC_{50} values of 198 ± 53 µM and 63 ± 10 µM (n = 3 to 8), respectively. Thus, at the concentration of 50 µM GYKI used to block AMPA receptor mediated EPSCs, GluR7 mediated currents are inhibited by about 50 %. Heteromeric GluR6/GluR7 receptors were found to

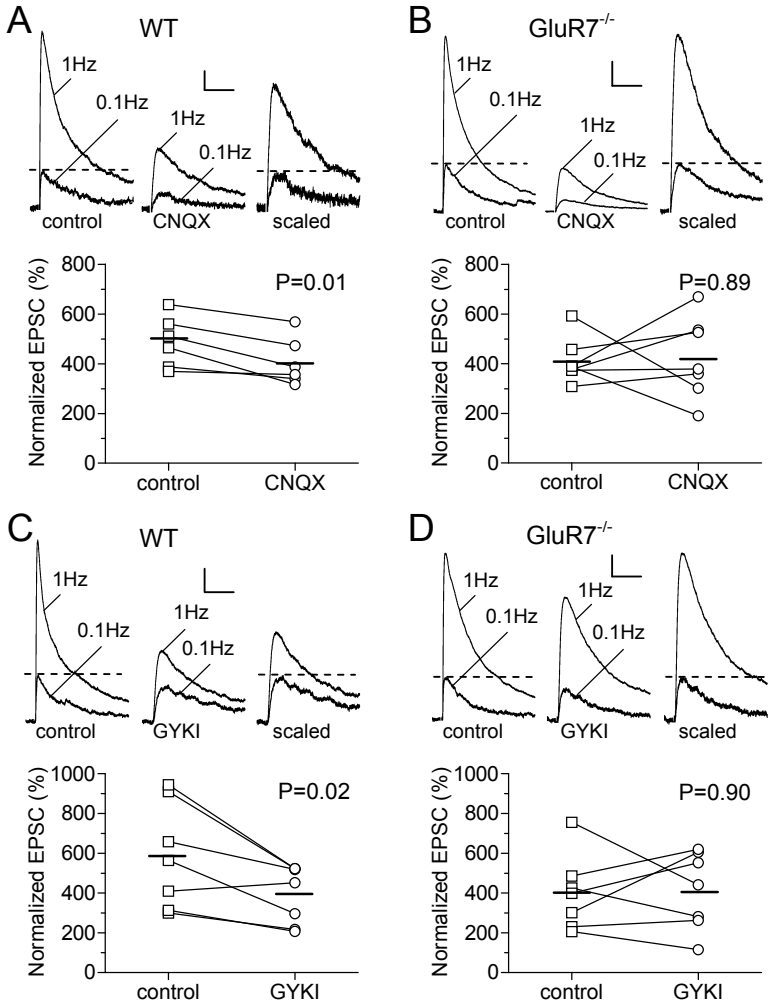

Figure 5.10 – Facilitatory GluR7-containing presynaptic kainate receptors are blocked by CNQX and GYKI 53655. **(A)** Top panel: representative traces of mossy fiber EPSCs from slices of wildtype mice recorded at +40 mV in control conditions or in the presence of 50 μM CNQX when shifting stimulation from 0.1 Hz to 1 Hz. In the presence of CNQX the fast AMPA component is abolished. Scale bars: y axis, 10 pA; x axis, 40 ms. Bottom panel: summary graph of the average percent facilitation of mossy fiber EPSCs when shifting stimulation from 0.1 Hz to 1 Hz showing that facilitation is significantly reduced in the presence of CNQX. **(B)** Top panel: representative traces of mossy fiber EPSCs from slices of GluR7$^{-/-}$ mice recorded at +40 mV in control conditions or in the presence of 50 μM CNQX when shifting stimulation from 0.1 Hz to 1 Hz. Scale bars: y axis, 33 pA; x axis, 40 ms. Bottom panel: summary graph of the average percent facilitation of mossy fiber EPSCs when shifting stimulation from 0.1 Hz to 1 Hz showing that not only facilitation is not changed in the presence of CNQX in GluR7$^{-/-}$ mice but also that its levels are similar to those of wildtype mice when in the presence of CNQX. **(C)** and **(D)** Representation of similar experiments as in (A) and (B) showing that in the presence of 50 μM GYKI frequency facilitation, when shifting the stimulation frequency from 0.1 Hz to 1 Hz, is significantly reduced in slices from wildtype mice [shown in (C); scale bars: 25 pA x 40 ms] but is not changed in slices from GluR7$^{-/-}$ mice [shown in (D); scale bars: 22 pA x 40 ms]. Furthermore, in GluR7$^{-/-}$ mice the levels of facilitation are similar to those of wildtype mice in the presence of GYKI.

as sensitive as homomeric GluR7 receptors (IC50 = 74 ± 26 µM; n = 4) (Figure 5.9D, E). Therefore, GYKI 53655, usually considered as a selective AMPA receptor antagonist, also inhibits GluR6/GluR7 recombinant receptors and can thus affect synaptic transmission at the presynaptic level at mossy fiber synapses.

