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Characterization of *de novo* generated synapses in the adult brain – effects of melanocortin receptor activation

Degree Project in Medicine

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ABSTRACT

Introduction

In neurodegenerative diseases, there is a loss of synapses due to excessive elimination. On the other hand, formation of new synapses is increased by certain anti-depressants and endogenous hormones, including the melanocortin-4 receptor agonist melanotan II (MTII). In the developing brain, newly formed synapses are AMPA-silent (i.e. they lack functional AMPA signaling), whereas not much is known about newly formed synapses in the adult brain. The aim of this study was to induce formation of new synapses in the adult hippocampus using MTII, and to investigate if these *de novo* generated synapses are AMPA-silent.

Materials and Methods

Hippocampal slices from adult rats were treated with MTII (by either incubation or wash-in) for 1-3 hours. Extracellular field recordings with stimulation at test-pulse frequency (0.2 Hz) were performed in the hippocampal CA1 region, and the magnitude of the field excitatory postsynaptic potential (fEPSP) was measured.

Results

During test-pulse stimulation for 10 minutes, fEPSP magnitude decreased by ~ 15 %. The decrease was fully reversed by 20 minutes of inactivity, and was induced again by further stimulation. Wash-in of MTII led to an initial increase in fEPSP magnitude by ~ 40 %, and a subsequent decrease of ~ 15 % during test-pulse stimulation.

Conclusions

MTII induced a synaptic depression in the adult hippocampus resembling that in the developing hippocampus, i.e. induced by very low frequency stimulation (0.2 Hz) and

showing full recovery upon inactivity. This depression is likely due to loss of functional AMPA signaling in newly formed synapses. The mismatch in potentiation by wash-in of MTII and the subsequent depression by test-pulse stimulation is likely explained by an increased number of AMPA receptors in existing synapses.

Keywords: AMPA receptor, hippocampus, neurodegeneration, silent synapse, synaptic depression

POPULÄRVETENSKAPLIG SAMMANFATTNING

Den vuxna hjärnan utgörs av ett enormt nätverk av ca 100 miljarder nervceller ihopkopplade i många mindre nätverk. Alla dessa nervceller skickar signaler mellan varandra genom speciella kopplingar kallade synapser. Den samtida aktiveringen av olika synapser som sker när vår hjärna stimuleras utgör grunden för våra tankar, minnen och beteenden.

I en frisk hjärna bildas hela tiden nya synapser samtidigt som lika många försvinner, men i en hjärna drabbad av en så kallad neurodegenerativ sjukdom, som till exempel Alzheimers sjukdom, förloras fler synapser än det bildas nya. Samtidigt har man sett att nybildningen av synapser ökas av viss antidepressiv behandling och av vissa hormoner. För att kunna använda denna kunskap i utvecklingen av nya sätt att behandla neurodegenerativa sjukdomar är det viktigt att veta mer om de nya synapser som dessa behandlingar ger.

I detta projekt har jag undersökt egenskaperna hos nybildade synapser i den vuxna hjärnan. Hos nyfödda vet man att synapser till en början saknar en specifik receptor som är mycket viktig för att signaler ska kunna skickas mellan nervcellerna. Detta gör att de till en början är icke-fungerande, och kallas därför för ”tysta” synapser. Dessa tysta synapser kan sedan utvecklas till fungerande synapser om de får tillräckligt med koordinerad stimulering. Målet med denna studie har varit att undersöka om nya synapser i den vuxna hjärnan till en början också är tysta, och på så sätt öka kunskapen om egenskaperna hos dessa synapser. För att undersöka detta har jag använt tunna vävnadsskivor från hippocampus (ett område i hjärnan viktigt för minne och inlärning) hos vuxna råttor, och behandlat dessa med ett syntetiskt hormon som visats ge bildning av nya synapser. Genom elektrisk stimulering har jag sedan kunnat registrera aktiviteten hos de nybildade synapserna. Det mönster jag har sett i aktiveringen av dessa synapser liknar det man sett tidigare i hjärnan hos nyfödda råttor, och detta tyder på att även dessa synapser är tysta.

Min slutsats är att nybildade synapser i den vuxna hjärnan uppvisar liknande egenskaper som nybildade synapser i hjärnan hos nyfödda, i form av att de till en början sannolikt är tysta. För att bekräfta detta krävs vidare studier där man tittar på aktiveringen av enstaka nybildade synapser. Förhoppningsvis kan denna kunskap utgöra en grund för bättre förståelse för hur antidepressiv behandling fungerar samt bidra till nya idéer kring behandling av neurodegenerativa sjukdomar.

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
α-MSH	α-melanocyte-stimulating hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis
CNS	Central nervous system
DG	Dentate gyrus
ECT	Electroconvulsive therapy
Ent ctx	Entorhinal cortex
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
fEPSP	Field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GluA	Glutamate AMPA receptor subunit
KAR	Kainate receptor
LTD	Long-term depression
LTP	Long-term potentiation
MC4R	Melanocortin-4 receptor
MF	Mossy fiber
mGluR	Metabotropic glutamate receptor

MTII	D-Tyrosine melanotan II
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
POMC	Pro-opiomelanocortin
PP	Perforant pathway
PTX	Picrotoxin
SC	Schaffer collateral
SEM	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitors
Sub	Subiculum
TrkB	Tropomyosin receptor kinase B

INTRODUCTION

The brain is perhaps one of the most impressive aspects of human physiology, and even though a lot of progress has been made in understanding its anatomy, function, and complex governing of the rest of the human body, there is still so much that is yet to be understood. The focus of this project was on the formation of new synapses in the adult brain, specifically in the hippocampus, and on the properties of these synapses as compared to synapses in the developing brain. In order to better grasp the fundamentals of this project, an introduction to some of the basics of synaptic function will be given.

Synaptic transmission

In the nervous system, communication between neurons is essential for the development, maintenance and functioning of the complex neural networks that make our brain work. This communication takes place at specialized junctions called synapses, where information generally is transferred from one neuron (presynaptic) to another (postsynaptic). Transmission of signals across the synapse occurs through either a direct flow of ions through intercellular channels (gap junctions) between the pre- and postsynaptic cells (electrical transmission) or, more commonly, through the presynaptic release of a neurotransmitter which binds to receptors on the postsynaptic cell, where this intercellular chemical signal is converted to an intracellular signal (chemical transmission) [1, 2]. Most neurons in the central nervous system (CNS) function as relay stations, receiving signals (input) from many presynaptic neurons that are then integrated and potentially forwarded by means of action potentials (output).

The action potential is the way for signals in the nervous system to propagate and carry information over distances. At rest, the intracellular fluid in the neuron is negatively charged, whereas the extracellular fluid is positively charged. The difference between the inside and outside gives rise to a membrane potential. The action potential occurs when ions (usually

Na^+) flows into the cell, causing a depolarization of the membrane where the inside of the cell temporarily becomes less negatively charged. If the depolarization is large enough to reach a threshold level, an action potential is triggered. When this happens, so called voltage-gated Na^+-channels open and cause another influx of positive charge which in turn depolarizes the segment of membrane just ahead. In this manner, the action potential propagates along the axon of the neuron, like the burning of a fuse, until it reaches the presynaptic terminal. There, the depolarization causes voltage-gated Ca^{2+}-channels to open, and the subsequent influx of Ca^{2+}-ions will increase the probability of neurotransmitter release from presynaptic vesicles into the space between the pre- and postsynaptic cells (the synaptic cleft). The transmitters may then activate postsynaptic receptors so that the signal can be integrated into the postsynaptic neuron and further forwarded [1].

The glutamatergic synapse

There are several different types of synapses in the CNS, but the most common is the excitatory glutamatergic synapse, which uses the amino acid glutamate as its neurotransmitter. As this study focused solely on the glutamatergic synapse, other types of synapses will not be discussed.

Typically, when a signal reaches the presynaptic terminal of a glutamatergic synapse, influx of Ca^{2+} will increase the probability of glutamate being released from presynaptic vesicles, as described above. Glutamate can then act on four different postsynaptic receptors – AMPA receptors (AMPARs), NMDA receptors (NMDARs), kainate receptors (KARs) and metabotropic glutamate receptors (mGluRs). AMPARs, NMDARs and KARs are ionotropic receptors, i.e. ligand-gated ion channels that allow ions to pass through the postsynaptic membrane in response to the binding of glutamate. The typical glutamatergic synapse

contains AMPARs and NMDARs, clustered together in the postsynaptic membrane, whereas KARs are only found in some synapses [3].

