AMPA-type glutamate receptors and behavioural neuroplasticity: Bidirectional role of GluA1 subunits

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ABSTRACT

Neuroplasticity is defined as an ability of the nervous system to change when it responds to modified environmental or internal conditions. Functional neuroplasticity enables a change in behaviour and ultimately provides a major mechanism for enhanced survival in a given environment. There are two main types of plasticity, positive and negative plasticity. Positive plasticity describes abilities and brain processes that benefit an animal. Negative plasticity is related to processes that tend to be harmful. In this study, positive neuroplasticity was investigated in relation to aggressive behaviour and processing of novel signals; processes that are both required for successful survival in a changing habitat. Negative plasticity was studied in several stages of addiction, a psychiatric disease in which normal plasticity is interfered by drugs of abuse eventually leading to compulsive drug use. The neurotransmitter glutamate has a well-established role in chemical signalling between neurons, and an emerging role as a major component regulating neuroplasticity. Glutamate receptors of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type are tetramers composed of subunits GluA1-4 and they mediate a majority of the fast synaptic neurotransmission, that is also implicated in neuroplastic processes. Here, the AMPA receptor subunit GluA1-deficient mouse line (GluA1-KO mouse line) was studied using behavioural and neurochemical approaches. GluA1-KO mouse line provides an alternative approach to study the importance of AMPA receptors in neuroplasticity, as there are no subunit-selective pharmacological ligands available. GluA1-KO mice displayed less propensity to change their agonistic behaviour when exposed to an altered social environment in comparison with wild-type (WT) control mice, increased responsiveness towards spatial novelty followed by deficient habituation, decreased capability to develop tolerance to benzodiazepine flurazepam, and defective morphine-induced state-dependency. Taken together, these results propose a bidirectional role for GluA1 subunit in positive and negative neuroplastic processes. In summary, these results strengthen the role of GluA1 subunit in neuroplasticity, and may assist future drug development in disorders in which neuroplasticity plays a role.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred in the text by their roman numerals:

I Vekovischeva OY, **Aitta-aho T**, Echenko O, Kankaanpää A, Seppälä T, Honkanen A, Sprengel R, Korpi ER. Reduced aggression in AMPA-type glutamate receptor GluR-A subunit-deficient mice. Genes Brain Behav. 2004;3:253-65.

II Procaccini C*, **Aitta-aho T***, Jaako-Movits K, Zharkovsky A, Panhelainen A, Sprengel R, Linden AM, Korpi ER. Excessive novelty-induced c-Fos expression and altered neurogenesis in the hippocampus of GluA1 knockout mice. Eur J Neurosci. 2011;33:161-74.

III **Aitta-aho T**, Vekovischeva OY, Neuvonen PJ, Korpi ER. Reduced benzodiazepine tolerance, but increased flumazenil-precipitated withdrawal in AMPA-receptor GluR-A subunit-deficient mice. Pharmacol Biochem Behav. 2009;92:283-90.

IV **Aitta-aho T**, Möykkynen TP, Panhelainen AE, Vekovischeva OY, Bäckström P, Korpi ER. Importance of AMPA receptor GluA1 subunit for morphine state-dependency. Submitted.

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ABBREVIATIONS

5-HT	5-hydroxytryptophan, serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BrdU	bromodeoxyuridine
CA	*cornu ammonis* of the hippocampus
CaMK2A	calcium/calmodulin-dependent protein kinase II alpha
CNS	central nervous system
CS	conditioned stimulus
DA	dopamine
DG	dentate gyrus
DL-AP5	racemic (2*R*)-amino-5-phosphonovaleric acid
DNA	deoxyribonucleic acid
EAAT	excitatory amino acid transporter
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
GAD	glutamic acid decarboxylase
GPCR	G-protein-coupled receptor
HPLC	high-performance liquid chromatography
IEG	immediate-early genes
KO	knockout
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
mRNA	messenger RNA
NAc	nucleus accumbens
NSF	N-ethylmaleimide-sensitive factor
NMDA	*N*-methyl-D-aspartate
PCR	polymerase chain reaction
PICK1	protein interacting with C kinase 1
RNA	ribonucleic acid
SHIRPA	SmithKline Beecham-Harwell-Imperial College-Royal London Hospital-phenotype assessment
SYM 2081	(2S, 4R)-4-methylglutamate
US	unconditional stimulus
VGLUT	vesicular glutamate transporter
VTA	ventral tegmental area
WT	wild-type

1 INTRODUCTION

Neuroplasticity is defined as an ability of the nervous system to change when it responds to modified environmental conditions, brain trauma or degenerative change. Plasticity also can occur within the brain network itself, which can furthermore be initiated by external stimuli. Behaviour related to social interaction, processing of novel signals and to drugs of abuse is subject to change in response to experience-related stimuli. These changes are based on modifications between the basic units of communication in the brain, the neurons. Glial cells have also been suggested as becoming increasingly important in the modulation of synaptical communication. Two main signal modalities exist that mediate the information from neuron to neuron. These include electrical signals that propagate within the neurons, and chemical ones that send messages between the neurons across a synapse.

An outcome-based division of plasticity describes two directions of plasticity. Positive plasticity describes changes that benefit an animal while negative plasticity describes changes that eventually tend to be harmful. Aggression and processing of novel signals are related to the positive plasticity. For example, a change in a social environment might warrant activation of aggressive behaviour in order to survive, and proper responding towards novelty promotes orderly perception that enables learning. In contrast, the latter type of plasticity can be caused by drugs of abuse. Drug use modifies brain function by causing an array of processes underlying the behavioural changes known as addiction.

Glutamate is an amino acid that is primarily responsible for the main excitatory signalling. Chemical neurotransmission is initiated by the arrival of electrical action potential to the presynaptic neuron, where glutamate is released in highly controlled manner. When glutamate reaches the postsynaptic neuron, it activates a diverse class of glutamate receptor proteins. Of these, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type receptors mediate the fast excitatory neurotransmission leading to excitatory postsynaptic potential in response to cation influx to neurons. However, AMPA receptors are instrumental in neuroplasticity underlying changes in behaviour. The foundation of activity-dependent change between the neurons involves either insertion or removal of AMPA receptors at synaptic cell membrane leading to long-term potentiation (LTP) or long-term depression (LTD), respectively. These models of activity-dependent change in neuronal communication are thought as cellular correlates of neuroplasticity in its various forms.

2 REVIEW OF THE LITERATURE

2.1 BEHAVIOURAL PLASTICITY

By definition, plasticity is an ability to change over time. Behavioural plasticity, a change in behaviour, is of primary importance as it allows adaptation to the changes in environment, and thus increases survival. Changes in environment can originate from a decrease in food sources, modified social constructs and an increase in the number of other individuals in the same habitat. In all of these cases, the change in behaviour promoting survival is of a positive type; and can include learning new rules, memory of events and appropriate responses to the novel environment. However, plasticity can also have negative consequences. Environmental changes can lead to sustained stress, which can in-turn increase the risk of developing anxiety and depressive conditions. Activities such as games, drug use and shopping can interfere with the control of the underlying mechanisms of plasticity which can lead to behaviours related to addiction.

2.2 CELLULAR AND MOLECULAR FOUNDATION FOR BEHAVIOURAL PLASTICITY

Experience-related stimuli continuously modify the brain neuronal networks, a process that eventually results in changes in behaviour. These modifications underlying behavioural plasticity take place at various levels, including brain regional, morphological and molecular one levels. Changes in the volume of distinct brain regions have been observed as being linked to neuroplastic events such as learning or drug abuse (Anderson *et al.* 2002; Bartzokis *et al.* 2000). At the morphological level, neuronal activity can promote dendritic outgrowth, synapse elimination, and remodelling of dendritic spines (for review, see McKinney 2010). However, these changes are under the control of molecular determinants of plasticity including modifications in e.g. receptors, neurotrophic factors, second messengers, immediate-early genes (IEGs) and gene expression.

At the cellular level, the most widely studied forms of neuroplasticity are LTP and LTD (Bliss and Lømo 1973). These represent a long-lasting change in the synaptic strength between two neurons that is due to repeated and synchronous stimulation of them. These phenomena occur in various kinds of synapses. It has even been suggested that all the neuronal connections possess an ability to show synaptic LTP and/or LTD (reviewed in Malenka and Bear 2004). Typically, LTP is generated by a brief electrical stimulation of presynaptic neuron that activates postsynaptic glutamate N-methyl-D-aspartate

(NMDA) receptors. This results in the elevation of intracellular Ca^{2+} for a time period as short as 2-3 seconds (Malenka *et al.* 1992). Thereafter, rapid insertion of AMPA receptors to the plasma membrane by trafficking mechanisms (see chapter 2.7.4) causes the expression of LTP (Hayashi *et al.* 2000), while removal of plasma membrane AMPA receptors leads to the expression of LTD (Beattie et al. 2000). After the acute phase of increase in synaptic efficacy, the sustained potentiation will require protein synthesis (Frey *et al.* 1988). Apart from plasticity occurring at the synaptic level, neuron-scale intrinsic plasticity has also been shown to exist in the central nervous system (CNS) (for review, see Zhang and Linden 2003).

At the molecular level, change in the number and properties of receptors is one of a diverse class of mechanisms behind neuroplasticity. These are reviewed in chapter 2.7, with a special reference to AMPA receptors. There are also other well-established molecular substrates involved in neuroplasticity. IEGs are an example of this and are a heterogeneous family comprising hundreds of members including, transcription factors, enzymes, cytokines and neurotrophic factors (Herschman 1991; Fowler *et al.* 2011). Alterations in IEGs can correlate with the plasticity-inducing experience. Some of these genes include activity-regulated cytoskeleton-associated protein (*arc*), *c-fos*, *fosB* and *deltaFosB* (Bramham *et al.* 2008; Lanahan and Worley 1998). Of the neurotrophic factors, brain-derived neurotrophic factor (BDNF) is probably best studied in relation to plasticity (for review, see McAllister *et al.* 1999). The role of BDNF in plasticity relates to several lines of evidence. This being that BDNF is present in brain areas known for their role in plasticity such as hippocampus and cerebral cortex, that brain activity regulates BDNF messenger RNA (mRNA) expression in these brain regions, and that BDNF has been shown as having a role in synaptic plasticity in LTP (for review, see McAllister *et al.* 1999; Malenka and Bear 2004). Finally, also cyclic adenosine monophosphate and its response element binding protein regulate gene expression that affects biosynthesis of a variety of proteins leading to enduring changes in the CNS (Chrivia *et al.* 1993).

2.3 APPROACHES TO STUDY POSITIVE AND NEGATIVE PLASTICITY

Appropriate aggressive behaviour in mice is instrumental in survival and reproduction as it enables both an increase, in the rank of individual as a member of a habitat (e.g. by assuring better sources for nutrition) and the possibility of finding a suitable mating partner (Bronson 1979). Novel signals provides the opportunity for finding better resources thus promoting survival (Panksepp 1998). Proper processing of information elicited by novel signals provides a platform for perception and subsequent encoding of these signals (Vinogradova 2001). Plasticity due to drug abuse leads to behaviours that are the core characteristics of addiction, compulsive and continuing drug use

despite the negative consequences. These harmful outcomes can be, but not limited to, an increased risk of mental and physical disorders, job loss, loss of custodial rights and committing of crimes leading to legal actions (World Health Organization 1999).

2.3.1 AGGRESSIVE BEHAVIOUR

Aggressive behaviour can be hostile, destructive and injurious actions that are directed towards the self, others or objects (Swann 2003). In humans, aggression can occur comorbidly with several psychiatric illnesses or disorders. Aggressive behaviour can be caused by defects in perception and cognition in psychosis, by traumatic events in patients with post-traumatic stress disorder, by psychopathy in antisocial personality disorder and by increased emotional sensitivity in borderline personality disorder (Siever 2008). Aggressive behaviour is part of agonistic behaviours that are social behaviours related to fighting (Scott 1966). Agonistic behaviour is a more extensive term than aggression, since it also covers behaviours (such as threatening, displays, postures and defensive behaviours) that show the intention of, not the actual act of aggression (Scott 1966).

Brain circuitry mediating aggression consists of several components. Evaluation and decision-making processes which regulate the action of aggression stem from the prefrontal cortex (Davidson and Irwin 1999). Lesions in these areas may lead to increase in aggressive behaviour (Damasio *et al.* 1994). Cortical areas signal the limbic system, most notably to amygdala, which is critical in associative learning related to aggression (Hollander and Gallagher 1999). Thus, the amygdala enables the linkage of unconditional aggressive events with the cues that remind of these events. This process can be seen as classical conditioning (Hollander and Gallagher 1999). Lesions that damage amygdala cause dysfunctions in recognition of facial expressions, also those related to aggression (Adolphs *et al.* 1994). In contrast to this, patients with borderline personality disorder (see above) show overactivation of the amygdala in response to positive, neutral and negative facial expressions (Donegan *et al.* 2003). Finally, the action of aggression is suggested to originate from the ventromedial hypothalamus (Lin *et al.* 2011).

Several neurotransmitter systems and hormones have been linked to aggression. Serotonin, by acting on 5-HT_2 receptors, activates prefrontal cortical regions and thereby increases control over aggressive emotions (Swann 2003). Increases in serotonin transmission by selective serotonin reuptake inhibitors decreases aggressive behaviour (Coccaro and Kavoussi 1997). This may be mediated by an increased activation of prefrontal areas which in turn inhibit the amygdala (Davidson *et al.* 2000). Inhibitory and excitatory neurotransmitters γ-aminobutyric acid (GABA) and glutamate, have been implicated in the modulation of aggressive behaviours. $GABA_A$ receptor positive modulators such as neurosteroid allopregnanolone (Fish *et al.* 2002), benzodiazepines (Takahashi *et al.* 2010) and ethanol (Miczek 2004) increase

aggression in low doses while high doses decrease aggression possibly via sedative actions. Increased glutamatergic tone leads to augmentation of aggressiveness in many species (Bullock and Rogers 1986; Munoz-Blanco *et al.* 1986). The steroid hormone testosterone, and similar chemical entities, can also enhance aggression (Archer 1991).

2.3.2 PROCESSING OF NOVEL SIGNALS

Novelty, irrespective of the stimulus modality, evokes a response that has an elementary role in biology. Response to novelty, or orienting response, was first described as "что ето такой" or "what is this" reaction (Pavlov 1927). Its purpose is to direct an animal's attention to perceive a change in the environment. Response to novelty can be detected in a wide variety of bodily functions such as dilatation in the pupil of the eye, sweat gland activity, respiration, heart rate and vascular activity. In the brain, novelty can be observed in various responses. Using electroencephalography or magnetoencephalography, changes in the activity of the alpha frequency (Barry 2009) and in the event-related potentials related to novelty detection, such as P3 (reviewed in Friedman *et al.* 2001) and mismatch negativity (reviewed in Kujala and Näätänen 2010), can be detected. Of these, mismatch negativity is mediated by glutamate receptors of NMDA type (Javitt *et al.* 1996).

Organisms react preferentially to novel information over familiar information. Response to novelty often undergoes subsequent decrease when the novel stimulus is repeated or constantly presented (Rankin *et al.* 2009), thus finally the stimulus elicits no response. This is known as habituation. The significance of habituation lies in making sure the system remains reactive to novel stimuli, that is, preserving a positive signal-to-noise ratio. Habituation has been considered as one of the most simple forms of learning, although this notion probably only includes the phenomenon of habituation. The neuroplastic mechanisms of habituation remain, however, largely unexplained (Rankin *et al.* 2009).

The detection of novelty and habituation of the novelty response are mediated by various circuitries in the CNS, although the hippocampus has received most of the attention. In subjects with hippocampal damage, novelty-related brain responses (see above) are not detected (Knight 1996), and hippocampal lesions render subjects incapable of habituating to novelty (Vinogradova 2001). Thus, the hippocampus has been suggested to act as a detector of novel signals, comparing these signals with the older ones (Vinogradova 2001). The hippocampus is strongly innervated by glutamatergic neurons, and thus not surprisingly, response to novelty and habituation are mediated by glutamatergic signalling (Leussis and Bolivar 2006). In the midbrain, the ventral tegmental area (VTA) contains dopamine (DA) neurons that provide a substantial response to novelty (Kovacs 1998). Finally, the nucleus accumbens (NAc), is a brain region postulated to play a role in

novelty-related behaviours and is a place of convergence for glutamate and dopamine (DA) neurons (Beckstead 1979).

Novelty-induced response evokes patterns of activation in the CNS that can also be detected using IEG-based strategies (Kovacs 1998). Among these genes, *c-fos* is probably the most widely used marker of neuronal activity. The most common approach used to study this gene are to detect either its mRNA *c-fos* or its protein product c-Fos.

Novelty induces a wide array of effects in brain regional c-Fos expression. Increased expression of c-Fos protein has been localised to the hippocampus, amygdala, cortex, thalamic regions and midbrain regions (Kovacs 1998; Ryabinin *et al.* 1999). In addition to the use as a marker of neuronal activation, c-Fos has also been implicated in the processes of plasticity (Fleischmann *et al.* 2003). Thus, experiences that modify the expression of *c-fos* might have plasticity-related effects through this gene.

2.3.3 DRUG ADDICTION

Addiction is a chronic and relapsing disorder that causes direct and indirect harms to an individual and for society, in social and medical problems as well as loss of productivity. It has been estimated that in the year 2010, costs related to addiction were 66 billion euros (Olesen *et al.* 2012). In Finland, 13% of the population from the age of 15-69 years reported to have used an illicit drug at some point of their lives, and 90% of population reported to use of alcohol (National Institute for Health and Welfare, 2010). However, disparity exists between the prevalence of drug use and drug abuse. In Finland, the prevalence of illicit drug abuse is estimated at 1%, and for alcohol 6% (Halme *et al.* 2008; National Institute for Health and Welfare, 2010). For benzodiazepines, 6% of the Finnish population has been estimated to use these drugs annually, and while there are no statistics of benzodiazepine dependence in Finland, dependence has been estimated to be around 1% (Vorma 2003).

