Interplay between BDNF/TrkB signalling and GABAergic inhibition in the visual cortex of mice

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Abstract

The visual cortex represents one of the most attractive model systems for studying the molecular mechanisms underlying synaptic plasticity in the brain. It has been shown that the neurotrophin Brain-Derived-Neurotrophic-Factor (BDNF) and the GABAergic inhibition play a pivotal role in controlling the timing of the visual cortical plasticity during normal brain development. BDNF signalling is mediated by the activation of TrkB receptors. Besides BDNF, there are not many TrkB agonists available. The flavonoid 7,8-Dihydroxyflavone (7,8-DHF) is a new recently identified molecule, which possesses a potent neurotrophic activity. In the first part of the present thesis, the effects of this agent on synaptic transmission and intrinsic neuronal properties were examined, by performing whole-cell patch clamp recordings from layer 2/3 pyramidal neurons in the mouse visual cortex. It was found that incubation of acute cortical slices for 30 minutes in 20 μM of 7,8 DHF attenuates the strength of the inhibition, without affecting the glutamatergic transmission. Moreover, 7,8-DHF was also able to alter the intrinsic neuronal excitability, by increasing spike frequency and input resistance. The selective action of 7,8-DHF on the GABAergic system was also confirmed after a chronic *in vivo* administration.

In the context of a lesion, the functional interplay between the BDNF-TrkB signalling and the GABAergic inhibition is less understood. One of the most frequently reported electrophysiological alterations post-lesion is the suppression of the GABAergic transmission. In order to investigate the contribution of the BDNF/TrkB signalling to this partial disinhibition, in the second part of the present thesis a well established "ex vivo-in vitro" model of focal laser lesion in the visual cortex of WT and BDNF (+/−) mice was employed. *In vitro* electrophysiological recordings revealed that the impact of a laser lesion on the GABAergic transmission in the mouse visual cortex is dependent on the initial concentration of BDNF. Indeed, in WT animals, the lesion caused a reduction in the frequency of mIPSCs, accompanied by an increase in the Paired-Pulse Ratio (PPR), indicating a presynaptic mechanism of action. Conversely, in BDNF (+/−) mice, an increased amplitude of mIPSCs and a prolonged decay time of eIPSCs strongly suggest that the lesion affected the function of the postsynaptic $GABA_A$ receptors. Moreover, it was shown that an acute pharmacological block of the TrkB receptor completely reproduced the lesion-induced alterations of GABAergic transmission, and that these alterations are not mediated by a reduction in the expression of BDNF.

Taken together these findings demonstrate that the activity of the BDNF/TrkB signalling seems to be fundamental in the functional reorganization of the GABAergic transmission post-lesion. The possibility to pharmacologically modulate this pathway could be important for the development of future therapeutic strategies of recovery from an injury.

Zusammenfassung

Der visuelle Kortex ist eine der attraktivsten Modellsysteme zur Untersuchung der molekularen Mechanismen der synaptischen Plastizität im Gehirn. Es hat sich gezeigt, dass der Wachstumsfaktor *brain-derived-neurotrophic-factor* (BDNF) und die GABAerge Hemmung während der Entwicklung eine essentielle Funktion in der Regulierung der synaptischen Plastizität im visuellen Kortex besitzen. BDNF bindet u.a. an TrkB Rezeptoren, die das Signal intrazellular an unterschiedliche Effektormoleküle weiter vermitteln. Außer BDNF sind auch andere TrkB-Rezeptor Agonisten in der Literatur beschrieben. Einer davon ist das kürzlich identifizierte Flavonoid 7,8-Dihydroxyflavone (7,8-DHF), welchem eine neurotrophe Wirkung zugeschrieben wird. Im ersten Abschnitt der vorliegenden Doktorarbeit wurde der Effekt dieses Agonisten auf die synaptische Übertragung und intrinsischen Zelleigenschaften im visuellen Kortex der Maus untersucht. Dies wurde mit Hilfe der *whole-cell patch clamp* Methode durchgeführt, wobei die synaptischen Eingänge der Pyramidalzellen der kortikalen Schicht 2/3 von besonderem Interesse waren.

Eine 30 minütige Inkubationszeit der kortikalen Schnitte mit 7,8 DHF (20µM) erzielte eine signifikante Reduktion der GABAergen Hemmung, während die glutamaterge synaptische Übertragung unverändert blieb. Des weiteren konnte in Gegenwart von 7,8 DHF eine Veränderung der intrinsischen neuronalen Zellmembraneigenschaften beobachtet werden. Dies wurde deutlich in der Erhöhung des Eingangwiderstandes und der Frequenz der induzierten Aktionspotentiale. Die chronische Applikation von 7,8 DHF *in vivo* bestätigte die selektive Wirkung von 7,8 DHF auf das GABAerge System.

Die Rolle des BDNF-TrkB-Signalweges in der GABAergen Hemmung nach kortikalen Verletzungen ist bisher wenig verstanden. Eine häufig beschriebene elektrophysiologische Veränderung nach kortikaler Verletzung ist eine Reduktion in der GABAergen Hemmung. Im zweiten Abschnitt dieser Doktorarbeit wurde hierzu die Funktion des BDNF-TrkB-Signalweges auf die GABAerge Hemmung nach kortikaler Verletzung untersucht. Es wurde ein "*ex-vivo/in-vitro*“ Laser-Läsions Modell verwendet, wobei mittels eines Lasers im visuellen Kortex von WT und heterozygoten BDNF (+/−) Mäusen eine definierte, reproduzierbare Läsion induziert wurde. Nachfolgende elektrophysiologische Messungen ergaben, dass die Auswirkung einer Verletzung des visuellen Kortex auf die GABAerge Funktion signifikant von der basalen BDNF Konzentration im Kortex abhängt. Des weiteren konnte beobachtet werden, dass nach kortikaler Verletzung in WT Mäusen sowohl die

Frequenz der basalen inhibitorischen, postsynaptischen Potentiale (*mIPSCs*) reduziert war, als auch ein erhöhtes *Paired-Pulse* Verhältnis vorlag. Diese Ergebnisse deuten auf Veränderungen der präsynaptischen Funktion inhibitorischer Synapsen auf Pyramidalneurone hin. Im Gegensatz dazu konnte in BDNF (+/−) mice Mäusen eine erhöhte und gleichzeitig verlängerte mIPSC-Amplitude beobachtet werden, induziert durch Reizung afferenter Nervenfasern. Hieraus lässt sich schließen, dass kortikale Verletzungen in BDNF (+/−) mice Mäusen Auswirkungen auf die Eigenschaften von postsynaptischen $GABA_A$-Rezeptoren haben. Die nachfolgende Gabe eines TrkB-Rezeptor Antagonisten bestätigte diese Ergebnisse für das GABAerge System post-Läsion. Dies zeigt auch, dass die Änderungen der synaptischen Hemmung nicht auf eine Reduktion der BDNF-Konzentration zurückzuführen sind. Zusammengefasst zeigen die Ergebnisse der vorliegenden Arbeit, dass der BDNF-TrkB Signalweg eine wichtige Rolle in der Reorganisation der GABAergen Hemmung nach kortikalen Verletzungen spielt. So könnte ein TrkB-Rezeptor Agonist, wie das kürzlich entdeckte 7,8-DHF, über eine Modulation der BDNF-TrB Signalkaskade pharmakologisch die funktionelle Reorganisation des Kortex nach einer fokalen Gehirnverletzung fördern.

Abbreviations

7,8-DHF	7,8-Dihydroxyflavone
ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate
AMPARs	AMPA receptors
AP	action potential
CA1/CA	hippocampal subfields 1-3 of the ammon horn
cAMP	cyclic adenosine monophosphate
CCC	cation chloride cotransporters
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CREB	cAMP response element binding protein
DAG	diacylglycerol
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
DMSO	Dimethylsulfoxide
DNQX	6,7-dinitroquinoxaline-2,3-(1H,4H)-dione
eEPSCs	evoked excitatory postsynaptic currents
EGTA	ethylene glycol tetraacetic acid
eIPSCs	evoked inhibitory postsynaptic currents
Erk	extracellular signal regulated kinase
GABA	γ-aminobutyric acid
$GABA_A$Rs	γ-aminobutyric acid receptors type A
$GABA_B$ Rs	γ-aminobutyric acid receptors type B
GABAT	GABA transaminase
GAD 65/67	glutamic acid decarboxylase 65/67
GAT	GABA transporter
GFAP	glial fibrillary acidic protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IP3	inositol 1,4,5 triphosphate
ISI	inter-stimulus interval
K252a	tyrosine kinase inhibitor
LGN	lateral geniculate nucleus
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
NFkB	nuclear factor kB
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMDARs	NMDA receptors
NT	neurotrophin
p75NTR	pan neurotrophin receptor
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositide
PKC	protein kinase C
PLC γ	phosholipase Cγ
PPR	paired-pulse ratio
PTX	picrotoxin
QX-314	N-(2,6-Dimethylphenylcarbamoylmethyl)-triethylammonium bromide
Ras	GTP binding protein
RGCs	retinal ganglion cells
R_i	input resistance
SEM	standard error of the mean
SH2	src homology domain 2

TrkA/B/C	tropomyosin-related kinase A/B/C
TTX	tetrodotoxin
VGAT	vescicular GABA transporter
V_m	resting membrane potential
WT	wild type

1. Introduction

1.1 The mouse visual system

Over the last few decades, the majority of vision studies were carried out in primates. In recent years, however, more and more groups started using mice as animal model. Despite their poor visual acuity, mice have a highly developed visual system, which has most of the elements found in primates (for review see: Huberman and Niell, 2011) (Fig. 1.1). As in primates, the light photons activate particular photopigments (opsines) located in the photoreceptors (rods and cones) of the retina. The mouse retina is rod-dominated (only 3% are cones), and thereby specialized for vision under low light (scotopic) conditions. The activation of the opsines in the photoreceptors is responsible for the conversion of the photons into electrical signals, which contain the visual information. After photoreceptors convert light into electrical impulses, this information is filtered through the three major classes of retinal interneurons: horizontal, bipolar and amacrine cells. These interneurons filter and transmit the visual information to the retinal ganglion cells (RGCs) (for review see: Huberman and Niell, 2011). The axons of RGCs project from the retina of both eyes via two optic nerves, which cross at the optic chiasm, located at the base of the hypothalamus. After they passed the optic chiasm, the axons continue as the optic tract. The information for the left visual field is carried in the right optic tract, while information from the right visual field is carried in the left optic tract.

The majority (90%) of the retinal ganglion cells project to the dorsal lateral geniculate nucleus of the thalamus (dLGN), which is the visual part of the thalamus, and sends information directly to the primary visual cortex. The role of the dLGN is to process and to relay visual information to the cortex for conscious visual perception. The right LGN receives information from the left visual field, while the left LGN receives information from the right visual field. A minority of the RGCs send their axons to a midbrain structure, the superior colliculus (SC), primary involved in the control of eye movements (saccades), to the pretectum (which lies between the thalamus and the midbrain), for the pupillary light reflex and to the suprachiasmatic nucleus (SCN) in the hypothalamus, implicated in the control of circadian rhythms and sleep (for review see: Huberman and Niell, 2011).

From the LGN, visual information is relayed to the visual cortex. Most of the axons from the LGN form the optic radiation, which terminates in the visual cortical areas. The visual cortex

is located in the occipital lobe of the cerebral cortex in the posterior part of the brain, and it is specialized in processing the visual stimuli. It can be subdivided in primary visual cortex (also known as V1) and extrastriate visual cortical areas such as V2, V3, V4 and V5, which further integrate the visual information and contribute to the final perception of the visual scene. The primary visual cortex corresponds to the Brodmann area 17 (for review see: Huebener, 2003). There is a visual cortex in each cortical hemisphere. The left hemisphere receives signals from the right visual field and the right visual cortex from the left visual field. Moreover each V1's hemisphere receives information directly from its ipsilateral LGN.

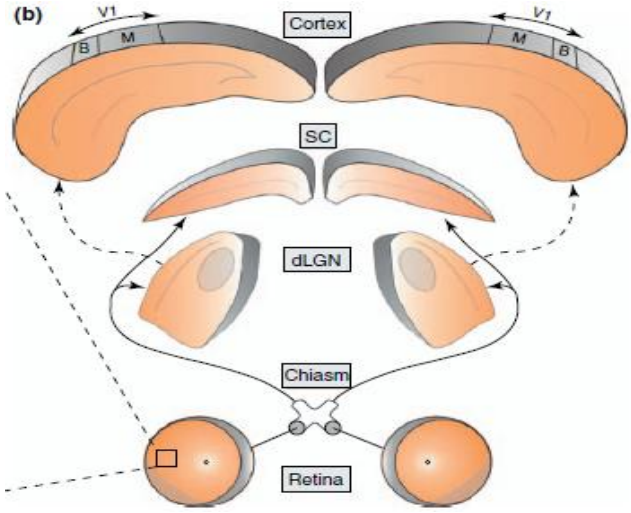

Fig. 1.1 Basic architecture of mouse visual system.
Schematic diagram of the mouse visual pathways, showing direct retinal projections to the dorsal lateral geniculate nucleus (dLGN) and to the superior colliculus (SC) as well as geniculocortical pathways from the dLGN to the visual cortex (from Huberman and Niell, 2011).

1.1.1 Structure and circuits of the primary visual cortex

In the rodents visual cortex there are two main categories of neurons, distinguishable by the shape of their soma and by the appearance of their dendritic tree: pyramidal and non-pyramidal neurons, including spiny stellate cells and smooth or sparsely spinous interneurons (Coogan and Burkhalter, 1993).

The primary visual cortex consists of six distinct horizontal layers, normally identified with Roman numbers: VI, V, IV, III, II, I (from the white matter to the pia mater) (Coogan and Burkhalter, 1993).

Layer I is located just below the cortical surface and consists of small non-pyramidal neurons, which possess short dendrites and axons that arborize only locally.

Layer II and III are hardly separable, and usually are considered a unique entity: the layer II/III. They contain many small soma and dendrites of excitatory pyramidal cells. The dendrites of most pyramidal cells are thick and have several spines. The axons of some pyramidal cells project to the white matter, while collateral branches reach the layer I and layer V. Others are confined to the layer where they originate, and establish horizontal connections with adiacent cortical columns. In this layer there are also few non-pyramidal cells, which present short dendrites and locally arborizing axons.

Layer IV contains both pyramidal and non-pyramidal neurons. The apical dendrites of pyramidal neurons reach layer I without branching. Star pyramidal cells and spiny stellate neurons represent the main excitatory cells in this layer. The axons of star pyramidal cells reach layer V, giving off a small number of terminal branches. The spiny stellate cells dendrites are instead confined in layer 4 and their short axons cover almost exclusively the region around the dendritic tree.

Layer V has several large and small excitatory pyramidal neurons, which innervate the LGN and provide feedback to this relay area. The cell body of these cells has a broad base with prominent basal dendrites extending horizontally and vertically. The most part of their axons reach layer I. A variety of non-pyramidal cells are also present throughout the layer.

Layer VI consists of small and medium sized pyramidal neurons. Some of these cells project to the ipsilateral LGN. Non-pyramidal cells are also present in this layer (Hubel and Wiesel, 1962; Coogan and Burkhalter, 1993).

The cortical layers are strongly interconnected. Layer IV receives the most part of the visual inputs coming from the LGN. From layer IV precise afferents project to layer II/III, and then

the information is sent to extrastriate areas and to the controlateral hemisphere via the corpus callosum. Cells in the layer IV project also sparsely to layer V and VI. Layer VI neurons establish synapses with layer IV neurons through their distal dendrites. The main output of layers II/III is layer V. Layer V project to layer VI and back to layer I and II/III (for review see: Khan et al, 2011). Visual inputs undergo different levels of signal integration and processing through these interlaminar vertical connections (Burkhalter, 1989).

1.2 Plasticity in the visual cortex

The term "plasticity" refers to the ability of the nervous system to structurally and functionally reorganize its connections in response to changes in environmental experience (for review see: Spolidoro et al, 2009).

The capability of the visual cortex to undergo plastic changes, made it one of the most attractive model systems for studying the molecular mechanisms underlying synaptic plasticity in the brain. In rodents, the majority of the plastic changes occur in a limited time window in the early postnatal life called "critical period" (absent at eye opening (P14-P15), peaks at P28-P35 and decline over weeks). During the critical period, the neural circuits display an increased synaptic plasticity in response to the external stimuli, that are essential for the normal brain development. After the end of the critical period, neural plasticity dramatically wanes. In the adult brain in fact, the plasticity is extremely limited (for review see: Levelt and Hubener, 2012).

The first detailed investigation of the critical period at the neuronal level was conducted by Wiesel and Hubel in the early 1960s (Hubel and Wiesel, 1962). They discovered that in the visual cortex the information coming from both eyes converges at the level of single neurons, and many neurons in this area can be driven by stimulation through either eye. Typically, a neuron fires more action potentials when identical visual stimuli are presented to one eye versus the other. This preference for inputs coming from one eye is called "ocular dominance (OD)" (for review see: Levelt and Hubener, 2012). Since then, ocular dominance plasticity served as an important model system to study how cortical circuits are shaped by experience.

In the visual cortex of cats, ferrets and primates, neurons are clustered together according to the eye by which they are preferentially driven, forming the so called "OD columns". The rodents visual cortex lacks OD columns but individual neurons in the binocular part show OD (Espinosa and Stryker, 2012) (Fig. 1.2a).

OD can change during the development, for example, due to refractive error, cataract or misalignment of the eyes, or under experimental conditions during so called "monocular deprivation (MD)". MD, as one prominent type of experience dependent plasticity, implies the closure of one eye for a limited period early in life. This transient closure alters the OD in the visual cortex, resulting in the temporarily closed eye becoming less effective in driving the cortical neurons, whereas inputs from the nondeprived eye become more dominant. At the level of OD columns, this is reflected in an expansion of the open eye's columns at the expense of the columns associated with the closed eye. In other words the MD causes an OD shift towards the open eye, which is accompanied by an extensive rewiring of thalamocortical and intracortical synaptic connections. Similar to higher mammals, a shift in the OD distribution can also be detected in rodents after monocular deprivation. Taken together, the above-described changes are termed OD plasticity (Fig. 1.2b) (for review see: Hensch, 2005). Emerging body of evidence points to the neurotrophin BDNF and the GABAergic inhibition as key mediators of experience-dependent plasticity in the visual cortex (for review see: Berardi et al, 2003).