The focus of this study was on AMPAR-mediated synaptic transmission, which is the most common type of excitatory transmission in the CNS. The AMPA receptor is a tetramer composed of four subunits, GluA1-A4, in different combinations. Depending on the combination of subunits, AMPARs can exhibit different properties, including the ability to traffic to and from the postsynaptic membrane. The AMPAR is permeable to Na^+ and K^+ ions. When glutamate acts on the AMPAR, Na^+ ions are allowed to flow through the postsynaptic membrane. Subsequently, the postsynaptic membrane is depolarized, and the signal is forwarded [3].

Like the AMPAR, the NMDA receptor is permeable to Na^+ and K^+ ions, but it is also permeable to Ca^{2+} ions, a characteristic important in synaptic plasticity. Moreover, in addition to being ligand-gated, the NMDAR is actually also voltage-gated, as the ion channel is normally blocked by extracellular Mg^{2+}. This means that at the resting membrane potential, the NMDAR will not be activated [3, 4]. In order for it to be activated, the postsynaptic membrane must first be depolarized, e.g. by activation of AMPARs, after which the Mg^{2+} block is relieved. Hence, the most important role of the NMDAR in synaptic transmission is in the induction of plasticity of AMPAR-mediated transmission (see Synaptic plasticity below) [3].

The metabotropic glutamate receptor is G-protein coupled and located both pre- and postsynaptically. Its role in synaptic transmission is in modulating the probability of glutamate release and the excitability of the postsynaptic membrane, and in synaptic plasticity [3].

As mentioned above, the glutamatergic synapse is an excitatory synapse. This means that when the synapse is activated, the postsynaptic membrane is depolarized and the likelihood of action potentials being generated is increased. In this way, an excitatory synapse forwards the input signal and stimulates the postsynaptic target, as opposed to an inhibitory synapse (e.g. the GABAergic synapse) in which the membrane is hyperpolarized and the postsynaptic target inhibited. The depolarization of the postsynaptic membrane creates an excitatory postsynaptic potential (EPSP) over the membrane [1]. The EPSP can be measured, and subsequently represents the postsynaptic activity of the glutamatergic synapse.

Synaptic plasticity

Both newly-formed and mature synapses can be strengthened or weakened depending on their level of activity. Basically, the more signals the neurons in a synapse receive, the stronger the synapse will be, provided the signals are correlated with each other. More specifically, when pre- and postsynaptic neurons are strongly activated at the same time (i.e. the pre- and postsynaptic activity is correlated), the connection between these neurons is strengthened, making the involved neurons more likely to survive, whereas weak connections will make the involved neurons more likely to be eliminated [1, 5]. This is the basis for the formation and maintenance of functional neural networks in a healthy brain ("cells that fire together wire together") [1].

The strengthening of a connection between neurons in a synapse is called long-term potentiation (LTP), whereas the weakening of such connections is called long-term depression (LTD). Experimentally, LTP can be induced by subjecting an excitatory pathway in the brain to short, high-frequency stimulation (~ 10-100 Hz), which will lead to a long-lasting strengthening of the synapses involved [6]. Conversely, LTD can be induced by low-

frequency stimulation (~ 1-3 Hz) [7]. LTP and LTD are both long-lasting forms of synaptic plasticity widely considered essential for memory formation and learning.

Hippocampus and electrophysiology

The hippocampus has a fairly simple structure, making it ideal for studying synaptic transmission. It is made up of two main parts folded onto one another – the dentate gyrus (DG) and the hippocampus proper (or cornu Ammonis, CA) (*Fig. 1A*). There is also the subiculum (Sub) and the entorhinal cortex (Ent ctx), which is the major source of input and target of output for the hippocampus. The hippocampus proper is divided into four regions, CA1-CA4, where CA1 and CA3 are the most important areas for electrophysiological study. Furthermore, the CA-regions are made up of several different histological layers (*Fig. 1B*).

The main neural circuit within the hippocampus starts in the entorhinal cortex, which sends signals to the DG via the perforant pathway (PP). The DG connects to neurons of CA3 through axons called mossy fibers (MF). The neurons in CA3 in turn projects to CA1 through axons called Schaffer collaterals (SC). The SCs synapse upon CA1 pyramidal neurons (found in the stratum pyramidale) in the stratum radiatum. Finally, the CA1 neurons project to the subiculum, whose neurons project back to the entorhinal cortex.

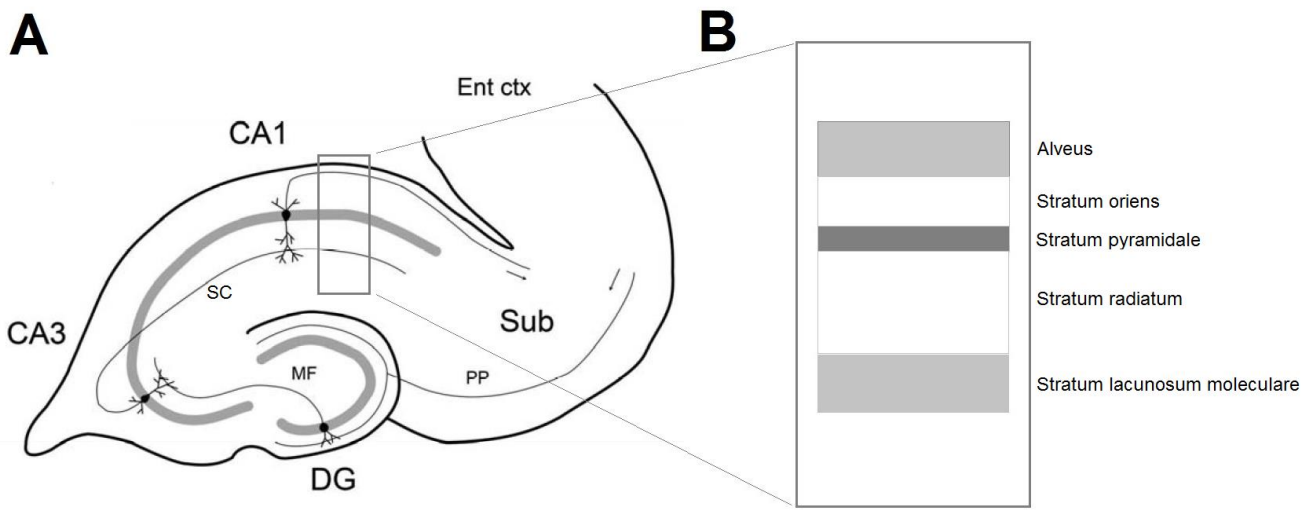

Figure 1. *The hippocampus.* **A.** *Schematic of the hippocampus with the main intrinsic circuit illustrated (used with permission from J. Strandberg).* **B.** *Basic outline of the layers of CA1 (modified with permission from A. Thorén). Abbreviations: CA – cornu ammonis, DG – dentate gyrus, Ent ctx – entorhinal cortex, MF – mossy fibers, PP – perforant pathway, SC – Schaffer collaterals, Sub – subiculum.*

A common way of studying synapses in the hippocampus is using extracellular field recordings, in which the glutamatergic SC-CA1 synapse is the target. First, thin slices of rodent hippocampus are prepared and set up in a recording chamber (for details, see Methods and Materials below). By placing stimulating electrodes in the stratum radiatum of the hippocampal slice, a population of hundreds of SC axons are stimulated (*Fig. 2*). The resulting action potentials propagate to the SC-CA1 synapses, leading to synaptic transmission and subsequent activation of postsynaptic CA1 pyramidal neurons. By placing a recording electrode between the two stimulating electrodes (but closer to the stratum pyramidale), one can record the postsynaptic activation as a field potential that results from the activation of many postsynaptic pyramidal neurons. Such a field potential, generated by glutamatergic synapses, is called a field excitatory postsynaptic potential (fEPSP). Essentially, the field potential is a summation of the postsynaptic activity of all the activated CA1 neurons.

The main part of an extracellular field recording is the fEPSP, but two other components are also recorded (*Fig. 2*) – the stimulation artefact, which simply represents the current from the stimulation going straight through the surrounding solution rather than through the slice, and the volley. The volley represents the presynaptic activity of the SCs, and arise from action potentials produced by the stimulated SCs as the signal propagates towards the pyramidal cells of CA1 (as described above, the fEPSP, on the other hand, represents the postsynaptic activity of the SC-CA1 synapses). As the volley represents presynaptic SC activity, its magnitude is proportional to the number of presynaptic SC axons that are stimulated. This means that the volley can be used as an internal control of how much activity is produced by the stimulation. In field recordings, the external stimulation is not changed during the experiment, and therefore the volley should remain mostly constant throughout. If the volley is unstable and changes a lot during the course of the experiment, the recorded data might not be reliable (smaller fluctuations are generally acceptable). Therefore, it is important to measure the volley and take it into account during analysis of the recorded data. A common way to estimate the size of the fEPSP and volley is to measure the initial slope of the curve seen in figure 2 (over ~ 0.3 ms for the slope and ~ 0.8 ms for the fEPSP). It is also possible to use the amplitude to estimate the size of the fEPSP and volley [3].

