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UNIVERSITY OF HELSINKI, FINLAND

Neurophysiological mechanisms of plasticity induced in adult brain

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ACADEMIC DISSERTATION

To be presented, with the permission from the Faculty of Veterinary Medicine, University of Helsinki, for public examination in lecture room 2402, Viikki Biocenter 3 on 18th of December 2015 at 12 noon.

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ISBN 978-951-51-1839-4 (paperback)

ISBN978-951-51-1840-0 (PDF)

ISSN

Hansaprint, Helsinki, Finland, 2015

To my family

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ABSTRACT

Dynamic modifications of synaptic connectivity enables the brain to adequately respond to environmental challenges. This ability, known as synaptic plasticity, peaks during the early postnatal period, yet it is maintained throughout life. Interestingly, antidepressants (ADs) and AD-like drugs can promote neuronal plasticity in the adult brain, a phenomenon recently suggested to contribute to the mood-improving effects of ADs. However, the mechanisms underlying AD-induced neuronal network refinement are still poorly understood.

The main goal of this thesis was to advance our understanding of the mechanisms associated with pharmacologically-enhanced plasticity in the adult brain. Two pharmacologically distinct compounds with AD-like actions, namely the selective serotonin reuptake inhibitor fluoxetine (Flx) and the volatile anesthetic isoflurane were used to enhance synaptic plasticity in the rodent cortex and hippocampus. After drug exposure, behavioral, molecular, histological and in vitro electrophysiological approaches were utilized to investigate the effects of Flx and ISO on synaptic function and plasticity. Using electrophysiological recordings in brain slices, we show that chronic Flx treatment results in increased short- and long-term plasticity as well as enhanced basal transmission in excitatory CA3-CA1 synapses in the hippocampus. These changes were paralleled by an activity-dependent enhancement in the expression of proteins related to vesicular trafficking and release, such as synaptophysin, synaptotagmin 1, mammalian uncoordinated protein 18 (Munc 18) and syntaxin 1. Moreover, Flx treatment reduced the percentage of parvalbumin-expressing GABAergic neurons, increased the expression of polysialylated-neural cell adhesion molecule (PSA-NCAM) and decreased the expression of the potassium-chloride co-transporter 2 (KCC2)

in the basolateral amygdala and in the medial prefrontal cortex (mPFC). All the above findings are likely to be attributed to increased dynamic range of synaptic plasticity induced by Flx. Our behavioral findings demonstrate that long term Flx administration in combination with extinction training results in long-term loss of fearful memories while the Flx treatment alone failed to influence fear behavior. These data suggest that behavioral training is indispensable for the guidance of Flx-induced network plasticity.

Exposure to isoflurane promotes long-term synaptic plasticity and enhances basal synaptic transmission in excitatory CA3-CA1 synapses in the mouse hippocampus. These changes were correlated with increased tropomyosin receptor kinase B (TrkB) signaling through the mammalian target of rapamycin (mTOR) pathway in the prefrontal cortex and hippocampus and led to rapid antidepressant-like behavioral effects in the forced swim test.

Taken together, our findings highlight that Flx and isoflurane enhance synaptic plasticity in hippocampal and cortical excitatory synapses, however, the underlying molecular mechanisms as well as behavior improvements were different. In conclusion, the results described in this work provide a mechanistic background for adult brain plasticity and network tuning, with high practical significance to the design of clinical therapy.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in text by their Roman numerals

I. Karpova, N., Pickenhagen, A., Lindholm, E.,. Tiraboschi, E., Kulesskaya, N., Agustsdottir, A., Antila, A., Popova, D., Akamine, Y., Sullivan, R., Hen, R., Drew, J.L., Castren, E., "Fear Erasure in Mice Requires Synergy Between Antidepressant Drugs and Extinction Training", Science (2011).

The candidate performed most of the immunohistochemical experiments, participated in experiments with Lentivirus and in vivo stereotactic injections and contributed to data analysis of these experiments.

II. Popova, D., Ágústsdóttir, A., Lindholm, J., Mazulis, U., Akamine, Y., Castrén, E., Karpova, N., "Combination of fluoxetine and extinction treatments forms a unique synaptic protein profile that correlates with long-term fear reduction in adult mice", Eur. Neuropsychopharmacology (2014).

The candidate designed and performed most of the immunohistochemical experiments, completed most parts of data analysis and participated in manuscript preparation together with N.K.

III. Popova, D., Castren, E., and Taira, T., "Chronic antidepressant fluoxetine predispose CA3-CA1 hippocampal synapses to accentuated plasticity", Submitted to European Neuropharmacology journal.

The candidate designed and performed experiments, analyzed the data and wrote the manuscript together with T.T and E.C.

IV. Antila, H., Sipilä, P., Popova, D., Yalcin, I., Guirado, A., Lindholm, J., Autio, H., Vesa, L., Kislin, M., Khiroug, L., Taira, T., Castrén, E and and Rantamäki, T., "Isoflurane anesthesia rapidly activates TrkB receptor signaling and produces antidepressant-like behavioral effects", Submitted to Nature Neuroscience

The candidate designed and performed electrophysiological experiments together with T.T and analyzed the data.

LIST OF ABBREVIATIONS

AD - antidepressant

AMPA - a-amino-3-hydroxy-5-methyl-4-isozazolepropionic acid receptors

BDNF - brain derived neurotrophic factor

BLA - Basolateral Amygdala

Ca^{2+} - calcium

CA - Cornu Ammonis

CaMKII - calcium/calmodulin dependent protein kinase II

CREB - cAMP response element-binding protein

CS - conditioned stimuli

CSPGs - chondroitin sulfate proteoglycans

DG - dentate gyrus

ECM - extracellular matrix

ECT - electroconvulsive therapy

EE - environmental enrichment

EPSPs - excitatory postsynaptic potentials

fEPSPs – field excitatory postsynaptic potentials

FC - fear conditioning

FF – frequency facilitation

Flx - fluoxetine

FST - forced swim test

GABA - gamma-aminobutyric acid

Gat1 - GABA Transporter 1

iPlasticity - induced in adult brain plasticity

IPSP - inhibitory postsynaptic potentials

KAR-kainate receptors

KCC2 - potassium-chloride co-transporter 2

LH - learned helplessness test

LTP - long term potentiation

mTOR - mammalian target of rapamycin

Munc 18 - mammalian uncoordinated protein 18

NMDA - N-methyl-D-aspartate

NT - neurotransmitter

NSF - novelty suppressed feeding

PC - prefrontal cortex

PiX - picrotoxin

P_r - probability of neurotransmitter release

PKA - cyclic adenosine-monophosphate dependent protein kinase

PKC - protein kinase C

PLCγ - phospholipase γ

PNNs - perineuronal nets

PPD - paired pulse depression

PPF - paired pulse facilitation

PSA-NCAM - polysialylated-neural cell adhesion molecule

PSD95 - post synaptic density protein 95

PV - parvalbumin

SC - Schaffer collateral

SSRI - selective serotonin reuptake inhibitor

STP - short-term plasticity

Sptg1 - synaptotagmin 1

STDP - spike timing dependent plasticity

Stx 1 - syntaxin 1

SYP - synaptophysin

TrkB - tropomyosin receptor kinase B

TST - tail suspension test

US - unconditioned stimuli

VGAT - vesicular GABA and glycine transporter

VGLUT1 - vesicular Glutamate Transporter 1

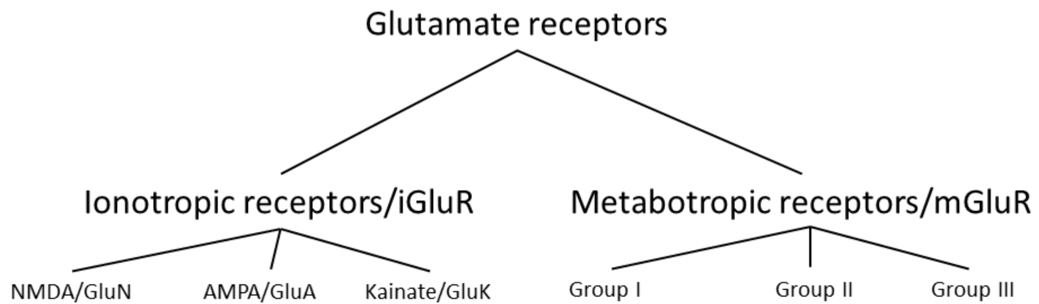
1. REVIEW OF THE LITERATURE

1.1 FEATURES OF NEURONAL TRANSMISSION

Neurons in the mammalian brain communicate with each other mainly by using electrical and chemical signals, utilizing specialized compartments known as synapses. At electrical synapses, current flows through gap junctions, which are membrane channels that connect two cells. In contrast, chemical synapses enable communication via the secretion of specific molecules, neurotransmitters (NT), which are released into the synaptic cleft from the presynaptic terminal, triggered by an influx of calcium (Ca^{2+}), usually as a consequence of an action potential (AP). NT bind to receptors at the postsynaptic membrane, which leads to depolarization (excitatory postsynaptic potential, EPSP) or hyperpolarization (inhibitory postsynaptic potentials, IPSP) of the target neuron and thus making them more or less likely to fire an AP.

The major excitatory transmitter in the brain is glutamate, which exerts its postsynaptic actions via activation of both ionotropic (iGluRs) and G-protein-coupled metabotropic (mGluRs) glutamate receptors (Scannevin & Huganir 2000). Fast excitatory neurotransmission in the hippocampus is mediated by tetrameric glutamate-gated cation channels (iGluRs): N-methyl-D-aspartate (NMDA) receptors (GluNRs), a-amino-3-hydroxy-5-methyl-4-isozazolepropionic acid (AMPA) receptors (GluARs) and kainate receptors (KARs) (Scheme 1). All glutamate receptors are mostly permeable to sodium (Na^{+}) and potassium (K^{+}), while NMDARs are also permeable to Ca^{2+}. The Ca^{2+} permeability of other iGluRs depends on their subunit composition and mRNA editing.

Scheme 1. *Classification of glutamate receptors*



Transmission efficacy and dynamics at glutamatergic synapses can easily be altered by ongoing neuronal activity in a process called synaptic plasticity. By virtue of this property, the glutamatergic synapse can thus act as a 'cellular memory device' containing information about the previous activity history of the neuronal circuitry (Malinow & Malenka 2002; Malenka & Bear 2004; Markram et al. 1997).

Whilst glutamatergic synapses convey information in long projective neuronal pathways, GABAergic networks (operated mainly by gamma-aminobutyric acid (GABA)) control excitability and coordinate spatiotemporal integration properties of principal neurons. Inhibitory GABAergic transmission is mediated via ionotropic $GABA_A$ receptors ($GABA_ARs$) and metabotropic $GABA_B$ receptors ($GABA_BRs$). $GABA_ARs$ are ion channels permeable to chloride (Cl^-) and bicarbonate ions (HCO_3^-) and mediate both fast and tonic inhibition (Farrant & Nusser 2005; Capogna & Pearce 2011). Inhibitory GABAergic neurons (interneurons) perform several types of inhibition (Fig. 1). In feedforward inhibition a principal cell and an interneuron receive excitatory inputs from the same presynaptic source (Sik et al. 1994). The interneuron then outputs its inhibitory signal to the principal cell. Thus, upon activation, the principal cell receives two types of input, one excitatory and

one inhibitory, separated by a brief delay due to interneuron integration. In the feedback mechanism, the principal cell receives excitatory input first and then outputs back to the interneuron (Anderen et al. 1964). Feed-back inhibition is mediated primarily by the perisomatic inhibition of pyramidal neurons. An extension of feedback inhibition is lateral inhibition (Freund & Buzsáki 1996). This occurs when the activation of a principal cell recruits an interneuron, which, in turn, suppresses the activity of surrounding principal cells.

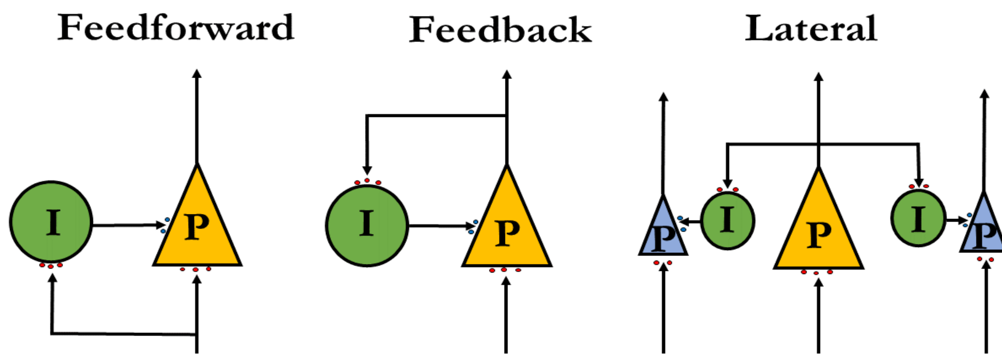


Figure 1. *Types of inhibition in central nervous system. I-inhibitory neuron, P-principal cell.*

1.2 SYNAPTIC PLASTICITY

In the brain, all neurons are structured in a complex system of neuronal networks. They consist of assemblies of excitatory and inhibitory neurons whose work is delicately synchronized and aimed at efficient processing of information coming from inside and outside of the brain. Effectiveness of information processing in the nervous system is very much dependent on the ability of the nervous system to reorganize its connections functionally and

structurally in response to changes in environmental experience, which is referred to as neuronal plasticity (Baroncelli et al. 2011).