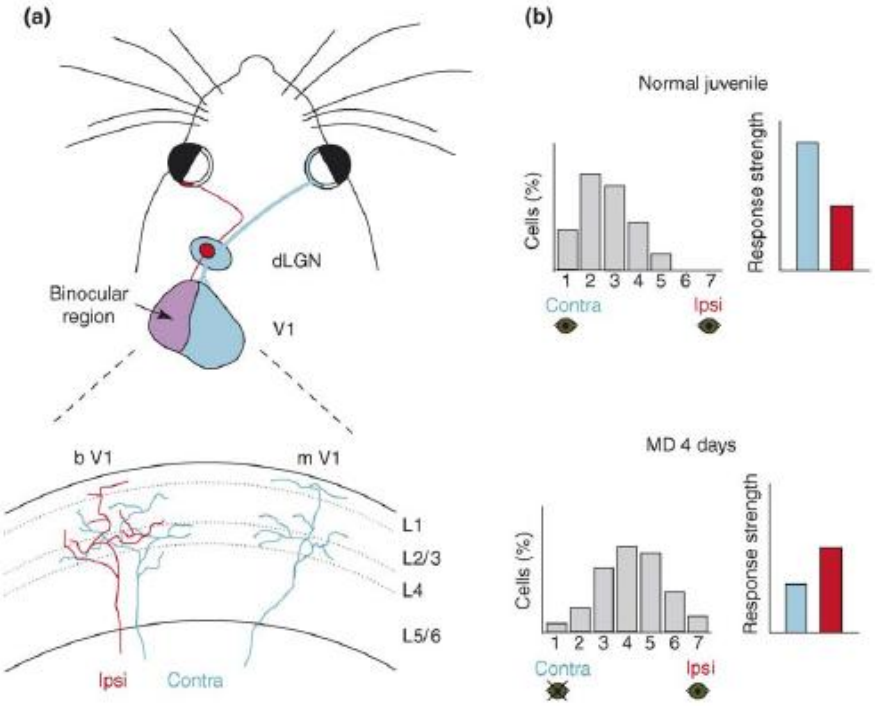

Fig. 1.2 Ocular dominance (OD) plasticity in mouse visual cortex
a) Scheme showing the mouse visual system. The major part of the visual cortex (V1) receives input only from the contralateral retina (light blue projections). The third lateral of V1 is innervated in addition by ipsilateral projections (red). Although neurons in the binocular zone are dominated by contralateral eye input, most neurons respond to both eyes. Thalamocortical axons from the dorsal lateral geniculate nucleus (dLGN) reach not only layer 4 (L4) but also the superficial layers (L1-3). b) 4 days of monocular deprivation (MD) in young mice (around P28) lead to changes in binocular cortical responses: the OD distribution of neurons shifts toward the open eye (ipsilateral eye). OD classes from 1-7 indicate the relative responsiveness of neurons to contralateral and ipsilateral eye stimulation (1 or 7, cells respond only to controlateral or ipsilateral eye, respectively; 4, equal response to both eyes). The OD shifts are caused by a strong weakening of responsiveness and a partial strengthening of non-deprived eye responsiveness, as measurement of population response strength with visually-evoked potentials and intrinsic signal imaging (from Hofer et al, 2006).

1.3 The GABAergic system

1.3.1 GABA (γ-amminobutyric acid)

GABA (γ-amminobutyric acid) is the main inhibitory neurotransmitter in the CNS. It is implicated in many neuronal processes: neuronal excitability, generation of oscillations, propagation of neuronal activity within neuronal networks and neuronal plasticity. At the cellular level, it mediates, by hyperpolarizing the membrane and thereby decreasing the probability to elicit an action potential, an inhibition in the adult brain. It also plays an important role in cognition, regulation of sleep states, control of anxiety and executive functions (for review see: Kilb, 2012).

GABA does not penetrate the Blood Brain Barrier (BBB); it is synthesized directly in the brain from glutamate by the enzyme glutamic acid decarboxylase (GAD), which exists in two different isoforms, GAD65 and GAD67. These two GADs are products of two separate genes, and exhibit different subcellular localizations and functions. GAD65 is concentrated in the nerve terminals, where it synthesizes GABA for neurotransmission purposes, while GAD67 catalyzes mainly cytoplasmatic GABA pools (for review see: Soghomonian and Martin, 1998). After being synthesized, GABA is accumulated in synaptic vesicles by the vesicular inhibitory amino acid transporter (VGAT). It is released into the synaptic cleft by Ca2+ dependent exocytosis, diffuses out of the cleft, and it is afterwards reuptaken by a selective Na/Cl dependent GABA transporters (GAT) into nerve terminals and glial cells. After the reuptake GABA is metabolized by the enzyme GABA transaminase (GABAT) in both neurons and glia (for review see: Farrant and Kaila, 2007).

1.3.2 GABA receptors

GABA predominantly binds to two different types of receptors: the ionotropic $GABA_A$ and the metabotropic $GABA_B$ receptors.

$GABA_A$ receptors are ligand-gated ion channels, permeable to chloride and to a less extent to bicarbonate (HCO3). They are members of Cys loop superfamily (so called for a conserved motif in the amino-terminal domain in which a pair of cysteines forms a disulphide bridge), which also includes Ach, glycine and 5HT3 receptors (Fig. 1.3a). The receptor consists of distinct subunits (each with four transmembrane domains; M1–4) which form a pentamer. To date, 19 different subunits, grouped in 8 families (α, β, γ, δ, ε, θ, π) have been cloned. The subunits composition determines the functional properties and pharmacological modulation of the receptors. The majority of the receptors generally contain 3 different subunits. However, combinations of α and β subunits are sufficient to have functional $GABA_A$ receptors. In the brain the most abundant $GABA_A$ receptor subtype is α1β2γ2. Binding of two GABA molecules at the extracellular interface between α and β subunits is required to trigger the conformational change, which opens the internal pore of the channel, allowing the ions to pass through the receptor pore (for review see: Farrant and Kaila, 2007) (Fig. 1.3b).

$GABA_B$ receptors are G-protein coupled receptors which are localized on both pre- and postsynaptic membranes. Postsynaptic $GABA_B$ activation causes the activation of inwardly rectifying K channels (GIRK or Kir3) that hyperpolarize the cell. Presynaptic $GABA_B$ receptors act as autoreceptors, diminishing the release of neurotransmitter through the inhibition of voltage-gated Ca2+ channels. Activation of $GABA_B$ receptors can also modulate the production of cAMP. Fully functional $GABA_B$ receptors are heterodimers, that require the assembly of the 2 subunits $GABA_{B1}$ and $GABA_{B2}$ (Ben-Ari et al, 2007).

A third subclass of ligand-gated ionotropic GABA receptors, insensitive to typical allosteric modulators of $GABA_A$ receptor channels such as benzodiazepines and barbiturates, has been recently identified. These receptors are known as $GABA_C$ or $GABA_A$-rho receptors (for review see: Chebib, 2004). They belong to the $GABA_A$ receptors family and are entirely composed of rho (ρ) subunits. These receptors are expressed in many areas of the brain, but are especially expressed in the retina. They consist of 5 subunits, forming a central pore permeable to chloride ions. Binding of GABA to the receptor results in opening of the channels and in a hyperpolarization of the cell (for review see: Chebib, 2004).

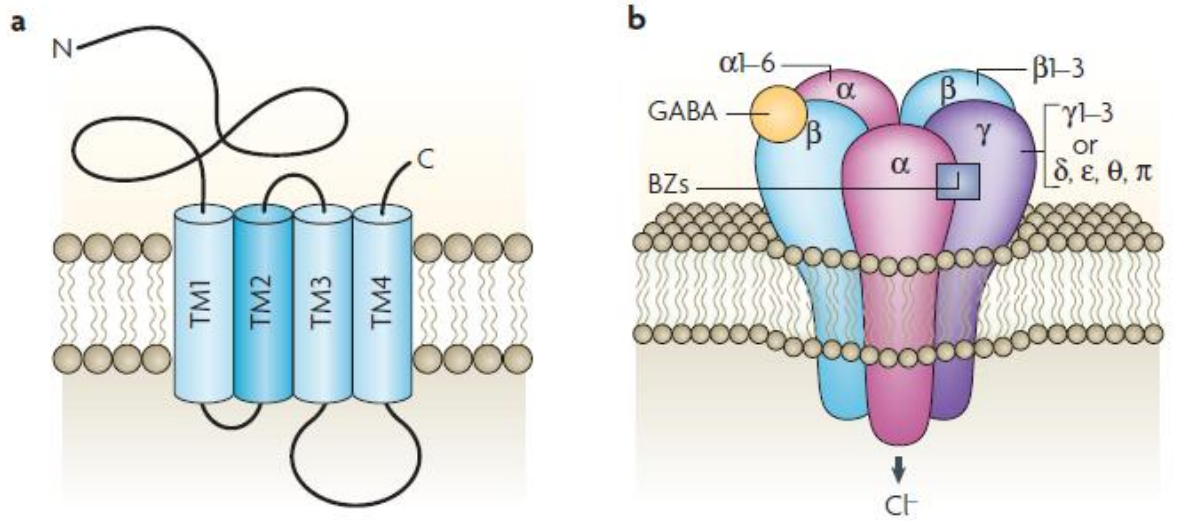

Fig. 1.3 $GABA_A$ receptor structure and neuronal location
a) $GABA_A$ receptors are members of the ligand-gated ion channel superfamily. They consist of 4 hydrophobic transmembrane domains (TM1-4). TM2 is considered the pore of the channel. The large extracellular amino terminus is the site of GABA binding and also contains the binding site of psychoactive drug , such as benzodiazepine (BZ). Each subunit also has a large intracellular domain between TM3 and TM4, which is the site for protein interactions as well as for post-translational modifications that modulate receptor activity. b) 5 subunits from 7 different subfamilies (α, β, γ, δ, ε, θ and π) assemble to form a heteropentameric Cl^- permeable channel. The majority of the receptors consist of 2 α, 2 β and 1 γ subunits. GABA binding site is located at the interface between α and β subunits. Binding of the neurotransmitter triggers a rapid Cl^- influx. BZ bind at the interface between α and γ subunits and potentiate GABA-induced Cl^- influx (from Jacob et al, 2008).

1.3.3 The role of Chloride Transporters on GABAergic function

Activation of $GABA_A$ receptors can also result in opposite effects, depending on the maturation state of the GABAergic system and on the distribution of the Cl^- ions across the plasma membrane. In adult neurons, the intracellular Cl^- concentration is low due to active Cl^- extrusion. Therefore, after the opening of $GABA_A$ receptors, Cl^- ions tend to flow into the cell, thereby hyperpolarizing the membrane. Contrarily, in immature neurons the intracellular Cl^- concentration is much higher than in adult neurons due to an inefficient Cl^- extrusion system (Rivera et al, 2005). For this reason in immature neurons, upon $GABA_A$ receptor activation, Cl^- ions tend to flow out of the cell, thereby eliciting depolarizing IPSPs. The GABAergic system during its development undergoes a "developmental switch": the

depolarizing action of GABA in developing neurons turns to hyperpolarizing in adult neurons (For review see: Ben-Ari, 2002).

Slight changes in the intracellular Cl^- concentration can have profound consequences on the normal physiology of the cell (i.e.: alterations in the resting membrane potential, lowering the threshold for action potential generation etc.). Thus, the maintainance of the Cl^- homeostasis is crucial. Neuronal Cl^- homeostasis is chiefly controlled by two cation-chloride co-transporter molecules (CCC): KCC2 and NKCC1.

The CCCs are glycoproteins of 120-200 kDa. They consist of 12 transmembrane segments, flanked on one side by a small intracellular amino-terminus and by a large intracellular carboxy-terminus on the other. They do not consume ATP, but rather obtain the energy for Cl^- translocation from the Na+ and K+ gradients generated by the Na-K-ATPase (for review see: Blaesse et al., 2009).

KCC2 is a neuron-specific plasma membrane protein that extrudes 1 Cl^- ion together with 1 K+ driven by the K+ gradient. It exists as mono- and oligomers. It is the major CCC in mature central neurons and the only one expressed exclusively in the CNS. It has been found in the cell body and in the dendrites but not in the axons (Gulyas et al, 2005). The GABA developmental switch is based on a decrease in the intracellular Cl^- concentration. The developmental upregulation of KCC2 seems to be the mechanism underlying this change. BDNF is an important modulator of KCC2 expression. In developing neurons BDNF upregulates KCC2 (Aguado et al, 2003), whereas in mature neurons BDNF, through the TrkB receptor downregulate KCC2 expression (Rivera et al, 2002). KCC2 activity can be inhibited by Zn2+ (for review see: Blaesse et al., 2009; Ben-Ari et al., 2012).

NKCC1 is driven by Na+ and K+ gradients and it is responsible for the intracellular Cl^- accumulation by transporting 1 Na+, 1 K+ and 2 Cl^-. NKCC1 is predominantly expressed in immature neurons, where it keeps Cl^- concentration high. In adult neurons KCC2 takes over and overcomes NKCC1 activity, thus promoting the GABA shift. In immature neurons NKCC1 is located in somata, whereas in mature neurons in mainly dendritic. NKCC1 can be selectively blocked by diuretic drug bumetanide, which does not affect KCC2 (for review see: Kahle et al, 2008; Blaesse et al., 2009).

1.4 Brain Derived Neurotrophic Factor (BDNF)

Brain Derived Neurotrophic Factor (BDNF) is the most studied and well-known member of the Neurotrophin family, which also includes the Nerve Growth Factor (NGF), Neurotrophin 3 (NT-3) and Neurotrophin 4/5 (NT-4/5). The Neurotrophins are small homodimeric polypeptides (~ 120 aa), all structurally related, sharing approximately 50% of the amino acidic sequence (for review see: Huang and Reichardt, 2001).

BDNF was isolated and sequenced for the first time from the pig brain by Ives Barde and his colleagues (Barde et al., 1982). It is implicated in several aspects of the CNS function: cell differentiation, neuronal survival, migration, dendritic arborization, synaptogenesis, neurotransmitter release, myelination, activity-dependent forms of synaptic plasticity (for review see: Gottmann et al, 2009).

The BDNF gene consists of multiple 5' untranslated exons (10 in humans, 8 in rodents, 6 in lower vertebrates) and one protein coding 3' exon, which are under the control of distinct promoters. The transcription of the gene results in BDNF mRNA transcripts containing one of the eight 5' exons spliced to the protein coding exon and in a transcript containing only 5' extended protein coding exon. Through the use of alternative promoters, splicing and polyadenylation sites, up to 24 transcripts can be produced and remarkably each transcript encodes an identical initial BDNF protein product. Some promoters maintain basal levels of BDNF expression necessary for neuronal survival and differentiation, whereas others drive developmental, tissue specific and activity-dependent BDNF expression. BDNF is expressed in many regions of the CNS, including the spinal cord, substantia nigra, amygdala, hypothalamus, cerebellum, hippocampus and cortex.

BDNF is synthesized as 32 k-Dalton precursor, the Pro-BDNF (containing a prodomain and a mature domain), which undergoes an enzymatic cleavage to release the mature 14 k-Dalton protein (mBDNF). ProBDNF is either proteolytically cleaved intracellularly by enzymes such as furin or pro-convertases and secreted as the 14 kDa mature BDNF (mBDNF), or secreted as proBDNF and then further cleaved by extracellular proteases, such as metalloproteinases and plasmin, to mBDNF (for review see: Lu et al, 2005).

Both proBDNF and mBDNF are sorted and packaged in vesicles belonging either to the constitutive or to the regulated pathway, and transported to the appropriate cellular compartment to be secreted. BDNF is present in pre- and postsynaptic compartments and it can undergo both retrograde and anterograde transport. Moreover, BDNF can act via autocrine and paracrine mechanisms (Greenberg et al, 2009).

Histological studies showed that BDNF is produced in hippocampal and neocortical pyramidal neurons, as well as the its receptor TrkB, whereas interneurons express TrkB but not BDNF (Marty et al., 1996; Gorba and Wahle, 1999; Aid et al, 2007).

Many non-neuronal cells, such as smooth muscle cells, fibroblasts and astrocytes, may not express molecular components of the regulated secretory pathway and, therefore, secrete neurotrophins only constitutively. Regulated secretion is prevalent in neurons. Neurotrophin-containing secretory granules are transported to dendrites and spines, and are secreted postsynaptically. On the other hand, neurotrophin containing large dense core vesicles (LDCVs) undergo anterograde transport to axonal terminals (for review see: Lu et al, 2005, Park and Poo, 2013).

The BDNF release is regulated by the level of neuronal activity: an increase in the neuronal activity leads to a massive Ca2+ influx into the cell, that ultimately triggers the release of BDNF (for review see: Lu et al, 2005; Park and Poo, 2013).

BDNF can exert its physiological actions via two different receptors types: the tropomyosin related kinase TrkB receptor and the pan neurotrophin receptor p75NTR (for review see: Lu et al, 2005).

1.4.1 BDNF receptors and signalling pathways

In the mammalian brain the four neurotrophins act via 4 different receptors: p75, TrkA, TrkB and TrkC. The p75 receptor binds with low affinity all neurotrophins , whereas each of the Trk receptors is activated with high affinity by a particular neurotrophin: NGF binds to TrkA, BDNF and NT4 bind TrkB and NT-3 (for review see: Reichardt, 2006)

The pan-neurotrophin receptor p75NTR is a member of the tumour necrosis superfamily (TNFR-SF), characterized by ligand binding domains consisting of four repeats of cystein-rich domains (CRD). Crucial for the binding of the neurotrophins and for the activation of the downstream signalling pathway is the presence of a disulfide linked p75NTR dimer, formed via cisteinyl residues within the transmembrane domains. Neurotrophin-dependent p75 NTR activation involves association of a neurotrophin dimer with CRDs 2-4 of the two extracellular domains of a p75 NTR dimer. It was demonstrated that proBDNF binds p75 NTR preferentially (Yang et al, 2009). The activation of p75NTR by BDNF initiates both pro-survival NF-kB and pro-apoptotic Jun kinase signalling cascades. p75NTR-mediated survival involves the activation of the NF-kB pathway via the association of p75NTR with

tumour necrosis factor receptor associated factor 4/6 (TRAF4/6) and receptor interacting protein-2 (for review see: Teng and Hempstead, 2004).

The Trks receptors are receptor tyrosine kinases. Their extracellular domain consists of a cysteine-rich cluster, followed by three leucine rich repeats, another cystein-rich cluster and two immunoglobulin-like domains. Each receptor has a transmembrane domain and terminates with a cytoplasmatic domain consisting of a tyrosin kinase domain surrounded by many tyrosine residues, which serve as a phosphorylation dependent docking sites for cytoplasmatics downstream signalling proteins like the SRC homologous and collagen like (Shc) adaptor protein and Phospholipase C-ϒ. The binding of the neurotrophins to the Trks receptors induces ligand-receptor dimerization and autophosphorylation of tyrosin residues in the intracellular domain of the receptor. Shc and PLC-ϒ together with other adaptors proteins such as Grb2, APS and Frs2 are phosphorylated by the activated Trk (Huang and Reichardt, 2003; for review see: Yoshii and Constantin-Paton, 2010).

Until now, it is known that virtually all BDNF effects are mediated by the TrkB receptor (Squinto et al, 1991). TrkB activation by BDNF triggers three different signalling pathways: phospholipase C (PLC-ϒ), phosphatidylinositol 3 kinase (PI3K) and mitogen activated protein kinase (MAPK) pathway (for review see: Schecterson and Bothwell, 2010) (Fig. 1.4).

In the MAP kinase pathway activation of Shc recruits the G-protein RAS, that, through adaptors Grb2 and SOS, starts a sequential activation by Phosphorilation of the MAP kinase cascade; whose elements are Raf (a serine threonine kinase), Mek (a mixed specificity kinase) and Erk (a MAP kinase). ERK is translocated into the nucleus, where it activates some transcription factor such as cAMP-response-element binding protein (CREB). The MAP pathway is fundamental for the normal neuronal development, neuronal survival and neurogenesis (for review see: Chao, 2003).

In the PLC-ϒ pathway, PLC-ϒ catalyzes the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2) to diacyl glycerol (DAG) and inositol (1,4,5) triphosphate (IP_3). DAG activates several protein kinase C (PKC) isoforms. IP_3 signaling through specific receptors promotes the release of Ca2+ from the intracellular stores. The rise in the intracellular Ca2+ concentration activates Ca2+ sensitive enzymes including Ca-calmoduline kinases II (CaMKII) and phosphatases, MAPKs and PI3K. PLC-ϒ pathway is important for many brain functions like NT-dependent regulation of synaptic plasticity and learning and memory. Moreover through the CaMKII signalling the BDNF regulates its own expression (Segal, 2003).

In the PI3 kinase pathway, activated Shc recruits PI3K via adaptor proteins Grb2 and Gab1; otherwise, PI3K can be activated by Trk receptors through adaptor protein IRS. PI3K phosporylates phosphoinositol (PI) lipids, generating 3,4,5-triphosphates (PIP_3). PIP_3 recruits two proteins to the plasma membrane by binding to pleckstrin homology (PH) domains. These proteins are Akt (PKB) and PDK-1. PI3K pathway is essential for NT-mediated neuronal survival (Kaplan and Miller, 2000).