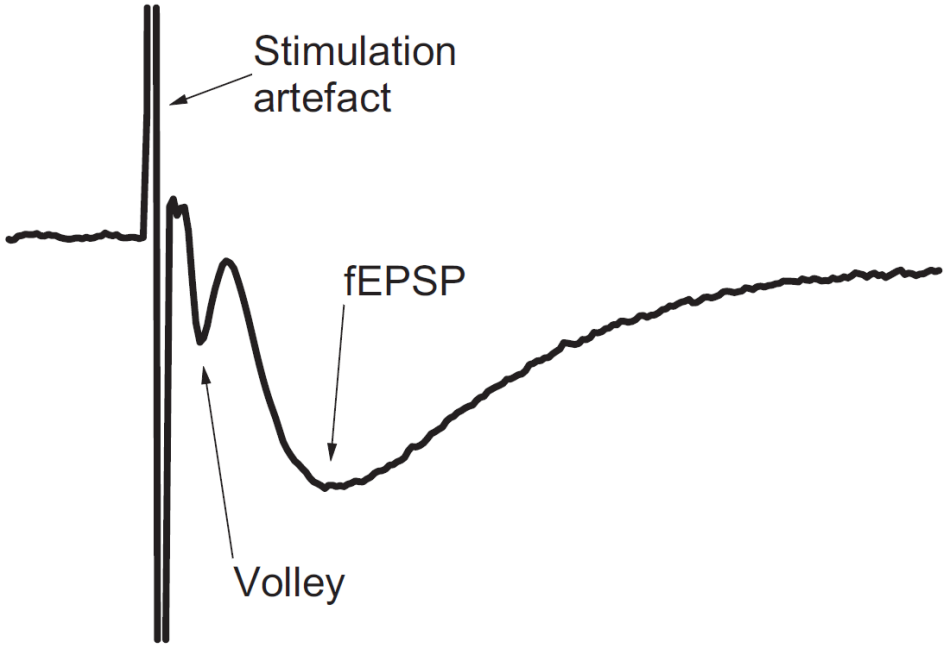

Figure 2. *Example of a field potential recording (used with permission from J. Strandberg). Abbreviations: fEPSP – field excitatory postsynaptic potential.*

Synaptic formation and elimination

In the brain, there is a constant synaptogenesis coupled with a constant synaptic elimination. During development, there is an overproduction of immature neurons and a subsequent formation of a vast number of synapses. In an activity-dependent manner, those neural connections that become functionally relevant are stabilized whereas those that do not are eliminated (e.g. those that only mediate random noise). In the cerebral cortex, this selection process (called synaptic pruning) takes place throughout development up until early adolescence, and is critical for the formation of a functional synaptic network [4, 8-10].

Initially, synaptogenesis occurs at a higher rate than elimination, but as we grow up, the formation of synapses slows, and elimination takes over (the density of synapses in the cerebral cortex of the adult brain is actually about half of that in the early developing brain). In the fully developed brain, the formation and elimination are balanced, making the total number of synapses more or less constant [8].

AMPA-silent synapses

Previous studies have shown that the mechanisms for basal transmission and synaptic plasticity of newly formed synapses in the developing brain are different from those of mature synapses [11-16]. Specifically, glutamatergic synapses are formed without functional AMPAR-mediated signaling (generally thought to be caused by a lack of postsynaptic AMPARs), and are therefore said to be AMPA-silent [4]. Basically, this means that the synapses are unable to forward a signal from the presynaptic to the postsynaptic neuron.

As described earlier, these newly generated, nonfunctional synapses are either eliminated or stabilized depending on the activity of the synapse [4]. Through LTP and LTD, AMPARs can traffic to and from the postsynaptic membrane, making it possible for silent synapses to gain postsynaptic AMPARs [17]. Specifically, correlated pre- and postsynaptic activity determines which synapses will gain functional AMPAR-mediated transmission. The conversion to functional synapses is called AMPA unsilencing, and is the first step in a multistep process towards stabilization of the synapse. Moreover, AMPA silencing of preexisting synapses has been implicated in the pathogenesis of neurodegenerative diseases (e.g. Alzheimer's disease) [4, 18].

Even though it was previously thought that glutamatergic synapses completely lack AMPARs when they are formed, recent research suggests that in their naïve form (i.e. before being activated), these synapses do have AMPARs, but in a very labile state. Therefore, as soon as they are activated, these synapses quickly lose their AMPARs [4, 15, 16]. This has been shown by stimulating the synapse with low-frequency (0.05-0.2 Hz) stimulation normally used as test stimulation (e.g. to test synaptic function or to establish a baseline in LTP experiments) [11, 14]. In addition to inducing silencing (represented by a depression of the fEPSP amplitude) using this so-called test-pulse stimulation, it was also shown that when

interrupting the stimulation, the silencing was completely reversed within 20 minutes (*Fig. 3-5*) [11].

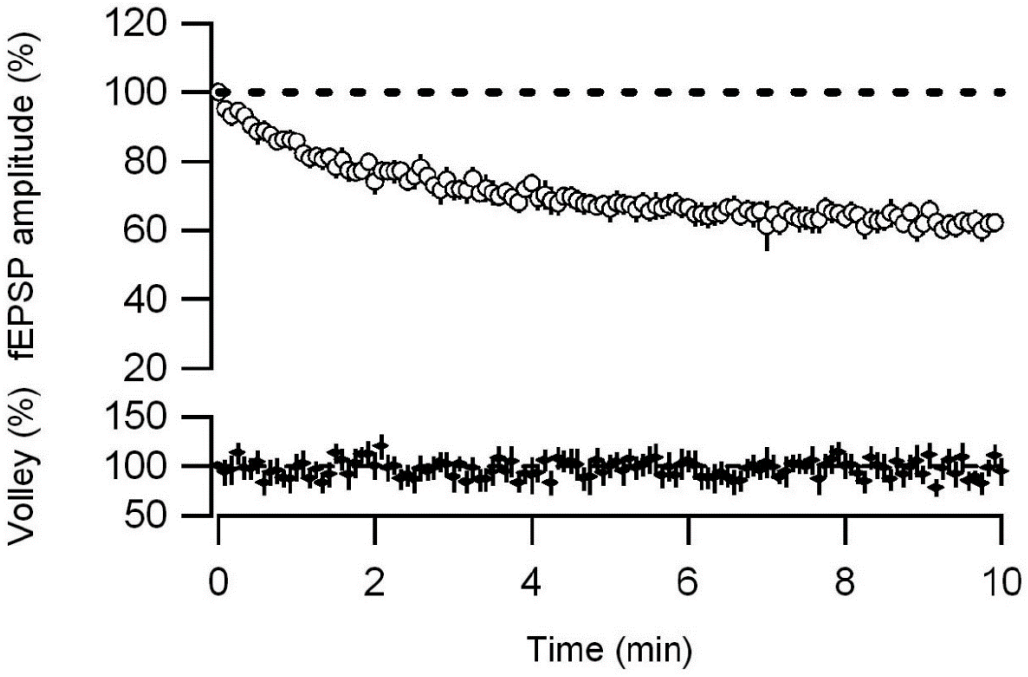

Figure 3. *fEPSP depression during 120 stimulations at test frequency (0.2 Hz, n = 16) (used with permission from T. Abrahamsson). Abbreviations: fEPSP – field excitatory postsynaptic potential.*