The disease of addiction has many causes that exist in parallel. These can be related to social issues, self-medication of a condition (e.g. pain or anxiety), or unintentional exposure to a drug of abuse (Marlatt et al. 1988; McLellan et al. 2000). When the process that eventually leads to addiction is presented in chronologically, the first event is the rewarding effect that drugs of abuse induce. The sense of well-being caused by drugs of abuse tend to have reinforcing properties, that is, the behaviour of drug taking is repeated (White 1989). Already, the first dose is able to modulate the brain in a morphological and functional way, thus possibly laying the foundation to the addictive behaviour (Ungless et al. 2001). When the drug use continues, an additional modification, the development of tolerance, takes place that leads to reduction in the effect of a certain drug dose. This type of plasticity also promotes continued drug use and increasing of the drug dosage. At the time of withdrawal the nervous system is in an adapted state of dependence, and usually the withdrawal symptoms that emerge are opposite to the initial drug

effects. Euphoria turns into dysphoria, anxiolysis turns into anxiety, liveliness turns into exhaustion, and confidence turns into uncertainty (World Health Organization 2007). Confrontation with such strong negative feelings may further encourage drug taking. However, the definition of addiction include yet another critical features, which are the relapsing nature of the disease despite long-lasting breaks in the drug use and compulsion in drug seeking and taking (World Health Organization 2007). These behaviours are not easily attributable exclusively to the rewarding and withdrawal-causing properties of abused drugs. As the drug use continues, the rewarding properties of the drug become harder to gain, and some classes of abused drugs like psychostimulants amphetamine and cocaine lead to non-existent, or at the most, mild withdrawal. Thus, as reward, tolerance and withdrawal are important features in addiction, a more complex neuroplasticity must underlie the addictive behaviour as a whole.

2.3.3.1 Sensitisation and tolerance

Sensitisation describes a plastic event in which a response caused by a stimulus is enhanced when the stimulus is repeated (Steketee and Kalivas 2011). In relation to drugs of abuse, behavioural sensitisation develops in consequence of repeated administration of the drug, leading to enhanced motor response in experimental animals. Although the induction and expression of sensitisation is normally caused by the same drug, different classes of drugs of abuse are able to sensitise each other's response, that is, cross-sensitisation occurs among wide array of drugs of abuse. This suggests a common neuronal substrate underlying sensitisation (for review, see Steketee and Kalivas 2011). The foundations of sensitisation have implicated the mesocorticolimbic system, more specifically to VTA, NAc, prefrontal cortex and amygdala. Between these regions, neurotransmitters glutamate, DA and GABA have an established role for sensitisation signalling (Kalivas and Weber 1988; Wolf *et al.* 1995; Tecuapetla *et al.* 2010). Addiction theory of incentive-sensitisation describes a proposed process in which the sensitivity to want the drug is enhanced (Robinson and Berridge 1993).

Tolerance describes a reduction in the response when the stimulus is repeated. Thus the phenomenon of tolerance is opposite to that of sensitisation, at least at the level of definition. Thus, tolerance is a plastic event, that might be related to the self-defensive process of the biological system to resist, or tolerate, a high exposure to an exogenous chemical. In the time-course of addiction, tolerance has a pivotal role. Due to the reinforcing properties of drugs of abuse, dose escalation tends to occur which in turn causes tolerance to the pleasurable effects of the drug (Hyman and Malenka 2001). Increases in drug taking may then enhance the propensity of the addiction-related plastic changes to develop. For certain drug classes like opioids, the neuroplastic processes have been extensively elucidated (reviewed in: Williams *et al.* 2001). In general, several different processes might co-exist

such as desensitisation of the receptor, downregulation of receptor protein, recycling of receptors, circuitry-level compensations and modifications in gene expression.

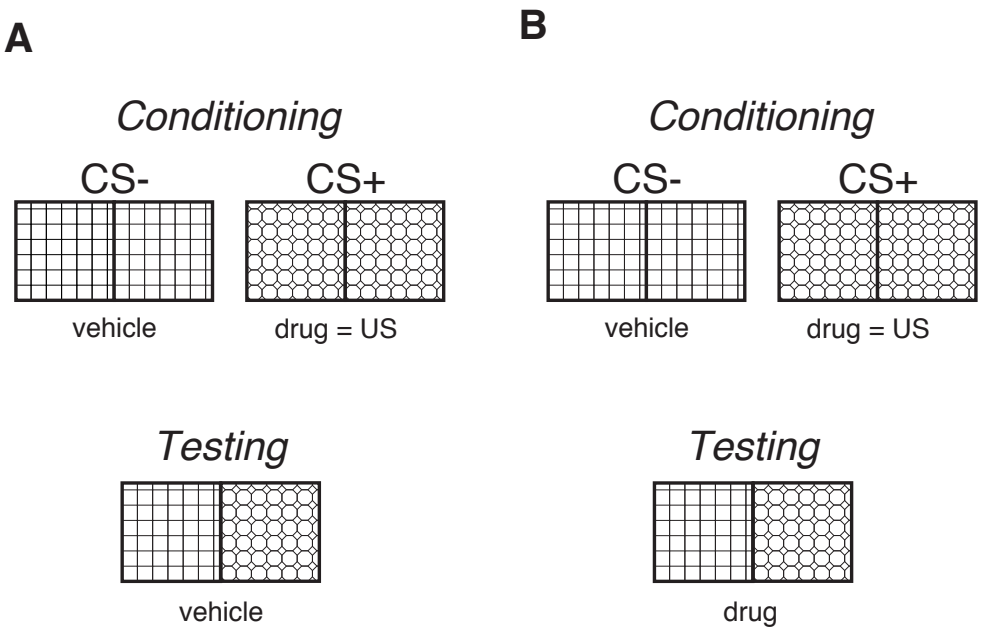

Figure 1. Place conditioning and state-dependency. (A) In place conditioning, a vehicle injection is paired with a neutral stimulus (squares) thus representing a conditioning stimulus minus trial, and drug injection with unconditional stimulus properties with another neutral stimulus (circles) thus representing a conditioning stimulus plus trial. In the test trial, both neutral stimuli are represented to choose from. If the drug-paired, previously neutral stimulus, elicits approach behaviour, it has acquired conditioned stimulus properties shown as place preference. The previously neutral stimulus has thus become conditioned stimulus. If the drug-paired stimulus elicits avoidance behaviour, it has acquired aversive stimulus properties shown as place avoidance. (B) Drug injection can produce an affective state which is present during the conditioning trial. State-dependency describes a situation when the test trial is performed in the same state as the conditioning. In contrast to that, testing can be performed in a vehicle state as shown in (A). Abbreviations: CS+, conditioning stimulus plus trial; CS-, conditioning stimulus minus trial; US, unconditional stimulus.

2.3.3.2 Pavlovian conditioning and state-dependency

In the conditioning process, a neutral stimulus lacking any clear response is paired with a stimulus that is able to produce a response in its own right thus termed an unconditional stimulus (US). After the pairing procedure, the then neutral stimulus acquires conditional stimulus properties therefore becoming conditioned stimulus (CS). When the CS is presented, it provokes a conditioned response (Pavlov 1927). Pavlovian conditioning, also termed classical or respondent conditioning, involves involuntary unconditional responses. In contrast to this, operant conditioning describes learning process in which an animal can control the behavioural consequences during the conditioning, consequently having voluntary actions playing a role in the process (Skinner 1981). In general, conditioning is a fundamental learning process that takes place in various fields of life and in several different species (Pavlov 1927; Hawkins *et al.* 1983; Schiller *et al.* 2009). Place conditioning procedure is described in Figure 1 as a type of Pavlovian conditioning.

Drug use and the rewarding feelings caused by it can be associated with environmental stimuli (also termed cues), through the process of Pavlovian conditioning (Shaham *et al.* 2003). The cue can be a certain place, people, music, smell or paraphernalia related to drug use such as needles and cigarette holder (Carter and Tiffany 1999). Thus, various common sensory modalities are covered, including visual, tactile, olfactory, gustatory and auditory. Usually the cue is not of a single modality, but a combination of many stimuli (Cunningham *et al.* 2006). Compound CS can have enhanced efficacy in acting as a CS compared to single element CS (Everitt and Robbins et al. 2005). After this process, the cues can also produce the same or similar effects as the drug. Drug-related cues can also precipitate relapse despite long periods of abstinence (Shaham *et al.* 2003), thus having a pivotal role in the critical feature of addiction. In addition to cue-induced relapse, also the drug itself can reinstate drug taking behaviour. In this case, a lower dose usually is able to produce the conditioned effect which may be attributable to the sensitisation of the processes (Robinson and Berridge 1993; Steketee and Kalivas 2011).

State-dependency describes a behavioural phenomenon in which the information retrieval is most efficient when an individual is in the same state as they were during the information acquisition. Usually the state is defined as an intrinsic affect, which can be formulated in a number of ways. Mood (Bower 1981), sleep (Patti *et al.* 2010) and drugs (Bruins Slot and Colpaert 1999) have been shown to create states which contribute to acquisition and retrieval of information. Drugs of abuse can therefore, form a state that can act as a cue in the learning process related to addiction. As a result, state-dependency may contribute to relapse behaviour when the drug-formed state is again achieved.

2.3.3.3 Neuroplastic properties of morphine and benzodiazepines

Opium, a white fluid derived from opium poppy, *Papaver somniferum*, is the etymological origin for the opioid neurotransmitter system that comprises four G-protein coupled receptors (μ, δ, κ and orphan) and several endogenously synthesised opioid peptides (for review, see Kieffer and Evans 2009). However, opium poppy-derived morphine is considered the prototypical ligand in the opioid system, with the effects of morphine well describing the wide functional distribution of the opioid system in the body. Sedative, euphoric, constipation-causing, immune system-acting, analgetic, antitussive and addictive properties of morphine indicate that the opioid system is having roles in both central and peripheral locations. Morphine binds to μ, δ, and κ opioid receptors which are linked to inhibitory G-proteins, causing inhibition of adenylate cyclase which then leads to decrease in second messenger cyclic adenosine monophosphate. Brain regional localisation of opioid receptor covers almost the entire CNS. For example, the hippocampus, thalamus, cortex, striatum, midbrain and spinal cord have strong expression of these receptors (Le Merrer *et al.* 2009).

Benzodiazepines are small-molecular weight synthetic chemicals that act as positive modulators in anion-permeable $GABA_A$ receptors (for review, see Möhler 2011). As GABA is the main inhibitory neurotransmitter in the brain, the enhanced inhibition enables the use of benzodiazepines as sedative, hypnotic, anxiolytic, muscle relaxant and an anticonvulsant. Benzodiazepine binding to $GABA_A$ receptors is determined by the subunit composition. The benzodiazepine binding site resides between the α and γ subunits (reviewed in Olsen and Sieghart 2009). Alpha 1 (α1), α2, α3 and α5 subunits render receptors sensitive to benzodiazepines. However, α4 and α6 subunits contain a histidine residue instead of arginine residue at a position participating in allosteric benzodiazepine binding site. This renders the receptor with these subunits insensitive to benzodiazepine binding (Wieland *et al.* 1992). Benzodiazepine binding sites are located throughout the brain in the hippocampus, thalamic areas, cerebellum, cortical structures and mesolimbic system (Braestrup and Squires 1982).

Morphine and benzodiazepines each target mesolimbic DA system in the VTA in a slightly different manner. Morphine preferentially binds to μ-receptors by inhibiting GABAergic interneurons, which in turn causes decreased modulatory inhibition of DA neurons (Johnson and North 1992), thus the net effect being excitation of DA neurons by the mechanism of disinhibition. This evokes increased DA release at the target areas of VTA DA neurons, most prominently in the NAc (Di Chiara and Imperato 1988). Benzodiazepines also activate VTA DA neurons via disinhibition by binding to the α1-subunit containing $GABA_A$ receptors in the interneurons (Tan *et al.* 2010).

One of the early and seemingly common plastic mechanism elicited by many drugs of abuse, thus also morphine and benzodiazepines, is the synaptic

potentiation in the VTA DA neurons (Ungless *et al.* 2001; Heikkinen *et al.* 2009). This LTP is observed as early as two hours after the drug administration and lasting beyond the actual presence of drugs and their metabolites in the brain. LTP is dependent on NMDA receptors, suggesting a similar underlying mechanism between VTA DA plasticity and the classical hippocampal LTP (Ungless *et al.* 2001; Heikkinen *et al.* 2009). However, drugs of abuse neither evoke LTP in the hippocampus nor at excitatory synapses on the VTA GABAergic cells. The specificity of this plastic event is further substantiated by the finding that other centrally acting drugs fluoxetine and carbamazepine do not cause the observed potentiation (Saal *et al.* 2003). These modifications seem to disagree with the premise that addiction is a chronic disease, since VTA DA neuron LTP was observable only for a week, even after the drug was repeatedly administered (Borgland *et al.* 2004). Therefore, other correlates of addiction-inducing plasticity must emerge.

2.4 GLUTAMATE, AN EXCITATORY NEUROTRANSMITTER

Neurotransmitter glutamate, released from the presynaptic neuron, mediates an excitatory response through its receptors thus leading to the activation of the postsynaptic neuron. Glutamate is found throughout the CNS and acts as the principal excitatory neurotransmitter in many neuronal networks (Fonnum 1984).

Apart from the role in plain excitatory neurotransmission, the glutamatergic system provides a platform for an organism to adapt to various dynamic situations. Neuronal adaptation, driven by modifications in the properties of the glutamate system, enables functions like learning and memory. Glutamatergic adaptations can lead to changes in the nervous system lasting from seconds to lifetime, thus suggesting a complex system.

2.5 CELLULAR TURNOVER OF GLUTAMATE

2.5.1 SYNTHESIS

Mismatch applies to glutamate synthesis and the site of its release as a transmitter in neurons. Although glutamate is released from neurons, most neurons contain no machinery to fully synthesise it from the precursor glucose (Hertz *et al.* 1999). More specifically, to utilise glucose in this process, an enzyme pyruvate carboxylase is required, which is expressed in glial cells of astrocyte type, but not in neurons (Yu *et al.* 1983). Astrocytes feed neurons with an amino acid glutamine (Albrecht *et al.* 2007) that has been synthesized

from glucose. The neuronal expression of enzyme phosphate-activated glutaminase subsequently enables neurons to transform glutamine to glutamate (Danbolt 2001). However, this classical view of neuronal inability to *de novo* synthesise glutamate has been challenged by providing evidence of neuronal puryvate carboxylase activity (Hassel and Bråthe 2000). Furthermore, microglial cells have also been shown to produce glutamate (Patrizio and Levi 1994).

Glutamate is also a precursor for the main inhibitory neurotransmitter GABA (see above). This transformation occurs by the use of glutamic acid decarboxylase (GAD) in GABAergic neurons (Martin and Rimvall 1993). Thus, interestingly, the principal excitatory and inhibitory neurotransmitter systems are closely interconnected at the metabolic level.

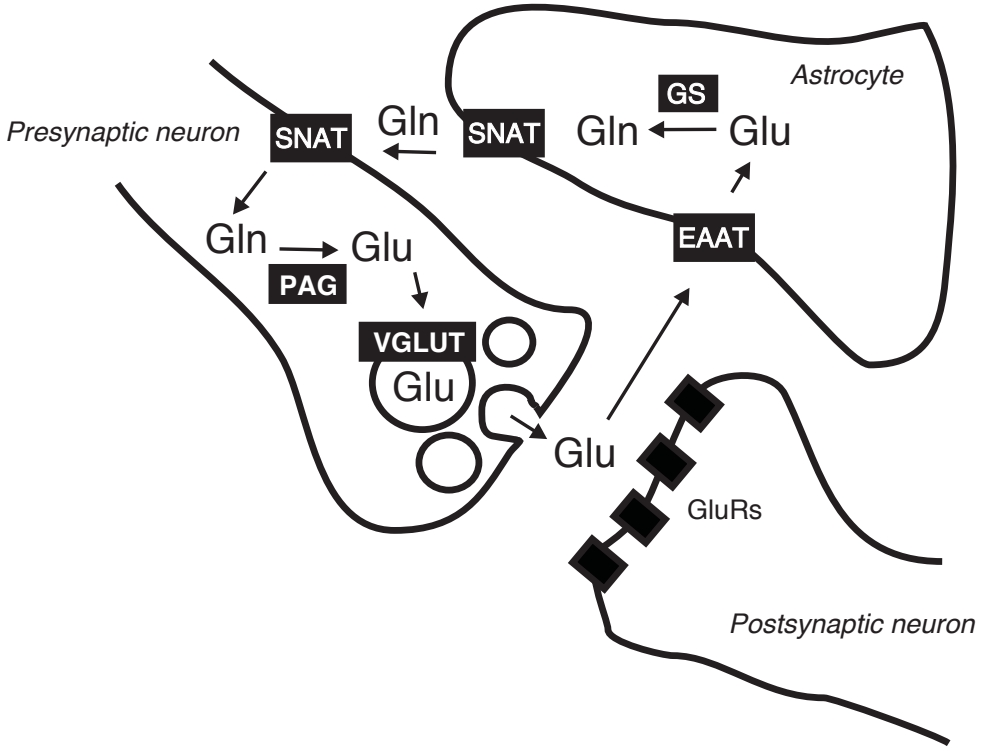

Figure 2. Glutamate-glutamine cycle. Glutamate is released from the vesicles to the synaptic cleft by exocytosis, followed by uptake to astrocytes by excitatory amino acid transporters. In astrocytes, enzyme glutamine synthetase converts glutamate to glutamine, which is transported to the extracellular space and finally to presynaptic neuron by system N/A transporters. In the presynaptic neuron glutamine is converted back to glutamate by enzyme phosphate-activated glutaminase. Glutamate is packed to presynaptic vesicles by vesicular glutamate transporter. Abbreviations: EAAT, excitatory amino acid transporter; Gln, glutamine; Glu, glutamate; GluRs, glutamate receptors; GS, glutamine synthetase; PAG, phosphate-activated glutaminase; SNAT, system N/A transporter; VGLUT, vesicular glutamate transporter.