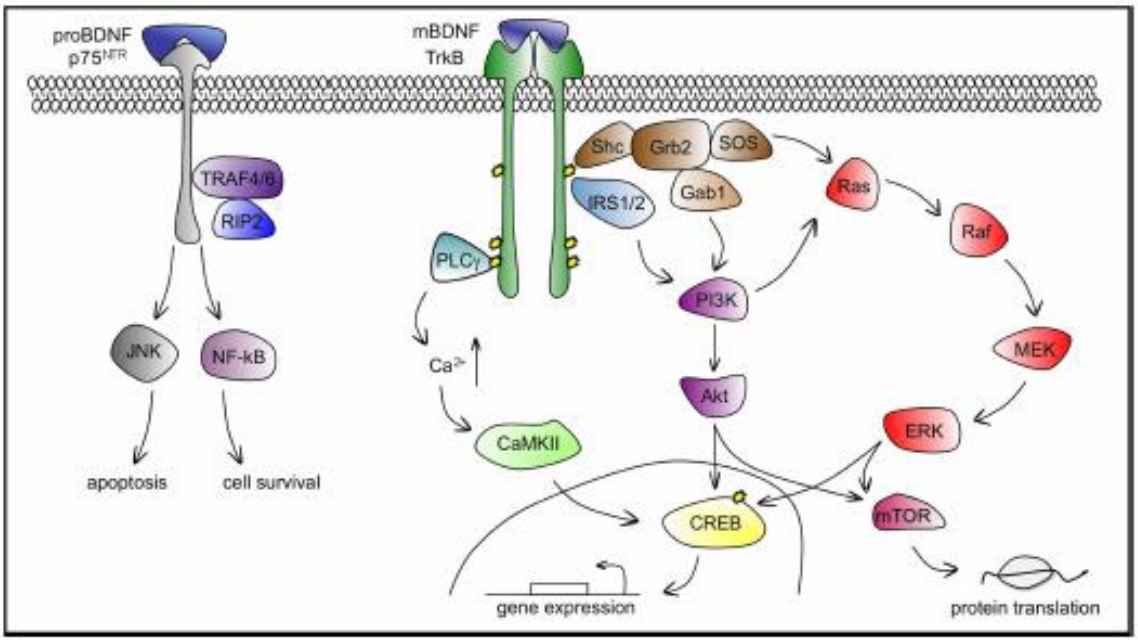

Fig. 1.4 Intracellular signal cascades triggered by BDNF receptors
BDNF binds to TrkB receptor with high affinity and induce its dimerization and autophosphorilation of tyrosine residues in the cytoplasmatic domain, which serve as docking sites for effector molecules, which trigger the activation of the 3 main signalling pathways: PLCγ, PI3K and ERK. Downstream in the nucleus, the ultimate goal is the phosphorylation and activation of the transcription factor CREB that mediates the transcription of genes, important for neuronal survival and differentiation. Recruitment of PLCγ increases the intracellular Ca^{2+} concentration that, leads to the activation of protein CaMKII, which phosphorylates CREB. PI3K can be activated via the Shc/Grb2/SOS complex through Gab1 and by IRS1/2. Lipid products generated by the activated PI3K, the phosphatidylinositides, bind and activate protein kinase Akt, upstream of CREB. The ERK cascade can be activated both by the Shc/Grb2/SOS complex and by PI3K. ERK phosphorylation leads directly to CREB phosphorylation. Both Akt and ERK activate mTOR, responsible for enhanced translation initiation. ProBDNF binds the p75 receptor and promotes apoptosis (from Cunha et al, 2010)

1.4.2 Effect of BDNF on GABAergic and glutamatergic synaptic transmission

BDNF exerts a pleiotropic effect on synaptic transmission in different brain regions (for review see: Gottmann et al., 2009). It is well accepted that BDNF acutely (i.e. within minutes) enhances the glutamatergic transmission. Several studies, performed in cultured neurons and acute slices, have demonstrated that this enhancement of the glutamatergic currents is mainly due to an increase in the presynaptic glutamate release (Lessmann et al, 1994; Akaneya et al, 1997; Carmignoto et al, 1997). Interestingly, the BDNF presynaptic effects are dependent on the maturation state of the glutamatergic synapses. Indeed immature synapses are more likely potentiated by BDNF than mature synapses (Berninger et al, 1999).

Apart from these presynaptic effects, BDNF also acts postsynaptically. It has been reported that BDNF induces a potentiation of the NMDA receptors activity through BDNF/TrkB/MAPK dependent phosphorylation of NMDAR subunit 2B, which leads to prolonged openings of NMDA receptor channels (Levine et al, 1998). In addition to the well known BDNF-induced Ca^{2+} release from internal stores via TrkB/PLC gamma signalling (Berninger et al, 1993), BDNF can also activate postsynaptic Na^{+} ascribable to the gating of the Nav 1.9 channels (Blum et al., 2002), and Ca^{2+} currents through the activaction of the TRPC channels (Li et al 1999; Amaral and Pozzo-Miller, 2007).

Conversely, acute application of BDNF has opposite effects on the GABAergic transmission, leading to suppression of the inhibition (Tanaka et al., 1997; Frerking et al, 1998). The majority of the effects on the GABAergic system are mediated by BDNF/TrkB dependent activation of PKC, which induces internalization of the postsynaptic $GABA_A$ receptors (Brunig et al, 2001). It has also been reported that BDNF attenuates the GABAergic inhibition by triggering postsynaptically the synthesis and the release of endocannabinoids, that acting retrogradely at presynaptic CB1 cannabinoids receptors, reduce the presynaptic GABA release (Lemtiri-Chlieh and Levine, 2010).

Furthermore, chronic administration of BDNF enhances the formation and functional maturation of both glutamategic and GABAergic synapses (for review see: Gottmann et al., 2009)

1.4.3 BDNF and long term plasticity

In the adult brain, the BDNF-TrkB signalling pathway plays an important role in synaptic plasticity. Synaptic plasticity can be defined as a series of changes in synaptic strength in response to a defined stimulus. The most studied form of synaptic plasticity is the long-term potentiation (LTP). LTP is typically induced by a high-frequency stimulation (HFS) of excitatory input, which results in an increase in the magnitude of the postsynaptic response (Lisman, 2003). The maintenance of LTP consists at least of two phases: early and late LTP. The early LTP (E-LTP), which lasts 1-2 h, does not require new protein synthesis but only modification of existing proteins and protein trafficking at the synapses. The development of late LTP (L-LTP), which could last several days, depends on de novo mRNA and protein synthesis. LTP is unanimously considered the neuronal correlate of learning and memory (for review see: Minichiello, 2009).

It has been reported that acute application of mature BDNF (mBDNF) facilitates LTP in the hippocampus (Figurov et al., 1996) and in the visual cortex (Akaneya et al, 1997). Conversely inhibition of BDNF activity by gene knockout (Korte et al, 1995), by application of TrkB-IgG (a molecular scavenger of endogenous BDNF) or by TrkB receptor blockade with K252a (a non specific TrkB receptor blocker), attenuates the LTP (Figurov et al, 1996; Meis et al, 2012).

BDNF regulates, via the TrkB receptor, both E-LTP and L-LTP through different mechanisms. During the induction of E-LTP BDNF stimulates the release of existing presynaptic BDNF-containing vesicles. By contrast L-LTP requires synthesis and release of ProBDNF and extracellular conversion of proBDNF to mature BDNF (Bramham and Messaoudi, 2005). It has also been demonstrated that the uncleaved proBDNF, through the p75NTR, promotes another form of synaptic plasticity: long-term depression (LTD) (Woo et al, 2005). Conversely the mature BDNF has been shown to impair the LTD induction (Akaneya et al, 1996).

Long-term changes in synaptic plasticity like LTP and LTD are always accompanied by structural and morphological alterations of synapses, particularly in the structure and density of dendritic spines. Many studies suggest that structural modifications of the synapses are associated with the bidirectional expression of long-term synaptic plasticity: LTP provokes new synaptic formation, whereas LTD is accompanied by synapse retraction (for review see: Lu et al, 2005). It appears that these morphological changes are mediated, at least in part, by BDNF. BDNF-TrkB signalling activation induces LTP and leads to axonal branching,

dendritic growth, increase in density and number of spines. Instead, proBDNF-p75NTR signalling pathway leads to spines retraction and synapses shrinkage, and induces LTD (Woo et al, 2005)

1.4.4 TrkB receptor agonists and antagonists

One commonly used TrkB receptor antagonist is K252a. It is an alkaloid isolated from of a fungus of the Nocurdiopsis species. It is an unspecific kinase inhibitor (Knuesel and Hefti, 1992) and a selective inhibitor of the tyrosine kinase activity of the TrkB receptor (Tapley, 1992).

Besides BDNF, there are not many molecules available, that are capable of activating the TrkB receptor. The ones which have been reported until now are: Amitriptyline, N-acetylserotonin (NAS) and 7,8 Dihydroxyflavone (7,8 DHF).

Amitriptyline is a tricyclic antidepressant. It acts as TrkA and TrkB agonist, promotes the heterodimerization of these proteins in the absence of NGF and has a potent neurotrophic activity (Jang et al., 2009).

N-Acetylserotonin, also known as normelatonin, is a chemical intermediate in the endogenous production of melatonin from serotonin. It produces robust antidepressant, neuoprotective and and neurotrophic effects, that are TrkB-mediated (Jang et al, 2010).

7,8 DHF (Fig. 1.5) is a new recently identified flavonoid, which binds and activates the TrkB receptor both *in vitro* and *in vivo*, and has shown several interesting biological effects (Jang et al, 2010). This makes it a good candidate for follow-up clinical investigations.

1.5 Flavonoids and 7,8-Dihydroxyflavone

The Flavonoids are the most common and abundant polyphenolic compounds (ie, several hydroxyl groups on aromatic rings) in the human diet. So far, over 6000 Flavonoids have been reported. The main sources of Flavonoids include a variety of fruits, vegetables, cereals, tea, wine, chocolate and fruit juices (for review see: Manach et al, 2004).

Flavonoids consist of two aromatic carbon rings, benzopyran (A and C rings) and benzene (B ring), and may be divided into six subgroups based on the degree of the oxidation of the C-ring, the hydroxylation pattern of the ring structure and the substitution of the 3-position: (1) flavonols (2) flavones (3) isoflavones (4) flavanones (5) flavanols (6) anthocyanidins. Initially, they have attracted much attention as free radical scavengers with antioxidant

activity. They are believed to play a significant role in reducing the risks of age- and lifestyle-related diseases such as cancer or cardiovascular disease (Spencer, 2008).

Due to their lipophilic structure they can cross the Blood Brain Barrier (BBB) and exert their effects directly on the brain, by modulating the activity of enzymes, receptors and signalling pathways (for review see: Youdim et al, 2004;).

They happened to be beneficial in counteracting functional age-related cognitive deficits by improving learning and memory; and they also appear to be neuroprotective due to their ability to suppress neuroinflammation, to protect neurons against stress-induced injury and to prevent neurodegeneration associated with Alzheimer's and Parkinson's diseases. Flavonoids of different classes are inhibitors of monoamine oxidase A or B, thereby working as antidepressants, while others have anxiolitic, sedative and anticonvulsant activities, by way of their interaction with the $GABA_A$ receptors (for review see: Jäger and Saaby, 2011, Rendeiro et al., 2012).

Recently Jang et al. (2010), screening a library of new chemical compounds, identified a small molecule, 7,8-Dihydroxyflavone (7,8 DHF). It belongs to the Flavonoids family and possesses a potent neurotrophic activity. This flavone can be obtained from the plant Godmania aesculifolia or through chemical synthesis.

The existing literature shows that 7,8-DHF shares many biological properties with the BDNF. *In vitro*, 7,8-DHF binds TrkB receptors with high affinity and provokes its dimerization and autophosphorilation, leading to the activation of the downstream intracellular signalling cascades (Jang et al., 2010). When administered systemically *in vivo*, 7,8-DHF causes a substantial activation of TrkB receptors in the entire brain (Jang et al., 2010). Furthermore, like BDNF, it has been shown to be neuroprotective against excytotoxic and ischemic damage, and to influence the emotional behaviour as well as rescuing memory and cognitive deficits in aged animals (Andero et al., 2011; Chen et al, 2011; Choi et al., 2010; Devi and Ohno, 2011). Despite all these findings, surprisingly almost nothing is known about the effects of this molecule on synaptic transmission and intrinsic neuronal excitability. Thus, the present thesis has addressed this point.

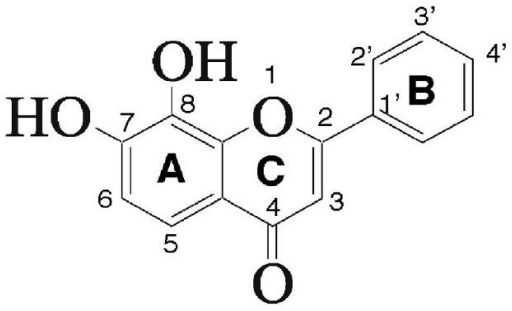

Fig. 1.5 7,8 dihydroxyflavone chemical structure (from Liu et al., 2010)

1.6 Interplay between BDNF and GABAergic inhibition (in the regulation of visual cortical plasticity)

Many studies have shown that BDNF and GABAergic inhibition play a pivotal role in controlling the timing of plasticity in the visual cortex (for review see: Hensch, 2004). Their mutual interaction is crucial in setting the time course of the critical period and in shaping the cortical neuronal circuits during development.

The initial evidence for the involvement of the GABAergic inhibition in regulating visual plasticity came from experiments in mice, in which GABA synthesis was reduced due to a deletion of the glutamic acid decarboxylase GAD 65. These animals failed to exhibit OD plasticity and the critical period could not be induced. A treatment with the benzodiazepine diazepam (a GABAA receptor agonist) rescued OD plasticity. Moreover, administration of benzodiazepines in normal animals accelerated both the onset and the closure of the critical period. (for review see: Baroncelli et al, 2011) (Fig. 1.6).

Compatible observations have been made in transgenic animals overexpressing BDNF. In these animals maturation of the GABAergic system was enhanced, leading to a premature closure of the critical period (Huang et al, 1999). BDNF is a protein, whose expression and release are regulated by neuronal activity. When wild-type mice are dark reared (housed in absence of light), BDNF expression is downregulated (due to a lack of stimuli coming from the external environment), the development of inhibitory innervation and critical-period onset are delayed (Morales et al, 2002). Furthermore, in BDNF knockout mice, lacking one copy of the BDNF gene, the GABAergic system appears immature and functionally impaired (Abidin et al, 2008). Surprisingly, in this animal model the OD plasticity during or after the critical period was unaffected (Bartoletti et al, 2002).

Taken together these findings clearly indicate that BDNF is an important factor in regulating the visual plasticity and highlights the functional interplay between the BDNF and the GABAergic inhibition in the developing visual cortex.

The GABAergic inhibition in the cortex is mediated by a heterogeneous population of interneurons. Interestingly not all GABA circuits are involved in the regulation of the critical period. Most evidence point to a subset of interneurons, the Parvalbumin (PV) expressing basket cells, as a primary candidate (Fagiolini et al, 2004). Blockade of the activity of a specific potassium channels (Kv3.1), important for the efficiency of the fast spiking behaviour and the release of GABA, slows down OD plasticity. Similar effect can be achieved by knocking down BDNF, which is crucial for the development of the PV interneurons. Furthermore, it has been shown that a particular GABAA receptor subtype containing the α1 subunits, located on the PV cells, plays an important role in the initiation of the critical period (Sale et al, 2010).

One long-lasting dogma in neuroscience claimed that brain plasticity is a phenomenon restricted to the early postnatal life. Recent studies have begun to challenge this dogma, demonstrating that the OD plasticity can also be induced, although to a lesser extent, in the adult cortex (Spolidoro et al, 2009).

It has been demonstrated that it is possible to restore OD plasticity by pharmacologically reducing the intracortical inhibition through infusion of either MPA (an inhibitor of GABA synthesis) or picrotoxin (GABAA receptor antagonist) directly into the visual cortex (Harauzov et al, 2010), or via a chronic treatment with the antidepressant Fluoxetine (Maya Vetencourt et al, 2008). An alternative strategy, reducing the GABAergic inhibition and enhancing plasticity in adulthood would be the environmental enrichment paradigm (EE). EE is a combination of complex sensory-motor stimulation, obtained by rearing large groups of animals in wide stimulating environments, where a variety of objects are used to promote exploration, cognitive activity, social interaction and physical exercise. EE has been shown to increase the BDNF levels. Interestingly, while during development environmental enrichment increases BDNF and accelerates the maturation of inhibition in the visual cortex, in adult animals housed in an enriched environment increased levels of BDNF are associated with reduced GABAergic inhibition (Baroncelli et al, 2011).

The reactivation of OD plasticity in the adult brain is associated with a structural rewiring of the GABAergic circuits. With age, the PV interneurons are progressively enwrapped in a perineuronal network (PNN) of the extracellular matrix (ECM). Disruption of PNN by using

for example the enzyme chondroitinase weakens the perisomatic inhibition, thus allowing the induction of the OD plasticity through MD (Pizzorusso et al, 2002).

At the synaptic level a recovery of plasticity in adult animals is accompanied by a restoration of the white matter-induced LTP in the layer 2/3 (WM-LTP), a form of plasticity which can be elicited only during the critical period (Harauzov et al, 2010).

Cortical plasticity has also been discussed in studies of functional recovery from brain injury. A deep understanding of the pathophysiology of the damaged tissue is crucial for the development of effective rehabilitation strategies post-injury. In the last few decades, a variety of lesion models have been established, in the attempt to reproduce different aspects of this pathological condition. In this study, a laser lesion model has been used, to investigate the cellular correlates of lesion-induced alterations in the mouse visual cortex.

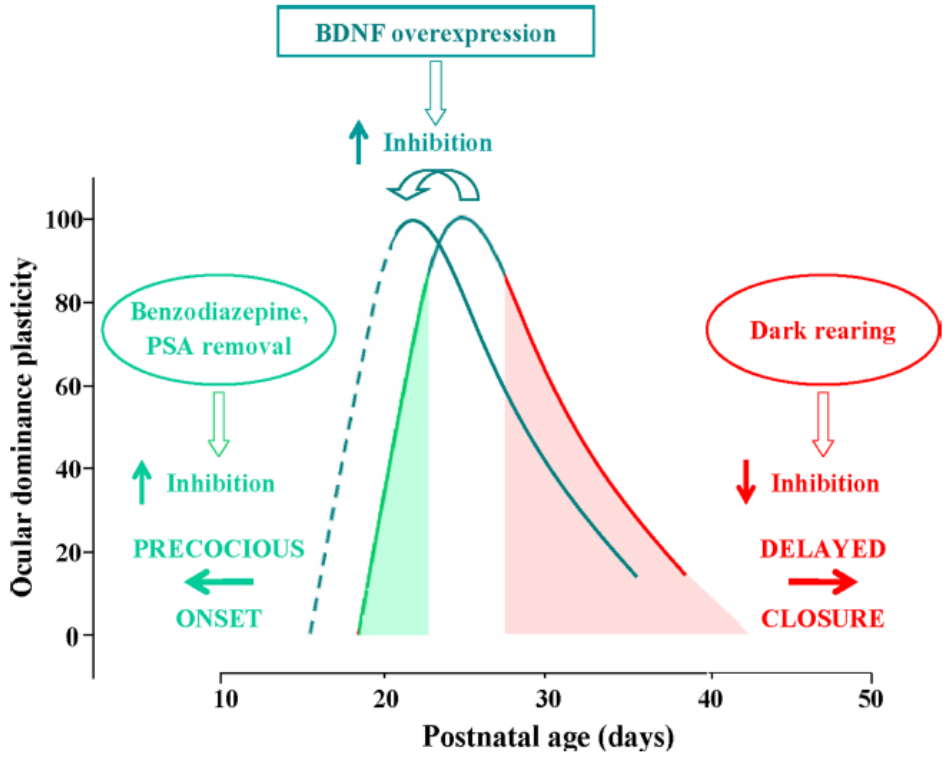

Fig. 1.6 Time course of the critical period (CP) for ocular dominance (OD) plasticity in response to monocular deprivation in rodents. OD plasticity is normalize to the CP peak's level.
CP is anticipated by increasing intracortical inhibition through Benzodiazepine treatment or Perineuronal network removal. Conversely, the end of the CP can be delayed by preventing the maturation of the GABAergic inhibition through dark rearing from birth. The effects of dark rearing can be counteracted by concomitant environmental enrichment. BDNF overexpression promotes a faster maturation of GABAergic interneurons, acting both on the onset and closure of the critical period, shifting leftward the plasticity curve (from Sale et al, 2010).