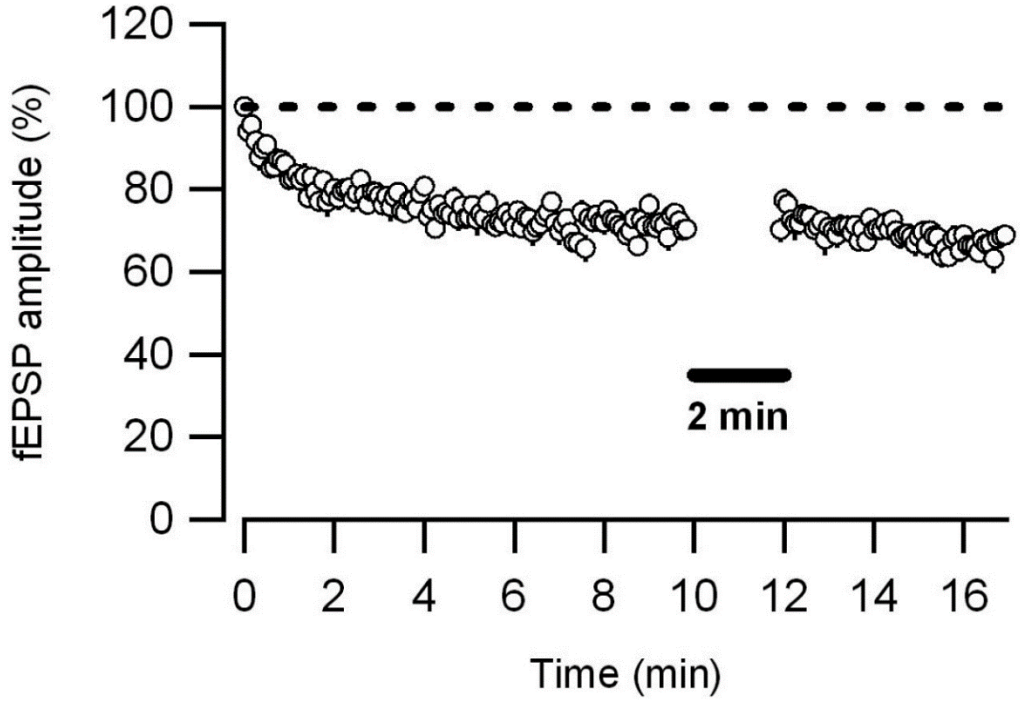

Figure 4. *Recovery of fEPSP depression by stimulus interruption for 20 minutes (n = 7) following stimulation at test frequency (used with permission from T. Abrahamsson). Abbreviations: fEPSP – field excitatory postsynaptic potential.*

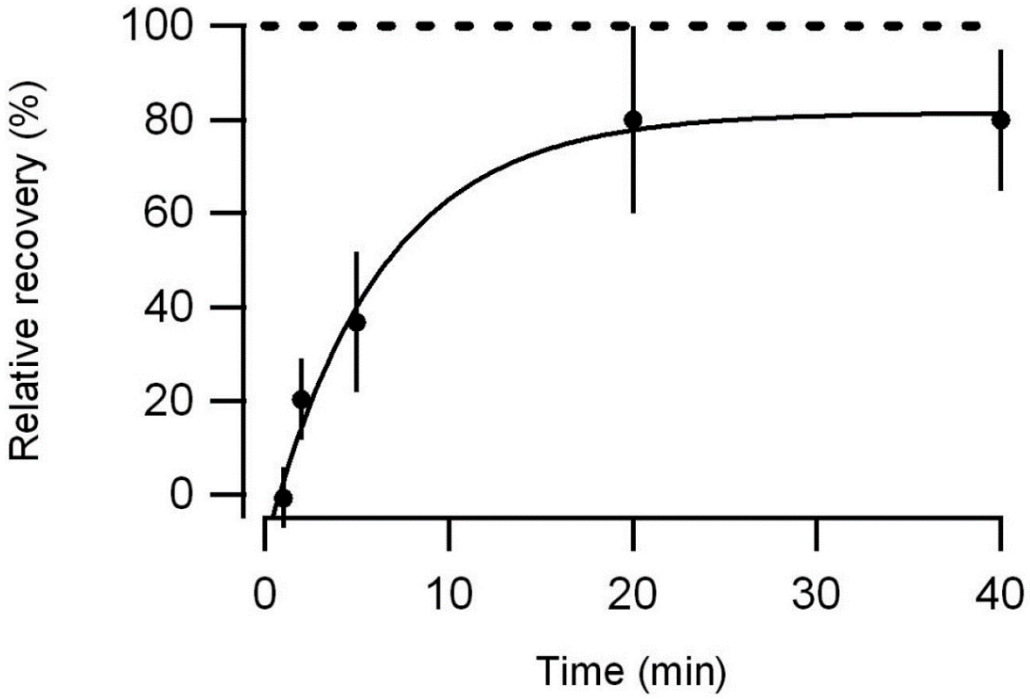

Figure 5. *Effect of stimulus interruption for 1 minute (n = 6), 2 minutes (n = 7), 5 minutes (n = 6), 20 minutes (n = 7) and 40 minutes (n = 6) on recovery of fEPSP depression following stimulation at test frequency (used with permission from T. Abrahamsson). Abbreviations: fEPSP – field excitatory postsynaptic potential.*

To summarize, new synapses in the developing brain are formed with AMPARs. As they are subjected to stimuli, they lose their AMPARs (silencing). If the stimulation is stopped, or if the synapses are subjected to LTP (through correlated pre- and postsynaptic activity), they can regain AMPARs, thereby becoming functional synapses (unsilencing).

Neurodegeneration and antidepressants

In a healthy adult brain, the total number of synapses remain fairly constant due to synaptic formation and elimination occurring at an equal rate. However, in certain diseases such as the neurodegenerative Alzheimer's disease [19, 20] and Huntington's disease [21, 22], as well as major depression [8, 23], this is not the case as the balance is shifted towards a net loss of synapses. Theoretically, one way to counter-act this is to increase synaptogenesis by, for instance, antidepressant treatments such as electroconvulsive therapy (ECT), selective serotonin reuptake inhibitors (SSRIs) and exercise [8, 24]. Furthermore, certain endogenous hormones such as melanocortins and estrogens have also been shown to increase synaptogenesis [25, 26]. The increase in synaptogenesis by these factors seems to occur through a joint mechanism, i.e. activation of the neurotrophin receptor TrkB through binding of brain-derived neurotrophic factor (BDNF), a growth factor critical to activity-dependent survival of neurons and synaptic plasticity [24, 27].

De novo generation of synapses

Whereas we know that newly formed synapses in the developing brain exhibit properties as mentioned above, the properties of newly formed synapses in the adult brain has not been as thoroughly investigated. A way to study this is to induce synaptogenesis in the hippocampus of adult rats and then carry out electrophysiological experiments on hippocampal preparations *in vitro.* A method for this has been described in a previous study by Shen et al., where the

synthetic melanocortin-4 receptor (MC4R) agonist D-Tyrosine melanotan II (MTII) was used to successfully induce synaptogenesis in the hippocampus of adult rats [25].

The melanocortin system is a collection of circuits in the CNS that play a major part in regulating the body's energy homeostasis. Its neurons express pro-opiomelanocortin (POMC), a precursor to several melanocortin peptides including adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH). Apart from regulating energy homeostasis, the melanocortin system has also been shown to have a role in memory formation, learning, mood and nociception [28]. It has also been shown that one of its receptors, MC4R, is present in the hippocampus of rodents. In the above mentioned study, stimulating this receptor led to formation of new synapses (observed as an increased number of dendritic spines) and to increased AMPAR-mediated signaling in existing synapses (by increasing the number of AMPARs and by the maturation of dendritic spines in pyramidal neurons of the hippocampus) in the SC-CA1 pathway of the hippocampus [25].

In this study, the purpose was to induce the formation of new synapses in the hippocampus of adult rats using MTII in order to analyze the electrophysiological effects of this drug's activation of the melanocortin-4 receptor, and to begin studying these *de novo* generated synapses with regards to their electrophysiological properties.

Hypothesis

We hypothesized that *de novo* generated synapses in the adult brain exhibit characteristics similar to those of newly formed synapses in the developing brain. Specifically, we hypothesized that glutamatergic SC-CA1 synapses generated *de novo* lack functional AMPAR-mediated signaling when they are formed.

MATERIALS AND METHODS

Ethics statement

In order to carry out these experiments in a way that best represents the hypothesized effects in humans, it is currently necessary to use animal models (where the hippocampus is structurally and functionally similar to the one in humans). This is mainly because it is not yet possible to achieve the correct morphology and neural network when using cultivated cells. The experiments were carried out in accordance with the Swedish Animal Welfare Act, and ethical approval was granted by a local ethics committee at the University of Gothenburg.

Drugs

Drugs were purchased from Tocris Bioscience, Bristol, United Kingdom.

Electrophysiology

Using methods well established in the field of neurophysiology and in our lab, electrophysiological experiments were carried out on hippocampal slices from adult (30-45 days old) Wistar rats.

Slice preparation

Animals were anaesthetized using isoflurane, and sacrificed by means of a guillotine. After decapitation, the brain was immediately removed and placed in an ice-cold (0-3 °C) solution (containing 26 mM $NaHCO_3$, 7 mM $MgCl_2$, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$, 219 mM glycerol and 11 mM glucose). Using a vibratome (Microm HM 650 V), 400 µm thick hippocampal transverse slices were prepared (still placed in the ice-cold solution). The slices were then directly moved to a storage chamber with artificial cerebrospinal fluid (ACSF), in which it is possible to keep slices viable for at least 8 hours. The ACSF contained 119 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$

and 10 mM D-glucose. The slices were stored in ACSF for 60-180 minutes, after which a single slice was transferred to a recording chamber with continuous circulation (at a flow of ~2 ml/minute) of ACSF at room temperature. The circulating ACSF contained 123 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$ and 10 mM D-glucose. For the purpose of blocking inhibitory $GABA_A$ activity, 100 µM picrotoxin (PTX) was added to the circulating ACSF. To further prevent the influence of spontaneous activity, the axons between CA3 and CA1 were cut during the slice preparation. All solutions mentioned were continuously perfused with 95% O_2 and 5% CO_2 at pH ~7.4.