One of the most intriguing questions in neuroscience concerns the manner in which the nervous system can modify its organization and ultimately its function throughout lifetime. Synaptic plasticity for over a century has been proposed to play a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces (Citri & Malenka 2008). Nowadays, many different forms of synaptic plasticity have been described; below the well-characterized plasticity principles will be elucidated.

1.2.1 HEBBIAN SYNAPTIC PLASTICITY

The plasticity rule proposed by Canadian psychologist Donald Hebb states that when one neuron drives the activity of another neuron, the connection between these neurons is potentiated (Hebb 1949). Nowadays, modifiable neuronal circuits are called "Hebbian" and the basic mechanism for synaptic plasticity, where an increase in synaptic efficacy arises from the presynaptic cell's repeated and persistent stimulation of the postsynaptic cell, is called "Hebbian plasticity"

1.2.1.1 SHORT-TERM SYNAPTIC PLASTICITY

Short-term plasticity (STP) refers to a phenomenon in which synaptic efficacy changes over time in a way that reflects the history of presynaptic activity. When two stimuli are delivered within a short interval, the response to the second stimulus can be either enhanced or depressed relative to the response to the first stimulus. If the second stimulus response is enhanced then the phenomenon is called paired pulse facilitation (PPF) and if depressed, paired pulse depression (PPD) (Katz & Miledi 1968; Zucker & Regehr 2002). Most

forms of STP are triggered by short bursts of activity causing a transient accumulation of Ca^{2+} in presynaptic nerve terminals. This increase in presynaptic calcium, in turn, causes changes in the probability of NT release (P_r) by directly modifying the biochemical processes that underlie the exocytosis of synaptic vesicles.

Short term plasticity depends on the initial P_r. Synapses with a high initial P_r tend to depress, whereas those with a low initial probability of release usually facilitate. Indeed, most synapses can show either facilitation or depression depending on the initial P_r. Several models have been proposed to account for short-term plasticity, including summation of residual Ca^{2+} with repetitive stimulation, and local saturation of calcium buffers. It has also been suggested that short-term changes in synaptic function is associated with the specific release sensor (Zucker & Regehr 2002).

Even if every synapse, examined in organisms ranging from invertebrates to mammals, exhibits numerous forms of short-term synaptic plasticity, the physiological meaning of STP still hasn't been fully recognized (Zucker & Regehr 2002), however, a few assumptions exist. Short term synaptic plasticity in mammalian brain may serve as high and low-pass filters influencing on function of information processing. For example, synapses with a low initial P_r function as high-pass filters, since they will facilitate during high-frequency action potential bursts while low-frequency bursts will not be transmitted with the same efficacy. In contrast, synapses with a high initial P_r function as low-pass filters, since they will depress during high-frequency bursts but will reliably relay low-frequency activity (Abbott & Regehr 2004).

1.2.1.2 LONG TERM SYNAPTIC PLASTICITY

Approximately two decades after Hebb published his postulate, Terje Lømo and Tim Bliss in 1968 showed that high-frequency electrical stimulation in the dentate gyrus of the rabbit hippocampus cause persistent growth of response amplitude and called this phenomenon long term potentiation (LTP) (Bliss & Lomo 1973). Over the past 40 years, long-lasting synaptic enhancement has been an object of intense investigation because it has been proposed that long term potentiation provides an important key for understanding the cellular and molecular mechanisms by which memories are formed and stored. Investigation of the mechanism of this phenomenon forced the study of LTP into the field of synaptic plasticity, in particular to *in vitro* studies of living hippocampal slices.

The hippocampal formation consists of different sections: subiculum, dentate gyrus (DG) and cornu ammonis (CA) and in rodents brain presented as folded structure of excitatory/principal and inhibitory/interneuron cells. The CA divides further into three different regions CA3, CA2 and CA1. A very important feature of the hippocampus is the relay organisation of synaptic transmission, the so called trisynaptic loop (Fig. 2), which starts in the entorhinal cortex where through granule cell fibers - perforant path - information is processed to DG. DG granule cells project to CA3 pyramidal cells synapses (mossy cell fibers). And then CA3 pyramidal cells form synapses on CA1 pyramidal cells, which cell bodies are organized in the thick band (striatum radiatum). Afferent fibers that connect CA3 and CA1 areas of hippocampus are called Schaffer collateral (SC). Pyramidal neurons in the CA1 area then synapse in the subiculum and and project to the entorhinal cortex. Collectively the DG, CA3 and CA1 areas of the hippocampus compose the trisynaptic loop.

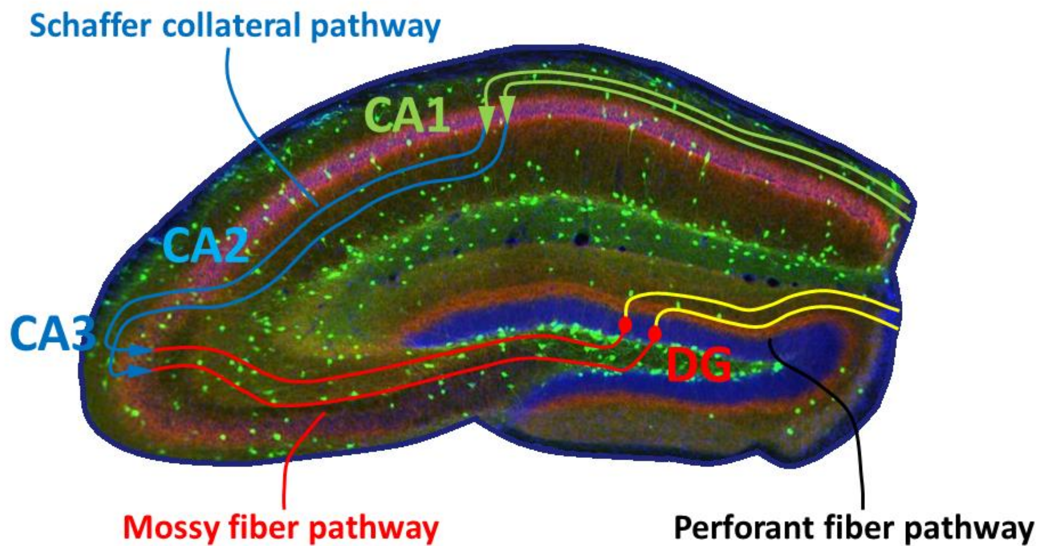


Figure 2. *Schematic representation of the synaptic connectivity in the transverse hippocampal slice. Hippocampal trisynaptic loop consist of DG-CA3-CA1 synaptic pathways there entorhinal cortex-DG pathways called perforant fiber pathway, DG-CA3 - mossy fibers, CA3-CA1 – Shaffer collateral.*

The classical way to observe LTP is via tetanic stimulation and recording of field postsynaptic potentials (fEPSPs) in hippocampal slices. However, there are other protocols for LTP induction. For example, the coupling of low frequency presynaptic stimulation with postsynaptic depolarization is one effective method used to induce LTP that can last for several hours (Gustafsson & Wigström 1988; Liao et al. 1995; Chen et al. 1999). Theta burst stimulation is another method of LTP induction, resembling the physiological events underlying the LTP. It takes its name from the theta rhythm observed on electroencephalogram (about 5 Hz) in living hippocampus. For instance, 10 trains consisting of 4 pulses at 100 Hz with 200 msec interval, effectively induces LTP in CA1 area of hippocampus (Larson et al. 1986; Staubli & Lynch 1987). It is important to notice that LTP occurs not only at excitatory synapses

of the hippocampus, but at many other synapses in a variety of brain regions (Yaniv et al. 2000; Grossman et al. 2002; Berretta et al. 2008; Caruana et al. 2012). Moreover, several properties of LTP match the properties of some forms of memory, suggesting that LTP may underlie cognitive functions. First, LTP is input-specific, which means that when it is generated in one synapse it doesn't normally occur in another. LTP is associative - weak stimulation of a pathway will not by itself trigger LTP. However, if one pathway is weakly activated at the same time that a neighboring pathway onto the same cell is strongly activated, both synaptic pathways undergo LTP. Although LTP is triggered rapidly, it last for hours in vitro and days in vivo and the late phase of LTP requires gene transcription and protein synthesis. Clearly, LTP reflects a mechanism that most likely contributes to memory formation by triggering long lasting, perhaps permanent changes in neuronal circuitry.

NMDA-dependent long term potentiation

In the CA1 region of hippocampus, as well as many other areas of the central nervous system (CNS), LTP induction requires a rise in postsynaptic Ca^{2+} via activation of NMDA receptors (Fig. 3). In order for the NMDA receptor channel to conduct, glutamate must bind to the receptor and the postsynaptic membrane must be depolarized. The basis for this is a voltage-dependent block of the ion channel by extracellular magnesium (Mg^{2+}). However when the postsynaptic cell is depolarized during induction of LTP, Mg^{2+} dissociates from its binding site within the NMDA receptor channel, allowing Ca^{2+} as well as Na^{+} to enter. It is now well accepted that trafficking of other glutamate receptors (AMPA receptors) to and away from synaptic plasma membrane plays an essential role in LTP induction, expression and maintenance. AMPA receptors are composed of four types of subunits GluA (1-4). Most AMPA receptors are heteromeric, consisting of symmetric 'dimer of dimers' complexes of GluA2 and either GluA1, GluA3 or GluA4 and, depending on

subunit composition of receptor; they may play distinct roles in neural communication. There are two general models explaining how the synapses acquire AMPA receptors during LTP. In the first model, glutamate receptors are freely moving via lateral diffusion into and out of the synapse (Opazo et al. 2012). In the second model, neuronal activity triggers exocytosis, which leads to insertion of GluARs into the synapse from an intracellular pool (Park et al. 2004). There are strong evidential data supporting both models, however further experiments are needed to clarify this issue.

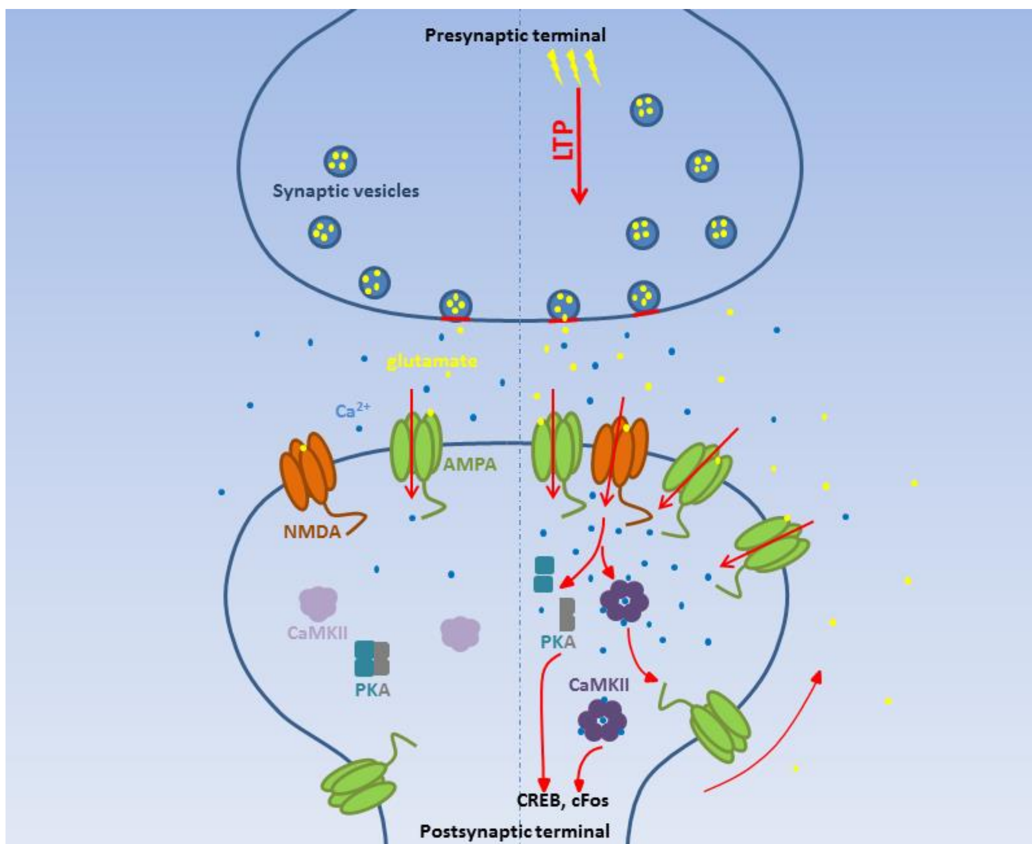


Figure 3. *Schematic representation of NMDA-dependent LTP. Robust stimulation of presynaptic terminal (or other protocols of stimulation) causes release of glutamate and consequent activation of AMPA and NMDA receptors. Depolarization induced CA^{2+} enter results in activation of downstream signaling molecules, transcription factors and internalization of AMPA receptors.*

Activation of AMPA and NMDA receptors, except NMDA-independent forms, seems essential for the induction of long term synaptic plasticity, but insufficient to elicit a stable form of LTP. Calcium/calmodulin dependent protein kinase II (CaMKII) is essential as a mediator for NMDA-dependent LTP. CaMKII is found in high concentrations in the postsynaptic density: the postsynaptic component of the dendritic spine that also contains glutamate receptors. Loading of cells with a constitutively active form of CaMKII enhances EPSCs, whereas genetic deletion of a critical CaMKII subunit blocks the ability to generate LTP (Gustafsson & Wigström 1988; Malenka et al. 1989). Several other protein kinases, including protein kinase C (PKC), cyclic adenosine-monophosphate (cAMP)–dependent protein kinase (PKA), the tyrosine kinase Src, and mitogen-activated protein kinase (MAPK), have also been suggested to contribute to LTP (Teyler & DiScenna 1987; Gustafsson & Wigström 1988; Larkman & Jack 1995; Wikström et al. 2003).