To test whether presynaptic kainate receptors were inhibited by agents that block GluR6/GluR7 recombinant receptors, we compared the effects of CNQX and GYKI 53655 on short-term plasticity at the mossy fiber synapse of wildtype and GluR7$^{-/-}$ mice. In the impossibility to record AMPA receptor-mediated EPSCs we recorded mossy fiber EPSCs at +40 mV to remove the Mg^{2+} block from NMDA receptors and we evaluated the extent of low frequency facilitation (shift in tonic frequency from 0.1Hz to 1Hz) in the absence of any antagonist of AMPA/kainate receptors. Application of CNQX (50 µM) fully blocked the fast AMPA component, and significantly decreased the magnitude of low frequency facilitation from 488 ± 42% to 406 ± 39% (P = 0.02 compared to control, paired *t* test; n = 7) (Figure

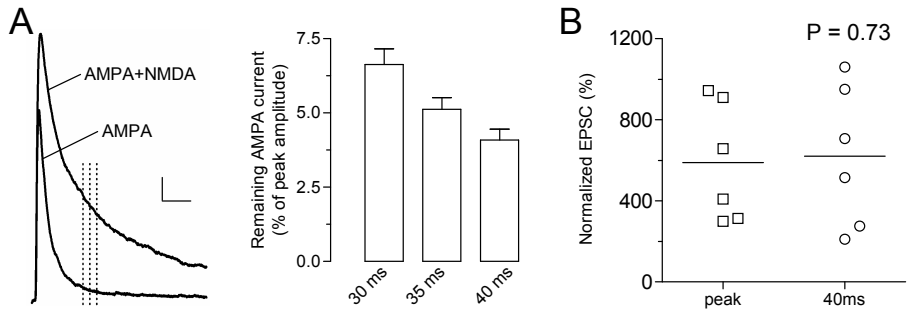

Figure 5.11 – The AMPA and the NMDA component of mossy fiber EPSCs show the same frequency facilitation. **(A)** Left panel: sample traces showing mossy fiber EPSCs recorded at +40 mV only in the presence of bicuculline (AMPA+NMDA) and the pure AMPA receptor-mediated EPSCs isolated by application of the NMDA receptor antagonist D-AP5 (50 µM). Dashed lines represent, from left to right, the time points of 30, 35 and 40 ms from the peak AMPA+NMDA EPSC. Scale bars: y axis, 20 pA; x axis, 20 ms. Right panel: percentage of pure AMPA receptor-mediated current relative to the peak response for the time points indicated. At 40 ms from the peak mossy fiber response only 4.1 ± 0.4% of the AMPA receptor mediated component is left. **(B)** Mossy fiber frequency facilitation, when measured at +40 mV, is the same when monitoring either the peak AMPA response or the NMDA component at 40 ms from the peak response, indicating that both display a similar degree of plasticity.

5.10A), a value close to that observed in control conditions in GluR7$^{-/-}$ mice. In addition, the inhibition of low frequency facilitation by CNQX was not observed in GluR7$^{-/-}$ mice (402 ± 34% for control and 423 ± 61% for CNQX; n = 7; P = 0.89, paired *t* test) (Figure 5.10B). Similarly, GYKI 53655 (50μM) inhibited low frequency facilitation in wildtype mice from 585 ± 100% to 391 ± 55 % (n = 7; p = 0.01 as compared to control, paired *t* test) (Figure 5.10C), but no inhibition was observed in GluR7$^{-/-}$ mice (400 ± 71% in control conditions, and 411 ± 74%, in the presence of GYKI 53655; n = 7; P = 0.90, paired *t* test) (Figure 5.10D). We also verified whether the magnitude of facilitation was similar for the AMPA component (measured at the peak of the EPSC), and for the NMDA component (measured 40 ms after the peak, when only 4.1 ± 0.4 % of the AMPA component is left in the presence of D-AP5; n = 3) and found no significant differences (Figure 5.11A, B). Overall, these experiments strongly suggest that both CNQX and GYKI 53655 attenuate short-term synaptic plasticity by acting on presynaptic kainate receptors containing the GluR7 subunit.