Extracellular field recordings

Extracellular fEPSP recordings were performed in the CA1 region (*Fig. 6*). Two tungsten microelectrodes (resistance ~0.5 MΩ) were used as stimulation electrodes and placed in the stratum radiatum of CA1, and SC axons in the stratum radiatum were subsequently stimulated with biphasic constant current pulses (20-50 µA, 200 + 200 µs) at a frequency of 0.2 Hz. Recordings were made using a borosilicate glass micropipette as a recording electrode (resistance ~2 MΩ). The pipette was filled with 1 M NaCl, connected to an AgCl electrode, and placed in between the two stimulating electrodes in the stratum radiatum. After positioning the stimulation and recording electrodes, the slice was left to recover for 10

minutes before starting the experiment.

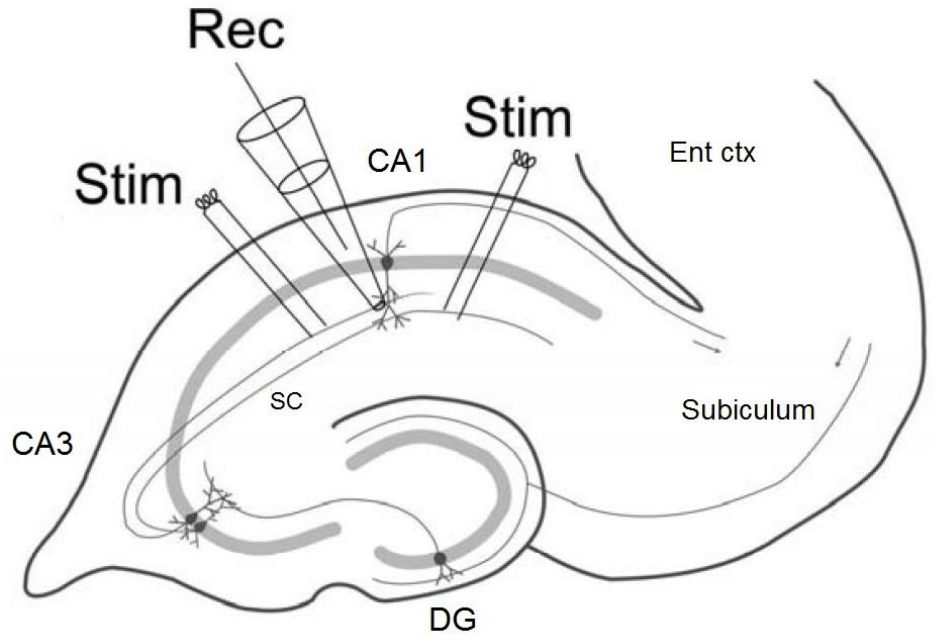

Figure 6. *Schematic of the positioning of recording and stimulation electrodes in the stratum radiatum of CA1 (used with permission from J. Strandberg). Abbreviations: CA – cornu ammonis, DG – dentate gyrus, Ent ctx – entorhinal cortex, SC – Schaffer collaterals.*

Stimulation protocol

As the purpose of this study was to examine the properties of newly formed synapses in the adult brain compared to those of the developing brain, test-pulse stimulation at 0.2 Hz (like the one used by Abrahamsson et al. [11]) was used for all experiments.

All slices were stimulated at 0.2 Hz for 10 minutes (for a total of 120 stimulations), followed by a 20-minute recovery period without any stimulation. After the recovery period followed another 10 minutes of stimulation at 0.2 Hz. In addition to experiments with MTII, control experiments in which no MTII was added were performed according to this protocol.

Two different types of experiments were performed – "incubation" and "wash-in". In the incubation experiments, MTII was added to the ACSF in the storage chamber, and slices were consequently incubated in MTII for 60-180 minutes prior to stimulation. After incubation,

slices were stimulated according to the protocol described above. In the wash-in experiments, slices were first stimulated at 0.2 Hz for 10 minutes, after which MTII was immediately added, or "washed in", to the circulating ACSF in the recording chamber. The slices were then left as they were in the recording chamber, without any stimulation, for 120-180 minutes. Stimulation then took place according to the protocol described above.

Data analysis

Data was recorded using an amplifier (Molecular Devices MultiClamp 700B), digitizer (Molecular Devices Digidata 1440A) and software program Clampex (Molecular Devices). fEPSPs were analyzed with software programs ClampFit (Molecular Devices) and IGOR Pro (WaveMetrics). Size of the recorded fEPSPs were estimated by measuring the 0.8 ms initial slope. The size of the volley was measured by the 0.3 ms initial slope and was not allowed to change more than 10 % for each slice. Therefore, experiments in which the volley changed more than 10 % were omitted from analysis. Furthermore, as test-pulse stimulation is not meant to induce LTP, experiments in which spiking (a sign of LTP-induction) was observed were also omitted. Because of small, unavoidable variations in the setup (e.g. electrode positioning, individual variations between slices), no two experiments are exactly the same. This will lead to differences in the resulting field potential between experiments. In order to compare the data, fEPSPs are therefore normalized to the baseline fEPSP at the very beginning of the experiment (i.e. the fEPSP evoked by the very first stimulation) and presented as percentages of this baseline.

Statistical analysis

Statistical analysis using two-tailed Student's t-test was performed in Microsoft Excel. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Incubation in MTII

Incubation in MTII induced a significant synaptic depression

The fEPSP magnitude for the first 120 stimulations following incubation (for 60-180 minutes) in MTII are shown in figure 7. fEPSP depression at the end of stimulation was measured as the average of the last 20 stimulations and is presented as a percentage of the first stimulation (the baseline fEPSP).

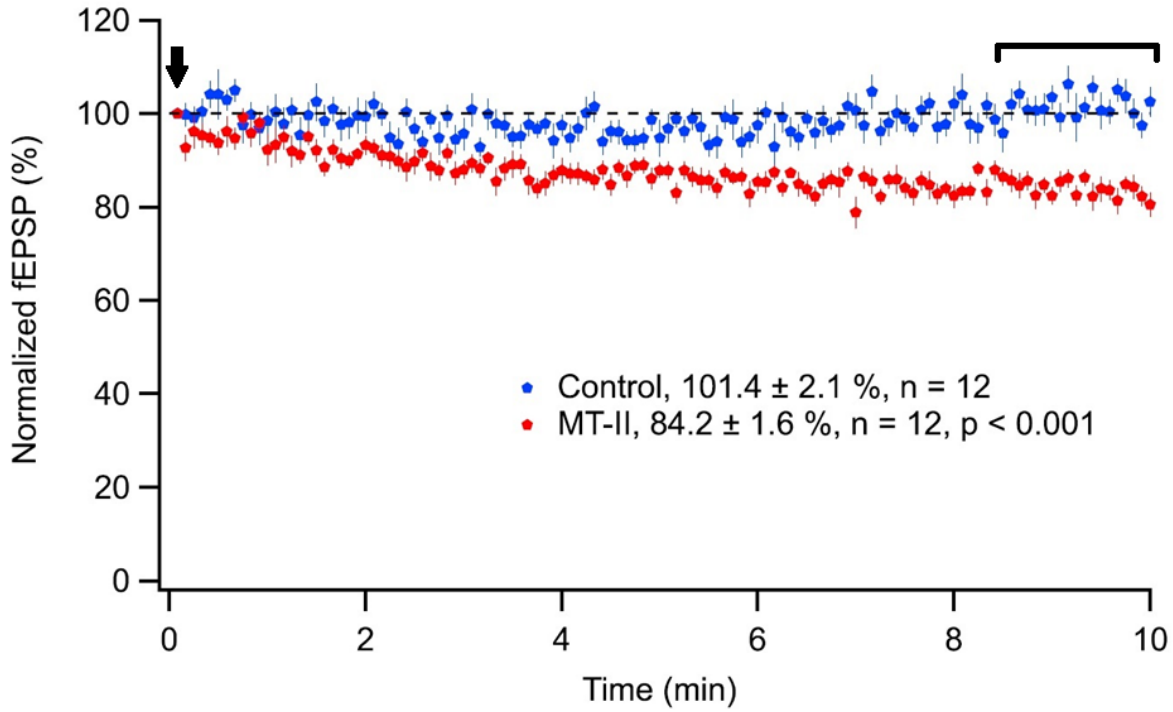

Figure 7. *fEPSP depression during 120 stimulations at 0.2 Hz following incubation in MTII. There was a significant difference ($p < 0.001$) in end fEPSP slope between controls (101.4 ± 2.1 %, n= 12) and MTII (84.2 ± 1.6 %, n = 12). End fEPSP slope was measured as the average of the last 20 stimulations (line), normalized to the first stimulation (arrow, dotted line). Abbreviations: fEPSP – field excitatory postsynaptic potential, MTII – melanotan II.*

As shown in figure 7, fEPSP magnitude decreased by ~ 15 % during test-pulse stimulation for experiments with MTII, whereas fEPSP magnitude was not decreased in control experiments. The difference in synaptic depression between MTII experiments and controls were

statistically significant ($p < 0.001$). The volley remained at ~ 100 % of baseline at the end of the first 120 stimulations for MT-experiments (99.6 ± 1.3 %, n = 12) as well as controls (99.8 ± 1.3 %, n = 12), and there was no significant difference in the volley at the end of 120 stimulations between MTII and controls ($p = 0.94$).