1.7 Focal laser lesion in the visual cortex

Lasers energy is commonly used in humans as treatment for injuries (Jallo et al, 2002). They have also been employed extensively in basic research to induce brain lesions, in order to study the reorganization of the neural circuits post-injury.

In the present study an "ex vivo in vitro" laser lesion model has been used in the visual cortex of mice. The laser lesion of the cortex, offers considerable advantages in comparison to other injury models: it is highly reproducible; it is a non invasive and highly precise procedure: the experimenter can deliver a circumscribed lesion of defined energy into the cortex through the intact dura mater and a thinly drilled skull. Moreover the border of the lesion is sharp and the gliosis reaction following the injury is spatially limited (for review see: Roll et al, 2012).

Different studies based on this lesion model have reported that laser-induced lesions in the rat visual cortex altered the synaptic plasticity in the vicinity of the injury (Imbrosci et al, 2010). These alterations in synaptic plasticity have been revealed mainly through in vitro electrophysiological techniques. The main findings were a facilitated long term potentiation (LTP) (Mittmann et Eysel, 2001) , associated with a modest increase in the intracellular Ca^{2+} concentration (Barmashenko et al, 2003), and an impaired long term depression (LTD) (Imbrosci et al, 2010). These plasticity changes seem to be, at least in part, mediated by the activity of NMDA receptor containing the NR2B subunits (Huemmeke et al 2004, Yan et al, 2012). Laser lesions were originally performed in cats. These experiments revealed a reorganization of receptive fields. A receptive field of a cell in the visual system is defined as the region of retina (or visual field), over which one can influence the firing of that cell. It is suggested that a structural reorganization of the cortical connections takes place after lesion (Eysel and Schweigart, 1999). Furthermore, it was demonstrated that laser lesions can trigger neurogenesis in the adult brain: An increase in proliferation neural stem progenitor cells (NSPCs) was detected within the lesion area (Sirko et al, 2009).

To date all lesions have been performed in cats and rats. However, this laser lesion model can be also transferred to the mouse cortex. Mouse models offer more advantages over the rat model due to the greater availability of genetically modified mouse models, which allow scientists to study the selective role of a single gene in response to lesions.

In the present study, the laser lesion has been perfomed for the first time in the mouse visual cortex.

1.8 Objectives of the study

The aim of the present thesis was to investigate the interplay between the BDNF-TrkB pathway and the GABAergic inhibition under physiological and pathophysiological conditions in the mouse visual cortex.

In the first part, the effects of the new recently identified TrkB receptor agonist, 7,8 Dihydroxyflavone (7,8-DHF), on synaptic transmission and on the intrinsic neuronal excitability were tested.

In the second part of this thesis, the lesion-induced alterations of the GABAergic transmission in WT and BDNF (+/−) mice were studied.

In particular, the following questions have been addressed:

- What is the contribution of BDNF/TrkB signalling to the functional reorganization of the GABAergic circuits post-lesion?
- Can the laser lesion model previously used in rats be transferred to mice?

The results could pave the way for the development of new therapeutical strategies for the recovery from a brain injury.

2. Materials and Methods

2.1 Animals

All experiments of the present study were performed in the mouse visual cortex.

C57BL/6 mice (n=62) at the age of P20-P24 days were used to investigate the effects of 7,8-DHF on GABAergic and glutamatergic synaptic transmission, and on the intrinsic neuronal excitability.

BDNF heterozygous (+/−) (n=57) and WT littermates (n=52) (P21- P27) were used to study lesion-induced alterations in GABAergic transmission.

Generation and genotyping of the BDNF (+/−) mice were described previously (Abidin et al, 2008, Korte et al, 1995). Briefly, in heterozygous knock-out mice one allele of the BDNF coding region is replaced by a neomycine-resistance gene (BDNF (+/−)) resulting in fully viable and fertile animals. Wild type littermates served as controls. The presence of the transgene was verified in each experiment by polymerase chain reaction (PCR) from tail tissue. PCR primers were used to recognize BDNF (5`-ACC ATA AGG ACG CGG ACT TGT AC-3`) and neomycine (5`GAT TCG CAG CGC ATC GCC TT-3`), while 5`-GAA GTG TCT ATC CTT ATG AAT CGC-3` was used as a reverse primer.

Since BDNF homozygous (−/−) mice usually die within the first 2 postnatal weeks, only BDNF heterozygous (+/−) mice of either sex were used for the experiments. BDNF (+/−) mice display deficits in feeding behaviour, aggressiveness and spatial learning (Kernie et al., 2000, Linnarsson et al., 1997; Lyons et al., 1999; Montkowski and Holsboer, 1997;), and show an impaired hippocampal and cortical LTP (Korte et al., 1995; Patterson et al., 1996). Despite these slight abnormalities they have normal growth, fertility and survival.

Mice were group-housed under standard conditions, with food and water available ad libitum and were kept on a 12-h day/night cycle.

In the lesion experiments the animals were divided in 4 groups: WT sham, WT lesion, BDNF sham, BDNF lesion.

2.2 In-vivo laser lesion in the mouse visual cortex

Infrared laser lesions were performed *in vivo* in the visual cortex of juvenile mice. BDNF (+/−) and WT littermates at the age of 21 days were anaesthetized by intraperitoneal injection

comprising a mixture of Ketamine (100 mg/kg) and Xilazine (8 mg/Kg). Subsequently, animals were fixed in a stereotaxic apparatus, and the skull was exposed and cautiously drilled above the right visual cortex parallel to the midline in a rectangular area 1 mm width beginning right anterior at the lambda suture and extending 3 mm towards the bregma without penetrating the dura mater. Cortical lesions were made through the translucent wet bone under visual control with a 810-nm infrared diode laser (OcuLight SLX, Iris Medical) attached to a binocular operating microscope (Leica, Germany). Multiple, partially overlapping round lesions (each 0.5 mm in diameter, 2 W intensity, ca. 4 s duration) were performed about 2 to 2.5 mm lateral from the midline in order to form an elongated lesion of 0.5 mm mediolateral width and 1 mm anteroposterior length starting anterior to the lambda suture in the visual cortex (areas V1M, V2ML V2MM). After surgery, the skin above the lesion was closed with histoacryl blue glue (Braun, Melsungen, Germany). The animals were carefully observed until they woke up, and post-surgery analgesia was performed if necessary. Age-matched sham-operated animals were treated similarly and served as control. In these animals after the skull opening no laser lesions were induced. The animals were sacrificed and the electrophysiological recordings were performed 3-6 days after lesion.

2.3 Electrophysiology

2.3.1 The patch clamp technique

The patch clamp technique is the method to studying the electrophysiological properties of biological membranes. It was established in the early 1980s by Erwin Neher and Bert Sakmann, who were awarded the Nobel Prize for it in 1991.

Patch clamp recordings are performed by employing a small glass micropipette, whose tip has a diameter of about 1 μm, permitting isolation of a small membrane surface area (“patch”) and measurement of the ionic currents flowing through the channels contained in the patch. The patch pipette is tipically filled with a specific intracellular solution and a chloride silver wire is placed in contact with this solution to conduct electric current to the amplifier.

Using this method, one can work either in voltage-clamp mode or in current-clamp mode. The voltage-clamp mode allows measurements of currents across the membrane by maintaining the transmembrane voltage constantly “clamped” to a command (or holding)

potential set by the experimenter. Alternatively, current-clamp mode allows to monitor changes in the membrane potential in response to defined current injection.

In order to record currents in the picoampere range with low background noise, it is necessary to form a high resistance seal ("Gigaseal", since the electrical resistance is in excess of $10^9\ \Omega$) between the pipette and the cell membrane. This is achieved by pressing the patch pipette against the cell membrane and by applying a gentle suction (negative pressure) to facilitate the adhesion of the pipette tip to the membrane. The result is the so called "cell attached", which is the precursor of all other patch clamp configurations. "Inside-out" and "Outside-out" configurations are called "excised patch techniques", because the membrane patch is excised (removed) from the main body of the cell. In general cell-attached and both excised patch techniques are employed to study the behaviour of individual ion channels in the section of membrane attached to the pipette. Whole-cell allows studying the electrical behaviour of the entire cell, instead of only single channels conductances.

In cell-attached configuration (Fig. 2.1a), the pipette is sealed to the patch of the membrane and the cell maintains its integrity. This allows the recordings of currents through single ion channels present in that particular patch, without disrupting the interior of the cell. The inside-out configuration (Fig. 2.1c) can be obtained by swiftly withdrawing the pipette from the cell, thus detaching the patch from the cell body, leaving it attached to the pipette and exposing the intracellular side of the membrane to the external bath solution. This configuration can be useful for example to study ion channels, which are activated by intracellular ligands. As an alternative to excising the patch, one can simply break the patch by applying a pulse of suction, thereby creating a hole in the membrane. The electrode is left in place on the cell and makes contact with the interior of the cell. This configuration is called "whole-cell" (Fig. 2.1b) and it is characterized by low access resistance (R_a= it is the resistance through the pipette to ground, which is affected by the geometry of the pipette in addition to anything that impedes current flow through the pipette tip, such as air bubbles, poorly conducting solution, debris in the bath, cellular debris, *etc.*), which enables voltage-clamping the whole cell. From the whole-cell configuration the pipette may be slowly withdrawn from the cell. This usually results in resealing of both the membrane of the cell and the patch at the pipette tip. After the patch excision the outside of the membrane will be facing the bath solution, leading to the "outside-out" configuration (Fig. 2.1d). This is usually used to examine the properties of an ion channel when it is isolated from the cell, and exposed to different solutions on the extracellular surface of the membrane.

All patch clamp recordings in the present study were performed in the whole cell configuration.

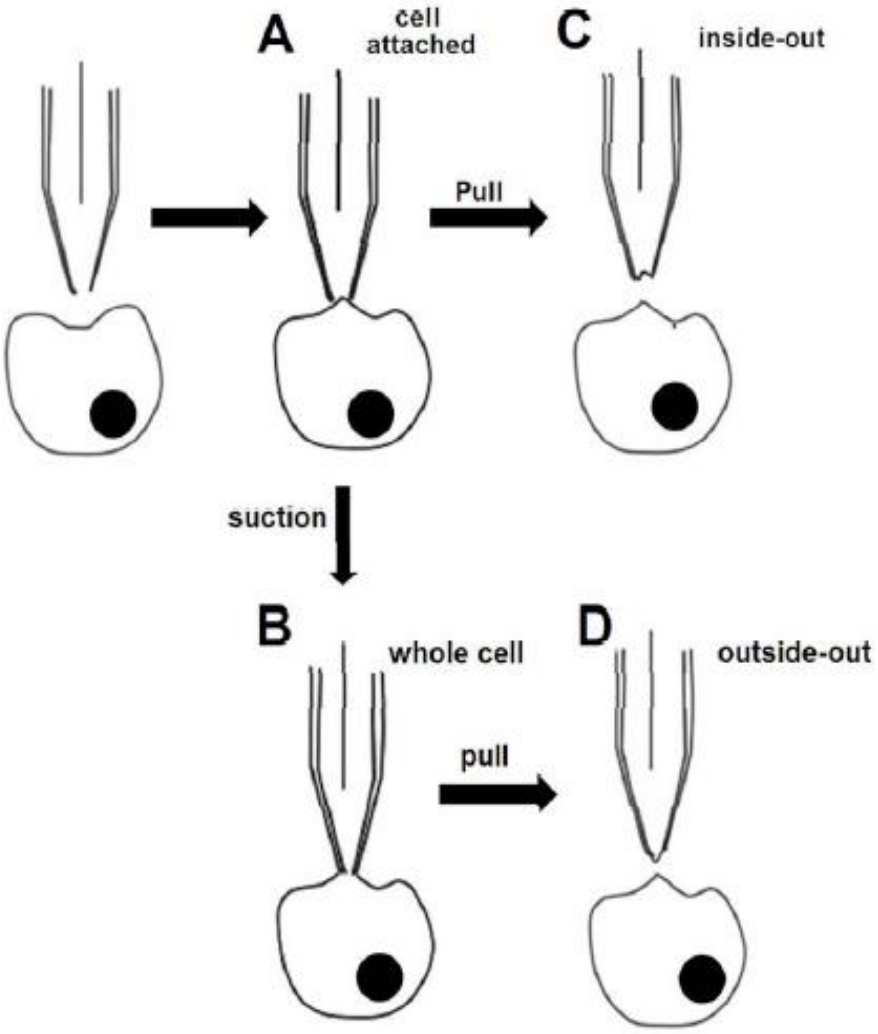

Fig 2.1 Schematic representations of patch clamp configurations
a) Cell –attached configuration: the electrode is tightly sealed to the cell membrane. A gentle suction facilitates the adhesion of the electrode tip to the membrane, allowing the establishment of the Gigaseal. Cell-attached is the precursor of all other patch clamp configurations. b) Whole-cell configuration: this configuration is achieved by applying a suction through the pipette, which leads to a rupturing of the plasma membrane, putting the pipette in direct contact with the interior of the cell. c) Inside-out configuration: After the formation of the Gigaseal, the pipette is quickly withdrawn from the cell, detaching the patch from the cell body, which remains attached to the pipette, and thus exposing the intracellular side of the membrane to the external bath solution. d) Outside-out configuration: from the Whole-cell configuration the pipette can be slowly withdrawn from the cell, which allows the outside of the membrane to face the external bath solution (from Yajuan et al., 2012).

2.3.2 Acute slices preparation

Mice were deeply anesthetized with isoflurane and decapitated. The brains were quickly removed and transferred into ice-cold artificial CerebroSpinal Fluid (aCSF) containing (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.5 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4 and 25 D-glucose (PH 7.4) and bubbled with 95% O_2 and 5% CO_2. Coronal slices of 300 µm thickness containing the visual cortex were prepared using a vibratome (VT 1000 S; Leica, Germany). The slices were allowed to recover in a submerged holding chamber with oxygenated aCSF at room temperature for at least 1h. Subsequently, single slices were transferred into a standard submerged recording chamber, superfused with oxygenated aCSF at 32 ± 2 ºC. The recording chamber was mounted on an upright microscope (Olympus-BX51WI, Olympus, Japan), equipped with 4X and 40X water immersion type objectives.

2.3.3 The patch clamp setup

Patch clamp experiments in this study were carried out using an upright microscope (Olympus BX51-WI, Olympus, Japan), placed on a vibration isolation table and enclosed in a Faraday cage. The microscope was equipped with differential interference contrast (DIC) and with 4X dry- and 40X water immersion objectives (Olympus) to visualize the brain slices. Video microscopy was performed with a CCD camera (Pco., Pixelfly), and the images were displayed on a connected monitor (Dell). The 40X water immersion objective allowed the identification of neurons in the tissue and the pyramidal shaped-soma, characteristic of pyramidal neurons, could easily be recognized.

The position of the recording pipette was controlled with the micromanipulator (MP-285, Sutter instrument). An amplifier (Multiclamp 700A, axon instrument) was used to record the electrophysiological signal. The aCSF was perfused into a submerged recording chamber through a tube pump system (minipulse 3, Gilson company, France). The aCSF was continuously bubbled with 95% oxygen and 5% carbon dioxide. The temperature of the aCSF was controlled by a waterbath (Lauda, ET20, Germany). During the experiments the temperature was kept constant at 32 ± 2 °C. After 1h incubation, single brain slices were transferred from the storage chamber to the recording chamber, placed on a nylon net and fixed with a thin silver wire.

The electrical signals were monitored with an oscilloscope (54602B, Hawlett Packard), digitized and filtered by an AD/DA board (Digidata 1322A, Axon Instruments) and stored

on a PC. pClamp9 (Axon Instruments) software was used for data acquisition and analysis. Synaptic stimulation was delivered through an extracellular glass electrode (resistance 3-5 MΩ), connected to a stimulation unit (Stimulus isolator A365, WPI, USA).

2.3.4 Glass micropipettes production

Patch pipettes were pulled from borosilicate glass capillaries (GB 150F-8P; Science Products, Germany), using a horizontal micropipette puller (DMZ-Universal puller, Zeitz Instr. Germany). The tips of the pipettes were heat polished, with an additional step, to improve the seal formation. The resistance of the pipettes for patch clamp recordings was in the range of 4-6 MΩ. The same procedure was used to produce glass electrodes for extracellular stimulations.

2.3.5 Intracellular solutions

Patch pipettes were filled with chemical solutions resembling the intracellular ionic composition. This is a crucial factor because after the cell opening the intracellular solution diffuses through the citosol diluting the intracellular content. Different intracellular solutions were used, depending upon the experimental purpose. For recordings performed in current-clamp mode the pipette solution contained (in mM): 140 K-gluconate, 8 KCl, 2 MgCl2, 4 Na2-ATP, 0.3 Na2-GTP, 10 Na-phosphocreatin and 10 HEPES. The intracellular solution used to measure miniature inhibitory postsynaptic currents (mIPSCs) contained (in mM): 120 CsCl, 2 $MgCl_2$, 2 $CaCl_2$, 2 Mg-ATP, 0.3 Na-GTP, 10 Hepes, 10 EGTA. Miniature excitatory postsynaptic currents (mEPSCs) and evoked postsynaptic currents were recorded by use of an intracellular solution containing (in mM): 125 Csgluconate, 5 CsCl, 10 EGTA, 2 MgCl2, 2 Na2-ATP, 0.4 Na2-GTP, 10 HEPES and 5 QX-314. The pH was always adjusted to 7.3 with either KOH or CsOH. QX-314 (a Na^+ channels blocker) was added to the intracellular solution to prevent the induction of action potentials upon electrical stimulation.

2.3.6 Performing a patch clamp recording

After optical identification of a cell of interest, a patch pipette was filled with the appropriate intracellular solution, fixed on the headstage and lowered under visual control into the bath. Positive pressure was continuously applied to the patch pipette, in order to keep the pipette tip clean. A chloride silver wire, immersed in the solution of the recording chamber, was

connected to the headstage and served as a ground electrode. Another chloride silver wire, in contact with the intracellular solution, transmitted the electric signals from and to the amplifier (Multiclamp 700A, axon instrument). This electrode was used as a voltage sensor as well as current-passing electrode.

A patch clamp amplifier operates as a "differential amplifier": it monitors if the cell membrane potential (V_m), detected by the recording electrode (relative to the ground electrode) is equal to the command (holding) potential (V_c) set by the experimenter. The current, flowing through the membrane ion channels, causes a change in the cell membrane potential (V_m), generating a potential difference (V_m- V_c) across the cell membrane. To compensate this potential shift, and to bring the membrane potential (V_m) to the command potential (V_c), the amplifier sends an output current through the recording electrode (which now works as current source) into the cell. This is the current measured during a patch clamp experiment in voltage clamp mode.