2.5.2 RELEASE

Figure 2 represents the route of glutamate from release, to uptake, and finally to vesicle loading. Continuous release of glutamate occurs from presynaptic neurons to the synaptic cleft, which is demonstrated by the accumulation of extracellular glutamate due to the blockade of uptake capability (Jabaudon *et al.* 1999). Glutamate release typically occurs by vesicular exocytosis in response to a rise in intracellular calcium (Bollmann *et al.* 2000). These vesicles are loaded with glutamate by vesicular glutamate transporters (VGLUT1-3) that are expressed in the presynaptic terminal on the cytoplasmic vesicles. VGLUT1 and VGLUT2 are expressed in the cortical and subcortical regions, while VGLUT3 is found in the raphe serotonergic, striatal cholinergic and cerebellar and hippocampal GABAergic neurons (Ni *et al.* 1994; Aihara *et al.* 2000; Fremeau *et al.* 2002). An estimate of the number of glutamate molecules per each vesicle has been proposed based on a combination of methods of electron microscopy and immunohistochemistry, although without a resolution of a single vesicle. Vesicle size is subject to change, and thus an approximation from 400 to 4000 molecules has been suggested (Shupliakov *et al.* 1992; Schikorski and Stevens 1997). Also, reversed transporter activity, that releases instead of removing glutamate from the synapse, has been speculated as a mechanism (Rossi *et al.* 2000), although this may only exist in pathological, excitotoxic conditions triggered by abnormal cell membrane ion gradients.

Two types of glutamate release take place in neuronal communication. These being evoked, action-potential-driven, and spontaneous release that gives rise to so called miniature excitatory postsynaptic potentials. Several features differentiate these types of release. First, release rate of vesicles is highly variable (>100 Hz and 0.02 Hz, for evoked and spontaneous release, respectively), as determined by optical dye-loading (Murthy and Stephens 1999) and electrophysiological (Geppert *et al.* 1994) methods. Second, evoked and spontaneously released glutamate has been suggested to activate different postsynaptic receptor pool (Atasoy *et al.* 2008). Third, the physiological role of spontaneously released glutamate may deviate from the evoked one by, for example, stabilising the neuronal connections due to fostering of dendritic spine morphology in an AMPA receptor-mediated way (McKinney *et al.* 1999).

Glutamate is co-released with other neurotransmitters in native neuronal networks (for various neurotransmitters co-released with glutamate, see El Mestikawy *et al.* 2011). This co-release further supports the role of glutamate as the main excitatory neurotransmitter in the CNS. Co-release is based on the notion that expression of VGLUT1-3 is also localised in areas other than principal glutamate neurons (see above). This implies that VGLUTs cannot be used as specific markers for glutamatergic neurons exclusively, but that the possible expression for other transmitters has to be confirmed.

2.5.3 UPTAKE

Once released, glutamate is quickly removed from the synapses for three main reasons. First, excess glutamate is neurotoxic due to its excitatory nature. Excitotoxicity is based on glutamate-initiated cascade of events, that can lead to neuronal death (Lau and Tymianski 2010). Thus, the main excitatory neurotransmitter, critical it is to brain activity, also possesses great potential to harm the brain. Second, in order to maintain a good signal-to-noise ratio of glutamate neurotransmission, the synaptic cleft must be cleaned from glutamate. Third, recycling of glutamate reduces the need for *de novo* synthesis (see above).

Molecular cloning has identified five subtypes of glutamate transporters that are responsible for the uptake of glutamate from the synapse. These have been named excitatory amino acid transporters (EAAT1-5), and an alternative nomenclature exists for the three first: glutamate-aspartate transporter, glutamate transporter and excitatory amino acid carrier, for EAAT1, EAAT2 and EAAT3, respectively (for review, see Slotboom *et al.* 1999). EAAT1 and EAAT2 mainly account for the uptake in the CNS with the most extensive expression in the CNS (Lehre *et al.* 1995). These transporters have been largely localised to astroglia, but their neuronal expression has been debated (for review, see Danbolt 2001). The other transporters are mainly neuronal, with EAAT3 being expressed in the hippocampus, cerebellum and basal ganglia (Conti *et al.* 1998), EAAT4 in cerebellar Purkinje cells (Yamada *et al.* 1996), and EAAT5 in retinal bipolar cells (Bringmann *et al.* 2009). Thus, a site-specific mismatch largely applies to glutamate release and uptake.

When glutamate is taken up by astrocytes, it is not directly re-released to the extracellular space (see above). Instead, glial enzyme glutamine synthetase converts glutamate to glutamine (Albrecht *et al.* 2007). This process closes the circle of the two amino acids glutamate and glutamine, and is named the glutamate-glutamine cycle (see Figure 2). The importance of glutamate transporters in the cellular turnover of glutamate has led to development of various ligands to pharmacologically modulate glutamate uptake (Shigeri *et al.* 2004).

2.6 GLUTAMATE RECEPTORS

Glutamate receptors are proteins binding glutamate and mediating the excitatory synaptic transmission in the CNS. The receptors fall into two main categories, ionotropic and metabotropic receptors (reviewed in: Traynelis *et al.* 2010; Niswender and Conn 2010; Granger *et al.* 2011). Ionotropic receptors are comprised of four variable subunit proteins forming a channel that is permeable to ions, which mediate the glutamate signal. These receptors mediate the fast component of the glutamatergic transmission in a millisecond scale. Metabotropic receptors are proteins that pass the glutamate signal using G-protein coupling and mediate slower transmission modulating the

glutamatergic tone. Both receptor classes are localised in the neurons and glial cells. The classification of glutamate receptors is based both on pharmacology and molecular biology. Nomenclature of glutamate receptors is displayed in Table 1.

Glutamate receptors can be targeted with various pharmacological ligands that alter the properties and function of the receptors. Understanding the crucial role of glutamate and its receptors in CNS, it is surprising how few of the glutamatergic ligands have reached the status of a therapeutic drug in medical practice. However, a great many of the ligands have had an important role as a research tool in biological sciences. Also see below for AMPA receptor pharmacology.

2.6.1 AMPA RECEPTORS

AMPA receptors have been named after α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a synthetic pharmacological ligand that exerts an agonistic effect on these receptors (Honore *et al.* 1982). These receptors mediate the fast glutamate signal. See chapter 2.7 for a more detailed review of AMPA receptor properties.

2.6.2 NMDA RECEPTORS

NMDA receptors have been named after N-methyl-D-aspartate, which is a synthetic ligand having agonistic effect on NMDA receptors (Curtis and Watkins 1960). NMDA receptors are comprised of 14 possible subunits including variants from alternative splicing, of which two GluN1 subunits are required together with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Monyer *et al.* 1992). The outcome is thus a four-subunit tetramer with a cation-permeable pore inside the complex (Laube *et al.* 1998). The opening of the channel pore requires three events to occur. This includes binding of glutamate to GluN2 subunits, glycine acting as a co-agonist to GluN1 subunits (Johnson and Ascher 1987), and membrane depolarisation to remove the voltage-dependent physical Mg^{2+}-block from the intracellular site (Mayer *et al.* 1984). All of these requirements are modified upon the subunit composition of the receptor to yield variable binding of the ligands and to affect the sensitivity of the voltage-dependency in removing the Mg^{2+} block (Kuryatov *et al.* 1994; Kutsuwada *et al.* 1994; Cull-Candy *et al.* 2001; Clarke and Johnson 2006). The carriers of the electrical current through NMDA receptor are ions Na^{+}, Ca^{2+} and K^{+} (MacDermott *et al.* 1986; Mayer and Westbrook 1987).

NMDA receptors are located ubiquitously throughout the CNS, but regional distribution of different subtypes exists that also is regulated during the development (Monyer *et al.* 1994). Subcellular localisation studies imply that NMDA receptors are found in both post and presynaptic sites (Pinheiro and Mulle 2008).

TABLE 1. GLUTAMATE RECEPTORS

RECEPTOR FAMILY	SUBUNIT NAME	PREVIOUS NAMES	HUMAN GENE NAME	HUMAN CHROMOSOMAL LOCATION
IONOTROPIC RECEPTORS				
AMPA	GluA1	GluR-A, GluR1	*GRIA1*	5q31.1
	GluA2	GluR-B, GluR2	*GRIA2*	4q32–q33
	GluA3	GluR-C, GluR3	*GRIA3*	Xq25–q26
	GluA4	GluR-D, GluR4	*GRIA4*	11q22
NMDA	GluN1	NR1	*GRIN1*	9q34.3
	GluN2A	NR2A	*GRIN2A*	16p13.2
	GluN2B	NR2B	*GRIN2B*	12p12
	GluN2C	NR2C	*GRIN2C*	17q25
	GluN2D	NR2D	*GRIN2D*	19q13.1
	GluN3A		*GRIN3A*	9q31.1
	GluN3B		*GRIN3B*	19p13.3
KAINATE	GluK1	GluR5	*GRIK1*	21q22.11
	GluK2	GluR6	*GRIK2*	6q16.3–q21
	GluK3	GluR6	*GRIK3*	1p34–p33
	GluK4	KA1	*GRIK4*	11q22.3
	GluK5	KA2	*GRIK5*	19q13.2
ORPHAN	GluD1	GluRΔ1	*GRID1*	10q22
	GluD2	GluRΔ2	*GRID2*	4q22
METABOTROPIC RECEPTORS				
GROUP I	mGluR1		*GRM1*	6q24
	mGluR5		*GRM5*	11q14.3
GROUP II	mGluR2		*GRM2*	3p21.2
	mGluR3		*GRM3*	7q21.1
GROUP III	mGluR4		*GRM4*	6p21.3
	mGluR6		*GRM6*	5q35
	mGluR7		*GRM7*	3p26
	mGluR8		*GRM8*	7q31.3

NMDA receptors, as well as AMPA and kainate receptors, are also located in several peripheral tissues. For example, pancreatic islet cells and bone tissue osteoclasts express them (Inagaki *et al.* 1995; Chenu *et al.* 1998).

The physiological role of NMDA receptors for neuronal plasticity is critical. In response to depolarisation of the membrane, the removal of Mg^{2+} block enables Ca^{2+} flow through the receptor leading to subsequent calcium-initiated effects that govern LTP of the synapse (for review, see Blundon and Zakharenko 2008). This process is seen as a putative molecular correlate of memory (Bliss and Lømo 1973). The NMDA receptor is an "observer" of temporally concomitant stimuli (Bliss and Collingridge 1993), and thus NMDA receptor has been named a "coincidence detector" of the CNS. Due to the Mg^{2+} block, a rather high threshold in membrane potential must be overcome to facilitate the glutamate-induced activation of NMDA receptors. Consequently, the basal synaptic transmission is not mediated by NMDA but AMPA receptors, in which no such depolarisation-dependent block exist (Collingridge *et al.* 1983). Furthermore, NMDA and AMPA receptors work together in neurotransmission such that the activation of AMPA receptors leads to sufficient membrane depolarisation which also finally activates NMDA receptors (Petralia and Wenthold 2008).

2.6.3 KAINATE RECEPTORS

Kainate or kainic acid is a molecule derived from the algae *Digenea simplex* which binds to, and produces an agonistic effect on kainate receptors (London and Coyle 1979). Five kainate receptor subunits have been cloned (Bettler *et al.* 1990; Werner *et al.* 1991; Egebjerg et al. 1991; Herb *et al.* 1992; Bettler *et al.* 1992), and splice variants have been found, too (for review, see Lerma 2003). GluK1-3 can assembly as homomers or heteromers, but GluK4 and GluK5 only form functional receptors when compiled with GluK1-3 subunits (for review, see Traynelis *et al.* 2010). The binding affinity of kainate is higher in cells transfected with GluK4 or GluK5 subunits compared to GluK1-3 subunits (Werner *et al.* 1991; Herb *et al.* 1992). The kainate receptor, when activated, enables a flux of Na^+ and K^+ ions to pass through its channel (reviewed in: Traynelis *et al.* 2010).

Kainate receptors are expressed throughout the CNS, with GluK2/GluK5 heteromeric receptors being the most common subtype (Ball *et al.* 2010). Determination of the subcellular location of kainate receptors has been problematic due to the lack of selective antibodies for immunohistochemical studies needed to perform successive electron microscopy. However, both pre- and postsynaptic localisations have been suggested based on functional studies (Lerma 2003).

Physiological importance of kainate receptors is a modulatory one: Kainate receptors bidirectionally regulate glutamate release as presynaptic autoreceptors (Kullmann 2001). Although glutamate levels at the synapse are rather low due to efficient uptake mechanisms (see above), kainate receptors detect ambient

glutamate concentrations and thus constantly regulate glutamate release (Lauri *et al.* 2005). Perhaps this feature is counterbalanced by the fast desensitisation of the kainate receptors seen in electrophysiological observations, at least in the hippocampal neurons (Patneau *et al.* 1993). While NMDA and AMPA receptors have an established role in formation and expression of LTP in neuronal communication, kainate receptors have not been suggested a similar direct part on LTP (Jane *et al.* 2009). However, recent studies implicate that kainate receptors are also involved in strengthening of the synaptic transmission (Bortolotto *et al.* 1999), as a regulatory type action (Lauri *et al.* 2001). Much of the work in clarifying the roles of kainate receptors in the functions of the CNS have been put forward by pharmacology, as receptor-specific ligands have become available, and been studied in the developing hippocampus (Jane *et al.* 2009).

2.6.4 ORPHAN RECEPTORS

Orphan receptors include GluD1 and GluD2-subunit containing ion channels, which have been cloned (Yamazaki *et al.* 1992; Lomeli *et al.* 1993). These subunits show approximately 20% sequence homology to other ionotropic glutamate receptor subunits. GluD1 subunit is expressed in the hippocampus at low levels, while GluD2 subunit localises to cerebellar Purkinje cells (Lomeli *et al.* 1993). However, in recombinant expression systems, the receptors composed of these subunits neither bind glutamate nor are activated by glutamate (Yamazaki *et al.* 1992; Lomeli *et al.* 1993). D-serine binds to recombinant GluD2 receptors, but does not activate the receptor (Naur *et al.* 2007). Thus, these receptors might not represent a functional role as ion channels. Perhaps a more plausible account for the orphan receptors might be in the regulation of AMPA receptor subunits. This has been suggested by data from GluD2-deficient mice (Yamasaki *et al.* 2011) which show reduced mismatch in the number of AMPA receptors between two types of Purkinje cell synapses. These data can also assist in explaining deficient motor coordination and impaired cerebellar synapse formation and LTD seen in this mouse line (Kashiwabuchi *et al.* 1995).

2.6.5 METABOTROPIC GLUTAMATE RECEPTORS

Metabotropic glutamate receptors (mGluRs) belong to the superfamily of G-protein-coupled receptors (GPCRs) and to the subfamily of class C receptors. These receptors, when activated by glutamate, propagate the signal using G-protein interaction followed by activation or inhibition of subsequent second messenger systems. Metabotropic glutamate receptors are divided into three groups based on ligand selectivity, G-protein coupling and amino acid sequence. Group I includes mGluR1 and mGluR5, group II includes mGluR2 and mGluR3, and group III includes mGluR4, mGluR6, mGluR7 and

mGluR8. Altogether 8 genes that encode the mGluRs have been identified, several splice variants exist that can further increase the heterogeneity of the mGluR system. (reviewed in Kew and Kemp 2005).

Metabotropic glutamate receptors are expressed in dimers, presumably requiring glutamate binding to both receptor units (Suzuki *et al.* 2004; Kniazeff *et al.* 2004; Kammermaier and Yun 2005), although these studies only cover mGluR1 homomeric assembly. Signal transduction is continued by recruiting the G-protein system. Group I receptors couple to G_q protein to activate phospholipase C, resulting in the production of the second messenger, inositol-1,4,5-triphosphate. Group II and group III receptors couple to $G_{i/o}$ proteins leading to inhibition of adenylyl cyclase and the decrease of the second messenger cyclic adenosine monophosphate. However, in addition to these, a multitude of pathways can be activated by both receptor groups thus increasing the complexity of the system (reviewed in Niewander and Conn 2010).

The majority of mGluRs are widely expressed in the CNS, with only mGluR6 being restricted to the retina (Nakajima *et al.* 1993). Localisation to both neurons and glia exists, and both pre- and postsynaptic subcellular localisation has been identified (Pinheiro and Mulle 2008).

Generally, group I mGluRs are located postsynaptically (Lujan *et al.* 1996) and group II and III mGluRs presynaptically. When postsynaptic group I receptors are activated, the outcome is depolarisation of the postsynaptic membrane and thus an excitatory response. Presynaptic group II and III receptors, however, inhibit glutamate release. Group I receptors are involved in LTP and LTD induction, either as direct inductors or facilitators (Anwyl 1999). In various brain regions, mGlu-mediated LTD provides another type of bidirectional synaptic plasticity that is not NMDA receptor-dependent (Lüscher and Huber 2010), although in some brain region like amygdala both sources of activation are required for LTD (Wang and Gean 1999). In addition, both group II and group III receptors are involved in LTD (Pinheiro and Mulle 2008). Group II receptors accompany the communication between neurons and glia (Bruno *et al.* 1999), and can efficiently reduce the glutamate excitation to postsynaptic neurons. Similar role has been detected for mGlu7 receptors, which possess low affinity for glutamate and are possibly sensors of excess glutamate (Niewander and Conn 2010).

2.7 AMPA RECEPTORS AND THEIR PROPERTIES

AMPA receptors form a cation channel that is permeable to Na^+ and K^+, and depending on subunit composition of the receptor, also to Ca^{2+} ions. Ion flux through the receptor depolarises the postsynaptic membrane, leading to excitatory postsynaptic potential (EPSP). Many properties of AMPA receptors establish mechanisms for adaptational modifications that can underlie changes in behaviour.