5.3. Discussion

The evidence presented in this chapter of our study sheds light, for the first time, on the role for the GluR7 subunit in synaptic transmission, and also impacts the current view of the way these receptors act in the modulation of synaptic transmission. This kainate receptor subunit was cloned more than a decade ago (Bettler et al., 1992), but has received little attention. Due to the lack of specific pharmacological tools for GluR7, the physiological function of kainate receptors comprising this subunit has never been addressed before. Using a combination of electrophysiological, pharmacological and biochemical analysis of wildtype and GluR7-deficient mice, we show that GluR7 is a key subunit of presynaptic kainate receptors

at the mossy fiber synapse. Our results also confirm that presynaptic kainate receptors at the mossy fiber synapse are fast-acting facilitatory autoreceptors accounting for a permissive role in short- and long-term synaptic plasticity. We propose that this action is mediated by GluR6/GluR7 heteromers localized very close to glutamate release sites, where the low affinity GluR7 subunit can sense the expected millimolar concentrations of glutamate necessary for its activation. We further propose that the facilitatory and inhibitory actions of kainate at the mossy fiber synapse are mediated by kainate receptors with distinct molecular composition, and probably distinct localization. Finally, our data provide directions that should help to clarify the discrepancies raised by using different pharmacological tools or knock-out mice.

A physiological function for the GluR7 subunit

GluR7 forms functional homomeric receptor channels with a 10-fold higher EC_{50} for glutamate as compared to other kainate or AMPA receptor subtypes (Schiffer et al., 1997), suggesting that the GluR7 subunit may play a unique role in synaptic transmission. We show here that GluR7 is a key component of presynaptic autoreceptors at the mossy fiber synapse and is involved in several forms of short and long-term synaptic plasticity. In $GluR7^{-/-}$ mice, paired pulse facilitation was markedly reduced for interstimulus intervals as small as 20 ms, in conditions of minimal stimulation intensity, suggesting that presynaptic GluR7-containing kainate receptors can be activated by a single glutamate release event. The involvement of kainate autoreceptors in this form of plasticity takes place only within short intervals, since no impairment is observed at intervals of 100 ms or greater. Consistent with a fast action of presynaptic kainate receptors our data show that GluR7 is present in the presynaptic web (Figure 5.8) and, as already suggested by the results presented in chapter

4, they imply that presynaptic kainate receptors are localized close to the site of glutamate exocytosis. This localization helps to understand how the synaptic concentration of glutamate can reach levels sufficient to activate GluR7 receptors, which display a very low affinity for this agonist. Estimates of the concentration of glutamate in vesicles range from 60 to 210 mM (Clements, 1996). The closer GluR7 containing receptors are localized to the vesicle release sites, the higher the glutamate concentration they will sense during release.

Frequency facilitation, another form of short-term plasticity, develops more slowly upon repeated stimulations at low frequencies, and depends on a rise in intraterminal Ca^{2+} and activation of Ca^{2+}/calmodulin-dependent kinase II (Salin et al., 1996). The extent of frequency facilitation was markedly decreased in GluR7$^{-/-}$ mice. Moreover, frequency facilitation of mossy fiber EPSCs recorded at +40 mV was inhibited by CNQX and GYKI 53655 in wildtype mice but not in GluR7$^{-/-}$ mice. These results demonstrate the contribution of the GluR7 subunit to this different form of short-term synaptic plasticity, probably by contributing to a sustained enhancement of Ca^{2+} in the mossy fiber terminal.

Finally, the presynaptic form of LTP typical of mossy fiber synapses was clearly impaired in GluR7$^{-/-}$ mice but could be rescued by either increasing excitability during the induction protocol or by using a more robust induction protocol. Thus, presynaptic kainate receptors containing GluR7 play a facilitatory role in short and long-term synaptic plasticity, rather than an inducing role (Contractor et al., 2001; Lauri et al., 2001; Lauri et al., 2003; Schmitz et al., 2001). A very similar presynaptic phenotype has been described in GluR6$^{-/-}$ mice (Contractor et al., 2001; Schmitz et al., 2003), suggesting that both subunits partially play the same role and/or co-assemble together to form functional receptors (see below). However, unlike GluR6, GluR7 is a purely presynaptic kainate receptor subunit at the

mossy fiber synapse, and is not involved in the inhibition of synaptic transmission by kainate (Contractor et al., 2000).