When the pipette entered the bath solution the pipette offset was set to zero to eliminate liquid junction potentials between the bath solution and solution inside the pipette. The resistance of the pipette was monitored by applying repetitive 3 mV pulses. As soon as the patch pipette reached the surface of the cell of interest, the positive pressure was quickly removed and replaced with a small negative pressure to establish a high resistance seal (Gigaseal). Pipette capacitance transients were erased using the fast compensation adjustment of the amplifier, and the holding potential was set. Disruption of the cell membrane, by applying a strong and short negative pressure into the pipette, led to the whole-cell configuration. After compensation of the whole-cell capacitance and the series resistance, the recordings started.

In this study, intrinsic membrane properties were investigated in current clamp mode. All other electrophysiological recordings were conducted in voltage clamp mode.

2.3.7 Data acquisition

Electrophysiological current signals are converted in the headstage to voltage and amplified via the amplifier Multiclamp 700A amplifier (Axon Instruments, USA) and transmitted as analogue voltage-signals. Initially the electrical signals were visualized with an oscilloscope (54602B, Hawlett Packard). The data were filtered at 2 kHz and digitized at 4 kHz using an analogue-to-digitial/digital-to analogue transducer (AD/ DA transducer; Digidata-1322A system, Axon Instruments). The Digidata was connected to a computer, where the raw data were stored and displayed via the PClamp 9.2 software (Molecular Devices, Sunnyvale, CA,

USA). This software was also exploited to control intracellular current injections and extracellular stimulations. The digital signals were inversely converted into analogue signals by the Digidata and then sent to either the patch electrode for intracellular stimulation or to a stimulation unit finally connected to an extracellular stimulating electrode placed in the region of interest on the brain tissue.

2.3.8 Design of the experiments

Whole-cell patch-clamp recordings were performed from layer 2/3 pyramidal neurons in the visual cortex under visual guidance with DIC optics and a 40X objective (Olympus 40X). The recordings were always started five minutes after establishing the whole cell configuration. Passive membrane properties and firing behaviour of neurons were studied in current-clamp mode by injecting hyperpolarizing and depolarizing current steps through the recording electrode, starting with a current step of -20 pA, then gradually increased the magnitude in steps of 20 pA.

All other experiments were performed in voltage-clamp mode. Voltage-clamp recordings were not corrected for liquid junction potentials and series resistance. Only cells with high seal resistance of > 1 GΩ and a series resistance of < 25MΩ were included in the analysis. Series- and input-resistance were controlled before and after each recording, and the cells were discarded if one or both parameters changed more than 20% throughout the experiment.

It was recorded from one single neuron in each brain slice, 3 to 5 slices per animal and at least 3 animals were used for each recording parameter and each experimental group. Finally, the mean of all recorded neurons was calculated.

2.3.9 Isolation of excitatory and inhibitory postsynaptic currents

Type-A γ-aminobutyric acid receptor ($GABA_AR$)-mediated IPSCs were pharmacologically isolated by bath application of the *N*-methyl-D-aspartic acid (NMDA) receptor blocker D-2-amino-5-phosphonopentanoic acid (DAP-5) (25 μM) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 μM). AMPA-mediated currents were pharmacologically isolated by bath application of the $GABA_A$ receptor antagonist picrotoxin (PTX) (100 μM) and D-AP5 (25 μM). NMDA-mediated currents were isolated by bath application of PTX (100 μM), and DNQX (20 μM). $GABA_AR$ and AMPAR mediated currents were acquired at the holding

potential of -80 mV, while NMDAR mediated currents were measured at the holding potential of +40 mV. To measure mIPSCs and mEPSCs, Tetrodotoxin (TTX) (1 μM) was added to the aCSF solution and recordings were performed for at least 5 minutes.

2.3.10 Stimulation protocols

Evoked postsynaptic currents were elicited by stimuli of 100 μs duration through an extracellular glass electrode (resistance 3-5 MΩ). The stimulation electrode was placed in layer 4 to activate afferent axonal fibres projecting onto the recorded pyramidal neuron in layer 2/3.

To precisely compare the evoked signals between different experimental groups, the stimulus intensity was adjusted to yield a response with similar amplitude (100 ± 20 pA). Paired-pulse ratios (PPRs) were derived from two consecutively evoked responses with interstimulus intervals (ISIs) of 30, 50 and 100 ms. An input–output (I/O) curve was created by firstly using a stimulation intensity, which induced a maximal amplitude response. Next, the stimulus duration was gradually reduced in steps of 20 μs from 200 to 20 μs to reveal the I/O-curve.

2.3.11 Analysis of electrophysiological data

PClamp 9.2 software was used for off-line analysis.

Miniature IPSCs and EPSCs (mIPSCs, mEPSCs): mIPSCs and mEPSCs were semi-automatically identified by Clampfit 9.2 and further validated through a careful visual inspection. Frequency and amplitude of mISPCs were calculated as the median of 600 events for each cell. Only those events with a clear signal to noise ratio, a defined baseline and smooth rising phase were used for the analysis.

Paired Pulse Ratio (PPR): The PPR of evoked IPSCs and EPSCs was calculated as the ratio between the peak amplitude of the second response to the first one. Here, averages of six to eight consecutive responses were used , which were repeated every 10 s. PPR represents a useful parameter to estimate the neurotransmitter release probability.

Kinetics of postsynaptic currents: The rise time of postsynaptic currents was calculated between 10% and 90% of the peak amplitude onset, while the decay time constant was obtained by fitting the decaying current to the following monoexponential function: $\mathbf{f(t) = Aie^{-t/\tau i} + C}$.

2.3.12 Drugs

Picrotoxin (PTX) (100 µM), d-(–)-2-amino-5-phosphonopentanoic acid (D-AP5) (25 µM), 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM), 7,8-Dihydroxyflavone (7,8-DHF), K252a (200nM) were obtained from Tocris Biozol (Eching, Germany); Tetrodotoxin (TTX) (1 µM) was from (ICS Munich, Germany), *N*-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314) (5 mM) was from Sigma-Aldrich (Munich, Germany). The specific concentrations of PTX, D-AP5, DNQX and K252a were chosen based on the fact that they selectively and completely blocked $GABA_A$, NMDA, AMPA or TrkB receptors activity. 7,8-DHF was dissolved in the organic solvent Dimethylsulfoxide (DMSO) and diluted with aCSF before application. When slices were incubated in 20 µM 7,8-DHF, the final (highest) DMSO concentration reached 0.1 %. aCSF containing a similar concentration of DMSO alone served as control for potential unspecific effects of the organic solvent.

2.3.13 7,8 DHF in vitro and in vivo treatment

The effects of 7,8 DHF on synaptic transmission and intrinsic neuronal excitability were investigated both in vitro and in vivo.

For in vitro experiments slices were incubated for 30’ in 20 µM 7,8-DHF before every electrophysiological recording.

For in vivo experiments 7,8-DHF was dissolved in phosphate-buffered saline (PBS) containing 17% dimethylsulfoxide (DMSO). Both WT (P14) and BDNF (P 21) received repeated intraperitoneal injections of 5 mg/kg 7,8-DHF or 17% DMSO vehicle once daily for 7 consecutive days. The last administration was performed 1h before the preparation of acute slices.

2.4 Histology

2.4.1 Nissl staining

Nissl staining is a method established by the German neuropathologist Franz Nissl (1860-1919). It is used to visualize morphological features of neurons. The method exploits the properties of some basic dyes such as cresyl violet, toluidinblue, thionine or aniline, which

bind and stain in blue/violet the negatively charged RNA and DNA present in the nuclei and in the ribosoms of neurons. Nissl stainings were performed in a population of slices for the morphological characterization of the lesion and to control the quality of the tissue.
The Nissl staining procedure consists of the following steps:

- Prepare acute slices (300 μm thickness) with a vibratome (VT 1000 S; Leica, Germany).
- Put the slices in fixative solution 4% PFA (Paraformaldehyde) overnight.
- Transfer the slices into a crioprotective solution, sucrose 30% (in phosphate buffer saline solution, PBS 0.1 M, pH 7.4).
- Coronal sections containing the visual cortex (30 μm thickness) were cut on a freezing microtome and collected in PBS, 0.1M.
- Wash slices 2X in PBS
- Mount slices on gelatine coated objective glass holders and let them completely dry
- Put objective containing the slices in alcohol gradient solutions: 30% EtOH-5 min, 50% EtOH-5min, 70% EtOH-5min, 100% EtOH-5min.
- Put the objectives in 0.1 % cresyl violet dye (Sigma Aldrich) based solution for 5-10 min
- Wash 3-5x the slices with distillate water till the tissue colour lightens.
- Let the objectives dry and store them at 4 ˚C in the refrigerator.
- Visualize the slices under the microscope

2.5 BDNF quantification

The visual cortex of BDNF (+/−) (P24) and WT littermates (P24) 3 days postlesion were excised, shock frozen in liquid nitrogen and stored at -80 °C. The amount of BDNF protein was determined by using the BDNF Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany). The samples were processed according to the kit instructions.

2.6 Statistical analysis

Parametric Student's *t* test was performed for statistical evaluation of the data between control and 7,8 DHF-treated groups. Quantitative differences between the WT sham, WT lesion, BDNF sham and BDNF lesion were evaluated statistically by one-way variance

(ANOVA), followed by post-hoc Fischer LSD test using SPSS software (version 15.0.0, Chicago USA). Results are presented as mean ± standard error of the mean (S.E.M.). The level of significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) is indicated by asterisks in each figure.

3. Results

3.1 Modulatory effects of the novel TrkB receptor agonist 7,8-Dihydroxyflavone on synaptic transmission and intrinsic neuronal excitability *in vitro*

3.1.1 7,8-DHF depresses GABAergic transmission

7,8-DHF attracted much attention for its ability to induce a robust activation of TrkB receptors and their downstream signalling pathways (Jang et al, 2010). Over the last few years this molecule has been the object of extensive studies, which have demonstrated its great therapeutic potential. Surprisingly, so far there is only little experimental data on the effects of 7,8 DHF on neurotransmission (Zeng et al., 2011; 2012). To bridge this gap, patch clamp recordings from pyramidal neurons in the layer 2/3 of the mouse visual cortex were performed. In line with previous studies (Jang et al., 2010; Mantilla and Ermilov, 2011; Zeng et al., 2011), acute slices were pre-incubated for 30 minutes in presence of 7,8 DHF before starting the experiment. Untreated slices were used as controls.

Initially the impact of 7,8 DHF on the GABAergic transmission was investigated. In order to quantify GABAergic inputs converging onto pyramidal neurons, the amplitude of the maximal evoked inhibitory postsynaptic currents (eIPSCs), elicited by layer 4 stimulation, was determined using an I/O-relationship. It represents a reliable index of the overall strength of the GABAergic system. In control slices a maximal eIPSCs amplitude of 500.2 ± 48 pA was yielded (Fig. 3.1A and 3.1B).

Various differing 7,8-DHF concentrations were tested. As shown in Fig. 3.1C, 7,8-DHF (1-40 µM) reduced the maximal amplitude of eIPSCs in a dosage-dependent manner. At concentrations of 1-10 µM no significant changes were observed (1 µM: 515.8 ± 55 pA, n=10 from 3 animals; 2 µM: 473.4 ± 40.3 pA, n=10 from 3 animals; 5 µM: 395.9 ± 11.1 pA, n=10 from 3 animals; 10 µM: 382 ± 43.4 pA, n=13 from 3 animals; $P > 0.05$). A significant reduction in eIPSCs amplitude was detected both at 20 µM (% of the control) 48.2 ± 8.9 (241.1 ± 21 pA, n = 12 from 4 animals, $P = 0.0001$) and at 40 µM, 50 ± 13.7 (250.2 ± 34 pA, n = 10 from 4 animals, $P = 0.001$). Since the concentration of 40 µM produced no further

reduction in the eIPSC amplitude, the concentration of 20 µM was used for all following experiments.

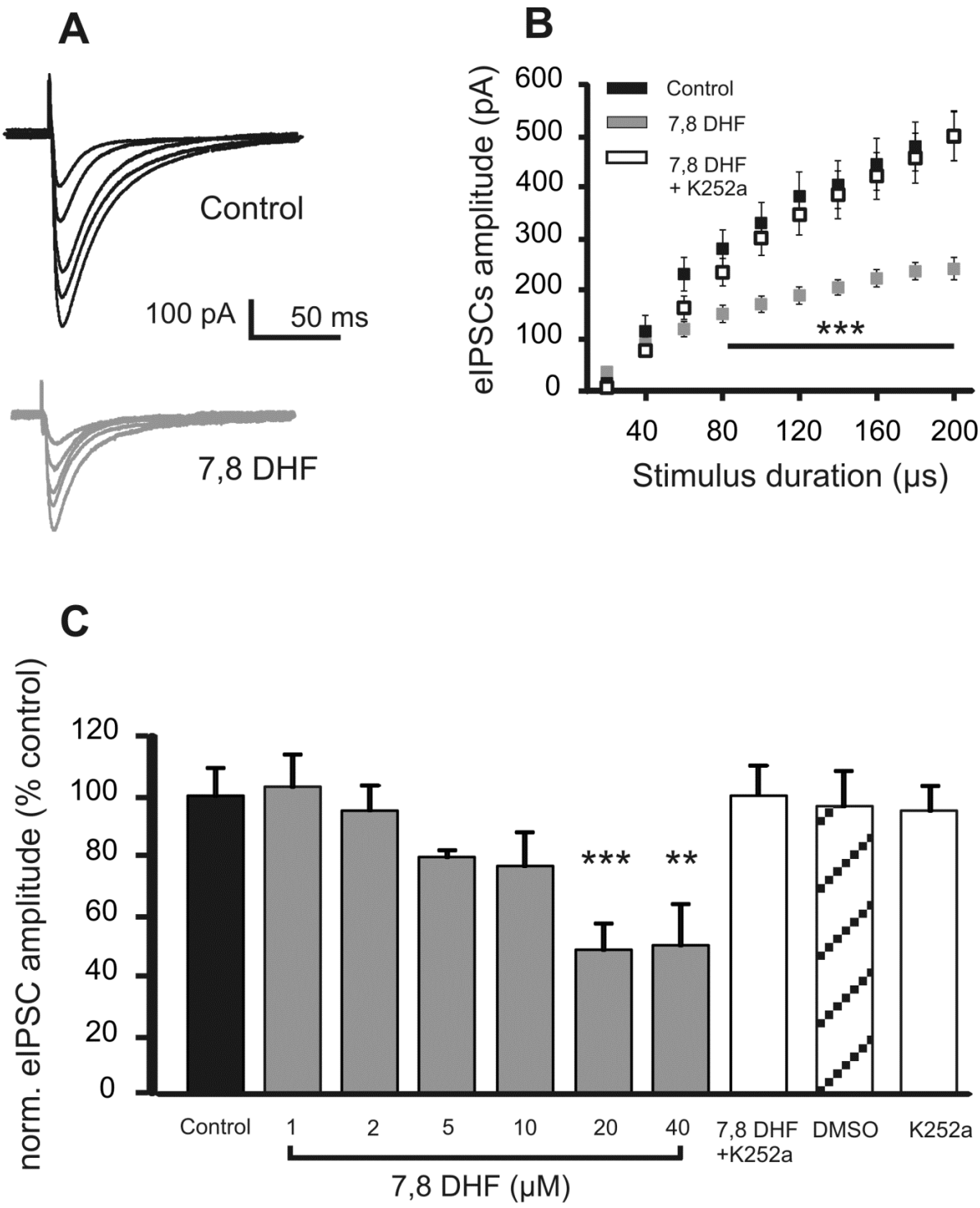

Fig. 3.1: 7,8 DHF reduced the amplitude of $GABA_A$ receptor-mediated eIPSCs.
a) Representative traces of maximal eIPSCs recorded in normal aCSF (black traces) or in presence of 7,8 DHF (20 µM, grey traces) at different stimulus durations (40, 80, 120, 160, 200 µs). b) Input-output curves of eIPSCs recorded in neurons in normal aCSF (control), 7,8 DHF or in 7,8 DHF + K252a treated neurons. c) Summary bar plot showing the concentration dependent inhibitory effect of 7,8 DHF on eIPSCs.

To ascertain if the effect of 7,8-DHF on the amplitude of eIPSCs was mediated by TrkB receptors, the slices were co-incubated with the TrkB receptor antagonist K252a (200 nM). In the presence of K252a, the effect of 7,8-DHF on eIPSCs was completely eliminated. The amplitude of the eIPSCs was 100.1 ± 9.8 (% of the control) (500.8 ± 49 pA, n = 12 from 4 animals, P > 0.05). As an additional control, the slices were incubated in the isolated presence of the vehicle DMSO or K252a, in order to exclude any possible modulatory effects of these two compounds on synaptic transmission. Neither DMSO (96.5 ± 12 % of the control; 488.8 ± 43 pA, n = 12 from 4 animals, P > 0.05) nor K252a (94.7 ± 8.8 %; n = 10 from 3 animals, P > 0.05) exerted a significant effect on the eIPSCs amplitude (Fig. 3.1C). These results indicate a direct involvement of TrkB receptors in the action of 7,8 DHF.

To gain more insights into the effects of 7,8-DHF on GABAergic synaptic transmission, mIPSCs were recorded (Fig 3.2A). The mIPSCs amplitude was unaltered (control 18.0 ± 0.9 pA, n = 20; 7,8-DHF 18.4 ± 1.1 pA, n = 17; P >0.05) (Fig. 3.2B, 3.2D), whereas the mIPSCs frequency was significantly decreased in 7,8-DHF-treated slices in comparison with controls. (10.7 ± 0.7 Hz; n = 20 from 5 animals) (Fig. 3.2C). This finding was confirmed by the analysis of the cumulative probability of inter-event intervals (Fig. 3.2E), which revealed a rightward shift in the curve for the slices treated with 7,8-DHF. When slices were co-incubated with K252a, no difference in the frequency of mIPSCs could be observed (10.0 ± 0.5; n = 13 from 3 animals, P > 0.05). These results indicate that (1) 7,8-DHF reduces the basal GABAergic transmission, and (2) changes in frequency, but not in amplitude, suggest that this reduction is most likely due to changes in the presynaptic GABA release.

To further investigate potential changes in presynaptic GABAergic function, the paired-pulse ratio (PPR) of two consecutive eIPSCs, evoked by two successive stimuli at given interval (30, 50, 100 ms) was analysed (Fig. 3.3A). In agreement with my hypothesis, the PPR was significantly larger in presence of 7,8-DHF (30 ms: control 0.64 ± 0.05, n = 19 from 5 animals; 7,8-DHF 0.98 ± 0.07, n = 14 from 4 animals, P = 0.001; 50 ms: control 0.69 ± 0.05, n = 19; 7,8-DHF 0.98 ± 0.08, n = 14, P = 0.006; 100 ms: control 0.69 ± 0.05, n = 19; 7,8-DHF 0.98 ± 0.08, n = 14, P = 0.015). Once again, co-incubation with K252a completely blocked the effect of 7,8-DHF. The PPR in slices co-treated with K252a and 7,8-DHF was indeed indistinguishable from control values (30 ms: 0.69 ± 0.04, n = 16 from 4 animals; 50 ms: 0.71 ± 0.04, n = 16; 100 ms: 0.65 ± 0.02; P > 0.05) (Fig. 3.3B). These results corroborate the idea of a 7,8-DHF-induced suppression of presynaptic GABA release.

Subsequently, I tested whether 7,8-DHF affects the functional properties of $GABA_A$ receptors, by examining the kinetics of eIPSCs (Fig. 3.3C). The rise time of the currents was significantly increased (control: 2.11 ± 0.2 ms, n = 16 from 4 animals; 7,8-DHF: 3.12 ± 0.3 ms, n = 16 from 4 animals; P = 0.02) (Fig 3.3D). Moreover, a prolonged decay-time constant of eIPSCs in 7,8-DHF treated slices was detected (control: 14.3 ± 1 ms, n = 17; 7,8-DHF: 21.9 ± 2.4 ms, n = 16; P = 0.009) (Fig. 3.3E). The effects were not detected in slices co-incubated with K252a (rise time: 2.03 ± 0.2 ms, n = 13; decay time: 14.9 ± 1 ms, n = 13; P> 0.05) (Marongiu et al, 2013).