Synaptic depression was reversed by stimulus interruption

After 20 minutes of inactivity following the first 120 stimulations of the incubation experiments, the decrease in fEPSP magnitude seen in MTII-experiments was fully reversed (*Fig. 8*). This was determined by looking at the average fEPSP of the first three stimulations after stimulus interruption (*Fig. 8B*), presented as a percentage of the baseline fEPSP (i.e. the very first stimulation of the first round of 120 stimulations). In control experiments, the fEPSP magnitude remained at ~ 100 % of baseline fEPSP.

Reversed synaptic depression could be induced again

At the start of the second round of stimulation, the fEPSP magnitude had recovered to ~ 100 % of baseline fEPSP (*Fig. 8B*). During the second round of 120 stimulations at 0.2 Hz, which took place after 20 minutes of inactivity, fEPSP magnitude was again decreased by ~ 15 % (*Fig. 8C*). The volley remained at ~ 100 % of baseline at the end of stimulation for both MTII experiments (99.0 ± 2.1 %, n = 5) and control experiments (97.0 ± 1.5 %, n = 7).

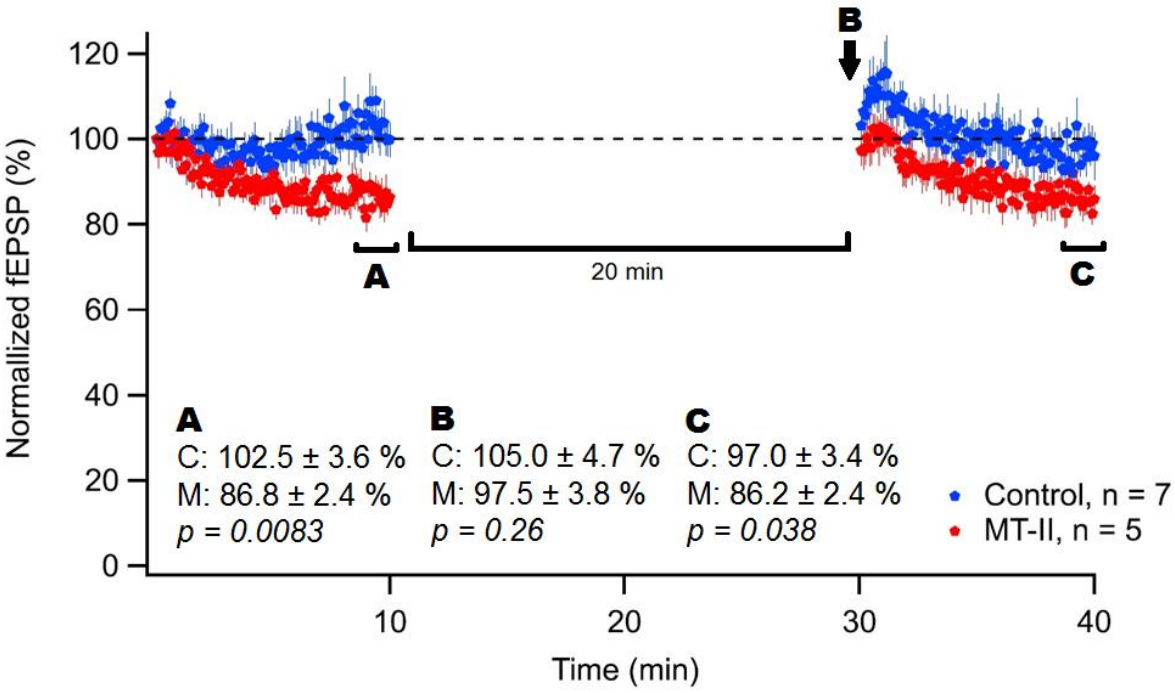

Figure 8. *fEPSP depression during two rounds of 120 stimulations at 0.2 Hz, with 20 minutes of inactivity in between, following incubation in MTII.* **A.** *End fEPSP slope after the 1st round of stimulation showed a significant difference between controls and MTII (p = 0.0083).* **B.** *fEPSP slope at the start of the 2nd round of stimulation (arrow) showed recovery to 97.5 ± 3.8 % for MTII. There was no significant difference in fEPSP slope between controls and MTII at this point (p = 0.26).* **C.** *End fEPSP slope after the 2nd round of stimulations showed a significant difference between controls and MTII (p = 0.038). End fEPSP slope was measured as the average of the last 20 stimulations (lines), normalized to the first stimulation (dotted line). Abbreviations: fEPSP – field excitatory postsynaptic potential, MTII – melanotan II.*

The most interesting time points when looking at the recovery of synaptic depression in these experiments are at the end of the first round of stimulation, at the start of the second round of stimulation, and at the end of the second round of stimulation. This data is summarized in table 1.

Table 1. *Summarized data of fEPSP slope at various time points in incubation experiments looking at recovery of synaptic depression. Data are presented as percentages of the fEPSP slope of the very first stimulation of the experiment.*

Time	Control (n = 7)	MTII (n = 5)	Two-tailed t-test
End of 1st round of stimulation	102.5 ± 3.6 %	86.8 ± 2.4 %	p = 0.0083
Start of 2nd round of stimulation	105.0 ± 4.7 %	97.5 ± 3.8 %	p = 0.26
End of 2nd round of stimulation	97.0 ± 3.4 %	86.2 ± 2.4 %	p = 0.038

Wash-in of MTII

In the analysis of the wash-in experiments, the fEPSP slope was adjusted for by changes in the magnitude of the volley.

Wash-in of MTII induced synaptic potentiation followed by depression

Before wash-in of MTII, slices were subjected to one round of 120 stimulations at test-pulse frequency. In figure 9, the baseline fEPSP is set as the average of the last 20 of these initial stimulations. At the start of stimulation following wash-in of MTII for 120 minutes (n = 4) or 180 minutes (n =3), fEPSP magnitude had increased by ~ 40 % from baseline (determined by comparing the average of the first three stimulations after wash-in to baseline) (*Fig. 9*). The increase in fEPSP magnitude was statistically significant (p = 0.021 for both 120 and 180 minute experiments). At the end of stimulation, fEPSP magnitude had decreased by ~ 15 % from this level (*Fig. 10*). Subsequently, the fEPSP magnitude was still ~ 20 % higher than at the very start of the experiment.

Synaptic depression could be reversed and induced again

After 20 minutes of inactivity following stimulation, the ~ 15 % decrease in fEPSP magnitude observed during stimulation was fully reversed to a level ~ 40 % above baseline (determined by looking at the average fEPSP evoked by the first three stimulations following inactivity).

During another round of 120 stimulations at 0.2 Hz, fEPSP magnitude was again decreased by ~ 15 % (*Fig. 10*). Data from the most interesting time points of the wash-in experiments are summarized in tables 2 and 3.