2.7.1 AMPA RECEPTOR SUBUNITS AND TOPOLOGY

AMPA receptors are tetramers constructed out of four distinct subunits, GluA1 to GluA4, which can be assembled in either homomers or heteromers (Hollmann *et al.* 1989; Keinänen *et al.* 1990). The molecular structure is similar between different subunits, and the subunits show approximately 70% homology in sequence. Each subunit consists of approximately 900 amino acids and has a molecular weight of 105 kDa. Figure 3 depicts AMPA receptor subunit structure.

Four main domains can be identified in each subunit. The amino-terminal domain, ligand binding domain, transmembrane domain and carboxyl-terminal domain (for review, see Traynelis *et al.* 2010). The amino-terminal domain is extracellularly located, has a large lobe consisting almost half of the subunits amino acids and that has been difficult to find a functional significance for. However, using molecular biological techniques, various mutations to the amino acid sequence have been made, enabling a closer study of this domain. These data suggest that amino-terminal domain is not necessary for receptor assembly and general function of the ion channel, but is needed for regulatory roles such as binding of extracellular proteins N-cadherin and pentraxins (O'Brien *et al.* 1999). The ligand binding domain, that is also located extracellularly, consists of two lobes, S1 and S2. These lobes create a clamshell-like binding pocket for glutamate. When bound, glutamate interacts with its receptor leading to a conformational change in the ligand binding domain, changing the channel structure and opening it (Erreger *et al.* 2004). Transmembrane domains TM1-TM4 of each of the four subunits form the ion channel of the receptor. TM2 differs from the others as it forms an intracellular hairpin loop thus not fully traversing the cell membrane (Molnar *et al.* 1994). Finally, the intracellular carboxyl-terminal domain contains interaction sites for intracellular proteins that function in trafficking of the AMPA receptors, an important step in activity-dependent modifications leading to LTP or LTD, and possibly in other forms of adaptation (see for review Henley 2003).

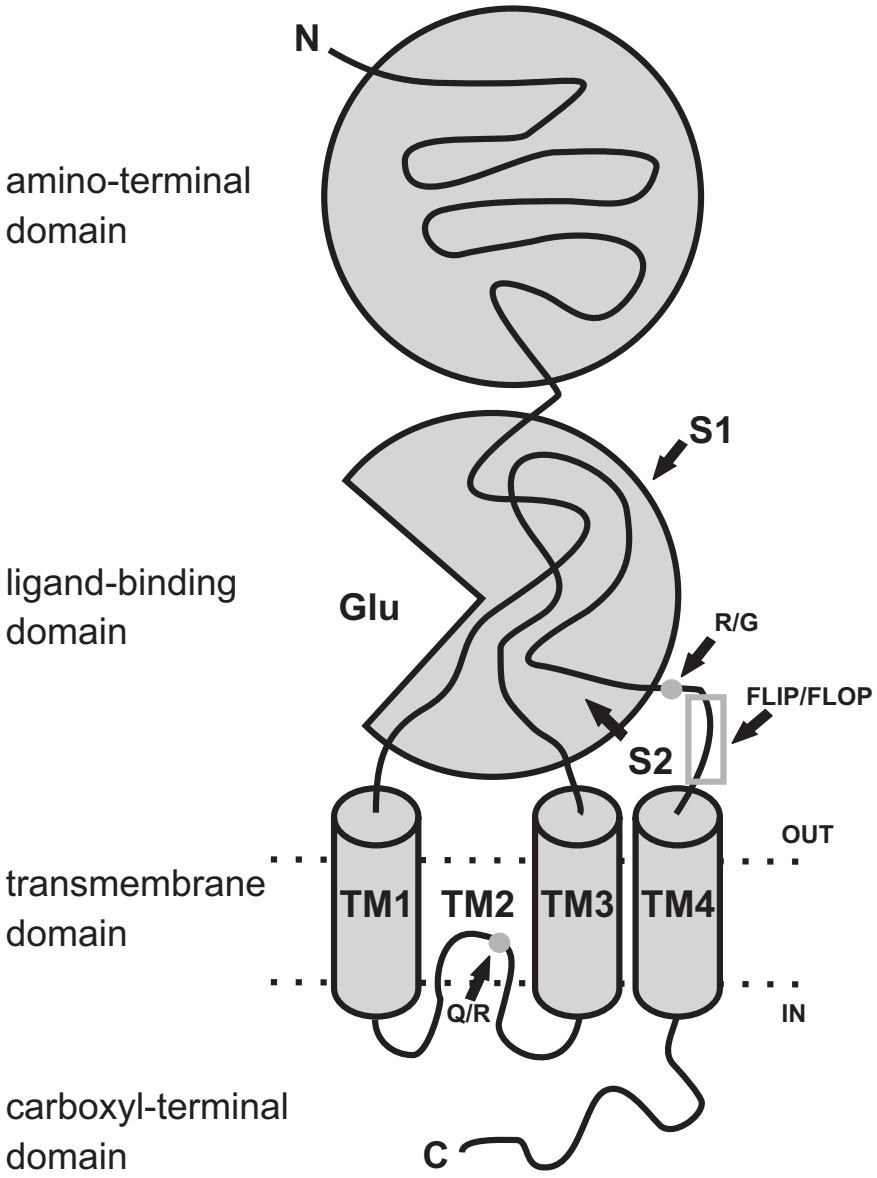

Figure 3. Topology of the AMPA receptor subunit. The four basic domains of the AMPA receptor subunit are shown. N-terminal domain comprises of nearly half of the amino acids of the subunit, but lacks a clear functional role. The ligand-binding domain consists of two lobes, S1 and S2, which form a binding pocket for glutamate. The binding of glutamate causes a conformational change in the transmembrane domain structure leading to opening of the ion channel. Carboxyl-terminal domain includes several locations for post-translational regulation such as phosphorylation. Alternative splicing occurs at flip/flop site where one of the two alternative exons is selected for the final mRNA. At the RNA editing sites the gene-encoded residue is changed post-transcriptionally to another one (R to G and Q to R). Abbreviations: Glu, glutamate; S1 and S2, ligand-binding domain lobes 1 and 2; TM1-4, transmembrane domains 1-4. “In” and “out” represent intracellular and extracellular spaces, respectively.

2.7.2 PROTEIN SYNTHESIS

Four genes encode AMPA receptor subunit proteins, *GRIA1-4* and *gria1-4*, for human and mouse genome, respectively. Each of the genes localises to a different chromosome (Collingridge *et al.* 2009). Subunits are produced and assembled in endoplasmic reticulum as dimers of dimers, and then a highly complex trafficking machinery moves them to the intracellular and synaptic sites (see below). Also, mRNA can be trafficked out to the dendritic regions, where a local machinery including polyribosomes, translational system and Golgi apparatus is expressed. The expression level of each subunit *in vitro* determines the composition of subunits (stoichiometry) in each receptor (Palmer *et al.* 2005). It remains to be determined how this occurs *in vivo*. However, RNA editing (see below) controls assembly to favor GluA2 as the most common partner (Greger *et al.* 2002). Transcription of the subunit mRNAs is regulated by various transcription factors, such as nuclear factor κB, cAMP response element, restriction element-1 and specific transcription factor-1. The gene expression of the subunits is dependent on neuronal activity, with AMPA receptors playing a role in neuronal signalling cascades regulating gene expression (Wang *et al.* 2007).

2.7.3 POST-TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION

During the protein synthesis of AMPA receptor subunits, the transcribed RNA can be modified to yield various forms of proteins that have differing functions. Furthermore, while the protein synthesis of AMPA receptor subunits takes place in the endoplasmic reticulum, the proteins undergo later modifications of phosphorylation, glycosylation, palmitoylation and ubiquitination in the Golgi apparatus. Both types of regulation have been shown a critical role in the adaptative changes that involve AMPA receptors (for review, see Henley *et al.* 2011).

2.7.3.1 Post-transcriptional regulation

Alternative splicing generates two isoforms, "flip" and "flop", of AMPA receptor subunits GluA1-GluA4 (Sommer *et al.* 1990). This modification is located extracellularly prior to TM4 (from the N-terminal) and it covers a region of 115 base pairs. The expression of these variants is dependent on the developmental stage. Flip isoform dominates during the embryonic phase, but progressively more flop isoform emerge before replacing the flip isoform at postnatal day 14 in the rat brain (Monyer *et al.* 1991). Flip isoform renders AMPA receptors less prone to desensitisation compared to flop isoform, but

affinity to AMPA remains unchanged (Mosbacher *et al.* 1994; Arvola and Keinänen 1996).

Another site of alternation in splicing produces the second type of isoforms, "short" and "long", in GluA1, GluA2 and GluA4 subunits with GluA3 lacking the specific site required for splicing to occur (Gallo *et al.* 1992). The modification site is located near C-terminal, intracellularly. GluA1 appears only in the long form, with GluA2 and GluA4 mainly in the short form (Köhler *et al.* 1994). Proteins interacting with AMPA receptors (see below) have their binding sites at the C-terminal domain and consequently alternative splicing generates different environment for these interactions (Dev *et al.* 1999).

RNA undergoes another type of post-transcriptional modification leading to changes in amino acid codons and thus amino acids themselves in the subunit proteins. RNA editing takes place at two sites and its occurrence is dependent on receptor subunit. In GluA2, adenosine deaminase acting on RNA (ADAR2) changes the codon for glutamine (CAG) to arginine (CIG, coding for arginine CGG) leading to change (Q/R) that is located in the TM2 (Higuchi *et al.* 2000). Arginine possess electrochemically positive motifs, and when inserted into the channel-forming region of AMPA receptor, results in receptors impermeable to Ca^{2+} (Sommer *et al.* 1991). Since the GluA2 subunit is predominantly found in the edited form and participates in most of the AMPA receptors assemblies, the usual AMPA receptor is impermeable to Ca^{2+}. The editing is, however, developmentally regulated leading to unedited GluA2 and thus to Ca^{2+}-permeable receptors during the embryonic phase (Kawahara *et al.* 2003). RNA editing at the second site located near the flip/flop splicing site results in a change from arginine to glycine (R/G) in the GluA2-GluA4 subunits (Lomeli *et al.* 1994). This editing modifies receptors desensitisation and resensitisation.

The variants of postranscriptional regulation taking place during development preferentially lead to receptors which contain properties that strengthen the glutamatergic neurotransmission. As discussed above, this can be achieved by decreasing desensitisation (alternative splicing and RNA editing at R/G), or by increasing Ca^{2+}-permeability (RNA editing at Q/R). The physiological significance of developmental promotion of the glutamatergic tone is not entirely known, but during development intense neurogenesis occurs, a process shown to be regulated by unedited GluA2-containing AMPA receptors (Whitney *et al.* 2008). Beyond the developmental phase, these activating processes may become unnecessary or also their role diminishes. Indeed, during adulthood, excessive Ca^{2+}-permeability of AMPA receptors causes problems in behaviour (Jia *et al.* 1996; Gerlai *et al.* 1998; Higuchi *et al.* 2000. In summary, AMPA receptors are involved in adaptational processes as early as the first stages of development.

2.7.3.2 Post-translational regulation

Phosphorylation modifies receptor subunits by the addition of a phosphate group to certain sites at the C-terminal domain. AMPA receptor subunits have their own respective phosphorylation features. GluA1 subunit is phosphorylated by protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II at serine 831, leading to increases in AMPA receptor currents. GluA1 is also phosphorylated by protein kinase A at serine 845 (reviewed in Palmer *et al.* 2005) resulting in potentiation of homomeric peak currents. GluA2 is phosphorylated by protein kinase C at serine 880, which facilitates internalisation of receptors from the synaptic membrane. GluA3 is phosphorylated at three sites, but no functional role has been suggested (reviewed in Traynelis *et al.* 2010). GluA4 is phosphorylated by protein kinase A at serine 842 leading to insertion of homomeric receptors to the synapses (Esteban *et al.* 2003). Regulation of phosphorylation is interconnected with synaptic activity so that phosphorylation of subunits modifies synaptic activity and *vice versa*. All of these phosphorylation processes, except for GluA3, are involved in LTP or LTD (reviewed in Lee 2006) through AMPA subunit surface expression (see below).

Glycosylation, a process of attachement of a carbohydrate residue, takes place at all of the AMPA receptor subunits at two sites located extracellularly. This form of regulation modifies AMPA receptor properties by protecting them from proteolysis and also modulates receptor trafficking (for review, see Palmer *et al.* 2005).

Palmitoylation, a process of fatty acid incorporation, takes place in all AMPA receptor subunits. Palmitoylation is required for correct AMPA receptor surface expression (Hayashi *et al.* 2005) through enabling interaction between AMPA receptors and their interacting proteins, in particular protein 4.1 (Shen *et al.* 2000) (see below).

Ubiquitination, a process of tagging ubiquitin to proteins, is involved in delivery of AMPA subunits to degradation (Schwartz *et al.* 2010).

2.7.4 PERISYNAPTIC TRAFFICKING AND INTERACTING PROTEINS

Once the AMPA subunits have been biosynthesised and assembled, they undergo trafficking to the synaptic membrane. The process of AMPA receptor turnover has been regarded as one of the mechanisms leading to LTP and LTD (for review, see Shepherd and Huganir 2007). The trafficking to synapses takes place using two different pathways in a subunit-specific manner, although a complete understanding of the trafficking machinery remains to be determined. A direct, exocytotic route involves the delivery of GluA2 subunits directly to the synapse. An indirect diffusion route involves preceding trafficking of GluA1 subunits to a more distal location before being finally moved to the postsynaptic density with association of synapse-associated

protein-97 (Hayashi *et al.* 2000; Passafaro *et al.* 2001; Shi *et al.* 2001). Activity-dependency is also apparent in that GluA2 receptors display constitutive perisynaptic movement associated with protein interacting with C kinase 1 (Xia *et al.* 1999), whereas GluA1 receptors, possibly also homomeric ones, show movement in response to NMDA receptor activation and an elevation in intracellular Ca^{2+}. This suggests that extrasynaptic AMPA receptors are mainly GluA1 subunit-containing, although this remains to be confirmed. Constitutive movement indicates that the turnover of AMPA receptors also includes a removal phase. In this process, GluA2-containing receptors undergo internalisation (Lee *et al.* 2004) which is based on interaction with N-ethylmaleimide-sensitive factor (NSF). On the contrary, NSF participates to insertion of GluA2 subunits to the synaptic membrane by detaching GluA2 from interacting with the protein interacting with C kinase 1 (PICK1); an interaction that stabilises AMPA receptors to the intracellular sites (Hanley *et al.* 2002). Stabilisation of AMPA receptor to the synaptic membrane, however, involves protein 4.1 (Shen *et al.* 2000) and transmembrane AMPA receptor regulatory proteins. These comprise a family of interacting proteins that not only direct receptor trafficking but also modulate the kinetics of the receptor ion channel. It has been suggested that the interactions occur on a specific site on the gaps between the subunits (Sobolevskiy *et al.* 2009).

2.7.5 LOCALISATION

AMPA receptors are expressed throughout the CNS with divergent levels across the brain regions. The distributional map of AMPA receptor expression has been determined by receptor autoradiographic (Monaghan *et al.* 1984), *in situ* hybridisation (Keinänen *et al.* 1990) and immunohistochemical (Petralia and Wenthold 1992) methods. Even though these methods measure receptors at different stages of protein synthesis (i.e. at the mRNA and at the protein level), overlapping results for the regional distribution have been gained. With the two latter techniques being capable of subunit level resolution, receptor subunit-specificity in the regional distribution has also been shown to occur. The most notable distinction to the other subunits is the very low level of GluA4 expression outside the cerebellum. In overall, the expression of the AMPA receptors in rodents is high in the hippocampal subregions (CA1, CA3 and dentate gyrus), the cortical regions, the basal ganglia, the olfactory bulbs and the amygdala.

AMPA receptors are localised in both neuronal and glial cells, although the expression takes place at lower level in the latter (Gallo and Ghiani 2000). Subcellular localisation of AMPA receptors indicates that 60-70% of the receptors are at intracellular sites, thus forming a reserve pool of receptors unless they possess an unknown duty distinct from the silent synapse theory (Lee *et al.* 2001). An extrasynaptic (Baude *et al.* 1994) and presynaptic (Pinheiro *et al.* 2003) subcellular localisations have also been suggested.

2.7.6 PHARMACOLOGY OF AMPA RECEPTORS

Glutamate is the endogenous agonist for AMPA receptors, kainate and NMDA receptors. NMDA cannot activate AMPA receptors, and AMPA cannot activate NMDA receptors. Similar selectivity does not occur between AMPA and kainate receptors (for review, see Traynelis *et al.* 2010). Indeed, kainate is an agonist at AMPA receptors, and AMPA is a partial agonist at kainate receptors (Herb *et al.* 1992; Sakimura *et al.* 1992; Sommer *et al.* 1992). Despite the highly conserved ligand-binding domain, there are other structural differences between kainate and AMPA receptors, thus warranting the search for novel ligands to better discriminate between these receptors (Sobolevskiy *et al.* 2009) with some examples already suggested (Lauri *et al.* 2001).

Antagonist pharmacology of AMPA receptors includes competitive, noncompetivite and uncompetitive ligands. Competitive, glutamate-site antagonists contain 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), 6,7-dinitroquinoxa- line-2,3-dione (DNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX), of which NBQX shows more selectivity for AMPA receptors over kainate receptors, and is lacking NMDA receptor glycine-site antagonism (Nikam and Kornberg 2001). Of these classical antagonists CNQX and DNQX act as partial agonists in the presence of transmembrane AMPA receptor regulatory proteins (TARPs). This implies that the results obtained in heterologous expression systems *in vitro* may deviate from those performed in native systems *in vivo*. Noncompetitive antagonist, 1-(4-amino- phenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI-53655), a negative allosteric modulator (see below), shows selectivity for AMPA receptors over kainate receptors (Gitto *et al.* 2003). Noncompetive, use-dependent, antagonists include polyamines argiotoxin, Joro spider toxin and spermine (Herlitze *et al.* 1993). All of these exhibit actions on AMPA receptors devoid of GluA2 subunits thereby making them important tools both in the study of Ca^{2+}-permeable AMPA receptors and in the identification of the subunit composition of AMPA receptors (Laezza *et al.* 2006). Besides the orthosteric pharmacology targeting glutamate binding site, there is an emerging variety of allosteric AMPA receptor modulators. These can be either positive or negative modulators (see above), and offer an increased possibility for subtype-selectivity due to binding with other than the conserved agonist binding site. Among the positive modulators, there are various classes of substances, e.g. cyclothiazide, aniracetam and 1-(1,4-benzodioxan-6-ylcarbonyl)piperidine (CX546), that differ in their potentiating-mechanims of action (reviewed in Traynelis *et al.* 2010).