Mechanisms of synaptic facilitation by kainate autoreceptors

The fast-acting effect of GluR7-containing kainate receptors on release probability, as judged by the extent of paired pulse facilitation, favors a presynaptic mechanism relying on the activation of the receptor channel, triggering depolarization of the nerve terminal membrane or direct Ca^{2+} influx. A rather direct evaluation of the role of presynaptic kainate receptors was recently given by imaging presynaptic Ca^{2+} and depolarization of the nerve terminal during short-term synaptic plasticity (Kamiya et al., 2002). These results are in favor of a depolarizing effect that thereby augments the action potential-dependent activation of voltage-gated Ca^{2+} channels. Presynaptic ionotropic receptors might enhance neurotransmitter release owing to Ca^{2+} entry through the receptor itself, by summating with Ca^{2+} entering through voltage-gated Ca^{2+} channels or triggering Ca^{2+} release from intracellular stores (Lauri et al., 2003). Given the non-editing and strong inward rectification of GluR7 receptors (Schiffer et al., 1997) direct permeation of Ca^{2+} through the receptor channels is a possibility. In fact, given the suggested proximity of GluR6/GluR7 receptors in relation to release sites, direct Ca^{2+} permeation through the receptors may predominate (Engelman and MacDermott, 2004). Additionally, mossy fiber synaptic plasticity is markedly reduced by philanthotoxin (Lauri et al., 2003), a compound that blocks unedited Ca^{2+}-permeable glutamate receptors (see Fletcher and Lodge, 1996), further arguing in favor of direct permeation of Ca^{2+} through GluR7 kainate receptors, that only exist in the unedited form. In favor of a depolarizing action of presynaptic kainate receptors, a slight elevation in the extracellular KCl concentration increases mossy fiber EPSCs and rescues mossy fiber LTP in $GluR6^{-/-}$ (Schmitz et al.,

2003) and GluR7$^{-/-}$ mice (this study). However, a KCl-induced depolarization involves different routes that account for Ca^{2+} entry and most probably simply mimics the outcome of activating presynaptic kainate receptors when these are not operating. In summary, it appears that presynaptic GluR7-containing kainate receptors facilitate neurotransmitter release probably through an ionotropic receptor action.

It is commonly proposed that the concentration of agonist activating presynaptic kainate receptors determines the outcome on neurotransmitter release; a small depolarization might enhance evoked release by bringing the membrane potential closer to threshold, while a larger depolarization might shunt or depress action potential amplitude to decrease release (Engelman and MacDermott, 2004). Such a dual mechanism has been postulated for presynaptic kainate receptors at the mossy fiber synapse (Lauri et al., 2001; Schmitz et al., 2001), implying that the same receptors could operate bi-directionally. Our data provide clear evidence that inhibition of glutamate release at mossy fiber synapses is not mediated by the same kainate receptors that contribute to enhancement of release, since inhibition of glutamate release by concentrations of kainate above 100 nM is lost in GluR6$^{-/-}$ mice but is not affected in GluR7$^{-/-}$ mice. It should be stressed that inhibition of glutamate release is observed at concentrations that also evoke consistent inward currents in postsynaptic CA3 pyramidal cells, raising the possibility that inhibition is indirectly dependent on the large change in network activity in response to kainate. It is interesting to point out that endogenous glutamate increases the efficacy of GABAergic synapses in CA1 pyramidal cells, whereas depression is only observed with high concentrations of kainate (Jiang et al., 2001), suggesting again in another system that the facilitatory and the inhibitory actions of kainate receptors might be mediated by distinct entities.

Subunit composition of presynaptic kainate receptors

Our results provide strong evidence that GluR7 is a presynaptic kainate receptor subunit at hippocampal mossy fiber synapses. However, the question still remains as to the full subunit composition of native kainate receptors at this synapse, which are probably heterotetrameric proteins. We propose that the presynaptic function at the mossy fiber synapse is mediated by heteromeric GluR6/GluR7 receptors. First, as mentioned above, the presynaptic phenotype of GluR6$^{-/-}$ mice and GluR7$^{-/-}$ mice are very similar. In contrast, no impairment of synaptic plasticity was observed in GluR5$^{-/-}$ mice (Contractor et al., 2001) and KA2$^{-/-}$ mice (apart from a change in heterosynaptic facilitation; Contractor et al., 2003). Second, both GluR6 and GluR7 are detected in preparations of synaptic junctions, a sub-fractionation of synaptosomes that comprise the postsynaptic density and the presynaptic web (Phillips et al., 2001). Due to the lack of specific anti-GluR6 and anti-GluR7 antibodies, we could not directly test if GluR6 and GluR7 readily associate in the brain. However, using transgenic mice expressing myc-GluR6a under the control of the α-CAMKII promoter on a GluR6$^{-/-}$ background (Coussen et al., 2002), we show that myc-GluR6 and GluR7 do co-immunoprecipitate. Finally, studies on recombinant kainate receptors have shown that GluR6 and GluR7 can co-assemble into heteromeric functional channels (Cui and Mayer, 1999), as also shown in the present study. In addition, immunoprecipitation experiments from transfected cells demonstrate a high degree of co-assembly of GluR6a and GluR7a (Jaskolski et al., 2005b).