Neurotrophins, specifically brain derived neurotrophic factor (BDNF), are of particular interest to synaptic plasticity because of the possibility that BDNF may serve as a mediator rather than simply as a modulator of LTP. The idea that BDNF might be involved in synaptic plasticity came from the observation that the expression of BDNF in the hippocampus can be induced by high frequency stimulation and that endogenous BDNF is required for LTP induction in hippocampal CA1 pyramidal neurons (Castrén et al. 1993; Patterson et al. 1996). Importantly, BDNF acts on synaptic transmission from both pre and post synaptic sites. Presynaptically, BDNF enhances glutamate release and increases the frequency of miniature EPSCs (mEPSCs) in hippocampus (Takei et al. 1998; Lessmann & Heumann 1998; Waterhouse & Xu 2009). On the postsynaptic site, BDNF increases NMDA single-channel open probability (Levine et al. 1998; Levine & Kolb 2000) presumably through tyrosine phosphorylation of the NMDA receptor subunits (Suen et al. 1997;

Lin et al. 1998) and regulate its expression by transcription dependent mechanisms (Caldeira et al. 2007; Carvalho et al. 2008).

The later stages of LTP are dependent upon both protein translation and gene transcription, which similarly involves the participation of multiple signaling pathways. During LTP, protein synthesis is required to supply functional and structural changes. In this regard, the mammalian target of rapamycin (mTOR) pathway was found to be important for LTP expression (Tang et al. 2001). mTOR is known to regulate both dendritic and somatic protein synthesis in neurons (Hoeffer & Klann 2010). Examples of mTOR translation targets include CaMKII, PSD-95 and GluR1 (Slipczuk et al. 2009). Another well studied transcription factor involved in LTP is cAMP response element-binding protein (CREB) (Bengtson & Bading 2012). CREB targets genes including *Bdnf* and its cognate receptor tropomyosin receptor kinase B (TrkB) (Deogracias et al. 2004), *Wnt2* (Wayman et al. 2006) and different glutamate receptor subunits (Wayman et al. 2006; Traynelis et al. 2010).

NMDA- nondependent long term potentiation

In most of the synapses, LTP requires the activation of NMDA receptors which are generally considered to be expressed postsynaptically. However, there are regions in the brain which undergo long term synaptic plasticity but the origin and basis of synaptic strengthening in those synapses are fundamentally different. Synaptic transmission and plasticity at the hippocampal mossy fiber synapse is unusual for several reasons, including low basal P_r, pronounced frequency facilitation and a lack of NMDARs involvement in LTP. Experimental evidence suggest that mossy fiber LTP does not need any postsynaptic activation but is triggered by an activity-dependent increase of Ca^{2+} in the presynaptic terminal (Katsuki et al. 1991; Maccaferri et al. 1998). Another set of findings suggest an important role of presynaptic kainate receptors in induction and maintenance of mossy fiber LTP. Thus, application

of a selective kainate receptor antagonist, which did not affect mossy fibre synaptic transmission, completely blocks the induction of mossy fibre LTP in a fully reversible manner (Bortolotto et al. 1999). Additionally, kainate receptors are found in higher levels in the CA3 region of the hippocampus.

Another non-conventional example of NMDA-independent form of long term synaptic plasticity is LTP in glycinergic synapses. Glycine receptors (GlyRs) are structurally related to $GABA_A$Rs and have a similar inhibitory role. In the superficial dorsal horn of the spinal cord, glycinergic synapses on inhibitory GABAergic neurons exhibit LTP (GlyRs LTP), which occurs rapidly after exposure to the inflammatory cytokine interleukin-1 beta. Notably, formalin-induced peripheral inflammation *in vivo* potentiates glycinergic synapses on dorsal horn neurons, suggesting that GlyR LTP is triggered during inflammatory peripheral injury (Chirila et al. 2014)

1.2.1.3 SPIKE TIMING DEPENDENT PLASTICITY

Another form of Hebbian long-term synaptic plasticity, spike-timing-dependent plasticity (STDP), depends on the relative timing of pre- and postsynaptic action potentials. A pioneering study by W. Levy and O. Steward (1983) demonstrated that stimulation of inputs from entorhinal cortex to the DG produced potentiation when the weak input preceded the strong input by less than 20 ms, and reversing the order led to depression (Levy & Steward 1983). Later on H. Markram and colleagues (1997), controlling pre- and postsynaptic spike timing, discovered that order and timing of pre- and postsynaptic spikes was critical for direction and magnitude of postsynaptic response (Markram et al. 1997) Although STDP describes new synaptic plasticity principles in neuronal network, the mechanisms underlying this phenomenon seems to be allied to classical Hebbian synaptic plasticity.

1.2.2 HOMEOSTATIC SYNAPTIC PLASTICITY

Homeostatic synaptic plasticity is a form of synaptic plasticity that acts to stabilize the activity of a neuron or neuronal circuit in the face of perturbations and complements Hebbian forms of plasticity where activity-dependent refinement of synaptic connectivity occurs. One of the most studied forms of homeostatic plasticity is synaptic scaling. In pioneering experiments by G. Turrigiano and colleagues (Turrigiano et al. 1998), it was demonstrated that chronic blockade of cortical culture activity increased the amplitude of miniature EPSCs (mEPSCs) without changing their kinetics. Conversely, blocking GABA - mediated inhibition initially raised firing rates, but over a 48-hour period mESPC amplitude decreased and firing rates reconciled to control values. This study demonstrates that, at least *in vitro*, homeostatic plasticity mechanisms are in place that function to keep activity relatively constant in the face of even major perturbations.

The major expression mechanism underlying homeostatic plasticity is through bidirectional accumulation of AMPA receptors. In spinal and neocortical neurons there are proportional changes in GluA1 and GluA2 subunits of AMPA receptors, after tetrodotoxin (TTX) blockade of neuronal activities (O'Brien et al. 1998; Wierenga et al. 2005), where studies on hippocampal neurons have reported enhanced GluA1 accumulation with smaller or absent changes in GluA2 (Thiagarajan et al. 2005; Sutton et al. 2006). Several studies have demonstrated the role of BDNF in synaptic scaling. BDNF is thought to be released by cortical pyramidal neurons in an activity dependent manner, and exogenous BDNF can prevent the effects of activity deprivation. Further, preventing activation of endogenous BDNF receptors mimics the effects of activity blockade (Rutherford et al. 1998; Copi et al. 2005). Evidently, there are likely multiple forms of synaptic homeostasis,

mediated by distinct signalling pathways and with distinct expression mechanisms (Ramakers et al. 1990; Corner & Ramakers 1992; Rutherford et al. 1998; Trasande & Ramirez 2007). And since the field of homeostatic synaptic plasticity is still relatively young it is expected that the cast of molecular players thought to be involved will rapidly accumulate over the time. Altogether, homeostatic synaptic plasticity serves as mechanisms to stabilize firing rates in the face of developmental or learning-induced changes in drive, and this contributes to the ability of central neuronal networks to maintain stable function and enables networks to maintain the specificity of synaptic changes that encode information.

1.3 NEURONAL PLASTICITY

1.3.1 TIMELINE OF PLASTICITY

Major challenges for the field of synaptic plasticity now include understanding when and why different forms of plasticity are present in real neuronal networks, and how these mechanisms interact with each other to generate flexible yet stable brain function.

Even though plasticity is an obvious phenomena in the brain, it is not present constantly throughout lifetime (scheme 2). During prenatal development and short time after a birth it is impractical for the genome to specify the connectivity of every connection in the brain. Later, connections are sculpted in response to internal and external events. During maturational stages in the lifespan of an organism nervous system is especially sensitive to certain environmental stimuli and these periods, which are called critical periods, are characterized with heightened plasticity. In the early 1960s David H. Hubel and Torsten Wiesel clearly demonstrated that sensory experience shapes

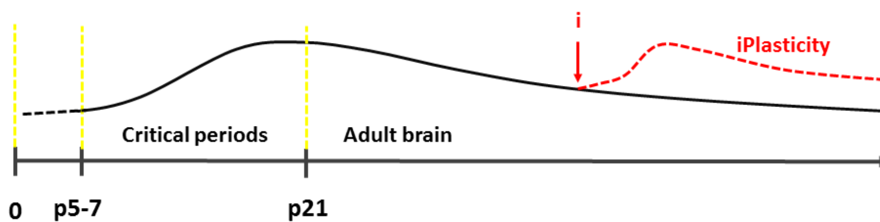
neuronal networks and that the degree to which the brain is changed by experience is variable and age dependent (Wiesel & Hubel 1963).

In primates and cats, visual inputs from each eye segregate into eye-specific regions in the primary visual cortex, called ocular dominance columns, where most of the neurons in columns are activated to some degree by both eyes, and about a quarter are more activated by either the contralateral or ipsilateral eye (Hubel & Wiesel 1963; Blakemore & Vital-Durand 1986). This segregation process takes place during a critical period of early postnatal development and requires the balanced use of both eyes (Wiesel 1982). However, if one eye is closed during the critical period, very few cells could be driven from the deprived eye and ocular dominance distribution is shifted such that all cells are driven by the eye that remained open. Such changes lead to development of poor vision or amblyopia. Importantly, if the patch is removed during the critical period and the use of the weaker eye is encouraged by patching the better eye, the vision of the amblyopic eye can be recovered. However, after the closure of this critical period, amblyopia becomes permanent and cannot be revised by patching of the better eye (Hubel & Wiesel 1963; Wiesel & Hubel 1965). Nowadays, it is widely accepted that similar processes govern the development and tuning of neuronal networks not only in visual system but in other brain regions. During normal development sensitive periods for the elaboration of sensory pathways (vision, hearing) and higher cognitive function elapse in humans to around 7 years of age and in rodents to day 21 of postnatal development. Important, timing of critical periods for different systems may significantly vary (Hensch 2003).

Use-dependent plasticity, plasticity which is sensitive to experience, continues to take place in adulthood. Adult brain plasticity is much more restricted in scope but still possible. Normal or naïve adult brain plasticity underlies our ability to form memories, learn and cope with changing environment (Kolb et

al. 2003; Robinson & Kolb 2004). Recent studies have shown, using mammalian visual cortex as an experimental model (Hensch 2005), that it is possible to reinstate much greater levels of plasticity in the adult visual cortex than previously suspected, employing various environmental and pharmacological strategies (Sale et al. 2007; Hensch 2003; Hensch 2005). This type of plasticity does not present in normal adult brain but can be induced by various interventions. It characterized with features similar to critical periods and thus is usually called reopened critical period or juvenile-like plasticity. We propose to use the term iPlasticity, which means induced plasticity, to describe structural and functional reorganizations of mature neuronal networks stimulated by intense environmental or/and pharmacological influence.

Scheme 2. *Timeline of plasticity.*



1.3.2 TOOLS TO TRIGGER iPLASTICITY

1.3.2.1 ENVIRONMENTAL ENRICHMENT

Exposure to complex environment, rich with sensory stimuli, so-called environmental enrichment (EE) – has been shown to induce plasticity. In neuroscience, EE refers to housing conditions of laboratory animals, where a combination of complex inanimate and social stimulation facilitates sensory, cognitive and motor function (Rozenzwieg et al. 1962). Enriched animals are

reared in large groups and maintained for at least three weeks in an environment where a variety of objects (e.g. toys, tunnels, nesting material and stairs) are present and change frequently.

It was demonstrated that EE restores plasticity of the visual cortex in adulthood (Sale et al., 2007).Exposure of adult rats to EE completely rescued the visual deficits associated with amblyopia (Sale et al., 2007). Consistent with this finding, it has been demonstrated that EE affected structural plasticity: increased dendritic branching and length, the number of dendritic spines, the size of synapses on some neuronal populations (Rosenzweig et al. 1964; Beaulieu & Colonnier 1987; Greenough et al. 1987) and synaptic plasticity: increased basal synaptic transmission and long term potentiation in hippocampus (Foster et al. 1996). At the level of behavior, environmental complexity enhanced learning and memory (Moser et al. 1997; Rampon et al. 2000; Tang et al. 2001; Lee et al. 2003), reduced memory decline in aged animals (Bennett et al. 2006), decreased anxiety and increased exploratory activity (Chapillon et al. 1999; Friske & Gammie 2005). Clearly, exposure to EE reinstates critical period plasticity and can be used as a tool to study iPlasticity.