All these AMPA receptor ligands are unable to discriminate between the subtypes of AMPA receptors. Lack of subtype-selective AMPA receptor ligands complicates the more specific study of these receptors. However, genetically engineered transgenic mouse and cell lines have provided an alternative approach to dissect the AMPA receptor system in more detail (see below).

2.8 GLUA1 SUBUNIT-DEFICIENT MOUSE LINE

GluA1 subunit-deficient mouse line (GluA1-KO mice) was generated by gene-targeted mutation of murine *gria1* gene (Zamanillo *et al.* 1999). The GluA1-KO mouse line has offered an alternative approach to study the role of the GluA1 subunit in the absence of subunit-specific pharmacological ligands.

2.8.1 BEHAVIOURAL PHENOTYPE

Table 2 represents the behavioural findings and Table 3 the electrophysiological findings in GluA1-KO mouse line. There have been two main categories in the study of GluA1-KO mouse line. These include, different aspects of learning and memory, and the modelling of psychiatric diseases. This is not surprising as the wide variety of phenomena in these categories are highly dependent on plastic events.

The immediate outcome of the deletion of *gria1* gene *per se* was perhaps a result less expected. Indeed, it was found out that the GluA1-KO mice are grossly normal and viable despite missing one of the main molecular substrates of fast neuronal transmission (Zamanillo *et al.* 1999). Even more intriguing was then the finding that the hippocampal neurotransmission actually was largely normal, although subsequent studies have shown deficits in hippocampal, amygdaloid and spinal neurotransmission (Humeau *et al.* 2007; Romberg *et al.* 2009).

The basic behavioural characterisation of the GluA1-KO mouse line speficied the baseline behavioural phenotype as surprisingly normal (Bannerman *et al.* 2004). However, novelty-related hyperactive phenotype (Vekovischeva *et al.* 2001) emerged as a very robust one, and it was reproduced in many laboratories studying the mouse line (Mead and Stephens 2003; Cowen *et al.* 2003; Bannerman *et al.* 2004; Dong *et al.* 2003; Wiedholz *et al.* 2008). Although a slight motor dysfunction in the behavioural level has been reported (Zhang *et al.* 2008), this does not seem to confound the locomotion of the GluA1-KO mouse line. This is evidenced in many locomotion-dependent tasks showing similar or greater locomotor activity for GluA1-KO mice compared to wild-type (WT) control mice.

In the processes of learning and memory, GluA1 subunit has been shown a distinctive role. GluA1-KO mice have intact spatial reference memory and disrupted spatial working memory (Zamanillo *et al.* 1999; Reisel *et al.* 2002). When these findings, repeated by different methods, are observed parallel with the non-existent hippocampal CA1 LTP in this mouse line, a dichotomy emerges between LTP and learning. However, the later studies of LTP in GluA1-KO mouse line have indicated that the absence of LTP is dependent on the electrophysiological parameters, and that there exists a GluA1-independent form of LTP existent in GluA1-KO mouse line.

TABLE 2. BEHAVIOURAL AND BASIC FUNCTIONAL FINDINGS OF GLUA1-KO MOUSE LINE

CATEGORY	BEHAVIOURAL PARADIGM	FINDING	REFERENCES
BODY TEMPERATURE	RECTAL TEMPERATURE	INCREASED	FITZGERALD ET AL. 2010
BODY WEIGHT		NORMAL	BANNERMAN ET AL. 2004
FOOD BURROWING		NORMAL	BANNERMAN ET AL. 2004
FOOD CONSUMPTION		NORMAL	BANNERMAN ET AL. 2004
GLUCOSE CONSUMPTION		DECREASED IN FEMALES	BANNERMAN ET AL. 2004
IMPULSIVE BEHAVIOR	CHOICE BEHAVIOUR	INCREASED	BARKUS ET AL. 2011
NOCICEPTION	TAIL-FLICK, HOT PLATE	NORMAL	VEKOVISCHEVA ET AL. 2001; HARTMANN ET AL. 2004; FEYDER ET AL. 2007; CHOURBAJI ET AL. 2008
INFLAMMATORY PAIN	PLANTAR CFA TEST, FORMALIN AND CAPSAICIN TESTS	DECREASED OR INCREASED	HARTMANN ET AL. 2004
LOCOMOTOR ACTIVITY	NOVELTY-INDUCED LA	INCREASED	VEKOVISCHEVA ET AL. 2001; COWEN ET AL. 2003; BANNERMAN ET AL. 2004; CHOURBAJI ET AL. 2008; WIEDHOLZ ET AL. 2008; FITZGERALD ET AL. 2010
LOCOMOTOR ACTIVITY	NOVELTY-INDUCED REARINGS	INCREASED	CHOURBAJI ET AL. 2008
LOCOMOTOR ACTIVITY	HOME CAGE LA	NORMAL	WIEDHOLZ ET AL. 2008
MOTOR PERFORMANCE	ROTAROD, TREADMILL, GRIP STRENGHT	DECREASED	ZHANG ET AL. 2008
MOTOR COORDINATION	ROTAROD, HORIZONTAL BAR, STATIC BARS	NORMAL OR DECREASED	COWEN ET AL. 2003; BANNERMAN ET AL. 2004; PALACHICK ET AL. 2008; ZHANG ET AL. 2008
NESTING		DECREASED IN MALES	BANNERMAN ET AL. 2004

TABLE 2. CONTINUED

CATEGORY	BEHAVIORAL PARADIGM	FINDING	REFERENCES
RESPONSE TO NOVEL OBJECT	RESPONSE TO NOVEL OBJECT	INCREASED	WIEDHOLZ ET AL. 2008
INFORMATION GATING	PREPULSE INHIBITION	DECREASED	WIEDHOLZ ET AL. 2008
SENSORY REFLEXES	SHIRPA TEST	NORMAL	FITZGERALD ET AL. 2010
SUCROSE PREFERENCE		NORMAL	BARKUS ET AL. 2011
ANXIETY	EPM; LDB; ZERO-MAZE	NORMAL, DECREASED OR INCREASED	BANNERMAN ET AL. 2004; MEAD ET AL. 2006; FITZGERALD ET AL. 2010
ANXIETY	HYPONEOPHAGIA	INCREASED LATENCY TO DRINK	BANNERMAN ET AL. 2004
ANXIETY	BLACK-WHITE ALLEY TEST	INCREASED IN FEMALES	BANNERMAN ET AL. 2004
DEPRESSION	LEARNED HELPLESSNESS	INCREASED	CHOURBAJI ET AL. 2008
DEPRESSION	FST	DECREASED IMMOBILITY	FITZGERALD ET AL. 2010
FEAR BEHAVIOR	AUDITORY AND CONTEXTUAL FEAR CONDITIONING	DECREASED IN GENDER-SPEFICIC MANNER	HUMEAU ET AL. 2007; DACHTLER ET AL. 2011; FEYDER ET AL. 2007
COGNITION AND EXECUTIVE FUNCTIONS	PUZZLE BOX	DECREASED	BEN ABDALLAH ET AL. 2011
INTERSESSION HABITUATION	LA	DECREASED OR NORMAL	VEKOVISCHEVA ET AL. 2001; COWEN ET AL. 2003; WIEDHOLZ ET AL. 2008
INTRASESSION HABITUATION	LA	DECREASED	VEKOVISCHEVA ET AL. 2001
NON-SPATIAL MEMORY	DRL	DECREASED	REISEL ET AL. 2005

TABLE 2. CONTINUED

CATEGORY	BEHAVIORAL PARADIGM	FINDING	REFERENCES
OBJECT RECOGNITION	OBJECT RECOGNITION	NORMAL	WIEDHOLZ ET AL. 2008; SANDERSON ET AL. 2011
RECOGNITION MEMORY, NON-SPATIAL	OBJECT RECOGNITION MEMORY	DECREASED	SANDERSON ET AL. 2011
RECOGNITION MEMORY, SPATIAL	Y-MAZE	DECREASED SHORT-TERM, INCREASED LONG-TERM	SANDERSON ET AL. 2009
REFERENCE MEMORY, SPATIAL	WATERMAZE, RADIAL MAZE, Y-MAZE	NORMAL, DECREASED REVERSAL	ZAMANILLO ET AL. 1999; SCHMITT ET AL. 2003; BANNERMAN ET AL. 2003; BANNERMAN ET AL. 2004; SCHMITT ET AL. 2004;
REFERENCE MEMORY, SPATIAL	WATERMAZE, Y-MAZE, HIPP LESIONS	NORMAL	REISEL ET AL. 2002
SENSITIZATION	LA	NORMAL; INCREASED AMPH	VEKOVISCHEVA ET AL. 2001; DONG ET AL. 2004
TRACE CONDITIONING MEMORY	T-MAZE	NORMAL	TAYLOR ET AL. 2011
WORKING MEMORY, SPATIAL	T-MAZE, Y-MAZE	DECREASED	REISEL ET AL. 2002; SCHMITT ET AL. 2003; SCHMITT ET AL. 2005; SANDERSON ET AL. 2007; SANDERSON ET AL. 2011

TABLE 2. CONTINUED

CATEGORY	BEHAVIOURAL PARADIGM	FINDING	REFERENCES
REINFORCEMENT	SA	NORMAL SA, NORMAL CUE-INDUCED REINSTATEMENT, OVER-RESPONDING IN EXTINCTION	COWEN ET AL. 2003; MEAD AND STEPHENS 2007; BARKUS ET AL. 2011
REWARD	CPP	COCAINE, DECREASED OR NORMAL; FOOD, NORMAL	DONG ET AL. 2004; MEAD AND STEPHENS 2005
REWARD PROCESSING	OPERANT REWARD CONDITIONING, FOOD	INCREASED ACQUISITION, NORMAL PIT, DECREASED OUTCOME-SPECIFIC PIT, NORMAL PAVLOVIAN APPROACH, DECREASED CONDITIONED REINFORCEMENT, DECREASED EXTINCTION, NORMAL DISCRIMINATION LEARNING, INCREASED REVERSAL LEARNING	MEAD AND STEPHENS 2003; JOHNSON ET AL. 2007; BARKUS ET AL. 2011
REWARD PROCESSING	OUTCOME-SPECIFIC DEVALUATION	DECREASED	JOHNSON ET AL. 2005
REWARD PROCESSING	CONDITIONED EMOTIONAL RESPONSE	NORMAL	MEAD ET AL. 2006
SOCIAL BEHAVIOR	NEUTRAL TERRITORY, ANOGENITAL AND NON-ANOGENITAL INVESTIGATION	ABNORMAL IN NOVEL, NORMAL IN HABITUATED	WIEDHOLZ ET AL. 2008; BARKUS ET AL. 2011
STRESS	LA, INJECTION OR RESTRAINT STRESS	INCREASED	FITZGERALD ET AL. 2010
STRESS	LDB, RESTRAINT-STRESS INDUCED	NORMAL	MOZHUI ET AL. 2010

ABBREVIATIONS: *AMPH*, AMPHETAMINE; *CPP*, CONDITIONED PLACE PREFERENCE; *DD*, LIGHTS OFF 24H CYCLE; *DRL*, DIFFERENTIAL REINFORCEMENT OF A LOW RATE OF RESPONDING; *EPM*, ELEVATED PLUS-MAZE; *FST*, FORCED SWIM TEST; *HIC*, HETEROGENEOUS INSTRUMENTAL CHAIN; *LA*, LOCOMOTOR ACTIVITY; *LD*, LIGHTS ON 12H - LIGHTS OFF 12H CYCLE; *LDB*, LIGHT-DARK BOX; *SA*, SELF-ADMINISTRATION; *SHIRPA*, SMITHKLINE BEECHAM-HARWELL-IMPERIAL COLLEGE-ROYAL LONDON HOSPITAL-PHENOTYPE ASSESSMENT

TABLE 3. ELECTROPHYSIOLOGICAL FINDINGS

CATEGORY	ANATOMICAL LOCATION	MANIPULATION	FINDING	REFERENCES
SYNAPTIC TRANSMISSION	HIPP, AMYG, ACC, SPINAL CORD	BASELINE	NORMAL OR DECREASED	ZAMANILLO ET AL. 1999; HOFFMAN ET AL. 2002; ANDRASFALVY ET AL. 2003; HARTMANN ET AL. 2004; HUMEAU ET AL. 2007; ROMBERG ET AL. 2009; TOYODA ET AL. 2009
SYNAPTIC PLASTICITY	SCHAFFER COLLATERAL CA3-CA1 LTP	BASELINE	DECREASED, PARAMETER-DEPENDENT	ZAMANILLO ET AL. 1999, MACK ET AL. 2001; HOFFMAN ET AL. 2002; JENSEN ET AL. 2003; FREY ET AL. 2009; ROMBERG ET AL. 2009
SYNAPTIC PLASTICITY	HIPP, THETA-BURST PAIRING LTP	BASELINE	NORMAL, INITIAL COMPONENT DECREASED	HOFFMAN ET AL. 2002
SYNAPTIC PLASTICITY	CA1 STP	BASELINE	DECREASED	ERICKSON ET AL. 2011
SYNAPTIC PLASTICITY	THAL-AMYG AND CTX-AMYG LTP	BASELINE	DECREASED	HUMEAU ET AL. 2007
SYNAPTIC PLASTICITY	ACC AND SSC LTP	BASELINE	DECREASED	TOYODA ET AL. 2009
SYNAPTIC PLASTICITY	VTA DA NEURON AMPA/NMDA RATIO	BASELINE, COCAINE, STRESS	MODIFIED	DONG ET AL. 2004
SYNAPTIC PLASTICITY	CA1 DISTANCE-DEPENDENT SCALING	BASELINE	DECREASED	ANDRASFALVY ET AL. 2003
NEUROMUSCULAR UNIT TRANSMISSION	SOLEUS MUSCLE	BASELINE	NORMAL	ZHANG ET AL. 2008

ABBREVIATIONS: ; *ACC*, ANTERIOR CINGULATE CORTEX; *AMYG*, AMYGDALA; *BLA*, BASOLATERAL AMYGDALA; *CA*, CENTRAL NUCLEUS OF AMYGDALA; *CTX*, CORTEX; *DA*, DOPAMINE; *HIPP*, HIPPOCAMPUS; *IHC*, IMMUNOHISTOCHEMISTRY; *LTP*, LONG-TERM POTENTIATION; *PFC*, PREFRONTAL CORTEX; *STP*, SHORT-TERM POTENTIATION; *SSC*, SOMATOSENSORY CORTEX; *STR*, STRIATUM; *THA*L, THALAMUS; *VTA*, VENTRAL TEGMENTAL AREA

TABLE 4. NEUROCHEMICAL, MORPHOLOGICAL AND METABOLIC FINDINGS OF GLUA1-KO MOUSE LINE

CATEGORY	METHOD, BRAIN REGION(S)	MANIPULATION	FINDING	REFERENCES
AUTORADIOGRAPHY, DAT	[^{3}H]WIN35,428	BASELINE	NORMAL	WIEDHOLZ ET AL. 2008
BIPOLAR DISORDER	pMARCS AND pNEUROMODULIN	LITHIUM, CHRONIC	DECREASED	FITZGERALD ET AL. 2010
CA^{2+} PERMEABILITY	COBALT LOADING; SPINAL CORD	BASELINE	DECREASED, LAMINAE I/II; NORMAL III/IV	HARTMANN ET AL. 2004
CELL ACTIVATION TO INFLAMMATION	IEG ISH	FORMALIN-INDUCED INTRAPLANTAR INFLAMMATION	DECREASED, REGION-SPECIFIC	HARTMANN ET AL. 2004
CELL ACTIVATION TO MECS	IEG ISH	MECS	NORMAL	ZAMANILLO ET AL. 1999
GENE EXPRESSION PROFILING	ALL MOUSE GENES; HIPP	BASELINE	CALCIUM-RELATED GENE AND PROTEIN CHANGES	ZHOU ET AL. 2009
HISTOCHEMISTRY	GLUTAMATE RECEPTOR SUBUNITS; HIPP, BLA, CA, SPINAL CORD	BASELINE	MODIFIED LOCALIZATION OF GLUA2 IN HIPP; INCREASED GLUA2/3 SOMAS IN BLA; INCREASED GLUN1 IN HIPP; OTHERS NORMAL	ZAMANILLO ET AL. 1999; MEAD AND STEPHENS 2003; HARTMANN ET AL. 2004; CHOURBAJI ET AL. 2008
HISTOCHEMISTRY	SUBSTANCE P AND ISOLECTIN-B4	BASELINE	NORMAL	HARTMANN ET AL. 2004
METABOLISM	MONOAMINE CONTENT; STR	DOPAMINE DEPLETION	NORMAL MONOAMINES, INCREASED 5-HIAA	FITZGERALD ET AL. 2010
METABOLISM	DA CLEARANCE; STR	BASELINE	DECREASED	WIEDHOLZ ET AL. 2008
METABOLISM	DA RELEASE; STR	AMPH-INDUCED	NORMAL	WIEDHOLZ ET AL. 2008
METABOLISM	GLUTAMATE; HIPP	BASELINE	INCREASED	CHOURBAJI ET AL. 2008
METABOLISM	5-HT AND NORADRENALINE; HIPP	BASELINE	DECREASED	CHOURBAJI ET AL. 2008