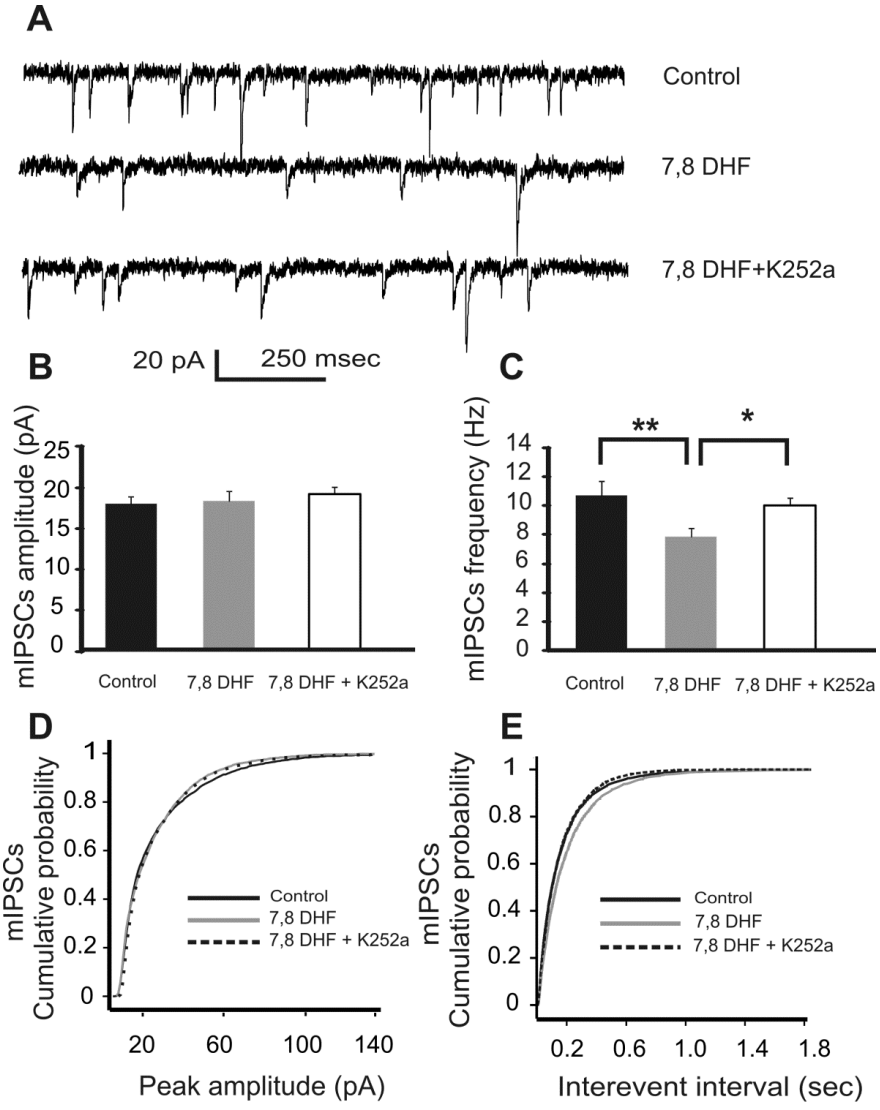

Fig. 3.2: 7,8 DHF decreased the frequency of mIPSCs a) Representative traces of mIPSCs recorded from cells in normal aCSF (control) and in presence of 7,8 DHF or 7,8 DHF+K252a. b, c) Bar graphs showing mean amplitude and frequency of mIPSCs. d,e) Cumulative probability plots showing peak amplitude and inter-event intervals of mIPSCs.

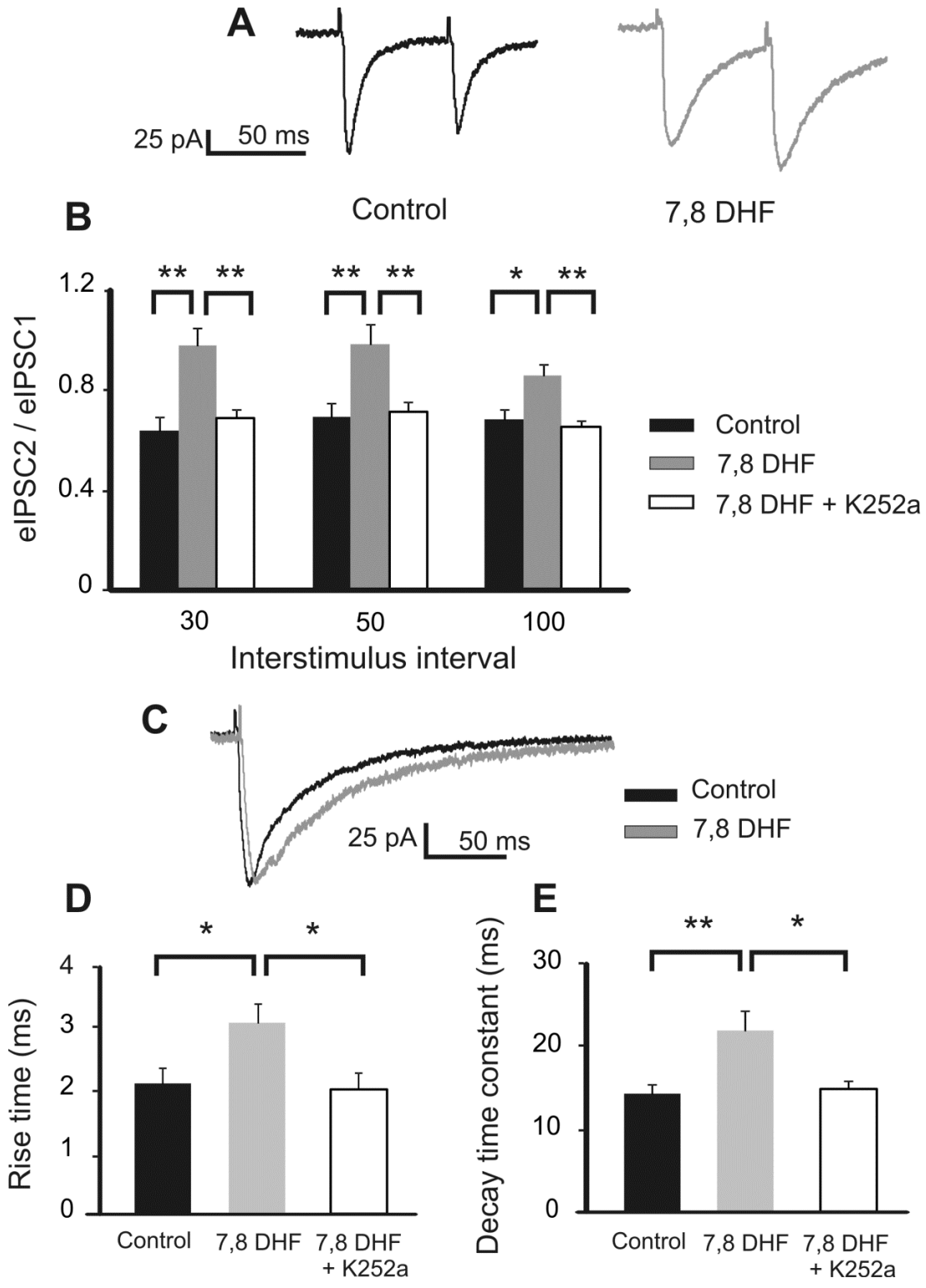

Fig. 3.3: Effect of 7,8 DHF on evoked GABA-A receptor mediated currents
a) Representative traces of $GABA_AR$-mediated currents evoked by pairs of synaptic stimulation with an inter-stimulus interval (ISI) of 50 ms in control and in 7,8 DHF-treated neurons. b) Summary diagram of the mean paired-pulse ratio (PPR) at different ISIs (30 ms, 50 ms and 100 ms) c) Superimposed traces of single eIPSCs recorded under control conditions and in presence of 7,8 DHF. d,e) Bar plots showing the means values of rise-time and decay-time constants of eIPSCs.

3.1.2 7,8-DHF does not affect the glutamatergic transmission

Activation of TrkB receptors also plays an important role in modulating the function of glutamatergic synapses (Carvalho et. al, 2008). In order to investigate whether 7,8-DHF affects the basal glutamatergic transmission, AMPAR-mediated mEPSCs were recorded (Fig 3.4A). Application of 7,8-DHF (20 µM) altered neither the amplitude (control 10.68 ± 0.3, n = 16 from 4 animals; 7,8-DHF 11.20 ± 0.3, n = 14 from 4 animals; P > 0.05) (Fig. 3.4B, 3.4D) nor the frequency of mEPSCs (control 11.48 ± 0.7, n = 15; 7,8-DHF 12.29 v 0.7, n= 13; p> 0.05) (Fig. 3.4C, 3.4E).

For the next step AMPAR-mediated evoked EPSCs (eEPSCs) were analyzed (Fig. 3.5A). The PPR (Fig. 3.5B), calculated from two consecutive evoked EPSCs (eEPSCs) at different interstimulus intervals (ISIs), (30 ms: control 1.41 ± 0.04, n = 16 from 5 animals ; 7,8-DHF 1.49 ± 0.05, n = 14 from 4 animals; 50 ms: control 1.48 ± 0.08, n = 16; 7,8-DHF 1.44 ± 0.08, n = 14, 100 ms: control 1.34 ± 0.06, n = 16; 7,8-DHF 1.35 ± 0.08, n = 14, P > 0.05), as well as the I/O-curve of eEPSCs were also unaltered. The maximal amplitude of the eEPSC in the control group was not different from the amplitude recorded in the 7,8-DHF-treated neurons (control: 1302.7 ± 143.8 pA, n = 11 from 4 animals; 7,8-DHF: 1334 ± 161.7 n=12 from 4 animals; P > 0.05; Fig. 3.5F, 3.5G). Moreover, no changes could be detected in the kinetics of the AMPA receptors (Fig. 3.5C). Both, rise time (control: 2.57 ± 0.2 ms, n = 20 from 5 animals, 7,8-DHF: 2.58 ± 0.2 ms, n = 20 from 4 animals; P > 0.05) (Fig. 3.5D) and decay-time constant (control: 7.18 ± 0.4 ms, n = 20, 7,8-DHF: 7.14 ± 0.3 ms, n = 20; P > 0.05) (Fig. 3.5E) were similar to controls values. These results strongly indicate, that 7,8-DHF does not exert any effect on presynaptic glutamate release and the basal function of AMPA receptors.

Several studies (Carmignoto et al., 1997; Kim et al., 2006; Kolb et al., 2005; Legrand et al., 2005; Michel et al., 2006) have reported that NMDA receptors represent another important downstream target of the TrkB signalling pathway. To shed light on the action of 7,8-DHF on NMDA receptor function, an input-output relationship of evoked NMDA-mediated currents was recorded. The peak amplitude of evoked NMDA-mediated currents in control slices was not significantly different from the peak magnitude measured in 7,8-DHF-treated slices (control: 391.9 ± 44.7 pA, n = 13 from 4 animals; 7,8-DHF: 399.5 ± 56.5, n = 10 from 3 animals; P > 0.05) (Fig. 3.6 C-D). The kinetics of evoked NMDA-mediated currents was also unaffected (control: 151.7 ± 13 ms, n = 15 from 4 animals, 7,8-DHF: 159.1 ± 14.4 ms, n = 10 from 4 animals; P > 0.05) (Fig. 3.6A, 3.6B). Taken together, these data suggest that 7,8-DHF does not affect the function of the glutamatergic neurotransmission (Marongiu et al, 2013).

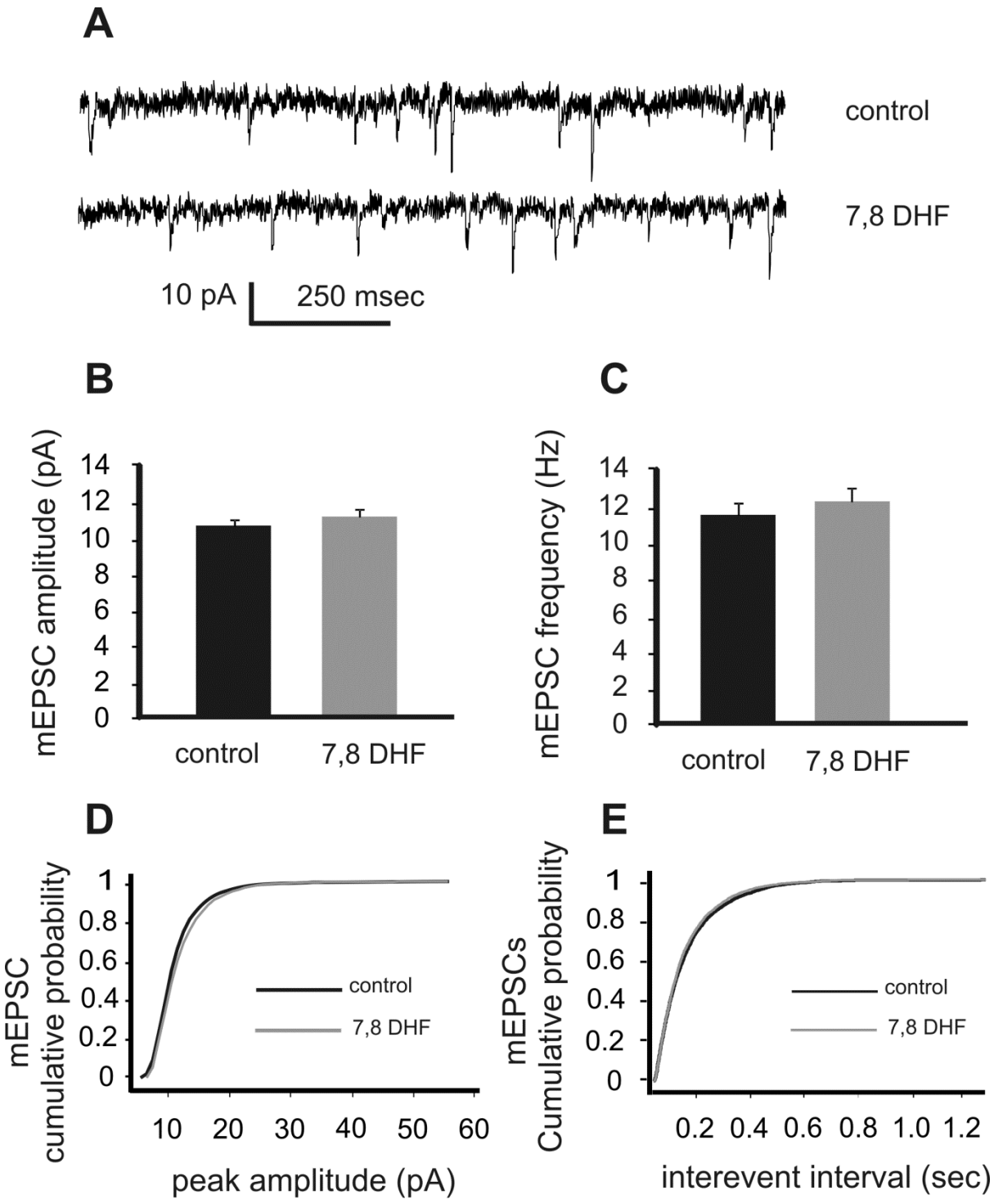

Fig. 3.4: No effect of 7,8 DHF on AMPAR mediated mEPSCs
a) Sample traces of AMPAR-mediated mEPSCs recorded in normal aCSF (control) and in presence of 7,8 DHF. (b,c) Bar charts showing amplitude and frequency of mEPSCs in control and in presence of 7,8 DHF d,e) Cumulative probability plots showing the peak amplitude and inter-event interval of the mEPSCs.

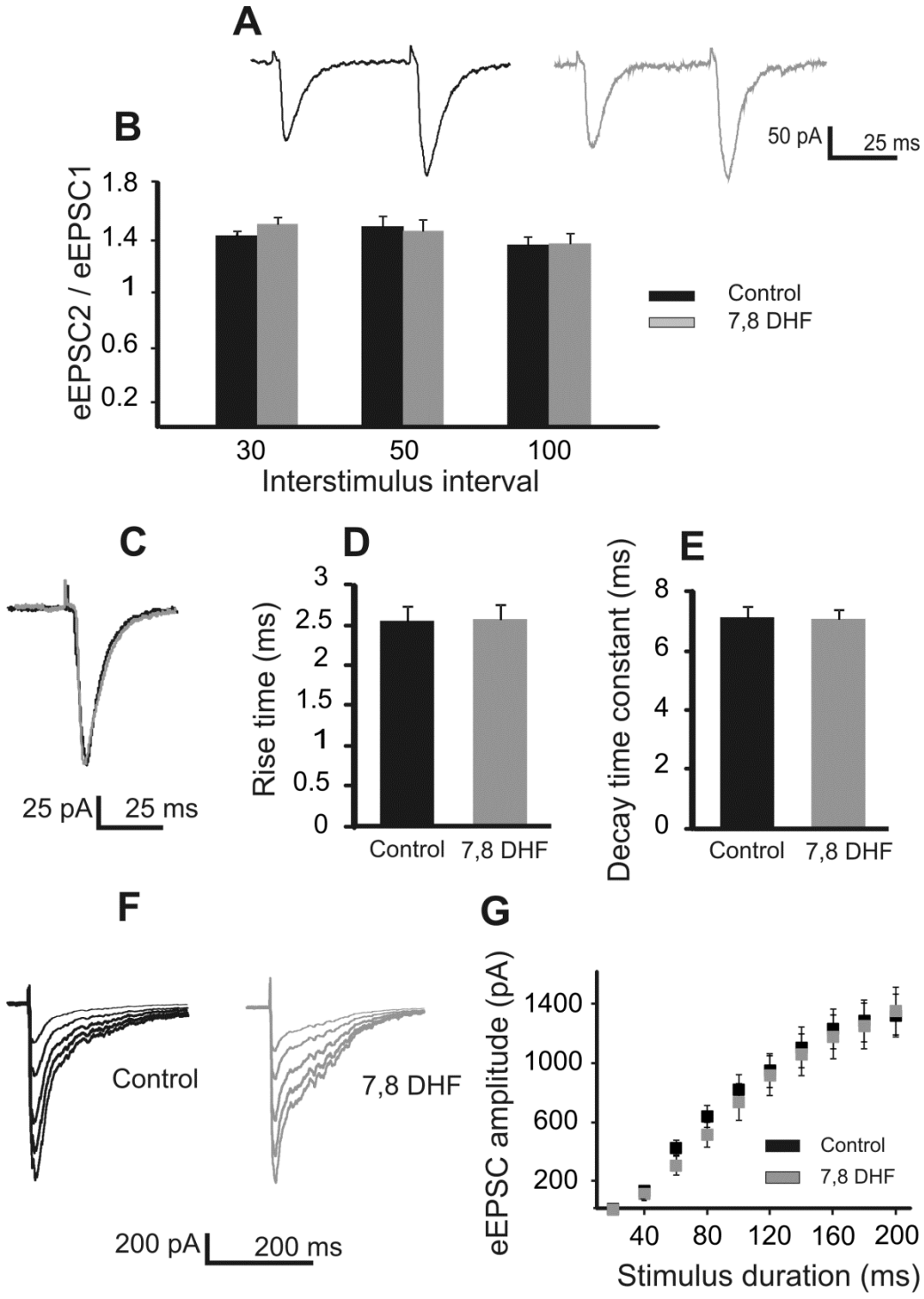

Fig. 3.5. 7,8 DHF did not alter evoked AMPAR-mediated EPSCs
a) Representative traces of AMPAR-mediated eIPSCs from control and 7,8 DHF-treated neurons, obtained in response to 2 consecutive stimulations separated by 50 ms. b) Bar plot showing PPR at different ISIs (30 ms, 50 ms, 100 ms) after 7,8 DHF treatment. c) Superimposed traces of AMPAR-mediated eEPSCs from control and 7,8 DHF- treated neurons. d,e) Diagram showing the kinetics of evoked AMPAR-mediated currents. f) Representative traces of maximal AMPA-mediated eEPSCs measured at different stimulus durations (40, 80, 120, 160, 200 μs) in control and 7,8 DHF treated neurons. g) The input-output relationship of AMPAR-mediated eEPSCs in control and in presence of 7,8 DHF.