If we assume that presynaptic kainate receptors contain both GluR6 and GluR7, we need to understand why the presynaptic function of these receptors as autoreceptors is abolished in mice with a gene deletion of one or the other subunit. The loss of presynaptic kainate receptor function in mice with a gene deletion of one or the other subunit might be due to miss-

targeting of GluR7 in the absence of GluR6 as its protein partner. Against this hypothesis, our biochemical experiments indicate that GluR7 is present in the synaptic junctions in the absence of GluR6. However, we did not show that GluR6 is correctly driven to the presynaptic active zone in the absence of GluR7, since the labelling seen for synaptic junctions in GluR7$^{-/-}$ mouse preparations might correspond to postsynaptic GluR6 subunits. We also do not know what the specific properties of the heteromeric kainate receptor combination that makes it a regulator of synaptic transmission are. One point that needs to be addressed is the Ca^{2+} permeability of GluR6/GluR7 receptors. Since GluR7 only exists in the non-edited Q form (Lomeli et al., 1992), Ca^{2+} permeability will depend on whether or not GluR6 is edited. Homomeric GluR6aR receptors are expected to have a low permeability to Ca^{2+}; however, the insertion of GluR7a subunits may confer some permeability to this cation. The presynaptic action of GluR6/GluR7 heteromers might also depend on specific interactions with presynaptic proteins that would require both subunits. For instance, a close interaction with the protein complexes involved in synaptic release might be required. Finally, presynaptic GluR6/GluR7 receptors may also combine with other kainate receptor subunits. An interesting third partner would be KA1, since ultrastructural immunogold staining for KA1 was observed at or near the active zones of mossy fiber terminals (Darstein et al., 2003).

Pharmacology of presynaptic kainate receptors

In order to determine a role for presynaptic kainate receptors in regulating mossy fiber synaptic transmission, several studies have monitored the amplitude of NMDAR-EPSCs. Our data show that the use of GYKI 53655 to inhibit AMPAR-EPSCs and reveal the NMDA component is problematic since this compound antagonizes GluR6/GluR7 receptors. GYKI 53655 inhibits short-term synaptic plasticity in wildtype but not GluR7^{-}

$^{/-}$ mice, providing strong evidence that GYKI 53655 is an antagonist of presynaptic GluR7-containing kainate receptors. CNQX binds to GluR7 with a slightly higher affinity than to GluR6 (Bettler et al., 1992) and, at the mossy fiber synapse, CNQX and NBQX have been reported to inhibit the presynaptic facilitation of the NMDA receptor-mediated EPSC in response to a short train of stimuli applied at 25 Hz and at 100 Hz (Schmitz et al., 2001). In these experiments, AMPA receptors were blocked with GYKI 53655 in order to record pure NMDA receptor EPSCs. In a set of preliminary experiments we tried to replicate this data by recording NMDA receptor EPSCs at +40 mV in the presence of bicuculline and GYKI 53655. We were unable to detect any changes in either high frequency facilitation (5 stimuli at 20 Hz) or low frequency facilitation (shift in tonic frequency from 0.1Hz to 1Hz) with CNQX. The reason for this discrepancy is unclear since experimental conditions did not appear very different, apart from species differences (rat *versus* mouse), and it is possible that presynaptic receptors have a different subunit composition or pharmacology between the mouse and the rat. Nevertheless, the fact that GYKI 53655 inhibits presynaptic kainate receptors likely explains the differences in the extent of synaptic plasticity when monitoring either AMPA receptor EPSCs or kainate receptor EPSCs at mossy fiber synapses (Ito et al., 2004).

The present work also supports the notion that the concentration of glutamate sensed by presynaptic kainate receptors is higher than that sensed by postsynaptic receptors, since GluR7 containing-kainate receptors are probably localized close to synaptic release sites. This could explain the relative insensitivity of LTP induction to competitive antagonists such as kynurenate (effective at 10 mM, but not 3 mM; Bortolotto et al., 1999). In other studies, LTP was not affected by 10-20 mM kynurenate (Castillo et al., 1994; Yeckel et al., 1999), raising the possibility that in these experiments, an increased level of excitability or stronger LTP inducing protocols can bypass the permissive function of kainate receptors (Schmitz

et al., 2003) by promoting Ca^{2+} entry through alternative routes such as L-type Ca^{2+} channels (Lauri et al., 2003).