TABLE 4. CONTINUED

PAIN	CA^{2+} RESPONSES; DORSAL HORN OF SPINAL CORD	CFA INJECTION	DECREASED CA-RESPONSES	LUO ET AL. 2008
PAIN	ERK PHOSPHORYLATION	FORMALIN, CFA, STIMULATION OF PRIMARY FIBERS	DECREASED	HARTMANN ET AL. 2004; TOYODA ET AL. 2009
PHARMACOKINETICS	ETHANOL; BLOOD	BASELINE	NORMAL	PALACHICK ET AL. 2008
PHARMACOKINETICS	MORPHINE; BLOOD AND BRAIN	BASELINE	NORMAL	VEKOVISCHEVA ET AL. 2001
PHARMACOKINETICS	LITHIUM; BLOOD	BASELINE	NORMAL	FITZGERALD ET AL. 2010
STRESS	CORTICOSTERONE; PLASMA	RESTRAINT STRESS, ACUTE; FST, ACUTE	NORMAL	FUMAGALLI ET AL. 2010; FITZGERALD ET AL. 2010
STRESS	GLUCOCORTICOID RECEPTORS; HIPP	RESTRAINT STRESS, ACUTE	NORMAL	FUMAGALLI ET AL. 2010
STRESS	ARC; HIPP AND PFC	RESTRAINT STRESS, ACUTE	INCREASED	FUMAGALLI ET AL. 2010
STRESS	CAMKII; HIPP AND PFC	RESTRAINT STRESS, ACUTE	INCREASED PHOSPHORYLATION	FUMAGALLI ET AL. 2010
STRESS	GLUN1, GLUN2B; HIPP	RESTRAINT STRESS, ACUTE	DECREASED PHOSPHORYLATION	FUMAGALLI ET AL. 2010
STRESS	SYN1; HIPP	RESTRAINT STRESS, ACUTE	NORMAL	FUMAGALLI ET AL. 2010
THROMBOSIS	BLEEDING TIME	BASELINE	DECREASED	MORRELL ET AL. 2008
MORPHOLOGY	FINE STRUCTURE OF DENDRITES AND SYNAPSES	BASELINE	NORMAL	ZAMANILLO ET AL. 1999
MORPHOLOGY	MOTOR NEURON MORPHOLOGY	BASELINE	VARIOUS MODIFICATIONS, E.G. DECREASED LENGTH OF DENDRITES	ZHANG ET AL. 2008
MORPHOLOGY	GASTROCNEMIOUS MUSCLE MORPHOLOGY	BASELINE	DECREASED DIAMETER AND NUMBER OF TYPE I FIBERS	ZHANG ET AL. 2008

ABBREVIATIONS: *5-HT*, 5-HYDROXYTRYPTOPHAN; *ACC*, ANTERIOR CINGULATE CORTEX; *AMPH*, AMPHETAMINE; *AMYG*, AMYGDALA; *ARC*, ACTIVITY-REGULATED CYTOSKELETAL-ASSOCIATED PROTEIN; *BLA*, BASOLATERAL AMYGDALA; *CA*, CENTRAL NUCLEUS OF AMYGDALA; *CAMKII*, CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE; *CFA*, COMPLETE FREUND'S ADJUVANT; *CTX*, CORTEX; *DA*, DOPAMINE; *DAT*, DOPAMINE TRANSPORTER; *HIPP*, HIPPOCAMPUS; *IEG*, IMMEDIATE-EARLY GENE; *IHC*, IMMUNOHISTOCHEMISTRY; *ISH*, IN SITU HYBRIDIZATION; *LA*, LOCOMOTOR ACTIVITY; *MECS*, MAXIMAL ELECTROSHOCK; *PFC*, PREFRONTAL CORTEX; *pMARCS*, PHOSPHORYLATED MYRISTOYLATEDALANINE-RICH C KINASE; *pSYN1*, SYNAPSIN 1; *SSC*, SOMATOSENSORY CORTEX; *STR*, STRIATUM; *THAL*, THALAMUS; *VTA*, VENTRAL TEGMENTAL AREA

TABLE 5. PHARMACOLOGICAL FINDINGS OF GLUA1-KO MOUSE LINE

PHARMACOLOGY	DOSE	PARADIGM	FINDING	REFERENCES
AMPH, ACUTE	2 MG/KG	LA	NORMAL OR INCREASED, PARAMETER-DEPENDENT	VEKOVISCHEVA ET AL. 2001; FITZGERALD ET AL. 2010
AMPH, PAIRED	2 MG/KG	LA	INCREASED	VEKOVISCHEVA ET AL. 2001
AMPH, UNPAIRED	2 MG/KG	LA	NORMAL	VEKOVISCHEVA ET AL. 2001
COCAINE, ACUTE	15 MG/KG	LA	NORMAL	DONG ET AL. 2004
COCAINE, REPEATED	15 MG/KG	LA	NORMAL	DONG ET AL. 2004
ETHANOL	1.75-3.5 G/KG	LA, ROTAROD, LORR, HYPOTHERMIA	NORMAL, DECREASED HYPOTHERMIA	COWEN ET AL. 2003; PALACHICK ET AL. 2008
ETHANOL, CHRONIC	ESCALATION DOSING	SA, ADE	NORMAL	COWEN ET AL. 2003
GYKI 52466	10 MG/KG	NOVELTY-INDUCED LA	NO EFFECT	FITZGERALD ET AL. 2010
HALOPERIDOL	0.3 MG/KG	NOVELTY-INDUCED LA	DECREASE	WIEDHOLZ ET AL. 2008
LITHIUM CARBONATE, CHRONIC	4 MG/KG	NOVELTY-INDUCED LA, EPM, BODY WEIGHT	DECREASED HYPERACTIVITY, DECREASED ENTRIES TO OPEN ARMS, NORMAL BODY WEIGHT	FITZGERALD ET AL. 2010
METHYLPHENIDATE	5 MG/KG	LA	INCREASED	FITZGERALD ET AL. 2010
MK-801	0.1-1 MG/KG	LA	NORMAL OR DECREASED	VEKOVISCHEVA ET AL. 2001; WIEDHOLZ ET AL. 2008

TABLE 5. CONTINUED

PHARMACOLOGY	DOSE	PARADIGM	FINDING	REFERENCES
MOR, ACUTE	3-30 MG/KG	LA	INCREASED	VEKOVISCHEVA ET AL. 2001
MOR, ACUTE	5 MG/KG	TAIL-FLICK LATENCY	NORMAL	VEKOVISCHEVA ET AL. 2001
MOR, CHRONIC	INCREMENTAL DOSING	TAIL-FLICK LATENCY	DECREASED TOLERANCE	VEKOVISCHEVA ET AL. 2001
MOR, CHRONIC	INCREMENTAL DOSING	WITHDRAWAL SYMPTOMS	DECREASED	VEKOVISCHEVA ET AL. 2001
SB216763, SUBCHRONIC	2 MG/KG	LA, FST	NO EFFECT	FITZGERALD ET AL. 2010
α-METHYL-p-TYROSINE METHYL ESTER	200 MG/KG	LA	INCREASED ACTIVITY	FITZGERALD ET AL. 2010

ABBREVIATIONS: *ADE*, ALCOHOL DEPRIVATION EFFECT; *AMPH*, AMPHETAMINE; *EPM*, ELEVATED-PLUS-MAZE; *FST*, FORCED SWIM TEST; *LA*, LOCOMOTOR ACTIVITY; *LORR*, LOSS OF RIGHTING REFLEX; *MOR*, MORPHINE; *OFT*, OPEN FIELD TEST; *SA*, SELF-ADMINISTRATION; *SHIRPA*, SMITHKLINE BEECHAM-HARWELL-IMPERIAL COLLEGE-ROYAL LONDON HOSPITAL-PHENOTYPE ASSESSMENT

Reward learning also is very specifically disrupted in GluA1-KO mouse line. While pavlovian conditioning and the ability of the cue to increase instrumental actions to obtain the reward (pavlovian-to-instrumental transfer) were functional in the GluA1-KO mice, the cue itself was not able to act as a conditioned reinforcer and second-order schedules of reinforcement (Mead and Stephens 2003). However, place conditioning is functional in these mice, as evidenced by place preference induced by drug (morphine, cocaine) and non-drug rewarding stimuli (food).

Aversive learning, studied as tone and contextual fear conditioning with foot-shock as an unconditioned stimulus, has been shown to be disrupted in GluA1-KO mice (Humeau *et al.* 2007). This deficit in fear conditioning was linked to absent amygdaloid LTP in both the subnuclei of the basolateral amygdala, basal and lateral nucleus. Interestingly, the basolateral amygdala is critically important for both reward and fear conditioning (Phillips and LeDoux 1992; Baxter and Murray 2002), but only the latter is disrupted in GluA1-KO mice. Thus, the conditioning processes mediated by the amygdala are complex ones, suggesting that there may be a GluA1-dependent dissociation between reward and aversive conditioning.

The emotional behaviour of the GluA1-KO mouse line has also been studied in anxiety-related paradigms. Several studies have utilised variable methods, with almost as many different outcomes (Table 2). Thus, the anxiety-related phenotype of the GluA1-KO mouse line has not been fully determined.

The pathophysiological basis of various psychiatric diseases has been studied in GluA1-KO mice. In addiction-related symptomatology, GluA1 subunit has been shown a critical role in mediating the conditioned reinforcement of reward, development of tolerance to drugs of abuse and finally in control-over behaviour evidenced as over-responding during extinction (Vekovischeva *et al.* 2001; Mead and Stephens 2003; Mead and Stephens 2007). As another core feature of addiction, relapse behaviour in response to cues previously associated with the drug reward, was not, however, modified in GluA1-KO mice. Stress is able to precipitate relapse behaviour, and while GluA1-KO mice show enhanced locomotor response to mild injection stress, no stress-induced relapse behaviour has been studied in this mouse line. However, VTA DA neurons show no cocaine-induced plasticity in GluA1-KO mice (Dong *et al.* 2004), a finding that might be linked to many of the behavioural phenotypes listed above.

GluA1-KO mouse line has been postulated to show various aspects of symptoms related to schizophrenia and bipolar disorder. Locomotor hyperactivity in response to novelty and mild stress, deficient prepulse inhibition and impaired working memory all represent symptoms found in these disorders (World Health Organization 1999). Additionally, depression-like phenotype has been suggested based on increased learned helplessness. However, decreased immobilisation detected in forced swim and tail suspension tests directs to an opposite direction, thus indicating a complex behavioural phenotype (Chourbaji *et al.* 2008).

2.8.2 NEUROCHEMICAL PHENOTYPE

Table 4 represents the neurochemical findings of GluA1-KO mouse line. Deletion of the GluA1 subunit from the mouse proteome provides an indication of the importance of subunit partnerships in the trafficking of neurotransmitter receptor subunits. While only the GluA1 subunit is absent, both the expression and the localisation of GluA2 subunit critically change so that less GluA2 is trafficked to the dendritic cell membranes while more of it stays in the somata (Zamanillo *et al.* 1999; Mead and Stephens 2003).

A decade after the generation of the GluA1-KO mouse line a whole genome expression profile was performed from the hippocampal tissue (Zhou *et al.* 2009). A total of 38 genes were found to be regulated more than 30%, with main part of these related to calcium signalling. Other studies have addressed calcium permeability in the absence of GluA1 subunit using cobalt loading in spinal cord slices *ex vivo*, finding decreased permeability in laminae I/II and unchanged permeability in laminae III/IV (Hartmann *et al.* 2004). Thus, deletion of GluA1 subunit and consequently the homomeric calcium-permeable channels results in calcium-related compensatory changes in the hippocampus.

The expression of IEGs *c-fos* and *arc* or their protein products were found to be increased in response to novelty hyperactivity and restraint stress in the hippocampus, and decreased in response to formalin-induced intraplantar inflammation in the spinal cord (Hartmann *et al.* 2004; Fumagalli *et al.* 2010). Maximal electroshock, however, failed to alter the expression of *c-fos*, *zif268*, *c-jun* and *RGS2* (Zamanillo *et al.* 1999). Restraint stress increased the expression of phosphorylation of Ca^{2+} / calmodulin-dependent protein kinase and decreased the phosphorylation of NMDA receptor subunits GluN1 and GluN2B, while the expression of these protein remained unmodified (Fumagalli *et al.* 2010). These results indicate that IEG signalling is functional in the absence of GluA1 subunit, and can be used as a marker of cellular activation (Kovacs 1998). These data also support the notion that GluA1-KO mice are supersensitive to rather small changes in the environmental status, while a powerful electroshock produces normal cellular activation. Thus, GluA1 subunit may be critical in mediating the changes of small magnitude, but not of large ones.

Monoamine metabolism in GluA1-KO mice has been determined in various brain regions at the basal state and after multitude of manipulations. In the hippocampus, serotonin (5-HT) content was found to be decreased without any manipulations (Chourbaji *et al.* 2008). The clearance of exogenous microdialytically applied DA was decreased in GluA1-KO mice without alterations in DA release or dopamine transporter expression (Wiedholz *et al.* 2008).

2.8.3 PHARMACOLOGICAL PHENOTYPE

Table 5 represents the pharmacological findings of GluA1-KO mouse line. The main interest in the pharmacological study of GluA1 mouse line has been in drugs of abuse. Acute locomotor effects of the stimulants amphetamine, methylphenidate and cocaine produce variable outcomes in GluA1-KO mouse line. Locomotor-activating effect to amphetamine is either normal or increased (Vekovischeva *et al.* 2001; Fitzgerald *et al.* 2010), to methylphenidate the effect is increased (Fitzgerald *et al.* 2010), and to cocaine the effect is normal (Dong *et al.* 2004).

Cocaine-induced sensitisation remains normal in GluA1-KO mice. However, amphetamine-induced sensitisation is increased when amphetamine is paired with the locomotor measurement context, but not when amphetamine is given in the home cage (Dong *et al.* 2004; Vekovischeva *et al.* 2001). Repeated tolerance-inducing administration of morphine leads to decreased tolerance in GluA1-KO mice (Vekovischeva *et al.* 2001). Thus, deletion of GluA1 subunit impairs these forms of drug-induced plasticity that suggest the importance of GluA1 subunit-containing AMPA receptors in addiction-related symptomatology.

Ethanol-induced behaviours are largely normal in GluA1-KO mice, with the exception that GluA1-KO mice were insensitive to ethanol-induced hypothermia (Cowen *et al.* 2003).

Pharmacokinetics of morphine, ethanol and lithium were found to be unaltered in GluA1-KO mouse line. (Vekovischeva *et al.* 2001; Cowen *et al.* 2003; Fitzgerald *et al.* 2010).

2.8.4 OTHER GENETICALLY MODIFIED AMPA RECEPTOR MOUSE LINES

GluA1(R/R) transgenic mice express a genetically modified GluA1 subunit with glutamine at the position 582 replaced by arginine, thus leading to receptors with very low channel conductance and no permeability to calcium (Vekovischeva *et al.* 2001, also see chapter 2.7.3.1). However, in the brain of these mice the GluA1 subunits are present and should successfully pair with GluA2 subunits, thus enabling membrane trafficking of GluA2 subunits. These mice have largely the same phenotype as the global GluA1-KO mice (Vekovischeva *et al.* 2001), suggesting that the phenotype of the GluA1-KO mouse line is due to the absence of functional GluA1 subunit-containing AMPA receptors.

GluA2-deficient mice (GluA2-KO) have been generated in two laboratories (Jia *et al.* 1996; Shimshek *et al.* 2006). Deletion of GluA2 subunit results in a decrease in reproductive performance, maternal agonistic behaviour, explorative behaviour, motor coordination and in an impairment in reference memory in the water maze task (Gerlai *et al.* 1998; Shimshek *et al.* 2006). The findings indicating impaired reference memory are at odds with the

electrophysiological recordings displaying highly increased hippocampal and anterior cingulate cortical LTP (Jia *et al.* 1996; Toyoda *et al.* 2009). GluA2-KO mice have modified responses in addiction-related, plasticity-requiring behaviours as evidenced by impairments in pavlovian-to-instrumental transfer, conditioned approach, conditioned emotional response and food-conditioned place preference, although they have spared conditioned place preference for cocaine (Mead and Stephens 2003; Mead and Stephens 2005; Mead *et al.* 2006). Inflammatory pain-induced behavioural responses are mainly increased (Hartmann *et al.* 2004). In summary, the pattern of behavioural findings observed in GluA2-KO mouse line appears to be the opposite to that of GluA1-KO mouse line.

GluA3-deficient mice (GluA3-KO) were generated and behaviourally characterized (Sanchis-Segura *et al.* 2006). The basic behavioural characterisation displayed no marked abnormalities in these mice, with the exception of slightly decreased novelty-induced locomotor activity and impairment in motor coordination. However, after an extinction/deprivation phase, these mice display both reduced number of ethanol-related lever-presses in response to the ethanol-paired cue and reduced ethanol drinking. However, self-administration neither in the voluntary ethanol drinking paradigm nor in the operant setting was changed. These data suggest a specific role for GluA3 subunit in ethanol relapse behaviour, although reduced levels of expression for other AMPA receptor subunits were detected (Sanchis-Segura *et al.* 2006). Despite the fact that synaptic potentiation measured as LTP in cerebrocortical to lateral amygdala connections was absent in GluA3-KO mice, these mice showed both spared auditory and contextual fear conditioning behaviour (Humeau *et al.* 2007). In inflammation-induced pain the GluA3 subunit appears to be disposable (Hartmann *et al.* 2004).

GluA4-deficient mice (GluA4-KO mice) have a specific hearing deficit related to sound localisation accompanied by impaired neurotransmission in calyx of Held synapses (Yang *et al.* 2011). GluA4-KO mice also display EEG-recordable spontaneous absence seizures, that are sensitive to ethosuximide treatment (Paz *et al.* 2011).