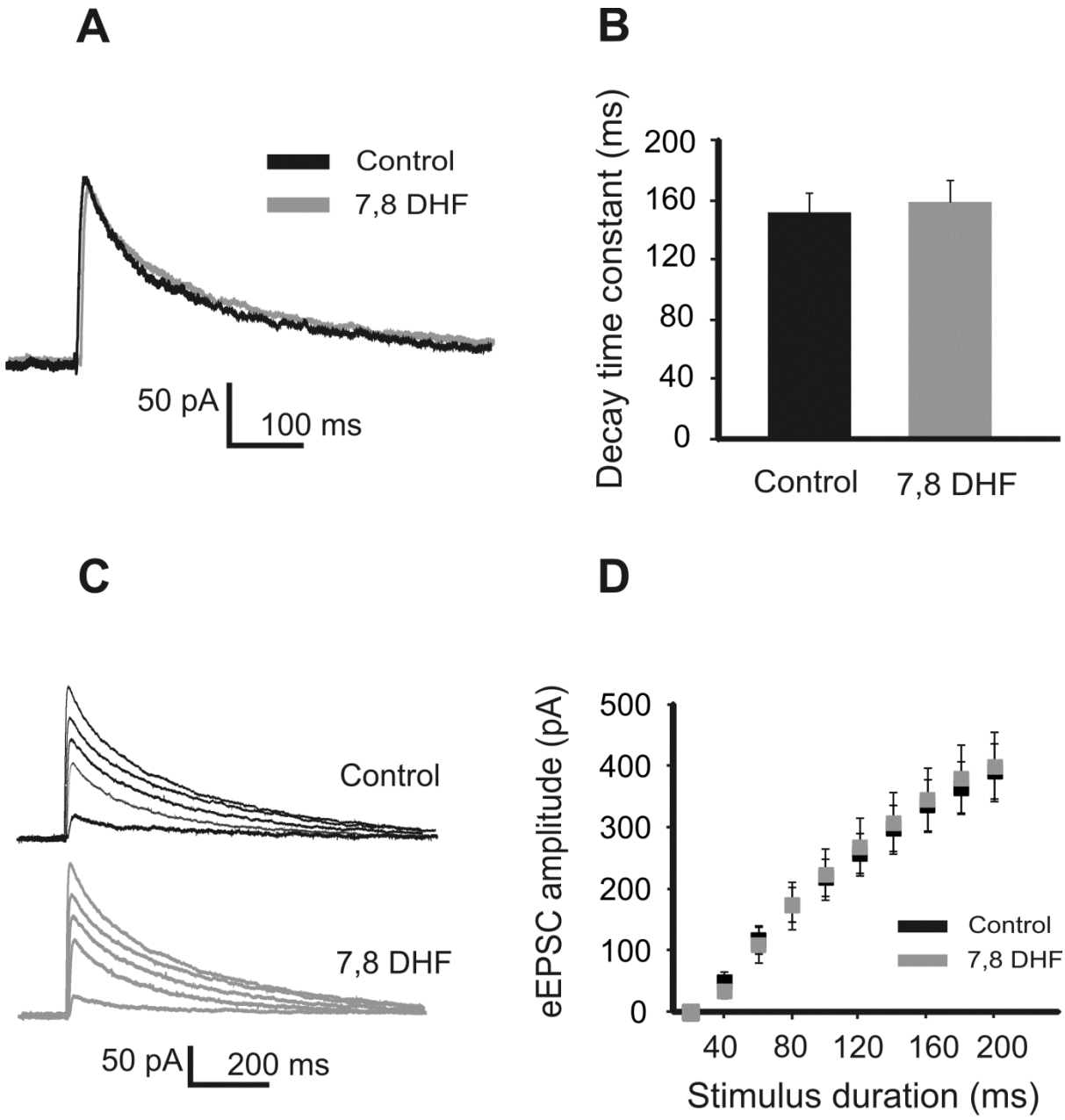

Fig. 3.6. Lack of effect of 7,8 DHF on NMDAR-mediated currents
a) Representative traces illustrating NMDAR-mediated eEPSCs recorded at a holding potential of +40 mV from control and 7,8 DHF treated neurons. b) Histogram displaying decay time constants from control and 7,8 DHF treated neurons. c) Examples traces of maximal NMDAR-mediated eEPSCs measured at different stimulus durations (40, 80, 120, 160, 200 μs) in control and 7,8 DHF treated neurons d) The input-output relationship of NMDAR-mediated eEPSCs in control and in presence of 7,8 DHF.

3.1.3. 7,8-DHF alters layers 2/3-pyramidal neurons intrinsic properties

Next, the effect of 7,8-DHF on the intrinsic membrane properties in cortical pyramidal neurons was investigated, by performing whole-cell patch-clamp recordings in current-clamp mode in the presence of blockers of synaptic transmission (PTX, 100 µM, D-AP5, 25 µM and DNQX, 20 µM). Typical membrane potential responses to a similar hyperpolarizing or depolarizing current step (+/- 20 pA, duration 1 s) are presented in Fig. 3.7A.

A significant increase in the spike frequency upon current injection was observed in slices treated with 7,8-DHF. As shown in Fig. 3.7B, this increment was significant up to a current injection of 100 pA (control: 6 ± 1.3 Hz , n = 15 from 4 animals, 7,8-DHF: 10 ± 1.2 Hz, n = 15 from 4 animals; P = 0.04). Once again, this effect was reversed when co-incubating slices with 7,8-DHF and K252a (6.4 ± 1.1 Hz, n = 15 from 4 animals; P > 0.05). While no changes in the resting membrane potential of the recorded neurons were observed (Fig. 3.7C) (control: -75.1 ± 1.3 mv, n = 17 from 4 animals, 7,8-DHF: -74.1 ± 1.2 mv, n = 19 from 4 animals; P > 0.05), 7,8-DHF caused a significant increase in the input resistance (Fig. 3.7D) (control: 243.75 ± 23.4 MΩ, n = 16 from 4 animals, 7,8-DHF: 338.5 ± 25.1 MΩ, n = 15 from 4 animals; P = 0.011). Again, this effect was absent in presence of TrkB antagonist K252a (235 ± 26.6 MΩ, n = 15 from 4 animals). Taken together, our findings suggest that 7,8-DHF modifies the intrinsic excitability of neocortical pyramidal neurons (Marongiu et al, 2013).

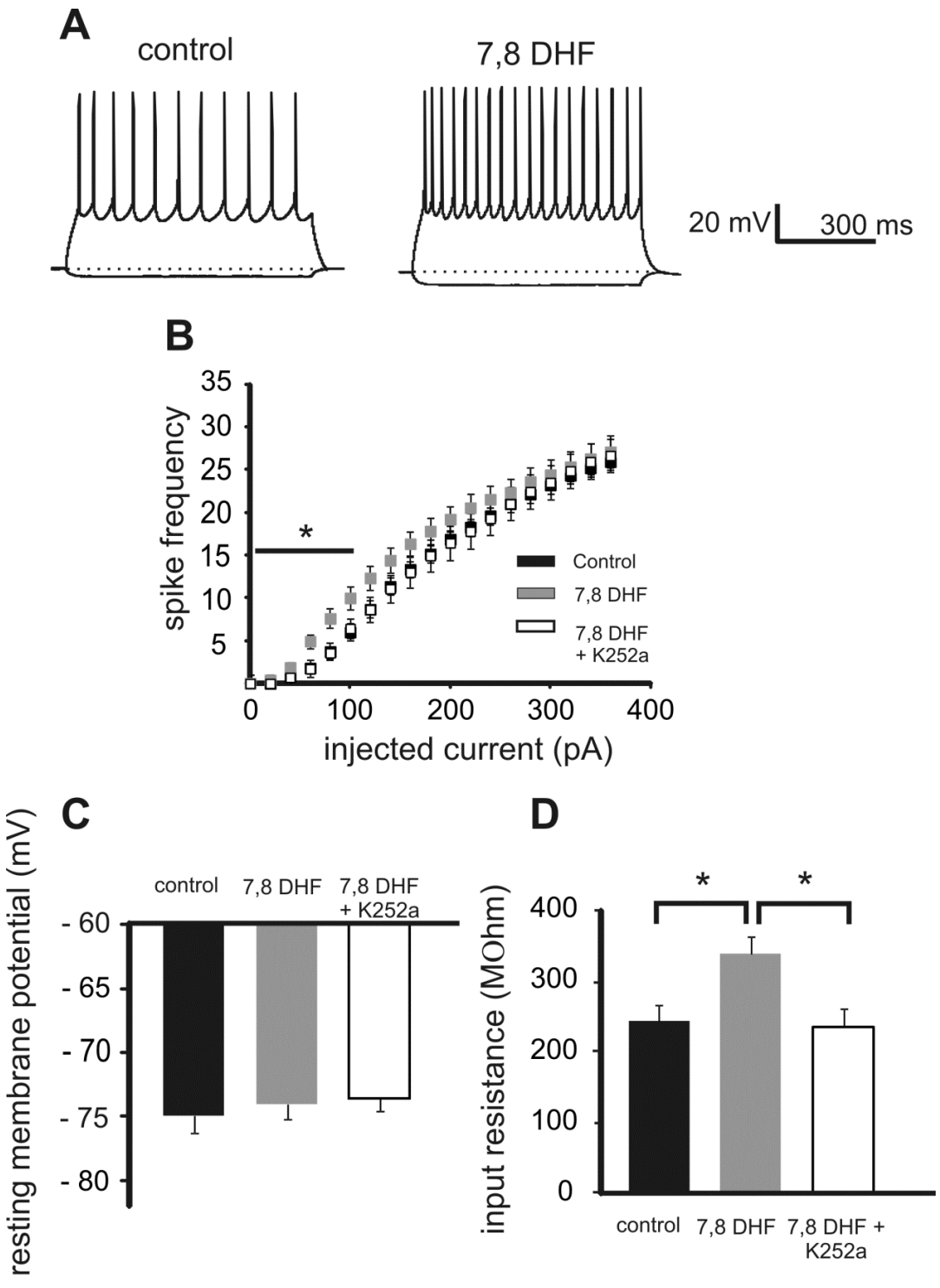

Fig. 3.7: Effect of 7,8 DHF on intrinsic properties of layer 2/3 pyramidal neurons
a) Membrane voltage deflection and spike trains in response to a small negative (-20 pA) or a suprathreshold positive somatic current injection (+ 140 pA) in control and 7,8-DHF-treated slices. b) Summary plot showing spike frequency of the recorded neurons in response to gradually increasing current injection, measured in control neurons, in 7,8-DHF and in 7,8-DHF+K252a treated pyramidal neurons. c, d) Mean resting membrane potential and input resistance in control and in presence of 7,8-DHF or 7,8-DHF + K252a.

3.2 Modulatory effects of the novel TrkB receptor agonist 7,8-Dihydroxyflavone on GABAergic transmission *in vivo*

3.2.1 Chronic administration of 7,8 DHF reduces $GABA_A$ receptors-mediated mIPSCs frequency

The BDNF-TrkB signalling pathway turned out to be involved in the pathogenesis of several psychiatric and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, schizophrenia and depression (Balu and Coyle, 2011; Dumas and Monteggia, 2006; Zuccato and Cattaneo, 2009). The possibility to pharmacologically manipulate this pathway would be a crucial breakthrough in the treatment of these disorders. BDNF has a poor pharmacokinetic profile, which limits its therapeutic usage. To date, 7,8-DHF represents the most promising candidate for the development of a new class of compounds capable of mimicking BDNF action *in vivo,* for its ability to penetrate the blood brain barrier (BBB) after systemic administration and to strongly activate TrkB receptors in different brain areas, such as the hippocampus, amigdala and cortex.

In vitro experiments revealed that acutely applied 7,8 DHF (30 minutes incubation) is able to selectively inhibit the GABAergic transmission in the visual cortex of WT animals. It is however still unknown whether the 7,8 DHF exerts similar effects also *in vivo,* and what is the chronical impact of this molecule.

In order to address these points, an *in vivo* treatment with 7,8 DHF was carried out, and mIPSCs were recorded to assess the consequences of 7,8 DHF administration on the basal GABAergic synaptic transmission (Fig. 3.8A).

WT mice (P14) received repeated intraperitoneal injections of 5 mg/kg 7,8-DHF or 17% DMSO vehicle once daily for 7 consecutive days. The last injection was performed 1h before preparation of acute slices. The dosage was chosen based on previous studies (Jang et al., 2010, Choi et al, 2010, Andero et al., 2011), which demonstrated that 7,8-DHF at this concentration exhibits the highest efficacy.

Chronic 7,8-DHF administration didn't have any noticeable effect on mIPSCs amplitude (control: 27.12 ± 2.1, n = 16 from 3 animals; 7,8-DHF: 26.4 ± 1.7, n= 15 from 3 animals; $p > 0.05$) (Fig. 3.8B). Also mIPSCs were not significantly, reduced in frequency in 7,8 DHF-treated animals (control: 6.98 ± 0.8, n = 16 from 3 animals; 7,8-DHF: 5.23 ± 0.5, n= 15 from 3 animals; $p= 0.105$) (Fig. 3.8C).

These data suggest that the 7,8-DHF *in vitro* acute effects are not reproducible in WT mice after a chronic *in vivo* treatment.

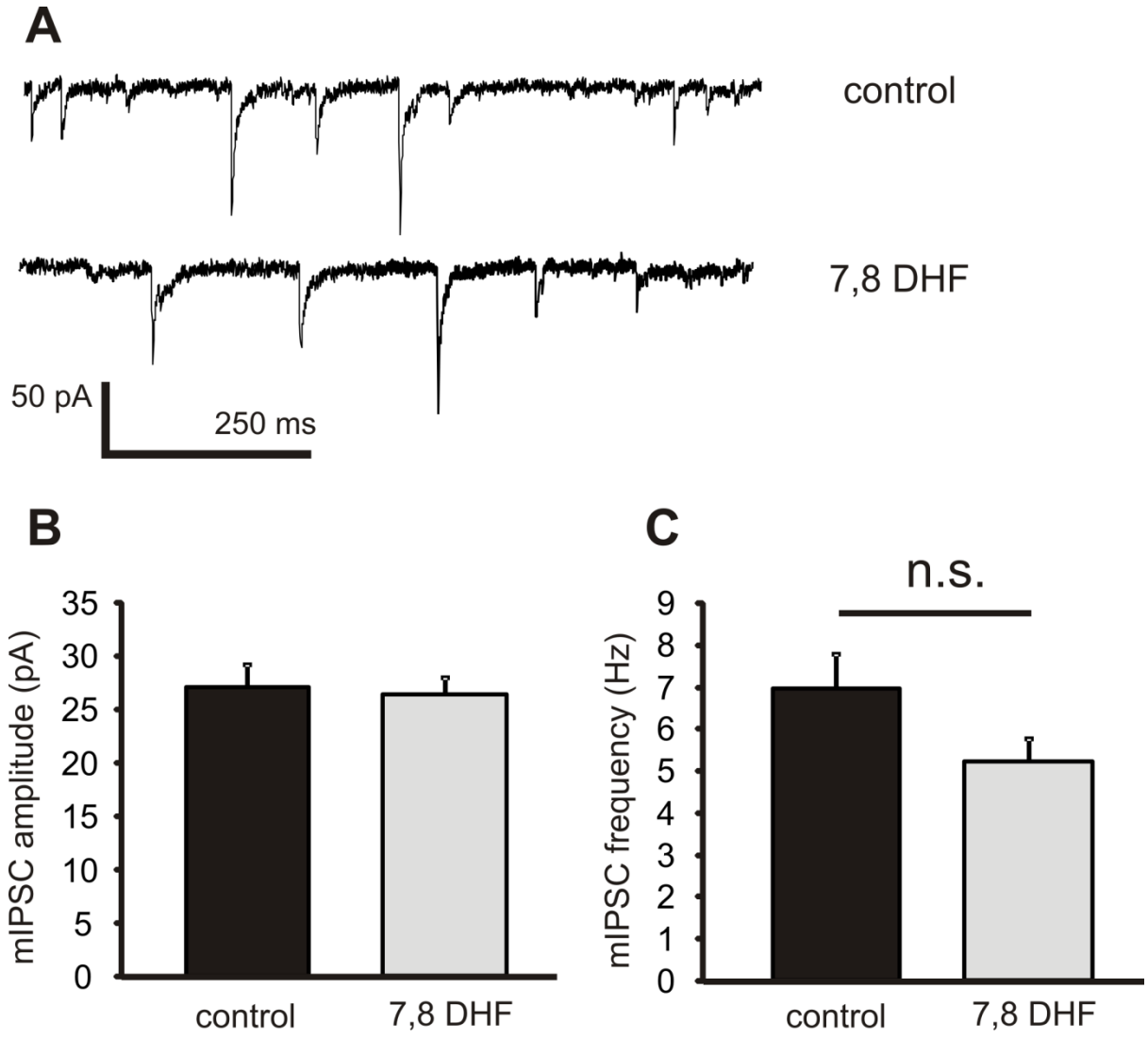

Fig. 3.8: Chronic administration of 7,8 DHF slightly decreased the mIPSCs frequency in WT mice
a) Representative traces of mIPSCs recorded from cells in control and 7,8 DHF animals. b, c) Bar graphs showing mean amplitude and frequency of mIPSCs.

3.2.2 Chronic administration of 7,8 DHF does not rescue the impairment of GABAergic transmission in BDNF (+/−) mice

Accumulating evidence shows that BDNF-TrkB pathway profoundly regulates the maturation and the function of the GABAergic system. The link between these two systems emerged from studies employing BDNF transgenic animals. BDNF overexpressing mice display an

acceleration in the maturation of the GABAergic system (Huang et al., 1999), while in BDNF (+/−) mice the inhibitory system is impaired (Abidin et al, 2008).

A key objective of this study was the attempt to rescue the impaired inhibitory synaptic strength in BDNF (+/−) mice, by chronically administering a TrkB agonist.

BDNF (+/−) mice (P21) underwent the same treatment performed in WT mice, and mIPSCs were recorded (Fig. 3.8A). Administration of 5 mg/kg 7,8-DHF once per day for 1 week failed to enhance the GABAergic transmission. Surprisingly, 7,8-DHF decreased mIPSCs frequency in BDNF (+/−) mice (control: 8.94 ± 0.9, n = 10 from 4 animals; 7,8-DHF: 4.38 ± 0.4, n= 14 from 4 animals; $p<0.001$) (Fig. 3.9C). mIPSCs amplitude was unaltered (control: 39.3 ± 2, n = 10 from 4 animals; 7,8-DHF: 38.4 ± 2.7, n= 14 from 4 animals; $p>0.05$) (Fig. 3.9 B).

These results clearly indicate that GABAergic transmission in BDNF (+/−) mice cannot be rescued to WT level through chronic 7,8 DHF application.