Another recurrent controversy lies in the use of antagonists directed at GluR5 receptors, such as LY382884 and UBP296. Because these antagonists attenuate short and long-term synaptic plasticity in the rat hippocampus (Bortolotto et al., 1999; Lauri et al., 2001; More et al., 2004), GluR5 was claimed to be the key subunit underlying the presynaptic actions of kainate receptors. However, a role for presynaptic GluR5 kainate receptors at the mossy fiber synapse has been disputed (Nicoll et al., 2000; Breustedt and Schmitz, 2004) since broad spectrum glutamate receptor antagonists have been reported not to block the induction of mossy fiber LTP (Ito and Sugiyama, 1991; Castillo et al., 1994; Weisskopf and Nicoll, 1995; Yeckel et al., 1999). Also, pharmacological studies with GluR5 receptor antagonists contradict the electrophysiological analysis of mutant mice (Contractor et al., 2001) and a likely explanation to reconcile the currently available data is that GluR5 antagonists may also be effective on kainate receptors containing the GluR7 subunit. This would be consistent with a selective presynaptic action of the antagonists at the mossy fiber synapse, since GluR7 was shown here to be active exclusively at presynaptic sites.

In conclusion, our data demonstrate a physiological function for the GluR7 subunit of kainate receptors. Given the high expression of GluR7 in the deep layers of the neocortex, the role of this subunit will likely extend to several efferent systems originating in this structure. GluR7 also seems to be expressed in populations of interneurons in the cerebellum or hippocampus. It will be interesting to determine for instance if GluR7 is involved in the presynaptic facilitatory actions of kainate receptors on GABAergic afferents (Jiang et al., 2001). Previous work has shown that the other kainate receptor subunits GluR5, GluR6 and KA2 play a role at both somatodendritic and axonal/presynaptic levels and it remains to be shown

whether GluR7 is, in contrast, a pure presynaptic subunit given its particular properties. Whereas the expression of GluR6 has a widespread distribution throughout the brain, that of GluR7 is more restricted. GluR7 is highly expressed in the deep layers of the neocortex (Bettler et al., 1992) which contain neurons that project to subcortical structures such as the thalamus or the striatum and it might be reasonable to think that presynaptic GluR7-containing kainate receptors may be present in the synaptic terminals of corticostriatal or corticothalamic afferents. Interestingly, a form of LTP described at the corticothalamic synapse shares a number of similarities with mossy fiber LTP; corticothalamic LTP is input-specific, NMDA receptor-independent, its induction is entirely presynaptic and Ca^{2+}-dependent and blocked by an inhibitor of the cAMP-dependent PKA (Castro-Alamancos and Calcagnotto, 1999). The same is observed in the cerebellum, at parallel fiber synapses (Salin et al., 1996b). It is thus tempting to speculate that presynaptic GluR7 kainate receptors might also be involved in LTP at the corticothalamic synapse. A crucial question will then be to understand what can be the molecular mechanisms underlying the polarized trafficking of GluR7 to the presynaptic compartment (Jaskolski et al., 2005b).

Chapter **6**

General Conclusions

Conclusões Gerais

Several conclusions can be drawn from the present work. First, we showed that the selective solubilization of synaptic proteins from hippocampal nerve terminals yields highly pure preparations of proteins from the presynaptic active zone, the postsynaptic density and from non-synaptic locations. These protein fractions can be used to investigate the subsynaptic distribution of glutamate receptors.

By using subsynaptic protein fractions we next showed that all NMDA receptors subunits analyzed had a predominantly postsynaptic localization with only residual labelling in the presynaptic active zone, whereas AMPA receptors may exist presynaptically and postsynaptically. We also detected high amounts of non-synaptic AMPA receptors, reflecting most probably receptors in cellular traffic and recycling processes. The distribution of Group I and Group II metabotropic glutamate receptors in the subsynaptic protein fractions is complex and hard to reconcile with the literature, most probably reflecting a limitation of the technique in resolving certain pools of receptors, while Group III mGluRs are localized mainly within the presynaptic active zone, as previously described. This technique seems, therefore, well suited to distinguish the pre- *versus* postsynaptic distribution of synaptic receptors.

We also concluded that presynaptic kainate receptors are localized within the active zone of hippocampal synapses. These receptors were found in the presynaptic active zone fraction of proteins, showing their close association with the presynaptic web. This subsynaptic distribution also puts forward their confinement close to glutamate release sites in hippocampal nerve terminals. These receptors are able to modulate the release of [^{3}H]glutamate and this is probably accomplished by direct ionic permeation through the receptor channel, since Ca^{2+} channel blockers only partially reduced the intracellular Ca^{2+} signal.