1.3.2.2 FLUOXETINE

Fluoxetine (Flx, also known as Prozac) is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class, which was discovered and developed by scientists from Eli Lilly and Company in 1974 (Wong et al. 1974). It is frequently used to treat major depressive disorder, obsessive-compulsive disorder, post-traumatic stress disorder, bulimia nervosa, panic disorder, premenstrual dysphoric disorder, trichotillomania etc. For a long time it was thought that mechanism of Flx action on the nervous system was associated with increasing serotonin levels by serotonin uptake blockade, which was very

much in line with the action of other antidepressants on monoamines balance in the brain. At the time it formed the basis for the monoamine theory of depression, which proposed that this condition was caused by a deficiency in monoaminergic neuromodulators and antidepressant drugs acted by replenishing them (Schildkraut 1995).

Recent studies showed that chronic treatment with Flx induced a plastic state in the visual cortex which closely resembles that observed at the peak of the critical period (Maya Vetencourt et al. 2008). When adult rats were treated with Flx, closing of one eye produced a dramatic shift in the sensitivity of visual cortical neurons in favor of the open eye, a response normally seen only during the early postnatal critical period (Maya Vetencourt et al., 2008). Furthermore, visual acuity of the amblyopic eye could be fully restored in adulthood when the eye was opened during Flx treatment and the previously open eye was simultaneously closed to encourage the use of the weak eye (Maya Vetencourt et al. 2008). Flx induced neuronal network changes that were also associated with reduced inhibition and enhanced expression of BDNF and TrkB (Saarelainen et al. 2003; Rantamäki et al. 2007; Maya Vetencourt et al. 2008). Importantly, only long term Flx administration enhances neuronal plasticity (Wang et al. 2008). Together these findings suggest that Flx action on neuronal networks may differ from its conventional role as an AD and may be associated with iPlasticity.

1.3.2.3 KETAMINE AND ISOFLURANE

Isoflurane is a halogenated ether used for inhalational anesthesia. Its use in human medicine is now starting to decline, however it is still frequently used for veterinary anesthesia. A pioneering study by Langer and colleagues (1985) revealed a rapid antidepressant effect of isoflurane on depressed patients (Langer et al. 1985). In their study treatment-resistant depressed patients who

primarily have been subjected to electroconvulsive therapy (ECT) were given a series of anesthesias with isoflurane and then tested for symptoms of depression. The authors hypothesized that a brief period of electrocerebral silence, which can be observed shortly after the grand mal seizure in ECT, may be, in itself, a crucial for the therapeutic effects of ECT. In this study, they clearly demonstrated rapid relief in depressive symptoms after isoflurane administration, which persist for several weeks. A study with another anesthetic, ketamine, in a placebo-controlled, double-blinded trial in humans demonstrated that a single ketamine administration significantly improves depressive symptoms within 72 hours after drug infusion (Berman et al. 2000).

While the mechanisms underlying isoflurane and ketamine rapid antidepressant action remain unclear, several lines of evidence suggest that treatment with ketamine also depends on plasticity. Thus ketamine produces a rapid antidepressant-like behavioral response in rodents subjected to chronic stress (Maeng et al. 2008; Li et al. 2010; Autry et al. 2011). However, because of its psychotomimetic properties, clinical use of ketamine is limited by abuse potentials (Machado-Vieira et al. 2009) and the lack of clinical studies of isoflurane action means it is currently not allowed to replace conventional ADs with fast acting anesthetics, but it is evidently possible to use these chemicals to study iPlasticity.

1.3.3 FEATURES OF IPLASTICITY

1.3.3.1 ROLE OF BDNF AND TRKB

The first member of the neurotrophin family, nerve growth factor (NGF) (Cohen & Levi-Montalcini 1956), was discovered in the early 1950s as a target-derived protein that promotes the survival and growth of sympathetic and

sensory neurons during development. The establishment of the neurotrophins family came with the purification and characterization of BDNF from pig brain by H. Thoenen laboratory (Barde et al. 1982). Since then, two other neurotrophins have been identified in the mammalian brain: neurotrophin 3 and 4 (NT3, NT4) (Lewin & Barde 1996). Neurotrophins are small proteins (molecular weight about 13 kDa) and like other secreted proteins, arise from precursors, proneurotrophins (30–35 kDa), which are proteolytically cleaved to produce mature proteins. Several studies showed that trophic factors are secreted in both mature (cleaved) and immature (non cleaved) forms (Mowla et al. 1999; Zhou et al. 2004). Two types of receptors for neurotrophins have been identified: $p75_{NTR}$ (Reichardt 2006), which belongs to the family of tumor necrosis factor (TNF) receptors and binds proneurotrophins; and one of the three tropomyosin-related kinase (Trk) receptors — NGF binds to TRKA, BDNF and NT4 bind to TRKB, and NT3 binds to TRKC. Through the differential expression and cellular localization of their receptors, neurotrophins can elicit diverse cellular responses in different types of neurons and at different cellular loci (Chao 2003; Reichardt 2006). But in general, interaction of mature trophic factors with Trk receptors leads to cell survival, whereas binding of neurotrophins precursors (proNGF and proBDNF) to $p75_{NTR}$ leads to apoptosis (Reichardt 2006; Chao 2003).

A solid body of data firmly established the role of BDNF and TrkB signaling in iPlasticity. Original observations from Ronald Dumans laboratory demonstrated that different classes of ADs significantly increased the expression of BDNF in the major subfields of the hippocampus (Nibuya et al. 1995). ADs have also been shown to increase BDNF protein levels not only in the hippocampus but in other brain regions (Altar et al. 2003; Calabrese et al. 2007; Balu et al. 2008; Maya Vetencourt et al. 2008). Phosphorylation of TrkB receptors have been also associated with iPlasticity. Different chemical

classes of ADs increased TrkB phosphorylation, resulted in an associated rise in phospholipase γ (PLCγ) and CREB acutely (within 30 min) and persist for at least 3 week of continuous treatment (Saarelainen et al. 2003; Rantamäki et al. 2007). Interestingly, it was found that increased TrkB phosphorylation by ADs is independent of BDNF (Rantamäki et al. 2011) and behavior effects induced by ADs are blunted in mice with reduced level of BDNF and inhibited TrkB signaling (Saarelainen et al. 2003; Guiard et al. 2007; Deltheil et al. 2008; Li et al. 2008).

1.3.3.2 ROLE OF EXTRACELLULAR MATRIX

Chondroitin sulfate proteoglycans (CSPGs) are components of the extracellular matrix (ECM) that inhibit axonal sprouting and growth. Their adult pattern of expression is very high. CSPGs condense around the soma and dendrites of a subset of neurons in the form of perineuronal nets (PNNs) (Köppe et al. 1997). The absence of PNNs is considered to be a key permissive factor that allows the induction of ocular dominance plasticity during the critical period (Pizzorusso et al. 2002a). The assembly of PNNs around parvalbumin (PV)-expressing inhibitory interneurons is thought to contribute to critical-period closure (Pizzorusso et al. 2002). PNNs preferentially surround cell bodies and proximal neurites of mature fast spiking PV-positive interneurons, which was suggested to limit PV cell plasticity by controlling the concentration of extracellular ions that surround these cells or by sequestering molecular factors which regulate plasticity (Härtig et al. 2001; Hensch 2003; Inoue et al. 2007). Consistent with this notion, the degradation of PNNs with chondroitinease ABC in adults allow the induction of ocular dominance plasticity in visual cortex (Pizzorusso et al. 2002a; Gogolla et al. 2009). Altogether, it is possible to conclude that iPlasticity is associated with

modified extracellular environment which is primarily, in the adult brain, directed to stabilize neuronal connections.

1.3.3.3 DISINHIBITION AS A MECHANISM OF IPLASTICITY

Inhibition plays a crucial role in shaping neuronal networks in response to changing environment. As it was described previously, the ocular dominance shift induced by Flx and EE was associated with an altered intracortical inhibitory-excitatory balance due to reduced GABAergic inhibition - disinhibition. Sale and co-authors revealed that three weeks exposure of amblyopic rats to EE promoted a complete recovery of both visual acuity and ocular dominance and this striking effect was associated with a threefold reduction in the basal level of GABA detected by in vivo brain microdialysis in the visual cortex (Sale et al. 2007). Reduced cortical inhibition was also found in synaptic plasticity levels, since the visual cortical slices of EE animals displayed full reinstatement of white matter-LTP, a phenomenon that is usually absent in the adults (Artola & Singer 1987; Sale et al. 2007; Maya Vetencourt et al. 2008). Moreover local and transient suppression of inhibition in adult brain restore critical period-like plasticity and promote ocular dominance plasticity in adult brain (Hensch 2003; Harauzov et al. 2010).

Recent studies elucidated the role of PV-positive inhibitory cells in mechanisms underlying iPlasticity. It was proposed that a transient suppression of PV cells may gate cortical plasticity. Mimicking a transient (24 h) reduction of inhibition upon eyelid suture by selective activation of designed receptors exclusively activated by designed drugs (DREADDs) within PV cells enables plasticity beyond the critical period (Kuhlman et al. 2013). Another study revealed that chronic Flx administration lead to reduced GABA release from PV positive basket cells (Méndez et al. 2012). All together,

these findings clearly demonstrate an important role of neuronal networks disinhibition as a mechanism of iPlasticity where PV positive inhibitory cells are a central hub.

1.3.3.4 Role of Neurogenesis

The environment has a striking influence on the rate of adult neurogenesis (Kempermann et al. 1997; Young et al. 1999; Uda et al. 2006). The iPlasticity has been associated with reinforced neurogenesis in DG. Chronic treatment with Flx and exposure to complex environment enhance neurogenesis and affect the maturation and functional integration of newborn neurons into hippocampal networks (Malberg et al. 2000; Olson et al. 2006; Kobayashi et al. 2010; Klomp et al. 2014). Flx induced plasticity enhances neurogenesis-dependent LTP in the DG and ablation of neurogenesis with x-irradiation completely block the effects of chronic Flx on synaptic function (Wang et al. 2008). Interestingly, it was also demonstrated that disruption of Flx-induced neurogenesis blocks behavioral responses to antidepressants (Santarelli 2003).

1.3.3.5 iPlasticity and behavior

Changes in the structural and functional properties of the brain reflect changes in behavior. Thus, enhanced neuronal plasticity not only improves learning and memory (Rampon et al. 2000; Lee et al. 2003; Bennett et al. 2006), reduces memory decline in aged animals, decreases anxiety-like behavior and increases exploratory activity (Chapillon et al. 1999; Friske & Gammie 2005), but also leads to better recovery following diverse pathological conditions. In order to study iPlasticity and behavior, a few common stress based models of depression-like behavior can be used.

The forced swim test (FST) is a model of “behavioral despair” used to study depression-like and antidepressant-like behavioral responses in rodents (Slattery & Cryan 2012). In FST, after the placement of rodents to beaker with water, despair behavior is analyzed as proportion of active response to immobility, where more animal spends in immobile state versus active the more depression it express. The novelty suppressed feeding (NSF) test can be used to measures anxiety-like behavior and study response to antidepressant treatment (Bodnoff et al. 1988; Santarelli 2003). In this paradigm latency to eat, for a food deprived animals, which were obliged to move into bright area in open field , is considered as anxiety-related behavior, and chronic, but not acute, antidepressant administration decreases the latency (Bodnoff et al. 1988; Santarelli 2003).

Another behavior paradigm, which can be implemented to study plasticity, is fear conditioning (FC) (Pavlov I, 1927). It recruits ability of animals to learn by experience that some stimuli precede danger. This in turn leads to formation of a life-long memory, which has made Pavlovian fear conditioning such a widely used and most intensively studied paradigm in translational neuroscience (Milad & Quirk 2012). In animal models, FC is mainly recognized as an associative learning task in which an aversive stimulus (unconditioned stimulus, US) is paired with a particular neutral context or stimulus (conditional stimulus, CS) resulting in the expression of fear responses to the originally neutral stimulus or context. During fear acquisition, the neutral stimulus alone starts to elicit the fear reaction, which can be measured as a freezing of an experimental animal. If the animal is after successful acquisition is repeatedly exposed to the CS alone without the US, freezing response gradually decreases, a process known as fear extinction (Milad & Quirk 2012). Extinction is regarded as a new type of learning in which extinction networks inhibit fear networks (Myers & Davis 2007).

However, extinction learning is not very effective: freezing response typically reappears after a few days (known as spontaneous recovery), especially if the animal is exposed to the environment where the pairing between the US and CS took place (fear renewal) (Milad & Quirk 2012).