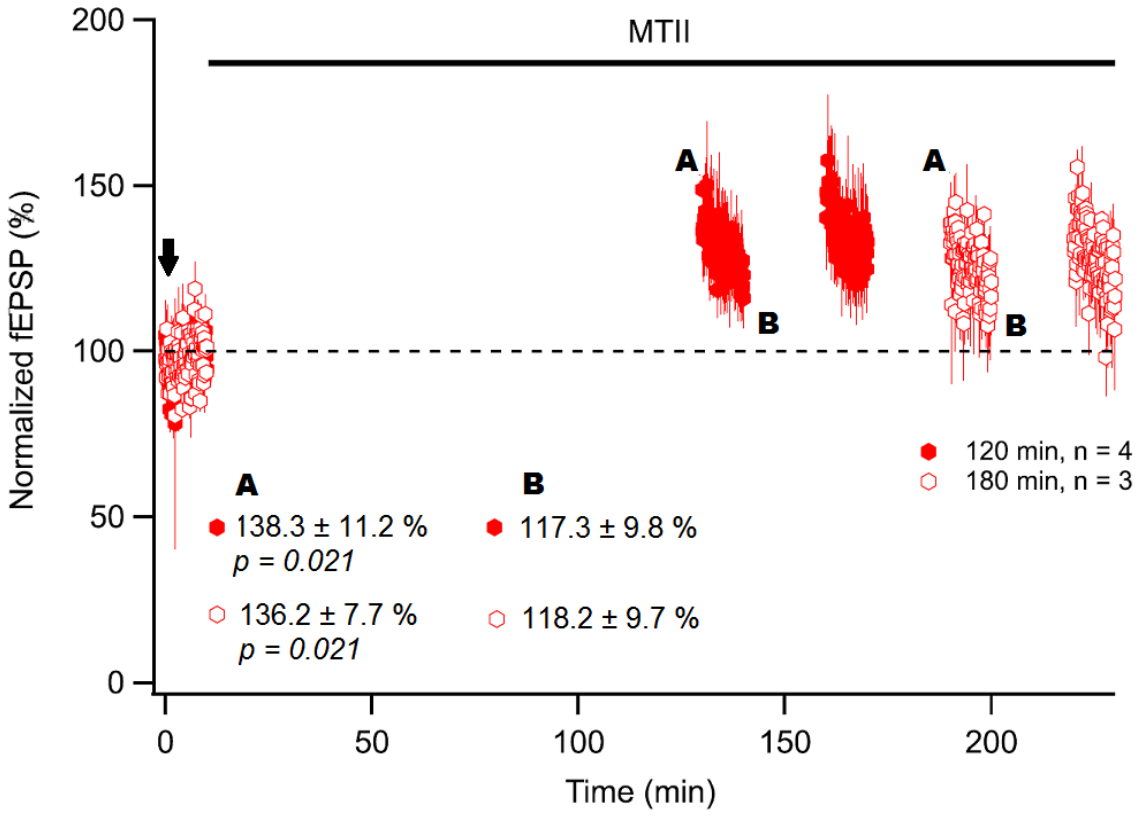

Figure 9. *fEPSP slope during one round of 120 stimulations at 0.2 Hz before, and two rounds after, wash-in of MTII. Baseline was set as the average of the last 20 stimulations before wash-in (arrow).* **A.** *fEPSP slope at the start of the 2nd round of stimulation had increased to 138.3 ± 11.2 % and to 136.2 ± 7.7 % of baseline for 120- and 180-minute wash-in, respectively.* **B.** *End fEPSP slope after the 2nd round of stimulation showed a decrease to 117.3 ± 9.8 % and to 118.2 ± 9.7 % of baseline for 120- and 180-minute wash-in, respectively. Abbreviations: fEPSP – field excitatory postsynaptic potential, MTII – melanotan II.*

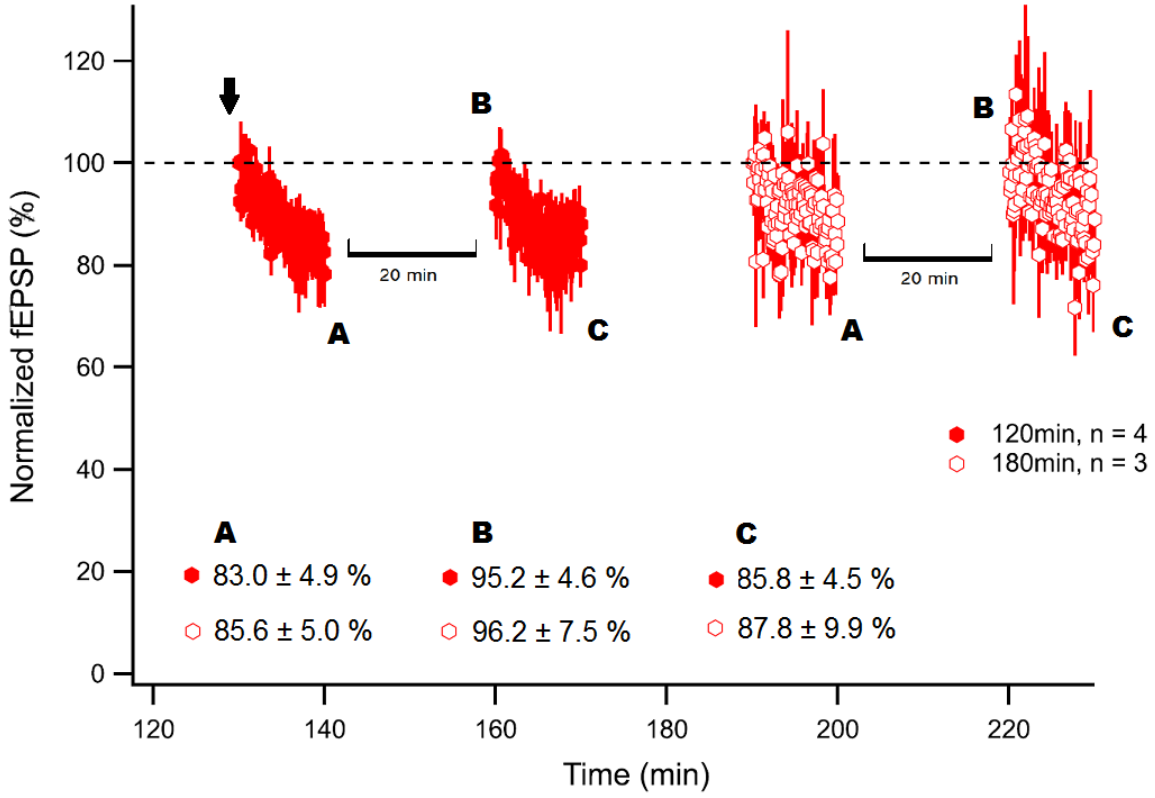

Figure 10. *fEPSP slope during two rounds of 120 stimulations at 0.2 Hz after wash-in of MTII. Baseline was set as the first stimulation after wash-in.* **A.** *End fEPSP slope after one round of stimulation showed a decrease to 83.0 ± 4.9 % and to 85.6 ± 5.0 % of baseline for 120- and 180-minute wash-in, respectively.* **B.** *fEPSP slope after 20 minutes of inactivity showed a recovery to 95.2 ± 4.6 % and to 96.2 ± 7.5 % of baseline for 120- and 180-minute wash-in, respectively.* **C.** *End fEPSP slope after two rounds of stimulation showed another decrease, to 85.8 ± 4.5 % and to 87.8 ± 9.9 % of baseline for 120- and 180-minute wash-in, respectively. Abbreviations: fEPSP – field excitatory postsynaptic potential, MTII – melanotan II.*

Table 2. *Summarized data of fEPSP slope at various time points during wash-in of MTII. Data are presented as percentages of the fEPSP slope of the very first stimulation of the experiment (before wash-in).*

Time	120 min (n = 4)	180 min (n = 3)
Start of 1st round of stimulation (before wash-in)	100 %	100 %
Start of 2nd round of stimulation (after wash-in)	138.3 ± 11.2 % p = 0.021	136.2 ± 7.7 % p = 0.021
End of 2nd round of stimulation	117.3 ± 9.8 %	118.2 ± 9.7 %

Table 3. *Summarized data of fEPSP slope at various time points during wash-in of MTII. Data are presented as percentages of the fEPSP slope of the first stimulation after wash-in.*

Time	120 min (n = 4)	180 min (n = 3)
Start of 2nd round of stimulation (after wash-in)	100 %	100 %
End of 2nd round of stimulation	83.0 ± 4.9 %	85.6 ± 5.0 %
Start of 3rd round of stimulation (after recovery)	95.2 ± 4.6 %	96.2 ± 7.5 %
End of 3rd round of stimulation	85.8 ± 4.5 %	87.8 ± 9.9 %

DISCUSSION

The initial lack of functional AMPAR-mediated signaling in immature synapses in the developing hippocampus is well established by now, but whether also newly generated synapses in the adult hippocampus exhibit this characteristic is yet to be determined. The possibility of inducing synaptogenesis in the adult rodent hippocampus using MTII has been described previously. Here, incubation in MTII led to a decrease in fEPSP magnitude by ~ 15 % during test-pulse stimulation. This decrease was fully reversed after 20 minutes of inactivity, and was induced again during another round of stimulation. Wash-in of MTII led to an initial increase in fEPSP magnitude by ~ 40 % prior to stimulation, followed by a decrease of ~ 15 % during test-pulse stimulation. Also in this case, the decrease was fully reversed upon inactivity, and could be induced again by further stimulation.