Furthermore, we studied a possible physiological role for the GluR7 subunit of kainate receptors in the brain by using GluR7 knockout mice. GluR7 was not identified functionally in postsynaptic sites at mossy fiber-CA3 pyramidal cell synapses. However, at the presynaptic level, the absence of GluR7 severely impairs low and high frequency-induced short-term synaptic plasticity. The involvement of these receptors in fast phenomena such as paired pulse facilitation lead us to suggest, in line with the results from chapter 4, their localization close to release sites. Probably only in such a location can GluR7 sense the elevated concentrations of glutamate required for its activation.

LTP and PTP are also severely impaired, but not completely absent, in animals lacking the GluR7 subunit and can be restored by protocols that increase excitability, showing a permissive role of presynaptic GluR7-containing kainate receptors in lowering the threshold for induction of these forms of plasticity. In the absence of GluR7 the forskolin induced enhancement of synaptic transmission is intact, further showing a deficit at the induction level of synaptic plasticity phenomena.

We also show that the facilitation of synaptic transmission by endogenous activation of kainate receptors and its inhibition by application of high concentrations of kainate are phenomena that seem to involve receptors of different subunit composition or different localization. Our studies also allowed us to conclude that the impairments in synaptic plasticity in GluR7$^{-/-}$ mice are not due to miss-targeting of the receptors in the absence of certain subunits, since GluR7 and GluR6 are found at synapses in the absence of each other. Importantly, since all the changes in presynaptic plasticity shown here for GluR7$^{-/-}$ mice are similar to the ones previously observed for GluR6$^{-/-}$ mice, and also because GluR6 and GluR7 co-immunoprecipitate from brain extracts, we conclude that presynaptic

receptors are formed by GluR6/GluR7 heteromers or, alternatively, that both GluR6 and GluR7 are essential for the presynaptic phenotype of mossy fibers.

In addition, we show that the AMPA receptor antagonist, GYKI 53655, is capable of antagonizing GluR6/GluR7 heteromeric receptors in transfected HEK cells and to reduce low frequency facilitation in brain slices of wild type but not GluR7$^{-/-}$ mice. This finding further supports the involvement of GluR6/GluR7 receptors in mossy fiber synaptic plasticity. Importantly, the present study may contribute to increase awareness about the interpretation of pharmacological data on kainate receptors since an antagonist thought to be selective towards AMPA receptors was shown to also antagonize GluR7.

As main conclusions we may summarize that:

-The technique to separate the various pools of synaptic proteins used in the present work may be used to study the subsynaptic localization of ionotropic and metabotropic glutamate receptors.

-Presynaptic kainate receptors are localized within the active zone, close to glutamate release sites, where they efficiently modulate the release of [^{3}H]glutamate probably through direct Ca^{2+} permeation.

-GluR7 is a kainate receptor subunit that plays a functional role at presynaptic but not postsynaptic receptors on mossy fiber terminals and is essential for the control of presynaptic forms of short- and long-term synaptic plasticity.

Conclusões Gerais

O presente trabalho permite a inferência de várias conclusões acerca dos resultados apresentados. Em primeiro lugar mostrámos que a solubilização selectiva das proteínas sinápticas a partir de terminais nervosos do hipocampo permite a obtenção de preparações enriquecidas em proteínas da zona activa pré-sináptica, da densidade pós-sináptica e de localizações não sinápticas.

Recorrendo às fracções proteicas sub-sinápticas mostrámos que as subunidades de receptores NMDA estudadas apresentavam uma localização predominantemente pós-sináptica, existindo apenas imunoreactividade residual nas proteínas da zona activa pré-sináptica, enquanto que os receptores AMPA podem estar presentes pré-sinapticamente. Foram também detectados elevados níveis de receptores AMPA não sinápticos reflectindo, provavelmente, receptores em processos de endereçamento para a membrana ou de reciclagem. A distribuição dos receptores metabotrópicos do Grupo I e II nas fracções proteicas é complexa e difícil de reconciliar com a literatura existente reflectindo, muito provavelmente, limitações da técnica na identificação de receptores com localização particular, como parece ser o caso de receptores localizados perisinapticamente. Os mGluRs do Grupo III, tal como previamente descrito, encontram-se localizados essencialmente na zona activa pré-sináptica. Esta metodologia simples parece, portanto, adequada para o estudo da localização pré-sináptica *versus* pós-sináptica de receptores localizados dentro da sinapse.