It is important to recognize that fear memories do not reside in a single anatomical locus but rather arise from interactions among a number of structures that compose a neural circuit. Anatomical, behavioral and electrophysiological evidence indicates that complex networks are involved in learning and expressing fear responses. These include, but are not limited to, the amygdala, the medial prefrontal cortex (mPFC) and the hippocampus (Duvarci & Pare 2014). The amygdala nuclei involved in fear learning can be divided into two main sub-areas that fundamentally differ in their anatomical and functional organization: the basolateral complex and the central amygdala. The basolateral complex consist of approximately 80% of glutamatergic spiny projection neurons and 20% GABAergic neurons and receives inputs from both subcortical and cortical sensory regions including thalamus, cortex and hippocampus (McDonald 1998; Swanson & Petrovich 1998). By contrast, the central amygdala is mainly composed of GABAergic neurons, many of which project to brain areas that are important for mediating defensive behaviors (Swanson & Petrovich 1998; Sah et al. 2003). Emerging literature suggests a role for the hippocampus and mPFC in the regulation of fear memories. It was demonstrated that ventral (infralimbic, IL) and dorsal (prelimbic, PL) mPFC play opposing roles in fear (Ji & Neugebauer 2012; Do-Monte et al. 2015). For example, activation of projections from the IL to the basomedial amygdala with channelrhodopsin-2 and light decreases the anxiety and promotes fear extinction (Adhikari et al. 2015). In contrast, activation of the PL increases fear responses and impairs extinction (Vidal-Gonzalez et al.). Finally, hippocampus has been shown to be

involved in indexing those associations to the contexts in which they occurred. Ventral hippocampus projects both to the mPFC and the basolateral amygdala and, depending on the experimental condition, may either enhance or inhibit fear extinction (Milad & Quirk 2012). For example, pharmacological inactivation of the ventral hippocampus prevents context-dependent fear renewal and interferes with context-dependent changes in extinction (Sparta et al. 2014).

2. AIMS OF STUDY

The major aim of this thesis was to advance our understanding of the mechanisms associated with plasticity induced in the adult brain. More specifically, the aims were:

1. To study Flx iPlasticity in pathology via examination of behavior and structural changes associated with Flx treatment after exposure to fear.

2. To study the mechanisms of iPlasticity in naïve mice induced by chronic Flx administration and single exposure to isoflurane.

3. To study hippocampal synaptic plasticity underlying long term Flx administration and single isoflurane exposure.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Adult male mice C57Bl/6JRcc.Hsd (Harlan, Netherlands) at 8-16 weeks old age were housed individually or in groups. Animals were kept under 12 h light/dark cycle (light on at 6 am). Food and water were available ad libitum. All animal procedures were done according to Animal Ethical Committee of Southern Finland and covered by ESAVI/7551/04.10.07/2013 license.

3.2 DRUG TREATMENT

Fluoxetine (study I, II, III)

Mice received Flx (Orion Pharma, Helsinki, Finland) via drinking water in light-protected tubes. Solutions were prepared fresh every day. Flx was dissolved in tap water at concentration of 0.08 or 0.016 mg/ml to achieve approximately 10-20 mg/kg per day dosing unless otherwise stated. The treatment was continued through all behavioral sessions until sacrifice (study I) or continued until the final day of experiments (study III).

Isoflurane (study IV)

Isoflurane (Vetflurane, Virbac) treatment was induced in a chamber with 4% isoflurane for 2 minutes, after which the mouse freely inhaled isoflurane via a mask (3.0 % for 1 min, then 2 % for maximum 30 minutes; airflow: 0.3-0.5 l/min). Body temperature was maintained by a heat pad throughout the treatment. Sham mice were kept in the induction chamber for 2 minutes without isoflurane.

3.3 BEHAVIOR

Marble burying (study III)

On day 20-21 of Flx or vehicle treatment mice were subjected to the marble burying test adapted from K. Njung'E and S. Handley (1991). Animals were placed individually into test cages (21×38×14cm) with 5 cm height of bedding. Twelve small marbles (15 mm diameter) were arranged on bedding in the form of an array. Mice were then exposed to marbles individually for 30 min and unburied marbles were counted. A marble was considered to be 'buried' if it was covered with bedding material more than 67% (i.e. two-third size). Behavior was then rated by counting the number of marbles buried and data was presented as % of buried marbles to control (before treatment) level.

Fear conditioning and extinction (study I, II)

Fear conditioning took place in context A (a transparent Plexiglas chamber with metal grids that was cleaned before each session with 70% ethanol). Freezing behavior was measured with an automatic infrared beam detection system which was placed on the sides of the fear conditioning chamber (TSE Systems GmbH, Germany). The mouse was considered to be frozen only if it was not moving for at least 3s, and this measure was expressed as percentage of time spent freezing. Every mouse was handled in the experimental room for 5–10 min during each of the 3 days prior to fear conditioning. On the day of acquisition, mice were exposed to context A for 2 min and conditioned using 5 pairings of the CS (Conditioned Stimulus; total duration 30s, 1Hz, white noise, 80dB) with the US (Unconditioned Stimulus; 1sf foot-shock 0.6mA, inter-trial interval: 20–120 s). The US was co-terminated with the CS. The freezing level during the first CS, preceding the first US, was taken as the baseline freezing during CS. Mice were then divided into four groups (two extinction (water and Flx drinking) and two no-extinction (water and Flx drinking) groups) with equal levels of freezing, two receiving Flx in their

drinking water until the end of the experiment and the other two receiving tap water. For the control group CTRL, mice were subjected to the same fear conditioning experimental protocol except that the CS was not followed by the US (non-conditioned, only context+ CS exposure group); then, the CTRL mice received tap water. Two weeks after the fear conditioning day, the mice from the CTRL and both no-extinction groups (n=6 per group) were sacrificed for subsequent immunohistochemical analysis. Fear extinction training, spontaneous recovery and fear renewal Two-day fear extinction training took place 2 weeks after fear conditioning in the context B (a black non-transparent Plexiglas chamber with a planar floor that was leaned before each session with 70% 2-propanol). Freezing behavior was measured as described above. On the first and second extinction days, conditioned mice received 12 presentations of the CS (total duration 30s, 1 Hz, white noise, 80dB, inter-trial interval: 20–60 s). One week after extinction, extinguished mice (n=6 per group) were sacrificed for subsequent immunohistochemical analysis. In parallel, additional mice (n=25 per each extinction group) were tested 7 days after extinction in context B and context A, respectively, using 4 presentations of the CS (inter-trial interval: 20–60 s) and were further used for Pearson's correlation analysis of context-dependent spontaneous recovery and fear renewal.

3.4 LENTIVIRUS PRODUCTION (STUDY I)

Time-specific BDNF overexpression in the basolateral amygdala was achieved using injection of lentivirus regulatable by doxycycline Tet-off system (33). To produce viral particles, the vector plasmid pTK431-BDNF, the packaging plasmid pΔNR and the envelope plasmid pMDG-VSV-G (ratio 4:3:1) were cotransfected into HEK293T cells as described previously (33). The viral particles were collected by ultracentrifugation and resuspended in MEM.

Virus titer was determined using p24 antigen ELISA as 0.21 mg/ml of p24 and viral solution was kept at -80°C in small aliquots.

3.5 STEREOTACTIC INJECTIONS (STUDY I)

Pilot experiments were performed to determine the stereotaxic coordinates of the basolateral amygdala: bregma -1.7, lateral ±3.6 and ventral -4.0 according to the Allen atlas (http://www.brain-map.org/). Mice were anesthetized with isoflurane and placed in a stereotaxic frame. Bilateral injection into the basolateral amygdala was performed using a 10 µl syringe with a stainless steel needle. On each brain side, 500 nl of the virus were infused at a speed of 3 nl/s. The needle was kept in place for 8 minutes after the infusion to improve the penetration of the viral solution into the tissue. As a control for the infection, additional mice were injected with the viral diluent solution (Sham) using the same protocol. The analgesic carprofen (5 mg/kg) was administered subcutaneously. After the surgery, mice were returned to their home cages and left to recover for 2 weeks.

3.6 WESTERN BLOTTING (STUDY III)

Following electrophysiological experiments hippocampal slices were homogenized in NP buffer (137mM NaCl, 20mM Tris, 1% NP-40, 10% glycerol, 48mM NaF, H2O, complete inhibitor mix (Roche), 2mM Na_3VO_4. After at least 15-minute incubation on ice, samples were centrifuged (16000g, 15 min, +4°C) and supernatant collected for further analysis. Protein concentrations were measured using Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Samples (25 µg protein) were separated with NuPAGe 4-12% Bis-Tris gel (Novex, life technologies, USA) and blotted to a polyvinylidene fluoride membrane (300mA, 1 hour, 4°C). Membranes were incubated with the following primary antibodies (Table 1): Synaptophysin (Sigma, USA, 1/200), Synaptotagmin (Sigma, USA, 1/1000), phospho-Synaptotagmin (Sigma, USA, 1/1000), CaMKII (Millipore, USA, 1/1000),

phospho-CaMKII (Millipore, USA, 1/1000), Syntaxin 1A (Cell Signaling, USA, 1/1000), CREB (Cell Signaling, USA, 1/1000), MUNC18 (Cell Signaling, USA, 1/1000) diluted in 5% BSA on TBS/0.1% Tween (TBST). Further, the membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibodies (1:10000 in non-fat dry milk, 1 hour at room temperature; Bio-Rad). After subsequent washes, secondary antibodies were visualized using enhanced chemiluminescence (ECL Plus, ThermoScientific, Vantaa, Finland) for detection by Fuji LAS-3000 camera (Tamro Medlabs, Vantaa, Finland).

Table 1. Summary of all proteins analyzed in study III.

Full name	Short name	Function related to LTP
Synaptophysin	SYP	vesicular membrane protein (Mullany & Lynch 1998)
Synaptotagmin 1	Sptg1	Ca^{2+}-sensor for synaptic vesicle exocytosis (Ahmad et al. 2012)
MUNC 18	MUNC 18	precede and/or regulate the formation of vesicles priming (Barclay 2008; Jurado et al. 2013)
Syntaxin 1	Stx1	membrane component of SNARE complex (Mishima et al. 2014; Davis et al. 2000)
Ca2+/calmodulin-dependent protein kinase II	CaMKII	protein kinase, initiates LTP-dependent Ca^{2+} cascade (Lisman 1994)
cAMP response element-binding protein	CREB	transcription factor, control memory consolidation and late LTP phase (Kida 2012)

3.7 IMUNOHISTOCHEMISTRY (STUDY I, II)

Immunostaining was performed using free-floating brain sections. After washing with PBS to remove the cryoprotective solution, the sections were incubated in a blocking reagent consisting of 5% goat serum (Vector Laboratories, UK), 3% bovine serum albumin (Sigma-Aldrich, Finland) and 0.4% Triton X-100 (Sigma-Aldrich, Finland) in PBS to prevent nonspecific binding of antibodies. Before blocking, an antigen retrieval step including an incubation in 0.1% pepsin (Sigma-Aldrich, Finland) in 5 mM HCl for 10 min at room temperature, was performed to increase the binding of the primary antibodies for $GABA_AR\alpha1$, $GABA_AR\alpha2$ and VGLUT1. Sections were incubated with one of the primary antibodies (Table 2) in PBS containing 0.4% Triton X-100 (PBST) overnight at +4 °C. Then, sections were washed in PBST and incubated with the appropriate secondary antibodies (Molecular probes, Invitrogen, Espoo, Finland) for 1 h at room temperature. Finally, sections were mounted on slides and covered with Prolong®Gold anti-fade reagent with DAPI (Molecular Probes, Invitrogen, Espoo, Finland).

Table 2. Summary of all proteins analyzed in study II.

Protein	Short name	Function
Synaptophysin	SYP	vesicular membrane protein
GABA Transporter 1	Gat1	GABA plasma membrane transporter (Heldt & Ressler 2007)
Glutamate receptor 1	GluA1	$Ca2^{+}$-permeable AMPA receptor subunit (Clem & Huganir 2010)
Glutamate receptor	GluA2	$Ca2^{+}$-impermeable AMPA receptor subunit (Kim et al. 2007)

Vesicular Glutamate Transporter 1	VGLUT1	vesicle-bound, sodium- phosphate dependent glutamate transporter (Wojcik et al. 2004)
Vesicular GABA and glycine transporter	VGAT	sodium- and chloride-dependent GABA transporter (Schoenfeld et al. 2013)
GABA A Receptor alpha 1	$GABA_AR\alpha1$	subunit of GAB_AR which agonist produce sedative effect (B Luscher et al. 2011)
GABA A Receptor alpha 2	$GABA_AR\alpha2$	subunit of GAB_AR which agonist produce anxiolytic effect (Bernhard Luscher et al. 2011)
NMDA receptor 2A	GluN2A	subunit of NMDARs abundantly expressed in adult brain (Walker et al. 2002)
Postsynaptic density protein 95	PSD95	major scaffolding protein of the excitatory post-synaptic density (Fitzgerald et al. 2015)

3.8 ELECTROPHYSIOLOGY (STUDY III, IV)

On the last day (21) of the Flx treatment or 24h after isoflurane exposurev mice were anaesthetized with pentobarbital (50mg/kg), decapitated and sagittal slices were cut from the hippocampi as described previously (Bortolotto et al. 1999). The slices were allowed to recover for 1-4 hours before the recordings were started. All recordings were done in an interface-type chamber (+32*C*°) which was constantly perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, NaH_2PO_4 1,25, $MgSO_4$ 4, $NaHCO_3$ 26, D-glucose 15, $CaCl_2$ 2, and gassed with 5% CO_2/95% O_2. Field excitatory postsynaptic potentials (fEPSP) were evoked with bipolar stimulating electrode placed within the Schaffer collateral pathway and the responses were recorded from CA1 stratum radiatum using ACSF-filled glass microelectrodes (2–5 MΩ). For baseline recordings square pulse (0.05

Hz/0.1) ms stimulation protocol was used. Input/output (I/O) curves were constructed using gradually increased stimulation intensities until the fEPSP reached plateau or visible population spike was seen. After I/O data were collected, the stimulus intensity was adjusted to evoke half-maximal (40-60%) fEPSP response. Long-term potentiation (LTP) was induced 10-15 minutes after baseline recording by 100Hz/1s tetanic stimulation. Post-induction responses were normalized to the final 10 min of the baseline recordings. The level of LTP was measured as a percentage increase of the fEPSP slope, averaged at a 1-min interval 50-60 min after the tetanus, and compared to the averaged baseline fEPSP slope recorded before tetanus. To examine short term plasticity we performed paired-pulse- (PPF) and frequency facilitation (FF) experiments. In PPF experiments interpulse intervals of 20, 60, 100, 150 and 200 ms were used. In FF experiments 100 pulses with either 1 or 100 Hz were applied.