3 AIMS OF THE STUDY

This study was performed to determine the role of glutamate AMPA-type receptor GluA1 subunits in various behavioural and neurochemical adaptive events. To accomplish these goals, a mouse line deficient in GluA1 subunit was utilised, and exposed to a range of challenges that require neuronal adaptation. The specific aims can be summarised as:

1. To perform a behavioural characterisation of the GluA1-KO mouse line

2. To investigate the role of the GluA1 subunit in encounters that require agonistic behaviour

3. To study the importance of GluA1 subunit in the processing of spatial novelty-related signals

4. To study the role of the GluA1 subunit in the acute and subchronic effects of the benzodiazepine flurazepam

5. To clarify the role of the GluA1 subunit in the morphine-induced state-dependency

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS

GluA1-KO mouse line was generated as described in Zamanillo et al. (1999). The male and female animals used in the experiments were produced by heterozygous breeding. Littermate WT mice were used as controls. The GluA1 mouse line was backcrossed to the C57BL/6J strain throughout the study so that initially the number of backcrosses was 5 and eventually reaching over 10 backcrosses. Genotype was determined using PCR from ear punch biopsy as described in Zamanillo et al. (1999). All experimental animal studies were approved by Southern Finland Provincial Government and Experimental Animal Committee of the University of Helsinki.

4.2 BEHAVIOURAL METHODS

The basic phenotypic profile (**I**) and the effects of flurazepam (**III**) were determined by SHIRPA behavioural screen which is based on visual observation of the animals (Rogers *et al.* 1999).

Agonistic behaviour (**I**) was studied in various paradigms by observing mouse behaviour from video recordings using Ethograph software. In resident-intruder test, the test (resident) mouse was isolated for various time periods followed by encounter with a group-housed intruder mouse in the home cage of the test mouse. The same test was also performed in male mice after sexual activity and in female mice after isolation during the period of gestation. Interline agonistic behaviour was studied by exposing isolated GluA1-KO and WT mice to a neutral territory. Agonistic behaviour in a group was assessed within each genotype by placing isolated mice in a large arena. The test was repeated until all aggressive mice were identified.

Anxiety behaviour (**I**) was studied in elevated plus-maze and light-dark exploration tests. Elevated plus-maze consisted of a central platform from which the mouse had access to two open arms and two closed arms. The maze was elevated to the height of 50 cm above floor level, and the closed arms offered mice a safe option to explore while the open arms were unprotected. The mice were placed in the central platform and their behaviour was observed. In the light-dark test the mice had access to either a light or a dark arena which were connected by an open door. The mice were placed in the light arena followed by observation of their behaviour.

Locomotor activity (**II**, **IV**) was determined by placing the animal in a polycarbonate box followed by detection of horizontal movements using Ethovision software.

Circadian rhythm of locomotor activity (**II**) was determined in home cages using photobeam activity frames. For the first seven days the mice were kept on a light-dark 12:12 h schedule, and thereafter the next seven days they were kept in constant darkness.

Forced swim test (**II**) was performed by placing the animal in a large beaker containing water. Tail suspension test (**II**) was performed by taping the mouse by its tail into a vertical metal bar. In both tests, the mouse behaviour was video-recorded and subsequently visually observed using Ethograph software. These tests have used to assess depression-like immobility phenotype, relieved by antidepressants (Porsolt *et al.* 1977; Steru *et al.* 1985).

Withdrawal symptoms (**III**) after subchronic flurazepam treatment were precipitated with 20 mg/kg (s.c.) flumazenil followed by visual observations of the symptoms that included jumping, digging, stretching, "wet-dog"—like shakes, forepaw tremor, paw treading, head twitching, writhing, circling behaviour, and pelvic elevation.

Conditioned place preference (**IV**) was studied by pairing a saline injection with either plastic or metal floor material in the first session of the day, and morphine injection with the other floor type in the second session of the day. After the conditioning sessions, the mice were provided a choice session in which both floor types were present. In the choise session, either saline or morphine was injected to study state-dependency. The spatial location and distance moved was determined by Ethovision software.

Morphine discrimination (**IV**) was conducted in operant chambers with two levers. Mice were trained to lever-press for sweetened condensed milk after injection of saline or morphine on a fixed ratio schedule. Subsequently the mice were tested for morphine-appropriate responding after injection of various testing doses of morphine.

4.3 NEUROCHEMICAL METHODS

Autoradiographic analyses (**I**, **IV**) were performed using various radioactively labeled ligands that bind to target proteins. The radioligands used were, [^{3}H]-S-AMPA to study binding to AMPA receptors, [^{3}H]SYM 2081 to study binding to kainate receptors, [^{3}H]MK-801 to study binding to NMDA receptors and GTPγ[^{35}S] to study opioid receptor signalling. For these analyses, mouse brains were dissected, frozen on dry ice, stored at -80 °C and later coronal sections were cut using a cryostat followed by thaw-mounting on glass slides. In general, the sections were incubated with a radioactive ligand in the presence or absence of a non-labeled ligand to determine specific and non-specific binding. After the incubation, the sections were exposed on a photographic film with plastic radioactive standards. The films were scanned for images, and the binding densities were determined by M5 software.

Brain monoamines (**I**) were determined from brain samples using HPLC with electrochemical detection. For the analysis, the brain samples were

dissected using a brain mould, frozen on dry ice, stored at -80 °C and homogenized using an ultrasonic processor.

Plasma testosterone (**I**) was determined using a radioimmunoassay. For the analysis, trunk blood was collected into heparinized tubes, centrifuged and plasma subjected for diethylether extraction prior to radioimmunoassay.

The expression of c-Fos (**II**) in various brain regions was determined by immunohistochemical method. For the analysis, the brains were dissected, frozen on dry ice, stored at -80 °C, cut into coronal sections using cryostat and thaw-mounted on glass slides. After preliminary preprocessing of the samples, the sections were incubated with primary goat anti-c-Fos antibody and secondary biotinylated horse anti-goat antibody. Photographed sections were counted for c-Fos-positive cells using Image Proplus 6 software. Primary rabbit anti-tyrosine hydroxylase antibody and secondary biotinylated donkey anti-rabbit antibody were added when performing double-immunostaining for c-Fos and tyrosine hydroxylase (**II**).

Flurazepam and desalkylflurazepam (**III**) were determined from blood plasma and brain tissue by liquid chromatography with mass spectrometric detection. For the analysis of blood plasma, trunk blood was collected into tubes containing ethylenediaminetetraacetic acid, centrifuged and plasma subjected for diethylether extraction. Brain tissue was homogenized using a mechanical processor.

4.4 ELECTROPHYSIOLOGICAL METHODS

AMPA/NMDA current ratio was determined *ex vivo* in the VTA DA neurons after systemic morphine injection. For the analysis, brains were dissected and midbrain slices were cut and studied with whole-cell patch-clamp recordings. The DAergic neurons were indentified by detection of hyperpolarisation-activated cation current. Baseline EPSC was evoked by electrical stimulation. AMPA receptor-mediated currents were pharmacologically isolated by blocking NMDA receptors with (2*R*)-amino-5-phosphonopentanoate.

4.5 STATISTICAL TESTS

The results were tested using analysis of variance (ANOVA), followed by *post-hoc* tests such as paired-samples *t*-test, independent-samples *t*-test, Bonferroni test, Newman-Keuls multiple comparison test, Fisher's test, Duncan test, least signifcant difference post-test, or Wilcoxon matched-pair test, depending the number of groups in question and whether the data were parametric or non-parametric. Correlations analysis was performed using Pearson's correlation. Statistical analyses were performed using SPSS and GraphPad Prism softwares.

5 RESULTS AND DISCUSSION

5.1 GLUA1 SUBUNIT-DEFICIENT MICE DISPLAY NORMAL PRIMARY BEHAVIOUR

GluA1-KO mice were inspected for abnormalities in the primary behaviour including sensory reflexes and functions, motor performance, muscle function and transfer arousal followed by instant locomotor activity (**I**; **III**, Fig. 1). No differences were found in any of the tests of SHIRPA phenotype screen, suggesting that GluA1-KO mice are viable without overt sensory, muscular, motor or neurological disturbances. These data are substantiated by similar findings found elsewhere (Bannerman *et al.* 2004; Wiedholz *et al.* 2008; Fitzgerald *et al.* 2010). In summary, the normal primary behaviour enables the use of the mouse line for further experiments. This is important as numerous behavioural tests are based on the locomotion of the mouse or detecting place of a mouse based on locomotor activity.

The phenotypic characterisation of the GluA1-KO mouse line was extended by observing the circadian rhythm of locomotor activity. The mice displayed a normal pattern of activity during light-dark 12:12 h (LD) schedule (**II**, Fig. 1C-D), as reported previously for mice (Schwartz & Zimmerman 1990). When exposed to constant darkness in the dark-dark 12:12 h (DD) schedule, the mice continued to show circadian rhythm obtained during the previous LD cycle which then underwent a gradual phase advance (**II**, Fig. 1E-F), which has also been reported for mice (Schwartz & Zimmerman 1990). These results are perhaps surprising considering the expression of glutamate AMPA receptor subunits in the suprachiasmatic nucleus, a main generator of the circadian rhythmicity in the mammalian brain (Ebling 1996).

Anxiety profile of the GluA1-KO mouse line was found to be somewhat less anxious than WT mice (**I**, Fig. 3). The anxiety-related phenotypes have been extensively studied in GluA1-KO mouse line. It seems that this phenotype is a rather complex one since unchanged, increased and decreased anxiety have all been proposed (Bannerman *et al.* 2004; Mead *et al.* 2006; Fitzgerald *et al.* 2010).

Neurochemical characterisation was performed to detect possible modifications in the glutamatergic system. [^{3}H]-S-AMPA binding was shown to be decreased in the hippocampi of GluA1-KO mice (**I**, Fig. 5), providing more evidence to decreased AMPA receptor expression (Zamanillo *et al.* 1999; Mead and Stephens 2003). In keeping with the protein blotting results (Zamanillo *et al.* 1999), no changes in hippocampal [^{3}H]MK-801 and [^{3}H]SYM 2081 binding were detected (**I**, Fig. 5), suggesting unaltered NMDA and kainate receptor expression, respectively.

In GluA1-KO mouse line, the *gria1* gene is non-functional for the entire age of the mouse, i.e. from the development to the adult age. In rats, AMPA receptor mRNA can be detected during the embryonic stage (Monyer *et al.*

1991). More specifically, the GluA1 subunit protein is expressed in rat brain at embryonic day E15.5, and the expression levels increase later at the postnatal stage (Martin *et al.* 1998). Synaptogenesis occurs in rodents at the first postnatal weeks, and functional AMPA receptors are present, forming synapses from the very beginning (for review, see Groc *et al.* 2006). Thus, the absence of GluA1 subunit in the GluA1-KO mouse line may lead to possible compensatory modifications of other genes, these genes' expression and post-transcriptional regulation of the resulting protein products. This issue has been addressed in a study performing a whole genome screen of the hippocampal tissue from the GluA1-KO mouse line (Zhou et al. 2009). The screening yielded 38 genes that were regulated more than 30%. Of these genes, the changed expression was confirmed in protein level for the obligatory NMDA receptor subunit GluN1 and calcium/calmodulin-dependent protein kinase II alpha (CaMK2A). Upregulation of GluN1 may suggest a need to compensate for the slightly reduced AMPA receptor-mediated transmission (Table 3 and section 2.8.1), and downregulation of CaMK2A may mirror a need to compensate for the increased calcium influx. Thus, some compensation occurs when one calcium regulator is absent. From this data it can also be seen that no other AMPA receptor subunits were found to be altered despite the lack of GluA1. Finally, although the GluA1-KO mouse line is viable without any gross abnormalities, one cannot exclude the fact that the *gria1* gene deletion-induced compensations that take place either during development or during adult age may affect the present results. Nevertheless, the role of this subunit in various forms of plasticity in the CNS has been specified and clarified by use of this mouse line.

5.2 POSITIVE PLASTICITY IN GLUA1-KO MOUSE LINE

5.2.1 GLUA1 SUBUNIT IS ESSENTIAL FOR PLASTICITY ENABLING NORMAL RESPONDING TO CHANGED SOCIAL STATE IN ENVIRONMENT

Agonistic behaviours were studied in GluA1-KO mouse line in various paradigms to acquire a wide-ranging picture of the involvement of GluA1 subunit in plasticity that is required for normal behaviour in mouse social interaction (Miczek *et al.* 2001). When GluA1-KO mice were isolated for various periods of time and then subjected to resident-intruder encounter as resident mice, they showed less agonistic behavioural elements towards the intruder than the WT littermate control mice (**I**, Fig. 1A and Table 1). Even long-lasting isolation of 30 days or agonistic encounter without isolation did not evoke normal aggressive behaviour in GluA1-KO mice (**I**, Fig. 1B and Table 2). Finally, the results from interline encounters on a new territory substantiated the previous findings and further specified the changed

behavioural strategy of GluA1-KO mouse line as more defensive-like in situations when attacked by another mouse (**I**, Fig. 2 and Table 2).

GluA1-KO mice showed aggressive behaviour in some social encounters suggesting reduced, but not completely absent aggressive behaviour in these mice. However, when the social encounter in resident-intruder test was repeated thus providing the mice new opportunities to change their behaviour (**I**, Fig. 1A and Table 2), GluA1-KO mice still showed remarkably reduced aggression which is a clear indication of the lacking ability to adapt to a new situation (Miczek *et al.* 2001). Thus, although the GluA1-KO mice were constantly at a disadvantage in the social interaction compared to WT mice, they showed no indication to enhance their status in the social situation. This might have fundamental deleterious influence on survival in the changing habitat of the mice. These results suggest that the plastic processes mediated by GluA1 subunit are required for successful encounter in changed social circumstances.

These data are supported by a range of pharmacological findings on mouse aggression. Non-competitive AMPA receptor antagonist GYKI 52466 was found to reduce aggression in a mouse line selectively bred for high aggression, while competitive antagonists CNQX and NBQX were less effective on aggressive behaviours (Vekovischeva *et al.* 2007). Although these antagonists are non-selective over various AMPA receptor subtypes, they nevertheless provide another piece of evidence that decreased activation of AMPA receptors is linked to reduction in aggression.

The lack of aggressive behaviour was found to be sex-dependent, as female mice of GluA1(R/R) line showed similar agonistic behaviour as WTs (**I**, Table 2). Thus, the deletion of GluA1 subunit renders only males deficient in aggressive behaviour. However, this was not caused by abnormal levels of plasma testosterone or brain monoamines (**I**, Fig. 4), despite both have been clearly linked to aggressive behaviour (Siever 2008). The normal testosterone levels in male GluA1-KO mice may be a surprising finding, since accumulating lines of evidence point to the role of AMPA receptors in regulation of testosterone levels. First, glutamatergic activation of gonadotropin-releasing hormone neurons is mainly mediated by AMPA receptors (Spergel *et al.* 1999). Second, AMPA receptors co-localise with androgen or estrogen receptors in hypothalamic neurons (Diano *et al.* 1997). Finally, to close the circle, gonadal steroids testosterone and estradiol regulate AMPA receptor expression in the hypothalamus (Diano *et al.* 1997). Moreover, deletion of the GluA2 subunit in gonadotropin-releasing hormone neurons failed to alter testosterone levels (Shimshek *et al.* 2006). Despite this, GluA2-KO mice show a profound lack of aggression (Shimshek *et al.* 2006), thus substantiating the findings that highlight the importance of AMPA receptors as mediators of agonistic behaviours.

There is another possible, although also a plasticity-dependent explanation for the reduced aggression observed in GluA1-KO mice. Agonistic behaviours largely develop in a social context over time, and usually significant experience with other mice is required before mice show agonistic behaviour as adults (King and Gurney 1954; King 1957; Miczek *et al.* 2001). Thus, it could be

assumed that there is a significant component of learning in the development of the aggressive behaviour in mice. In GluA1-KO mice, which show deficient learning in various tasks (Table 2), may also have a reduced capability to learn aggressive behaviours from their counterparts, or from their parents. Hence, the reduced aggression in GluA1-KO mice may be attributable to impaired imprinting behaviour (Spalding 1872), a phenomenon that enables learning critical behaviours from the parents and previously suggested to underlie aggressive behaviour in mice (Mugford and Nowell 1972). It should be noted that imprinting might be a consequence of deficient behaviour either in the parents or in the offspring. However, the former is not supported for two reasons. First, although the GluA1-KO and WT mice were bred using heterozygous mice, the WT mice showed robust agonistic behaviour in all behavioural situations of social interaction (**I**). Second, haploinsufficiency of GluA1 subunit spares social behaviour in mice (Wiedholz *et al.* 2008) implicating that heterozygous parents set a normal example of social behaviour to their offspring. Finally, imprinting has previously been suggested to depend on glutamate receptor-mediated plasticity (McCabe and Horn 1988).

5.2.2 SPATIAL NOVELTY INDUCES HYPERACTIVITY AND EXCESSIVE NEURONAL ACTIVATION IN GLUA1-KO MICE

Processing of novel signals was studied in GluA1-KO mice by exposing them to spatial novelty and then measuring their locomotor activity. GluA1-KO mice displayed increased locomotor activity in the novel cage from the beginning of the observation session, suggesting that these mice are hyper-responsive to this type of change in their environment (**II**, Fig. 1A-B). The strong novelty-induced response was sustained for 4 hours as compared to WT control mice, although gradually decreasing to the baseline level (**II**, Fig. 1A-B). This suggests that the intrasession habituation is somewhat impaired in these mice. The finding of novelty-induced hyperactivity is a very robust one, and has been documented in various paradigms (Table 2). In earlier studies also deficient intersession habituation has been detected (Vekovischeva *et al.* 2001; Wiedholz *et al.* 2008). These data suggest a critical role for GluA1 subunit-mediated neuroplasticity in normal processing of novel signals in a task of nonassociative learning (Leussis and Bolivar 2006).

The pharmacological data on AMPA receptor antagonists substantiates these findings by demonstrating that AMPA receptor antagonist CNQX reduces habituation when microinjected to the hippocampus (Izquierdo *et al.* 1993; Vianna *et al.* 2000). Furthermore, targeting NAc with another AMPA receptor antagonist DNQX reduces habituation, but interestingly DNQX also is able to decrease reaction to spatial novelty when injected just prior to exposure to novelty (Roullet *et al.* 2001). The reciprocal roles of GluA1 and GluA2 subunits in responding to novelty are supported by findings of GluA2-KO mouse line (Jia *et al.* 1996). Contrary to the behaviour of GluA1-KO mice,

GluA2-KO mice show hypolocomotion in response to spatial novelty, although this feature has not been studied in more detail (Gerlai *et al.* 1998).