Concluimos ainda que os receptores de cainato pré-sinápticos estão localizados dentro da zona activa. A sua localização na fracção proteica da zona activa pré-sináptica mostra uma associação estreita com proteínas da estrutura pré-sináptica e a sua restrição a locais próximos das

zonas de libertação de glutamato. Estes receptores modulam a libertação de [^{3}H]glutamato e tal modulação é, provavelmente, conseguida pela entrada directa de Ca^{2+} através dos receptores, uma vez que a aplicação de bloqueadores de canais de Ca^{2+} apenas reduziu parcialmente o aumento do Ca^{2+} intracelular.

Adicionalmente, procurámos identificar um possível papel fisiológico para a subunidade GluR7 dos receptores de cainato. Mostrámos que a subunidade GluR7 não participa na formação de receptores de cainato a nível pós-sináptico; no entanto, a nível pré-sináptico, a sua ausência leva a défices severos na plasticidade sináptica de curta duração induzida por estimulação a alta ou baixa frequência. O envolvimento em fenómenos de plasticidade sináptica rápidos levam-nos a sugerir, em conjunto com os resultados do capítulo 4, que estes receptores se encontram localizados próximo dos locais de libertação de glutamato. Provavelmente só em tais locais é que a subunidade GluR7 está exposta a concentrações de glutamato suficientemente elevadas para que a sua activação ocorra eficientemente.

Nestes animais, quer a LTP quer a PTP estão severamente reduzidas, mas não completamente ausentes, e podem ser restauradas para níveis semelhantes aos observados em animais controlo pelo uso de protocolos que aumentam a excitabilidade. Estes resultados demonstram que os receptores de cainato contendo a subunidade GluR7 desempenham um papel permissivo na indução destas formas de plasticidade, baixando o limiar para a sua indução. Na ausência da subunidade GluR7 o aumento da transmissão sináptica induzido pela aplicação de forscolina está intacto, reforçando o envolvimento de um défice ao nível dos mecanismos pré-sinápticos de indução da plasticidade e não da sua expressão.

Mostramos também que a facilitação da transmissão sináptica por activação endógena dos receptores de cainato, e a sua inibição pela aplicação de concentrações elevadas de cainato, envolve, muito provavelmente, receptores de cainato com subunidades ou localização cellular diferentes. Os nossos estudos também nos permitiram concluir que os défices na plasticidade sináptica em animais GluR7$^{-/-}$ não são devidos a problemas no endereçamento celular dos receptores de cainato para a sinapse, uma vez que as subunidades GluR6 e GluR7 são encontradas em junções sinápticas na ausência uma da outra.

Também de grande importância é o facto de que as alterações de plasticidade sináptica, a nível pré-sináptico, demonstradas neste estudo em animais GluR7$^{-/-}$ são iguais às observadas previamente em animais GluR6$^{-/-}$. Uma vez que as subunidades GluR6 e GluR7 co-imunoprecipitam em extractos de cérebro, os dados levam-nos a sugerir que os receptores pré-sinápticos de cainato a nível das fibras musgosas serão formados por heterómeros GluR6/GluR7. Alternativamente, ambas as subunidade poderão ser essenciais para o desenvolvimento do fenótipo pré-sináptico das fibras musgosas.

Adicionalmente, produzimos evidências de que o antagonista dos receptores AMPA, GYKI 53655, é capaz de bloquear receptores heteroméricos GluR6/GluR7 em células HEK transfectadas, e também de reduzir a facilitação em frequência em fatias de cérebro de animais de fenótipo selvagem, mas não de animais GluR7$^{-/-}$. Estas experiências reforçam o envolvimento de receptores heteroméricos GluR6/GluR7 em fenómenos de plasticidade pré-sináptica das fibras musgosas.

Resumidamente, podemos enumerar como conclusões principais deste estudo que:

- A metodologia que permite a separação das proteínas dos vários compartimentos sinápticos pode ser aplicada ao estudo da localização subsináptica de receptores ionotrópicos e metabotrópicos do glutamato;

- Os receptores pré-sinápticos de cainato estão localizados na zona active pré-sináptica, próximo dos locais de libertação do glutamato, onde podem eficientemente modular a libertação deste neurotransmissor, provavelmente pela entrada directa de Ca^{2+} através do receptor;

- A subunidade GluR7 dos receptores de cainato está presente e funcional a nível pré-sináptico, mas não a nível pós-sináptico, nas sinapses das fibras musgosas e desempenha um papel crucial no controlo de formas de plasticidade sináptica de curta e longa duração.

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