3.9 DATA ACQUISITION AND STATISTICAL ANALYSIS

Behavioral study

Statistical analyses of the behavioral tests were performed using repeated-measures ANOVA followed by Student’s paired or unpaired two tailed t-test. For the post-hoc matching analysis, the subjects with exactly matching the freezing levels at the “Acquisition” time point in control and Flx groups were selected. The bivariate Pearson's correlation and linear regression analyses were performed using Origin (OriginLab, Northampton, MA). A P-value < 0.05 was considered statistically significant.

Immunohistological study

Quantitative evaluation of immunostainings was performed by an investigator blind to the treatment groups; all slides were coded until the analyses were

finished. The images were obtained by the Imager M.1 fluorescent microscope (Zeiss, Germany) using AxioVision software. A minimum of 5 sections per brain area/per animal, as well as control sections "No primary antibody", were imaged using the same microscope and camera settings for all slides within each immunostaining experiment. Image processing was performed with ImageJ software. To estimate the difference in expression of proteins, brain regions were delineated and mean optical densities were measured. The mean optical densities of the controls "No primary antibody" were subtracted from obtained values for every brain area in all immunostaining experiments. All the values present as mean ± SEM and as percentage of control. Statistical analyses of protein levels were performed in Origin (OriginLab, Northampton, MA) using a two-way ANOVA with a post hoc Fisher's PLSD test. A p-value<0.05 was considered statistically significant.

Electrophysiological study

WinLTP (www.winltp.com) program was used for electrophysiological data acquisition and analysis. All the data are expressed as mean ± SEM and as percentage of control. For statistical analysis of I/O, PPF, LTP and FF two-way ANOVA for repeated measures were implemented. All statistical analyses were done using Origin (OriginLab, Northampton, MA). A p-value<0.05 was considered statistically significant.

Molecular biological study

Immunoblot bands were quantified using NIH ImageJ. All the data are expressed as mean ± SEM and as percentage of control. Statistical analyses were performed using Origin (OriginLab, Northampton, MA). For comparison between groups two-way ANOVA was used. The criterion for significance was set to p<0.05.

4. RESULTS

4.1 1PLASTICITY IN PATHOLOGY (STUDY I, II)

In order to study how plasticity induced by Flx account for successful recovery from fearful memories, we subjected mice to fear conditioning and then applied chronic Flx treatment. We analyzed how Flx administration influenced fear erasure and affected spontaneous fear renewal and recovery.

After successful fear acquisition, the mice were given either Flx or water for two weeks. Thereafter, both groups were subjected to extinction training and seven days later, the mice were tested for spontaneous recovery and fear renewal (study I, fig 2). This paradigm was utilized to explore whether fear reduction was permanent. Although fear extinction was seen in both control and Flx-treated animals, Flx-treated mice showed significantly faster extinction. However, whilst the control mice showed clear fear renewal and a tendency to spontaneous recovery, the Flx-treated mice showed no signs of renewal or spontaneous recovery. Moreover, mice not exposed to extinction training showed enhanced freezing regardless of their treatment group. Next, we examined the effect of Flx on fear reinstatement (study I, fig. 2). After successful extinction in the fear-conditioning context, mice were exposed to a foot shock five times without a CS and tested for freezing after a tone 24 hours later. Control mice showed a robust fear reinstatement whereas freezing in mice receiving Flx was significantly reduced. Our results highlight a previously undescribed principle of AD-treatment whereby long-term loss of fearful memories can be induced only by combined chronic Flx administration and extinction training.

In addition to the behavior study, we investigated whether Flx, extinction or their combination produced long-lasting changes in the expression of synaptic proteins in the well-studied fear networks: amygdala, hippocampus and mPFC (study II) (Quirk et al. 2010). We found extinction dependent and independent changes in the expression profile of pre- and postsynaptic proteins involved in glutamatergic and GABAergic synaptic transmission (study II, table I).

We found that fear conditioning significantly downregulated VGLUT1 and $GABA_A R\alpha 1$ expression induced by fear conditioning in the hippocampus and mPFC, and that chronic Flx-treatment accentuated these effects (study II, fig. 3). However, if combined with extinction training, Flx enhanced the expression of SYP in all investigated brain areas (study II, fig. 3). Concomitantly, the expression of VGLUT1, PSD95, GluN2A and GluA2 was increased in the amygdala and hippocampus (study II, fig. 3). Moreover, Flx increased the expression of both investigated $GABA_A Rs$ subunits in the mPFC and amygdala (study II, fig. 3). Thus, we demonstrated that combination of Flx and extinction treatments form a specific synaptic landscape permissive for long-term fear extinction facilitation and fear erasure in adult mice.

4.1.1 FEAR ERASURE DEPENDS ON BDNF IN AMYGDALA (STUDY I)

Chronic Flx treatment alone increased BDNF expression in many brain areas and after fear conditioning significantly increased BDNF mRNA level in the basolateral amygdala (BLA) (study I, fig 4A). To test whether overexpression of BDNF in the basolateral amygdala mimics Flx exposure, we used doxycycline regulated lentiviral infection to overexpress BDNF locally in the BLA from the end of extinction onward (study I, figs. S8 and S9). We showed that BDNF-overexpressing mice did not show fear renewal, induced through conditioned CS presentations in extinction and conditioning contexts,

whereas control mice showed robust freezing behavior following fear renewal (study I, fig. 4C). Thus, successful fear erasure depends on BDNF expression in amygdala.

4.2 IPLASTICITY IN HEALTH (STUDY I, III, IV)

4.2.1 FLX INDUCES IPLASTICITY IN NAÏVE MICE (STUDY I, III)

In order to investigate the mechanism of Flx induced neuronal network tuning, we performed experiments on naïve/healthy mice. In study I, in addition to the clinically relevant administration after exposure to fear, we tested an alternative paradigm of chronic Flx administration which precedes fear induction. Interestingly, we found that pre-treatment with Flx, as observed when Flx was applied after exposure to fear, did result in faster fear extinction (study I, fig. 2). In order to examine behavior sensitivity to chronic Flx in naïve mice, we tested mice digging activities using the marble burying test (study III). It was previously shown that mice digging behavior is sensitive to a variety of treatments, including anxiolytic drugs and serotonin-active compounds (Deacon 2006). We found that mice subjected to FLX administration exhibited significantly less burying activities compared to control animals (fig. 3). These results suggest that Flx not only promote recovery after pathological cues but apparently predisposes neuronal networks to cope with forthcoming pathological events.

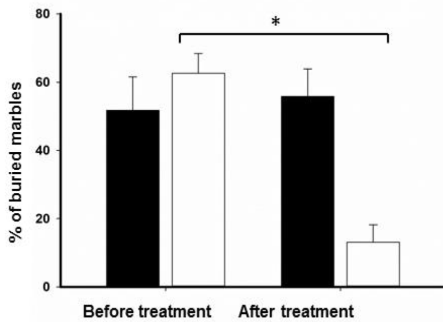


Figure 3. Fluoxetine treatment reduced marble burying behavior. Black bars-water treated animals, white bars-Flx treated animals

To evaluate the effect of Flx on naïve mice, we investigated the expression of histological neuroplasticity markers in hippocampal CA1 area, BLA, prelimbic and infralimbic mPFC (PL, IL). We found that chronic Flx did not change the absolute numbers of PNN-positive neurons in the BLA, hippocampal CA1 area, and IL (study I, fig. 3A, B and table S1). However, Flx treatment reduced the percentage of PNN neurons expressing parvalbumin in both the BLA and CA1 area of hippocampus (study I, fig. 3A, B), whereas no differences were found in PNN-positive interneurons containing calbindin or calretinin (study I, fig. S5 and table S1). Expression of PSA-NCAM, which is expressed in immature cortical cells and reduced with maturation, was increased by Flx treatment in the BLA (study I, fig. 3C). Concomitantly, Flx treatment reduced the expression of the K-Cl cotransporter KCC2 (study I, fig. 3D), which increases during postnatal development. All together, these data suggest that Flx induces plasticity in naïve mice, which can contribute to/explain the robust Flx effects on neuronal network reinstatement under pathological conditions (study I, II).

4.2.2 FLX FACILITATES SYNAPTIC PLASTICITY (STUDY III)

The results from studies I and II strongly suggested that Flx may affect the synaptic machinery involved in use-dependent synaptic plasticity in limbic areas. Therefore, we next studied the effects of Flx on neuronal transmission and plasticity at glutamatergic synapses in the area CA1 of the hippocampus.

Here, fEPSPs, evoked by Schaffer collateral stimulation, were recorded in the CA1 area of hippocampus. We found that chronic Flx treatment shifted the input-output curve to the left (study III, fig. 1A) thus implicating enhanced basal glutamatergic transmission. Moreover, LTP induced by tetanic stimulation (100st/1sec) was more prominent in hippocampal slices from Flx-treated mice (study III, fig. 2A). In parallel, PPF at 20ms and 50ms intervals as well as frequency facilitation (FF) by 1Hz/100 pulse and 100Hz/100 pulse stimulation protocols were both increased after Flx-treatment (study III, fig. 3, B and fig. 2.D).

We next investigated whether changes in the expression of synaptic proteins paralleled changes in synaptic function. To determine the molecular basis of activity dependent alterations in synaptic plasticity induced by Flx, we measured the expression levels of proteins related to vesicular trafficking and release and important mediators of LTP. Expression of investigated proteins was estimated with Western blotting in hippocampal slices where LTP was induced and maintained for 60 min; as a control, slices with 0.05Hz stimulation maintained under the same conditions were used.

We demonstrated that phosphorylation of CaMKII was enhanced in vehicle and Flx treated animals after LTP induction (study III, fig. 3A), while levels of CREB phosphorylation was increased by Flx treatment in control slices and after LTP in vehicle treated animals (study III, fig. 3B). Moreover, Flx accentuated expression of SYP, Sptg1, Stx 1 and MUNC 18 and also enhanced expression of SYP and Sptg1 in an activity-dependent manner (only after LTP) (study III, fig. 5). Thus, Flx administration predisposes hippocampal networks to activity-dependent plasticity, which is associated with accentuated presynaptic function and enhanced expression level of proteins related to vesicles trafficking and release.

4.2.3 ISOFLURANE IPLASTICITY (STUDY IV)

We found that brief isoflurane anesthesia induced rapid antidepressant-like effects: increased TrkB phosphorylation in the mouse mPFC, hippocampus and somatosensory cortex (study IV, fig. 1A-B, supplementary fig. 1-4). Isoflurane also rapidly activated the downstream signaling cascade of TrkB: induced the phosphorylation of CREB, Akt, mTOR and its downstream kinase p70S6K in the mouse mPFC (study IV, fig. 1C-F; supplementary fig. 5). Phosphorylation of CREB and p70S6K, but not Akt or mTOR, was also observed in the hippocampus (study IV, supplementary fig. 1). However, isoflurane had no rapid effects on BDNF mRNA or protein levels (study IV, supplementary fig. 6). Our molecular findings concerning isoflurane-induced neuronal plasticity was associated with changes in behavior. Thus, mice anesthetized with isoflurane for 30 min and tested 15 minutes later showed reduced immobility and increased latency to immobility (study IV, fig. 2A; supplementary fig. 7A) in the FST. Strikingly, when the isoflurane-treated mice were retested two weeks later, an antidepressant-like phenotype was still observed (study IV, fig. 2B; supplementary fig. 7B).

We also demonstrated that the rapid antidepressant actions of isoflurane were associated with changes in synaptic plasticity. Tetanic stimulation (100Hz/1s) of the Schaffer collateral produced significantly higher levels of long-term potentiation of fEPSPs in slices prepared from mice treated with isoflurane 24 hours before experiments than in the control slices (study IV, fig. 3A). Recordings of the input–output relationship showed that isoflurane exposure 24 hours before accentuated basal synaptic transmission in the hippocampus (study IV, fig. 3B). PPF was not affected by isoflurane indicating unaltered presynaptic function. Thus, our results demonstrate that single brief isoflurane anesthesia results in iPlasticity-type tuning of neuronal networks

which, in turn, may explain the antidepressant-like action and efficacy of isoflurane.