The decrease in fEPSP magnitude observed in both incubation and wash-in experiments likely represents a synaptic depression. As no decrease was seen in control experiments, this is likely a depression of the synapses formed by MTII. This depression has a similar time course as the one observed in the developing brain in previous studies by Xiao et al. and Abrahamsson et al. [11, 14]. In those studies, test-pulse stimulation of SC-CA1-synapses in the developing hippocampus also led to a similar decrease in fEPSP magnitude, as well as in EPSC (excitatory postsynaptic current) in whole-cell recordings. Through more in-depth study of individual synapses (by whole-cell recordings), this synaptic depression was shown to be caused by AMPA silencing [11, 14]. Synaptic depression is normally not seen when stimulating preexisting synapses in the adult brain in this fashion, and it is likely that the synaptic depression observed in the present study is the result of the newly formed synapses being AMPA-silent.

The complete reversal of the synaptic depression upon inactivity is similar to the one described by Abrahamsson et al. [11]. In their study, the reversal was determined to be caused by AMPA unsilencing, i.e. synapses reverting back to their naïve state where AMPARs are present. After reversal, the synapses were still labile and was silenced again upon stimulation anew [11]. It is likely that the reversal of the synaptic depression observed in the present study, too, can be explained by the same mechanism of AMPA silencing-unsilencing. This mechanism is different from LTP-induced unsilencing, where stronger stimulation at a higher frequency induces a more permanent unsilencing where synapses are no longer in that labile state where they are susceptible to become silenced again [11, 12].

When MTII was washed in after establishing a baseline, the fEPSP magnitude was increased by ~ 40 %. When reinitiating stimulation, there was a ~ 15 % depression after 120 stimulations. This is well in correlation with the results seen after incubation in MTII. However, there was a potentiation by MTII that was not depressed by the test-pulse stimulation. This potentiation could be due to newly formed synapses having stable AMPARs, or to potentiation of preexisting synapses. The results by Shen et al. [25] indicate that MTII actually also increases the number of AMPARs in preexisting synapses in addition to inducing new synapses, favoring the latter explanation.

To summarize, it seems likely that MTII successfully induced formation of new synapses. When stimulating at low-frequency, the newly formed synapses lost their AMPA signaling (expressed as a gradual synaptic depression throughout the round of 120 stimulations). When stimulation was interrupted, they regained their AMPA signaling (expressed as the complete recovery of the synaptic depression), but again quickly lost it upon further stimulation. Hence, these synapses exhibited the same lability in their naïve state as synapses in the developing brain, i.e. they cycled between losing and gaining functional AMPAR-mediated signaling.

In this study, the focus was not to show that MTII in fact induced formation of new synapses, as this has been shown previously by Shen et al. [25]. Hence, we cannot know for certain that the observed effects are really due to new synapses having been formed. However, based on the morphological and electrophysiological evidence of MTIIs effects presented in the study by Shen et al. [25], it is most likely that this is the case.

Methodological considerations

The method used to study synaptic function in the present study is well-established, and the animal model chosen is regarded as one of the most appropriate for studying the hippocampus. Using *in vitro* hippocampal slices rather than doing experiments *in vivo* has both advantages and disadvantages. Mainly, when using *in vitro* slices, it is easier to control the rather sensitive setup of the experiment, e.g. the positioning of recording and stimulation electrodes and the extracellular ion concentrations. Moreover, one does not have to take the blood-brain barrier into account when applying pharmacological treatments. On the other hand, all connections to other areas of the brain (with their potentially modulatory effects), are cut. Also, the dissection of the brain and slicing of the hippocampus may cause damage to the tissue, and the health of the slice is dependent upon the skill of the researcher performing the slice preparation.

The same stimulation protocol was used for all experiments. However, as can be seen from the figures above, the number of experiments where recovery was analyzed were fewer than the total number of experiments. This was due to changes in the volley (greater than 10 %) during stimulus interruption, and to spiking being observed during the second round of stimulation, after stimulus interruption. Therefore, it was necessary to disregard from that part of that particular experiment, as the resulting fEPSP likely would have been influenced by synaptic potentiation being induced. The most likely reason for spiking in these experiments

is the stimulation current being set slightly too high. The stimulation current must be strong enough to evoke a field potential, but not so strong that it induces synaptic potentiation. Even though test-pulse stimulation normally does not induce potentiation, the range of optimal stimulation current is usually narrow, and spiking may still occur if the current is set just slightly too high.

There are some potential weaknesses to note about this study. First, the relative inexperience of the author, mainly in preparing the slices and setting up and handling the sensitive equipment used in the experiments, could potentially have affected the results. However, training in animal handling, slice preparation, and setting up and carrying out experiments was given well before the start of the study. Additionally, a course in laboratory animal science was completed before independently handling the animals, as required by the Swedish Animal Welfare Act. Furthermore, as all experiments were carried out by the same person, any potential inexperience should have affected control experiments and experiments with MTII alike.

Another potential problem that can often occur in field recordings is fluctuations in the volley. As mentioned previously, the volley should remain mostly stable throughout an experiment in order for the data to be reliable. Fluctuations in the volley are usually adjusted for by either including those experiments in the analysis while compensating for the volley in each individual experiment, or by simply omitting experiments in which the volley changed more than a certain percent. Here, the latter was chosen for incubation experiments, as the volley is generally not expected to change during such short (30 minutes in total from the very first to the very last stimulation) experiments. Subsequently, any incubation experiments in which the volley changed more than 10 % were omitted. During longer experiments, the volley is expected to decrease slightly. Hence, we chose to adjust for the volley in the analysis of the

much longer (160-220 minutes from the very first to the very last stimulation) wash-in experiments. For more details, see Strandberg et al. [15].

As previously mentioned, the occurrence of spiking can also be an issue in field recordings in general. This can occur especially if researchers do not watch the recordings as they happen, or properly check them afterwards. When an experiment has begun, the software controls the equipment and takes care of stimulation and recording. Thus, it is easy to simply leave the room to do other things, and any potential unwanted irregularities in the appearance of the field potential, such as spiking, can easily be missed. Of course, it is still possible to check this afterwards, and an experienced researcher would easily detect any such occurrences. However, for an inexperienced researcher, it is still possible to miss this and include such experiments in the analysis Therefore, careful observations of ongoing experiments were made, and notes of any irregularities were taken immediately. Subsequently, some experiments were omitted, mainly because of the occurrence of spiking.

An important limitation to note is the choice of age group for the animals used, as rats at postnatal day 30-45 are, strictly speaking, considered prepubescent. Even though synaptogenesis has slowed considerably by postnatal day 30 [12], it is possible that some silencing might still be occurring, especially in the youngest animals, potentially masking the effects of MTII. On the other hand, if significant synaptic formation was still occurring in the animals used, signs of AMPA silencing should have been seen in control experiments as well. Still, limiting the study to older animals might have been more ideal for the intended purpose of the study.

There are two limitations with the present study specific to the wash-in experiments. For one, the number of experiments is relatively low (n = 4 and n =3 for 120 and 180 minute experiments, respectively). In order to obtain more reliable data, more of the same

experiments are needed. Furthermore, no control experiments in which untreated slices went through the full wash-in protocol were performed. Ideally, this should be done in the future to further solidify the results obtained in the wash-in part of the study.

The next step in the study of *de novo* generated synapses in the adult brain is to continue with further experiments of the same kind as in the present study. Specifically, more wash-in experiments, including controls, are needed. The observed results do give an indication that the newly formed synapses might be AMPA-silent. However, as this study was limited to only extracellular field recordings, it is not possible to definitely say that this is the case. Hence, maybe the most important next step would be to do studies that can confirm whether the observed results are actually due to AMPA silencing. The field recordings performed here are a good start, as characteristics similar to those of AMPA-silent synapses in the developing brain have been observed. However, in order to more thoroughly investigate the underlying mechanism of these findings, it is necessary to continue with electrophysiological and morphological studies on individual synapses, e.g. by whole-cell, patch-clamp recordings where only one neuron is recorded at a time.

CONCLUSIONS AND IMPLICATIONS

MTII induced a synaptic depression in the adult hippocampus resembling that in the developing hippocampus, i.e. induced by very low frequency stimulation (0.2 Hz) and showing full recovery upon inactivity. This depression is likely due to loss of functional AMPA signaling in newly formed synapses.

The mismatch in potentiation by wash-in of MTII and the subsequent depression by test-pulse stimulation is likely explained by an increased number of AMPA receptors in existing synapses.

In order to confirm that the observed results are in fact caused by AMPA silencing, further study of individual synapses by whole-cell, patch-clamp recordings are necessary.

Even though more studies are needed, these results lend promising support to the hypothesis that newly formed synapses in the adult brain are AMPA-silent. Hopefully, increased knowledge of the fundamental properties of these synapses will form a foundation for a better understanding of anti-depressive treatment and contribute to new ideas for treatment of neurodegenerative disorders related to a shifted balance between synaptic formation and elimination.

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