Exposure to novelty induced brain region-dependent increase in c-Fos protein expression (**II**, Table 1), creating a similar pattern of activation as described earlier (Kovacs 1998). c-Fos as a marker for the activated cells needed to fullfil the following criteria: (i) quick initiation of expression due to the rapid onset of hyperlocomotion of GluA1-KO mice in response to spatial novelty, (ii) maximal expression of the marker should have mirrored the time spent in novelty which was 2 h, and (iii) negligible expression in normal state of an animal. As the induction of *c-fos* mRNA occurs within couple of minutes, the maximal expression of c-Fos occurs between 1-3 h and expression is minimal in normal state, c-Fos can be summarised to reflect the acute spatial challenge in GluA1-KO mice (Kovacs 1998). The basal c-Fos expression remained unchanged in GluA1-KO mice with the exception of midbrain region substantia nigra in which GluA1-KO mice had more c-Fos positive cells although this region did not respond to novelty (**II**, Table 1). However, after novelty GluA1-KO mice had more c-Fos positive cells in certain regions compared with WT mice (**II**, Table 1). Especially the hippocampal subregions were more activated in GluA1-KO mice (**II**, Fig. 2). The main cortical input region to the hippocampus, the entorhinal cortex, was also activated more in GluA1-KO mice than in WT controls (**II**, Fig. 3). These data show that in GluA1-KO mice the higher responding to novelty is linked with pronounced neuronal activation of hippocampus and its main cortical input region, which might underlie the deficient detection of novelty (Vinogradova 2001).

Generation of new neurons (neurogenesis) in the adult hippocampal dentate gyrus was found to be reduced in GluA1-KO mice (**II**, Fig. 4), but the survival of the newly-born neurons increased (**II**, Fig. 4). The fate of the proliferated cells either to neurons or glia was not different between GluA1-KO and WT mice (**II**). These data are of interest because neurogenesis has been implicated in the etiology of depression (Jacobs *et al.* 2000), a psychiatric disorder the GluA1-KO mouse line has been suggested to model (Chourbaji *et al.* 2008). Also two other animal models show alterations in neurogenesis. In chronic mild stress model, both reduced hippocampal neurogenesis and GluA1 subunit levels were observed with normal levels of novelty exploration (Toth *et al.* 2008). In olfactory bulbectomy, reduced hippocampal neurogenesis was linked to novelty hyperactivity (Jaako-Movits and Zharkovsky 2005). However, in forced swim and tail suspension tests which both have predictive validity in depression studies (Steru *et al.* 1985), GluA1-KO mice show no depression-like phenotype (**II**, Fig. 6C). On the contrary, GluA1-KO mice display decreased immobility, rather suggesting acute mania-like behaviour. Indeed, spatial novelty-induced hyperactivity that undergoes clear habituation (spiking hyperactivity) has been documented in patients with bipolar mania (Perry *et al.* 2009). Also, in support of this, both acute and chronic treatment with the selective serotonin reuptake inhibitor escitalopram failed to alter novelty hyperactivity in GluA1-KO mice (**II**, Fig. 6A-B, D). Finally, novelty hyperactivity shows correlation with reduced proliferation of newly born cells

(Lemaire *et al.* 1999) suggesting that neurogenesis is functionally linked to processing novel information. However, in another study using mice with genetical reduction in proliferation, no alterations in novelty-induced locomotor activity were observed (Dupret *et al.* 2008).

Interestingly, AMPA receptor positive modulator CX546 failed to alter hyperlocomotion in GluA1-KO mice (**II**, Fig. 1B). However, the AMPA receptor antagonist NBQX resulted to partial rescue of normal response to novelty (**II**, Fig. 1A) further supporting the notion of glutamatergic overactivation in the brains of GluA1-KO mice.

Hippocampal LTP and LTD have previously been linked to novelty exposure (Manahan-Vaughan and Braunewell 1999; Li *et al.* 2003). These cellular plastic events represent putative correlates of learning and memory, and thus may show importance in the processing of novel signals in e.g. by encoding of spatial information. Although the classical hippocampal LTP is absent in GluA1-KO mice (Zamanillo *et al.* 1999), GluA1 subunit-independent LTP is largely spared (Hoffman *et al.* 2002; Romberg *et al.* 2009). Thus, this form of cellular plasticity may be recruited in GluA1-KO mice, and may underlie the eventual behavioural habituation to novelty in these mice (**II**, Fig. 1A-B). However, the facilitation LTP or LTD by exposure to novelty has not been studied in GluA1-KO mice (Table 3).

Furthermore, it is intriguing to note that the increased neuronal activation in the hippocampi of GluA1-KO mice may lead to increased cellular plasticity. In the behavioural level, this view is supported by data indicating that while short-term spatial memory is deficient in GluA1-KO mice, they show increased long-term spatial memory (Sanderson *et al.* 2009). Thus, an alternative explanation emerges, suggesting that in some instances GluA1-KO mice may have superior plasticity than WT mice. In line with this, *c-fos* has been reported to play a role in plasticity (Fleischmann et al. 2003).

5.3 NEGATIVE PLASTICITY IN GLUA1-KO MOUSE LINE

5.3.1 GLUA1-KO MOUSE LINE RETAIN SENSITIVITY TO ACUTE BENZODIAZEPINE FLURAZEPAM, BUT SHOW IMPAIRED DEVELOPMENT OF TOLERANCE AND INCREASED WITHDRAWAL

The pharmacology of flurazepam was studied in GluA1-KO mice using modified SHIRPA behavioural test battery as described (**III**, Table 1). First, acute sensitivity was determined by injecting flurazepam in acute 40 mg/kg (s.c.) dose and 30 min later observing mouse behaviour. Flurazepam elicited a clear sedative and muscle relaxant pattern of effects in motor performance, muscle relaxation, sensorimotor parameters and body temperature (**III**, Fig. 1) in both GluA1-KO and WT mice, with the exception that startle response was

somewhat decreased only in GluA1-KO mice. Concentrations of flurazepam and its main metabolite, desalkylflurazepam, were identical in GluA1-KO and WT mice 2 hours after the injection of 60 mg/kg (s.c.) (**III**, Table 2). These data suggest that the GABAergic system as the main inhibitory neurotransmitter system is largely normal despite the lack of GluA1 subunit, which is one of the main contributors to the counterbalancing excitatory glutamatergic system. Thus, this might suggest that no GluA1 deletion-induced compensations in the genes or their expression were extended to the GABAergic system.

Intact flurazepam sensitivity in GluA1-KO mouse line allowed determination as to whether the neuroplastic processes mediating chronic effects of flurazepam are dependent on GluA1 subunit. The subsequent behavioural investigations were twofold. First, recovery after an acute dose of flurazepam was determined. Second, development of tolerance to the effects of flurazepam was studied followed by observation of withdrawal.

After an acute 40 mg/kg (s.c.) dose of flurazepam, GluA1-KO mice needed more time to recover, as evidenced by gait and pelvic elevation tests (**III**, Fig. 2; Fig. 3A). There was a genotype-independent difference between the sexes; females recovering quicker than males. These effects were not due to pharmacokinetics of flurazepam, as blood and brain content of flurazepam were identical between GluA1-KO and WT mice after the recovery (**III**, Fig. 3B-C). Desalkylflurazepam was detected at higher blood and brain concentrations in WT male mice than in others (**III**, Fig. 3D-E). These data suggest that glutamate-driven plasticity is recruited already when mice are under the influence of flurazepam or its metabolites. More specifically, these data suggest that the lack of GluA1-dependent plasticity renders mice incapable of developing normal acute tolerance to the sedative and muscle relaxant effects of flurazepam already at the very first stage.

Subchronic treatment with high doses of flurazepam resulted in partial development of tolerance in both mouse lines (**III**, Fig. 4). However, GluA1-KO mice developed less tolerance than WT mice. The pharmacokinetic parameters after the subchronic treatment were identical between GluA1-KO and WT mice (**III**, Table 2). Forty-eight hours after the cessation of subchronic treatment, the flumazenil-precipitated withdrawal symptoms were scored. GluA1-KO mice had more severe withdrawal than WT mice (**III**, Fig. 5).

Changes in pharmacokinetics of flurazepam and desalkylflurazepam partly explain the development of tolerance in mice, although there were no differences between the pharmacokinetic parameters between GluA1-KO and WT mice. More specifically, the desalkylflurazepam to flurazepam concentration ratios were highly increased in both genotypes after the subchronic flurazepam treatment, documenting an induction of flurazepam metabolism.

These results demonstrate that GluA1 subunit-mediated neuroplasticity plays a role in both acute and repeated benzodiazepine administration. After an

acute dose, AMPA receptors have been implicated in benzodiazepine-induced plasticity localised to VTA DA neurons by showing increased AMPA/NMDA receptor current ratio and inward rectification (Heikkinen *et al.* 2009; Tan *et al.* 2010). This plasticity is thought to be mediated by GluA2-lacking AMPA receptors, which undergo rapid trafficking to the cell membrane and are permeable to calcium (Shi *et al.* 2000). The GluR2-lacking receptors are most likely GluA1 homomeric receptors, since GluA3 homomers show no rectification (Shi *et al.* 2001). Also, the hippocampus shows similar molecular and cellular plasticity (Hayashi *et al.* 2000), although acute benzodiazepine-induced plasticity has not been studied in this region despite it displays the most abundant AMPA receptor expression in the CNS.

Likewise, chronic treatment with flurazepam leading to dependence has been shown to cause an increase in GluA1 subunit-containing AMPA receptors in the hippocampus (Song *et al.* 2007), and pharmacological intervention using AMPA receptor antagonists reduces benzodiazepine treatment-induced withdrawal (Steppuhn and Turski 1993). However, the data showing reduced capability to develop tolerance but increased withdrawal in GluA1-KO mice are not well suited to these previous findings. In GluA1-KO mice, reduced tolerance due to deficient glutamate-mediated plasticity might have eventually resulted in greater inhibitory state, thus finally resulting in increased withdrawal when the positive modulation of $GABA_A$ receptors was removed by the benzodiazepine antagonist flumazenil.

5.3.2 MORPHINE-INDUCED STATE-DEPENDENCY IS ABSENT IN GLUA1-KO MOUSE LINE

GluA1-KO mice were studied in an associative learning paradigm of conditioned place preference to clarify the role of GluA1 subunit in morphine-induced neuroplasticity. GluA1-KO mice developed sensitisation to intermittently administered morphine (**IV**, Fig. 1-2). Although GluA1-KO mice did not show acute morphine-induced increase in locomotor activity, they regained this effect in subsequent trials (**IV**, Fig. 1-2). Place conditioning was evident in GluA1-KO mice as they approached morphine-paired conditioning stimulus in the test trial (**IV**, Fig. 3). Deficient (Dong *et al.* 2004) or intact (Mead and Stephens 2005) place preference to cocaine has previously been reported in GluA1-KO mice. The former study utilised a biased apparatus while both the latter and the current studies used an unbiased setting thus the difference in place conditioning results may be due to methodological differences (Cunningham *et al.* 2003). Morphine-induced state-dependency was probed in both saline and morphine states. While WT mice showed the greatest expression of place preference when tested in morphine state, GluA1-KO mice displayed no place preference (**IV**, Fig. 3). Both mouse lines showed a moderate place preference when tested in a saline state, that is, test was performed in a different state than the mice were conditioned to (**IV**, Fig. 3). An intact detection of morphine signal was demonstrated by normal morphine

discrimination (**IV**, Fig. 4), and also brain regional μ-opioid receptor signalling was unmodified in GluA1-KO mice (**IV**, Fig. 6). Taken together, glutamate neuroplasticity was suggested to play a role in state-dependency without modifications in the molecular target of morphine, the μ-opioid receptor.

Few molecular or cellular correlates of state-dependency have previously been described. Cortical muscarinic acetylcholine receptors may mediate state-dependency (Shulz *et al.* 2000), and morphine-induced state-dependency has been shown a receptor subtype-selectivity (Bruins Slot and Colpaert 1999). In the etiology of addiction, VTA DA cells have been shown an established role (Hyman and Malenka 2001). Therefore, it is interesting to note that the same area has been implicated in state-dependency (Zarrindast *et al.* 2005). The electrophysiological data presented a more detailed analysis of VTA DA cells as a putative cellular correlate of state-dependency by showing absence of capability for morphine-induced neuroplasticity in GluA1-KO mice (**IV**, Fig. 5), displaying deficient state-dependency.

6 SUMMARY

The understanding of the roles the main excitatory system in the CNS (in particular driven by neurotransmitter glutamate) plays in brain function has undergone a significant increase during the last decades. The glutamatergic system, that was initially thought to act as a mere molecular accelerator of neurons, is now believed to function as an important mediator of plastic changes of the CNS. At first, the functional significance of glutamate neuroplasticity was linked to the mechanisms of learning and memory (White *et al.* 1977). However, the list of issues that relate glutamate to plasticity is growing, but also plasticity *per se* has gained more interest as a general mechanism behind various physiological and pathological states.

The current studies expand these findings by describing the importance of glutamate AMPA receptor GluA1 subunit in two main types of plasticity. Positive plasticity, that is beneficial to the individual by enabling accomplishment of various tasks and eventually is important for survival, is dependent on GluA1 subunits. GluA1 subunit has been implicated in positive plasticity by demonstrating deficient working memory in GluA1-KO mice (Reisel *et al.* 2002) while reference memory is functional in these mice (Zamanillo *et al.* 1999). Negative plasticity, having potentially harmful outcomes, was shown to be attributable to GluA1 subunit-mediated neuroplastic processes caused by two different classes of drugs of abuse.

GluA1-KO mouse line has been linked to various psychiatric disorders (Chourbaji *et al.* 2008; Wiedholz *et al.* 2008; Fitzgerald *et al.* 2010). For example, GluA1 has been linked to the etiology of schizophrenia (Magri *et al.* 2006). More specifically, a reduction in GluA1 subunit mRNA expression has been reported in the post-mortem frontal cortex of schizophrenia patients (Sokolov 1998). Interestingly, the reduction was reversed in patients treated with antipsychotic medication. Thus, it may be possible to target GluA1 subunit-containing AMPA receptors with AMPA receptor positive allosteric modulators in schizophrenic patients, should subunit-selective ligands become available. In bipolar disorder, reduction in the expression of striatal GluA1 mRNA has been reported in post-mortem brains of patients (Meador-Woodruff *et al.* 2001).

Interestingly, no change in GluA1 subunit mRNA or protein level was detected in post-mortem VTA or NAc from cocaine overdose victims (Tang *et al.* 2003; Hemby *et al.* 2005), although GluA1 has been demonstrated a role in the effects of cocaine in rodents (Dong *et al.* 2003). However, in the post-mortem amygdala of cocaine and heroin abusers, a positive correlation was found between GluA1 subunit and postsynaptic density protein-95, while this correlation did not emerge in control subjects (Ökvist et al. 2011). This might suggest a dysregulated synaptic function in drug abusers, although the direct role of this positive correlation is unclear.

Specific aspects of learning and memory were shown to be GluA1-dependent. Non-associative learning in spatially novel place is deficient in GluA1-KO mouse line, and has previously been shown a glutamate-dependent component (Izquierdo *et al.* 1993). Although pavlovian conditioning is intact in these mice, GluA1 subunit is a mediator of state-dependency in this type of associative learning.

The mouse model that was used in the present studies is based on a global deletion of GluA1 subunit, that is, every brain region and every cell is devoid of GluA1 subunit-containing AMPA receptors from the development to the adult age. Since there are no subunit-selective pharmacological ligands available to target AMPA receptor subtypes, this genetical model offered an alternative way to study the present issues. However, it would be interesting to study the plasticity-related issues presented here with such ligands, since with a pharmacological approach it would be possible to avoid much, if not all, of the compensations that are caused by the global deletion of GluA1 subunit. Furthermore, these and other plasticity-related issues would need to be studied in more detail with other, e.g. conditional transgenic and optogenetic technologies, to elucidate the roles of specific neuronal populations.

Taken together, targeting GluA1 subunit may be beneficial in counteracting drug-induced malignant negative plasticity e.g. by possibly reducing relapsing behaviour in drug addictions. However, the results of this study on positive plasticity suggest a cautious approach on this matter, as there may be harmful effects on some fundamental behaviours such as learning and memory, processing of novel signals and responding in social environment. Furthermore, there is an increasing body of literature suggesting that enhancement of AMPA signalling with AMPA receptor positive modulators (see section 2.7.6) may be useful in disorders such as Alzheimer's disease, Parkinson's disease and depression (O'Neill *et al.* 2004). Although the present results suggest possible beneficial effect of targeting GluA1-containing AMPA receptors in counteracting addiction disorders, the potentiation of AMPA signalling may lead to increased vulnerability in addiction-related states. Thus, these results may be of importance for future drug development in highlighting both possible new avenues for new indications, and also possible adverse effects that might emerge. In summary, these results suggest that glutamate AMPA receptor GluA1 subunit plays a wide role in neuroplasticity in the CNS, and they further strengthen the role of glutamatergic system as a mediator of plastic processes.

7 CONCLUSIONS

1. The GluA1-deficient mouse line is viable and shows novelty-induced hyperactivity but no other overt changes in the basic behaviour or in the expression of other types of glutamate receptors.

2. GluA1 subunit is pivotal for plasticity that enables successful performance in agonistic encounters.

3. Normal response to spatial novelty requires GluA1 subunit. In the brain of GluA1-KO mouse, the increased novelty-induced activation is mainly localised to the hippocampus and its input area entorhinal cortex.

4. The acute pharmacological effects of benzodiazepine flurazepam are unchanged in the absence of GluA1 subunit, but development of acute tolerance to flurazepam requires the GluA1 subunit.

5. GluA1 subunit is necessary for functional morphine-induced state-dependency.

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10 ORIGINAL PUBLICATIONS