5. DISCUSSION

The aim of this thesis was at advance our understanding of the mechanisms associated with iPlasticity. Employing a wide range of techniques, we attempted to demonstrate how different pharmacological agents enhance synaptic plasticity and lead to behavior improvements in normal and pathological states in mice.

5.1 FLX AS AN ANTIDEPRESSANT

The clinical efficacy of Flx as antidepressant was demonstrated in many studies (Gaynes et al. 2009; Fournier et al. 2010). Initially, understanding of the underlying mechanism of action was associated with the reinstatement of monoamine deficit induced by pathological conditions. However, he plasticity hypothesis currently dominates explanations of the mode of Flx action.

Extinction training during a critical period in juvenile mice leads to permanent fear erasure (Gogolla et al. 2009; Kim & Richardson 2010). Since the antidepressant Flx induced plasticity in the visual cortex (Maya Vetencourt et al. 2008), we attempted to investigate whether reactivation of iPlasticity by Flx will lead to long term fear erasure if treatment alone is introduced or in combination with extinction treatment. We showed that chronic Flx treatment, given three weeks before or after exposure to FC, did cause faster extinction. Moreover, we demonstrated that these changes were permanent, because recovery, renewal and reinstatement of fear were attenuated in mice treated with Flx. In a pre-clinical investigation in mice by I. Branchi and colleagues, Flx administration, given in the enriched environment after exposure to chronic unpredictable stress (CUS), increased

saccharin preference, BDNF levels and decreased corticosterone which would account for the reduction of stress-induced symptoms observed. However, if Flx is given in a stressful environment, it lead to worse behavior outcomes (Branchi et al. 2013). Together with our findings, it is evident that the effect of Flx is not determined by drug per se but induced by the drug and driven by environment.

In parallel with the behavioral assessments of fear-conditioned mice, we demonstrated that the combination of Flx and extinction treatments formed a specific synaptic landscape permissive for long-term fear extinction facilitation and fear erasure in adult mice. We found that stress induced by fear conditioning had a long-lasting inhibitory effect on the expression of proteins related to excitatory transmissions: SYP and GluA2 in the hippocampal CA1 pyramidal layer and VGLUT1 in the whole hippocampus of both water-and Flx-treated animals. Previously it was demonstrated that exercise induces a switch from GluA2-containing to GluA2-lacking AMPA receptors (Park et al. 2014; Middei et al. 2014; Gan et al. 2015), which are commonly referred to as calcium-permeable (CP-AMPA receptors). Thus, transient CP-AMPAR expression may provide an important Ca^{2+} entry mechanism during synapse maturation and impart distinct short/long-term plastic properties to neuronal networks (Rozov & Burnashev 1999; Liu & Cull-Candy 2000).

We demonstrated that fear conditioning affected GABAergic transmission: reduced VGAT and sedation-associated $GABA_AR$ α1 expression in water drinking mice and down-regulated expression of anxiolytic $GABA_AR$ α2 in Flx treated animals. These data are very much in line with the finding where reduced glutamate function, due to VGLUT1 deficiency (Wojcik et al. 2004) or decreased GABA-receptor function was specifically implicated in anxiety and

fear-related disorders and depression- like behaviors (Tordera et al. 2005; Luscher et al. 2011).

Surprisingly, we could not detect robust changes in the expression profile of investigated proteins if Flx treatment alone was introduced after fear conditioning, whereas extinction affected the levels of synaptic protein expression much stronger then Flx treatment alone. Whilst Flx treatment, given after FC downregulated expression of $GABA_AR\alpha2$ in PFC and further reduced expression of VGLUT1 in the hippocampus, extinction alone enhanced expression of PSD 95 in BLA, VGAT in all investigated brain areas, GAT and $GABA_AR\alpha1$ in PFC and downregulated the expression of $GABA_AR\alpha2$ and GluA1:GluA2 ratio in amygdala.

We hypothesize that Flx iPlasticity promotes the effects of behavioral experience, such as extinction training. This, in turn, helps to reshape maladapted networks to better adjust to the environment. Therefore, our results provide a putative neurobiological basis for the enhanced effect of combining drug and psychological treatments and support the hypothesis that the chemical effect produced by administering antidepressants alone will not give full clinical benefit (Moncrieff & Kirsch 2005; Turner et al. 2008; Kirsch et al. 2008). Instead, drug treatments need to be combined with psychotherapy or other kinds of rehabilitation to optimize their mood-elevating effects.

In contrast, we found that combination of chronic Flx with extinction therapy resulted in a unique long-lasting remodeling of synaptic proteins expression. The synergic effect of Flx and extinction treatment was associated with enhanced GluN2A expression which is in agreement with a previous study, where D-cycloserine, an NMDA-receptor partial agonist, administration facilitated GluN2A expression and lead to successful fear erasure after FC

(Walker et al. 2002; Lin et al. 2010). Another important finding, associated with the formation of unique synaptic protein profiles, is accentuated expression of proteins related to glutamatergic transmission (VGLUT1, PSD95, GluN2A, and GluA1) and a mild change in inhibitory component of synaptic transmission, when compared to the effect of extinction alone. To address this question we further tested influence of Flx action on neuronal networks in naïve mode.

5.2 MECHANISMS OF FLX IPLASTICITY

5.2.1 FLX AND STRUCTURAL PLASTICITY

To understand the properties of Flx iPlasticity we investigated the effect of Flx administration on structural plasticity in the healthy state. Different molecules may mediate structural plasticity of neurons, particularly those involved in cell adhesion. Polysialated neuronal cell-adhesion molecule (PSA-NCAM) is expressed in immature cortical cells and reduced with maturation. PSA-NCAM-expressing neurons receive less synaptic contacts than those lacking this molecule and have reduced dendritic arborization and spine density (Nacher et al. 2002; Guirado et al. 2014), suggesting that PSA-NCAM plays a role in the developmental regulation of neuronal structure and function. In our study we observed that Flx administration increased PSA-NCAM expression in the basolateral amygdala (Fig. 3C, study I) In line with this findings, we demonstrated that Flx treatment reduced the expression of the K-Cl cotransporter KCC2 (Fig. 3D, study I), which is a key molecule responsible for the developmental switch from $GABA_A$ receptor -mediated depolarising to hyperpolarising action and whose expression dramatically increases with maturation of inhibitory systems (Rivera et al. 1999). Previously, it was demonstrated that disruption of PNNs with chondroitinease

ABC in the adult basolateral amygdala before, but not after, fear conditioning leads to fear erasure after extinction (Gogolla et al. 2009). In our study we assessed whether Flx treatment might disrupt PNNs and thereby facilitate extinction. Control and Flx-treated mice had similar numbers of PNN-positive neurons in all investigated brain areas. However, Flx treatment reduced the percentage of PNNs neurons expressing PV in the BLA and CA1 area of hippocampus. Previously, it was proposed that the developmental increase in PNNs may limit PV cells plasticity to allow these cells to strongly coordinate neuronal network through intense firing (Roux & Buzsáki 2015). These data suggest that Flx treatment selectively shifts the parvalbumin- and PNNs-containing neurons toward an immature state. Taken together we conclude that Flx iPlasticity induced a structural reorganisation of investigated neuronal networks which may form the basis for behavior improvements.

5.2.2 FLX AND SYNAPTIC PLASTICITY

Long-term plasticity can be bi-directionally modified by various cues. For example, enhanced LTP is observed after exposure to enriched environment and is associated with improved learning and memory. Other exposures, like chronic unpredictable stress, in turn suppress or block long term potentiation (Alfarez et al. 2003). In spite of having only two possible directions of change, the basis of such LTP regulation could vary greatly. We demonstrated in our study that chronic antidepressant Flx administration enhances basal synaptic transmission and long term plasticity in hippocampal CA3-CA1 synapses. Moreover we showed that LTP changes were accompanied with alterations in short term synaptic plasticity and dynamics in CA1 hippocampal synapses. To investigate short term synaptic plasticity, we measured paired pulse facilitation and frequency facilitation, which are frequently used to assess changes in underlying. We found that Flx alter P_r and frequency facilitation.

Flx action on presynaptic function was previously reported. Thus it was demonstrated that Flx restores depolarization-evoked glutamate release in hippocampus in animals exposed to prenatal stress (Marrocco et al. 2014), and increases the frequency of spontaneous EPSCs in the locus coeruleus. We hypothesize that altered by Flx P_r might give the synapses considerable leeway so the property of synaptic transmission can be carefully regulated. All together, these findings demonstrate that Flx enhance synaptic plasticity and one of the possible sources of such modulations could be altered presynaptic function.

We reported in our study that NMDA-dependent LTP (Kauer et al. 1988; Malenka et al. 1989) was enhanced after Flx in CA3-CA1 hippocampal synapses. However, there is considerable controversy regarding the effects of Flx on long-term synaptic plasticity. (Stewart & Reid 2000; Kobayashi et al. 2010; Rubio et al. 2013). The studies by Stewart and Reid, and Rubio and co-authors demonstrated that chronic Flx down-regulated long term synaptic plasticity in perforant path – DG synapses and CA3-CA1 hippocampal synapses, respectively. In the above experiments almost 20-fold lower dose of Flx treatment was employed, whereas in our study clinically relevant 20mg/kg/day dose of Flx treatment was used in order to reach same level of plasma Flx level as patients taking 20–80 mg Flx (Prozac) per day (Dulawa et al. 2004). In line with our data, several studies (Wang et al. 2008; Sale et al. 2007; Maya Vetencourt et al. 2008; Karpova et al. 2011) show that long term Flx administration enhances LTP in cerebral cortex, hippocampal perforant path and BLA and these findings were accompanied by altered maturation and survival of immature neurons in DG and enhanced expression of several plasticity-related markers.

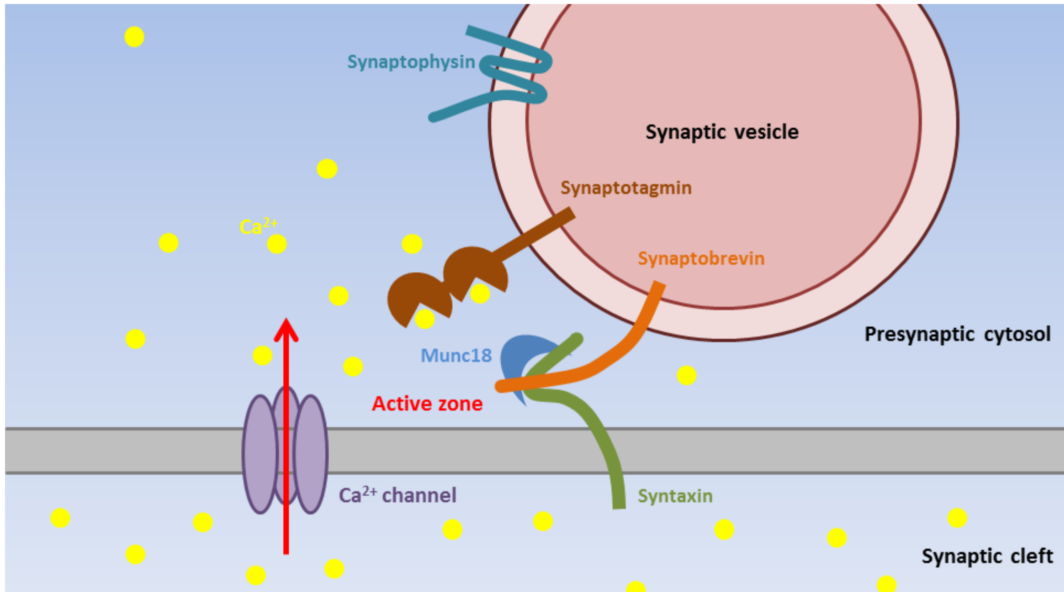


Figure 4. *Molecular protein complex that organize the secretory machinery at the presynaptic active zone.*

To investigate possible mechanism underlying altered synaptic plasticity, we measured expression of key downstream signaling molecules related to LTP: CaMKII/pCaMKII and CREB/pCREB and proteins associated with vesicular trafficking and release: synaptophysin, synaptotagmin 1, MUNC18 and Syntaxin1 (Fig.4). We also probed whether its expression depends on network activity by performing western blotting on slices from vehicle (water) and Flx treated animals where LTP was induced and maintained for 60min, (with control slices subjected to basal (0.05 Hz) stimulation only). CaMKII and CREB are involved in synaptic plasticity and have been previously shown to be targets of antidepressants (Celano et al. 2003; Tiraboschi et al. 2004; Bonanno et al. 2005; Barbiero et al. 2007). We found no difference in the expression of total CaMKII and CREB. Even though phosphorylation of both proteins was enhanced by LTP, the levels of phosphorylation were

unexpectedly lower in Flx treated animals than in vehicle treated ones. We conclude that synaptic plasticity enhanced by Flx is not associated with enhanced expression of downstream signaling molecules responsible for LTP. There are two possible explanations for these results, Flx: 1) recruits other downstream signaling proteins or 2) modulates synaptic plasticity in alternative way. Since our electrophysiological experiments identified changes in presynaptic function by Flx treatment, we probed a second hypothesis and measured expression of proteins related to vesicular trafficking and release. We choose synaptophysin, synaptotagmin, MUNC 18 and synatxin 1 because their roles in distinct stages of vesicles priming and fusion with the plasma membrane (fig. 4, table 2). SYP marks most of the vesicles in the brain. Synaptotagmin 1 acts as a Ca^{2+}-sensor for synaptic vesicle exocytosis. MUNC 18 regulates vesicle priming and Syntaxin 1, as part of membrane SNARE complex, reflects the overall amount of active zones. Our experiments showed that levels of expression of all investigated proteins were significantly higher in Flx treated mice then in controls. Interestingly, enhanced expression of SYP and Sptg1 was activity dependent and was enhanced only on slices from Flx treated animals where LTP was induced.

Even though we cannot exclude other possible explanations to account for LTP enhancement, we have indirect evidence that suggests the changes observed in synaptic plasticity following chronic Flx administration: 1) are associated with accentuated presynaptic function 2) partially emerged in an activity-dependent manner. In study I we showed that only a combination of Flx treatment with extinction therapy (but not treatment alone) produces behavior outcomes. Moreover, in study II we demonstrated that Flx in combination with extinction treatment resulted in enhanced expression of SYP, VGLUT1, PSD 95 and GluN2A. Thus, we propose thatlong-term Flx administration might predispose hippocampal networks to activity-

dependent plasticity, which is associated with accentuated long term synaptic plasticity and altered presynaptic function.

It is not clear why Flx, with its pronounced acute effect on neuronal networks (Rantamäki et al. 2007; Sung et al. 2008; Kim et al. 2013; Jung et al. 2014), induces iPlasticity only following chronic administration and, thereby, offering potential therapeutic outcomes to a variety of mental disorders. It is well known that the CA1 area of hippocampus is highly innervated by serotonergic inputs. Direct stimulation of raphe nuclei or local serotonin application (Otmakhova et al. 2005) results in suppressed excitatory synaptic transmission in CA1 pyramidal cells. Flx applied acutely to slices suppresses synaptic transmission and blocks LTP induction (Shakesby et al. 2002). However, chronic Flx administration not only enhances basal synaptic transmission, short and long-term synaptic plasticity, but also stimulates maturation and synaptic plasticity of adult born hippocampal granule cells (Wang et al. 2008), alters specific inhibitory circuits of hippocampus (Méndez et al. 2012) and restores juvenile-like plasticity in the visual cortex and BLA (Maya Vetencourt et al. 2008; Karpova et al. 2011) . Such adaptive responses support the idea of homeostatic synaptic scaling (Turrigiano et al. 1998) induced by long term elevation through blockade of serotonin reuptake by Flx. Prolonged shunting of excitatory synaptic transmission may cause adaptive homeostatic responses aimed at balancing changed properties of synaptic transmission by inducing plasticity in the brain. So enhanced long and short term synaptic plasticity induced by Flx may be nothing but adaptive tuning of the hippocampal network oriented to the reinstatement of inhibitory/excitatory balance after long-term serotonin augmentation and the so-called plasticity changes that we observe appear to be a consequence of an underlying homeostatic regulation induced by chronic Flx administration.

5.3 Isoflurane iPlasticity

In study IV, we assessed the role of another frequently used pharmacological compound, the volatile anesthetic Isoflurane, in iPlasticity. We found that brief isoflurane anesthesia induced a rapid and transient phosphorylation of TrkB and activation of CREB, Act and mTOR in different brain areas that have been linked to the antidepressant-like behavior in rodents. To elucidate the mechanisms underlying the rapid antidepressant actions of isoflurane, we examined synaptic plasticity and found that a single isoflurane administration, 24 hours before electrophysiological recordings, enhanced LTP and accentuated basal synaptic transmission in the CA3-CA1 hippocampal synapses (Fig. 3B, study 4). PPF was not affected by isoflurane indicating unaltered P_r and presynaptic function (Supplementary Fig. 10, study 4). These effects of isoflurane on synaptic strength resemble those of the antidepressant Flx: 1) antidepressant effects on behavior, 2) dependent on TrkB signalling, 3) enhanced basal synaptic transmission and long term synaptic plasticity. But to achieve all the indicated changes, long term Flx treatment is needed, whereas for isoflurane induced plasticity only a single exposure is required. Therefore, isoflurane action on neuronal networks more closely resembles the action of another compound with fast-acting properties on the nervous system - ketamine. Together with ketamine (Autry et al. 2011), isoflurane induced iPlasticity following a single administration, activates mTOR and lead to synaptic plasticity. However there are concerns that ketamine's addictive and psychotomimetic properties will hinder it from reaching its potential to treat human depression, while use of isoflurane in clinics is promising. It was already reported previously, that isoflurane produce rapid antidepressant effects in treatment-resistant depressed patients (Langer et al. 1985). We believe that isoflurane, with its ability to

quickly induce synaptic plasticity and antidepressant-like action will become a good substitute for a) ketamine as it is devoid of its psychotomimetic side effects and b) Flx with its time consuming action. Moreover this opens new potentials for clinical research in the field of human neuropsychiatric disorders where application of fast-acting antidepressants, alone or in combination with psychotherapy, might emerge as a therapeutic strategy in the near future.

5.4 SPECIFIC VS COMMON FEATURES OF FLX AND ISOFLURANE iPLASTICITY

As demonstrated, the investigated compounds, Flx and isoflurane, affected many aspects of neuronal plasticity and both induced synaptic plasticity. We showed that chronic Flx and single isoflurane exposure activated TrkB receptor and enhanced LTP. Moreover based on our results, we conclude that these compounds resulted in iPlasticity and can be used as tools to study it. However, not all the features of Flx and isoflurane iPlasticity are similar. First of all, to achieve behavior benefits, long term Flx administration is required. However for isoflurane action, only a single brief exposure is needed. A large number of studies into the action of Flx on the nervous system allow us to speculate that plasticity induced by Flx may be associated with homeostatic tuning of neuronal networks oriented to reinstate the inhibitory/excitatory balance caused by long-term serotonin augmentation. The poor knowledge concerning the action of isoflurane on the nervous system and relatively young field of fast acting antidepressants provides little insight into its role in rapid plasticity stimulation. More diversity arises from the effect of investigated drugs on BDNF expression. It is very well known that long term administration of Flx increases levels of BDNF and some of its action on

neuronal networks are BDNF dependent. Isoflurane, in turn, does not change BDNF levels. Taking into account that both investigated chemical compounds induce TrkB phosphorylation, the key receptor of BDNF, our findings propose different plasticity mechanisms underlie iPlasticity. In accordance with this hypothesis, our results may be dependent on the difference in effect of Flx and isoflurane on GABA function. As previously reported, isoflurane and Flx exert persistent effects on GABAergic transmission (Harauzov et al. 2010; Méndez et al. 2012; Guirado et al. 2014; Zurek et al. 2014). In our experiments (unpublished, fig. 5) designed to explore the origin of LTP changes and probe whether synaptic plasticity induced by Flx and isoflurane depends on GABAergic function, we measured LTP in presence of the $GABA_AR$ antagonist, picrotoxin (PiX). Surprisingly, we observed that whilst Flx induced LTP enhancement remained unchanged, isoflurane induced alterations in LTP were diminished in CA1 area of hippocampus.

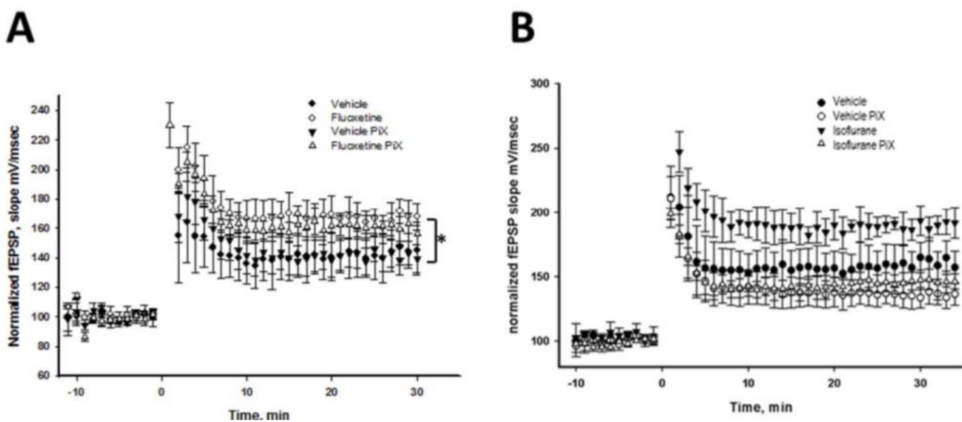


Figure 5. LTP induced by tetanic stimulation (100 Hz) in CA3-CA1 hippocampal synapses in presence of picrotoxin, study on Flx treated mice (**A**) and isoflurane treated mice (**B**).

6. CONCLUSIONS

In the present thesis, we described and analyzed a novel type of neuronal plasticity (iPlasticity), which, according to it features, resembles critical period plasticity but originates in the adult brain and required intense environmental or/and pharmacological guidance. To stimulate iPlasticity in our research we applied chronic treatment with antidepressant Flx and single brief exposure with volatile anesthetic isoflurane. We investigated the influence of this chemical compounds in pathology and health in mice.

1. Using parallel behavior and immunohistochemical approaches in fear conditioning paradigm, we demonstrated that for successful fear erasure, combination of chronic Flx administration with extinction treatment is required. Our findings resemble the principles of neuronal network tuning during critical periods, where establishing of proper network function is driven by environment. Thus we affirmed that Flx iPlasticity resembles critical period plasticity and manifested as juvenile-like plasticity or reopened critical periods.

2. We demonstrated that both investigated chemical compounds induced iPlasticity in naïve mice. Our immunohistochemical and molecular biological studies showed that Flx induced structural plasticity and stimulated BDNF-TrkB interaction and signaling, whereas isoflurane also stimulated TrkB and other plasticity related signaling but didn't influence BDNF expression. All findings were not restricted to certain brain region but were detected in different brain areas.

3. Enhanced synaptic plasticity was observed after Flx and isoflurane administration. Both drugs facilitated synaptic transmission and long term plasticity in CA3-CA1 hippocampal synapses, however differently affected

short term synaptic plasticity. Moreover in experiments with picrotoxin we detected GABA dependent (isoflurane) and independent (Flx) drugs action on synaptic plasticity.

In summary, in our studies we highlighted the principles and attempted to elucidate the mechanisms of iPlasticity. We demonstrated that iPlasticity induced by the investigated compounds underlie successful recovery from pathological cues and, in naïve animals, results in structural and functional reorganizations of neuronal networks suggesting that iPlasticity predisposes neurons to activity dependent changes. We also demonstrated that features of iPlasticity induced by Flx and isoflurane were not identical. Since both drugs are of particular use in humans, we expect our results will promote development and improve clinical strategies and practice in the use of these drugs.

7. ACKNOWLEDGEMENTS

This work was carried out at the Neuroscience center and Faculty of Veterinary Medicine University of Helsinki. Financial support by Center for International Mobility (CIMO), graduate school Brain and Mind and Sigrid Juselius Foundation is warmly acknowledged.

I would like to sincerely thank my supervisors Prof. Eero Castren and Prof. Tomi Taira for trust, enthusiasm, infinite patience and support. I am grateful to Nina Karpova who taught me innumerable lessons and insights into science and academic research. I thank Vernon Clarke for his expertise and unselfish assistance in writing and commenting of this thesis.

Thanks for reviewers of the thesis Iiris Hovatta and Petri Ala-Laurila for expert criticism and constructive and encouraging comment on this work. I acknowledge my thesis committee, Heikki Tanila and Petri Ala-Laurila, for professional help and practical comments during the course of my Ph.D.

My gratitude goes to Katri Wegelius, incredible coordinator of B&M graduate program and Sissi Pastel and Ann-Christine Aho, wonderful ladies from Biocenter 3 animal facility. It has always been a pleasure to work with you.

My warmest appreciations goes to former and present members of Eero's and Tomi's labs and personnel of Neuroscience center: Anna, Carol, Dima M., Dima T., Ester, Ettore, Frederike, Jonas, Johanna, Juzo, Jesse, Hanna, Katya, Liang, Madhu, Marina, Maria, Merve, Natalya K., Natalya L, Plinio, Prasanna, Ramon, Rimante, Sveta and Vernon. Discussions with each and every one of you was always fruitful, you provided never-ending source of support and motivation. I especially thank Outi and Sulo for all the time you have spent helping me with my work. Thanks to my dear friends Masha, Galya, Margarita, Ksenia, Kostya and Dima for supporting me in all the faces of life.

My warmest and sincere gratitude to my parents Larisa and Alexander and sisters Elya and Lisa for love, understanding and support whatever path I have chosen in my life. My deepest appreciations to my parents-in-law Tatiana and Sergei for infinite support, kindness and love. Without all of you it truly would not be possible!

Foremost, I express my deepest gratitude to my dear Misha, Anastasiia and Elena, whose love and support have kept me going even then it was not possible! Misha, your unbelievable love made me the happiest person in the word! Nastya and Lena, my little girls, you smiles make every day in my life precious! I love you, my family, with all my heart!

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