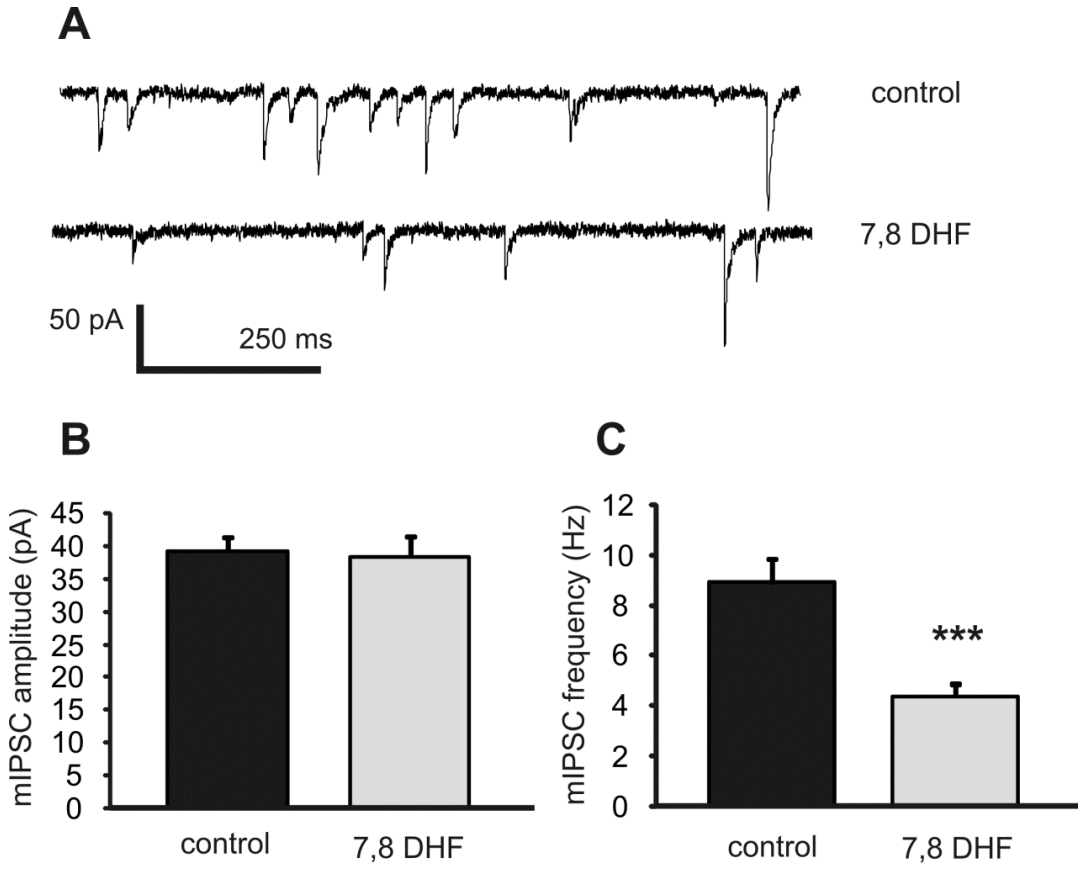

Fig. 3.9: Chronic 7,8 DHF decreased the mIPSCs frequency in BDNF (+/−) mice

a) Representative traces of mIPSCs recorded from cells in control and 7,8 DHF animals. b, c) Bar graphs showing mean amplitude and frequency of mIPSCs.

3.3 Lesion-induced alterations in GABAergic transmission in the visual cortex of WT and BDNF (+/−) mice

3.3.1 Histology of the lesion

To study the location and the morphological alterations of the tissue at the border of the lesion, Nissl stainings were performed between 3 and 6 days after lesion (n=3).

Nissl stainings revealed that the lesion was located in the primary and secondary visual cortices (V1M, V2MM, V2ML), in cortical slices prepared from the region Bregma -2.75 mm to Bregma -4.6 mm (Fig 3.10A). Slices containing a lesion within these areas were chosen for the experimental investigations.

In this part of this study the alterations in the GABAergic transmission post-lesion in WT and BDNF (+/−) mice were investigated.

All recordings were made from visually identified pyramidal neurons in the layer 2/3 of the visual cortex, located in a region 1 to 2 mm away from the lesion border.

To confirm that the recordings were carried out from layer 2/3 pyramidal cells, some of the patched neurons were labelled with lucyferase yellow (Fig. 3.10B). Pyramidal neurons could be easily recognized due to the typical pyramidal shape of their soma.

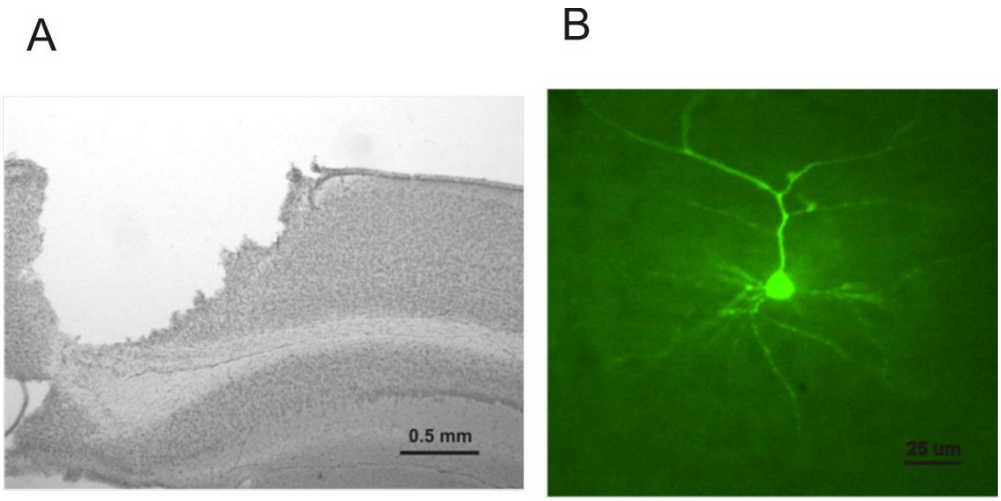

Fig. 3.10: Histology of the focal laser lesion in the visual cortex:
a) Nissl stained visual cortex slice from a 24 days old rat, 3 days post lesion. b) Intracellularly lucyferase yellow-labelled pyramidal neuron in layer 2/3 of mouse visual cortex

3.3.2 Unaltered intrinsic properties of pyramidal neurons in layers 2/3 post-lesion

BDNF (+/−) mice (n=57) and their WT littermates (n=52) (P21-P27) were used for all electrophysiological investigations. Each of these two groups was subdivided in SHAM and LESION animals. Thus a total of four experimental groups (WT SHAM, WT LESION, BDNF SHAM, BDNF) were employed in this study.

In the first set of experiments, the intrinsic properties of layer 2/3 pyramidal neurons post-lesion were analyzed by performing patch clamp recordings under current clamp conditions, in presence of the synaptic transmission blockers (PTX, 100 μM, D-AP5, 25 μM and DNQX, 20 μM) (Fig. 3.11A).

No significant differences were observed either in the resting membrane potential (V_m) (WT SHAM: -78.75 ± 1 mv, n= 20 from 4 animals; WT LESION: -77 ± 0.9 mv, n= 21 from 4 animals; BDNF SHAM: -78.4 ± 1.3 mv, n=23 from 4 animals; BDNF LESION: -78.4 ± 1.1 mv, n=21 from 4 animals; $p > 0.05$) (Fig. 3.11C) or in the input resistance (R_i) (WT SHAM: 162.5 ± 14.5 MΩ , n= 20 from 4 animals; WT LESION: 155.9 ± 8.9 MΩ, n=21 from 4 animals; BDNF SHAM: 150 ± 12.2 MΩ, n=23 from 4 animals; BDNF LESION: 153.6 ± 18.4 MΩ, n=21 from 4 animals; $p > 0.05$) (Fig. 3.11D).

To assess the intrinsic membrane excitability a voltage-current relationship was determined by measuring membrane potential changes in response to a series of depolarizing steps of 20 pA. The action potential frequency was plotted as a function of the amplitude of the injected current. Neurons from all 4 groups exhibited a regular and adapting spiking profile, which is characteristic of the pyramidal neurons (McCormick et al., 1995).

As shown in Fig. 3.11B the spike frequency did not differ significantly among the 4 experimental groups (WT SHAM: 29.65 ± 1.8 Hz , n= 20 from 4 animals; WT LESION: 29.4 ± 1.8 Hz, n=21 from 4 animals; BDNF SHAM: 28.6 ± 2.2 Hz, n=23 from 4 animals; BDNF LESION: 28.42 ± 2.4 Hz, n=21 from 4 animals; $p > 0.05$).

Taken together these data suggest that the lesion does not alter the intrinsic properties of the pyramidal neurons in the layer 2/3 of the visual cortex.

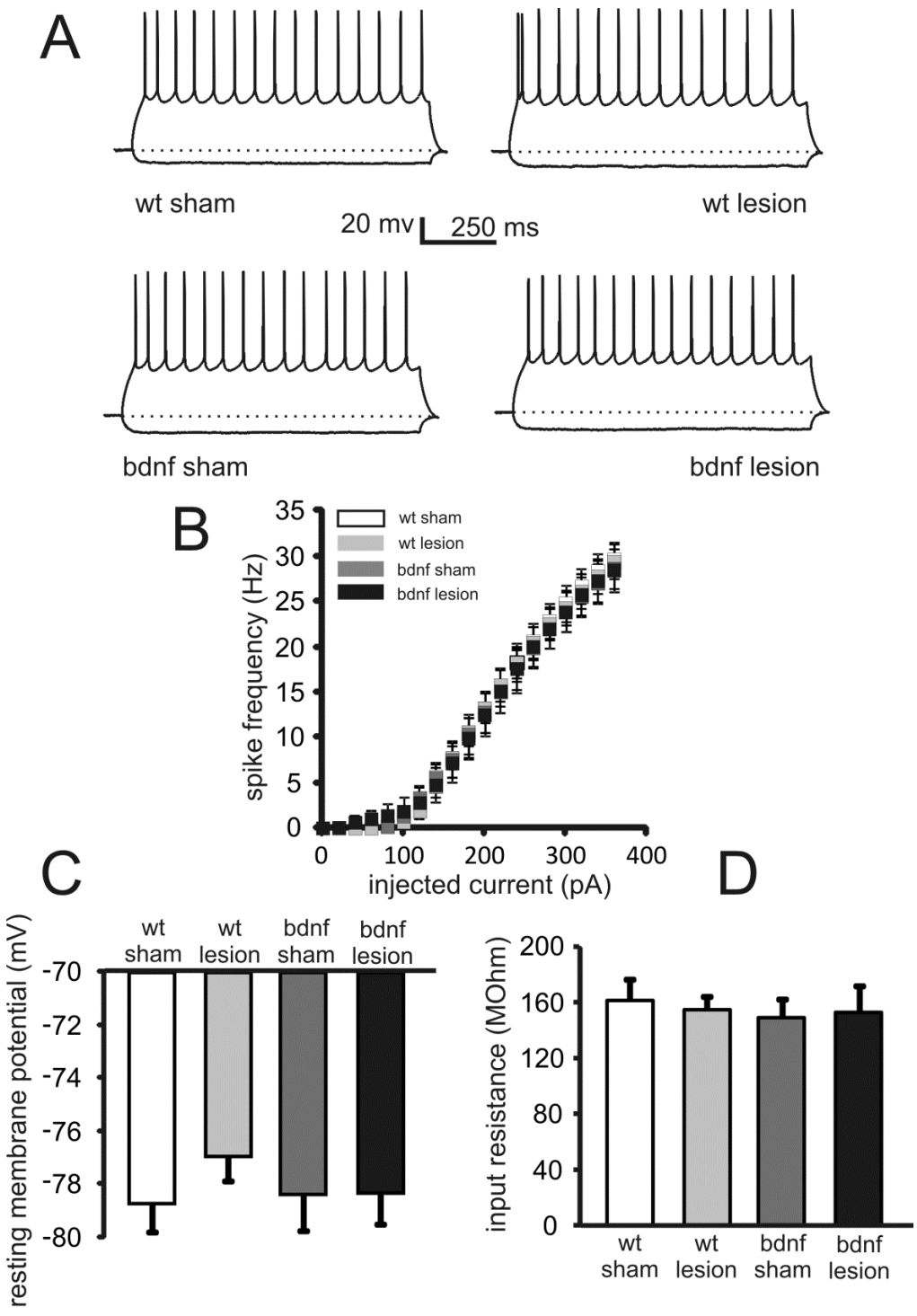

Fig. 3.11 Unaltered intrinsic properties of the layer 2/3 pyramidal neurons post-lesion
a) Voltage membrane responses to hyper- and depolarizing (increments 40 pA) current pulses from WT SHAM, WT LESION, BDNF SHAM and BDNF LESION animals. b) Graph showing the voltage-current obtained by plotting the action potential frequency as a function of the amplitude of the injected current. c, d) Histogram summarizing the mean values of resting membrane potential (v_m) and input resistance (R_i)

3.3.3 Lesion-induced effects on basal GABAergic synaptic transmission

In order to study the effects of the lesion on the basal GABAergic synaptic transmission, miniature inhibitory postsynaptic currents (mIPSCs) were recorded in presence of 1 μM TTX (Fig. 3.12A).

In a previous report Abidin et al. (2008) have shown that the frequency of mIPSCs in BDNF (+/−) mice was significantly reduced in comparison to the values measured in WT animals, due to a chronic lack of BDNF during development.

This finding was confirmed in the present study. Indeed the frequency of mIPSCs was significantly smaller in the BDNF SHAM group (4.9 ± 0.4 Hz, n= 14 from 5 animals; $P < 0.001$), when compared with the frequency of the neurons recorded from the WT SHAM group (10.2 ± 0.6 Hz; n= 14 from 5 animals) (Fig. 3.12C). On the contrary, the amplitude of mIPSCs was not significantly different between the two groups (WT SHAM: 24.3 ± 1.9 pA, n= 14 from 5 animals; BDNF SHAM: 22.6 ± 1.25 pA, n= 14 from 5 animals; $P > 0.05$) (Fig. 3.12B).

Imbrosci et al (2013) have demonstrated that a similar focal laser lesion in rats weakens the GABAergic inhibition in the visual cortex by reducing the phasic GABA release. To verify if this result could also be reproducible in mice, mIPSCs in WT lesioned mice was analyzed.

The lesion dramatically reduced the mIPSCs frequency (WT lesion: 7.3 ± 0.7 Hz, n=15 from 5 animals; $P = 0.001$) (Fig. 3.12C) with no changes in the amplitude (WT lesion: 23.1 ± 2.1 pA, n=15 from 5 animals; $P > 0.05$) (Fig. 3.12B).

As revealed by the statistical analysis, the impact of a chronic lack of BDNF on GABAergic transmission was stronger than the impact of a lesion. mIPSCs frequency in the BDNF SHAM group was significantly lower than the frequency in the WT lesion group (WT LESION: 7.3 ± 0.7 Hz, n=15 from 5 animals; BDNF SHAM: 4.9 ± 0.4 Hz, n= 14 from 5 animals; P= 0.005) (Fig. 3.12C). Once again the amplitude was not significantly different (WT LESION: 23.1 ± 2.1 pA; BDNF SHAM: 22.6 ± 1.25 pA; $P > 0.05$) (Fig. 3.12B).

The lesion did not affect mIPSCs frequency in the BDNF (+/−) mice. In the BDNF LESION group mIPSCs frequency was slightly but not significantly increased in comparison with the BDNF SHAM group (BDNF SHAM: 4.9 ± 0.4 Hz, n= 14 from 5 animals; BDNF LESION: 6 ± 0.5 Hz, n= 13 from 4 animals; $p > 0.05$) (Fig. 3.12C).

Surprisingly the amplitude of mIPSCs was significantly larger in the BDNF LESION group (31.3 ± 2 pA, n= 13 from 4 animals) when compared to the amplitude of all other

experimental groups (WT SHAM: 24.3 ± 1.9 pA; P= 0.017; WT lesion: 23.1 ± 2.1 pA; P= 0.005; BDNF SHAM: 22.6 ± 1.25 pA; P= 0.003) (Fig. 3.12B).

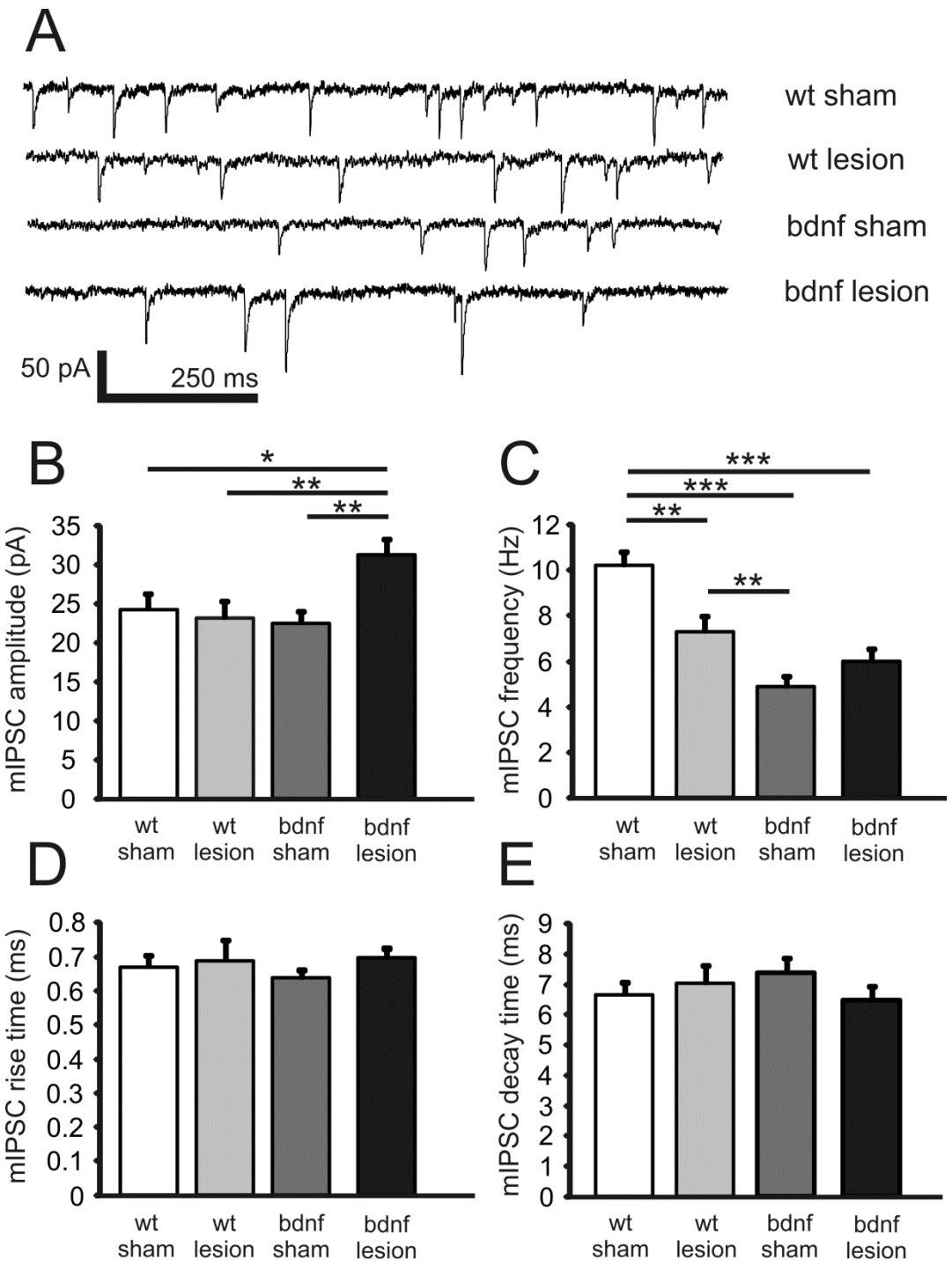

Fig. 3.12 Effects of the lesion on the basal GABAergic synaptic transmission a) Sample traces representing mIPSCs recorded in WT SHAM, WT LESION, BDNF SHAM and BDNF LESION animals. b, c) Bar plots showing the effects of a lesion on amplitude and frequency of mIPSCs from all 4 experimental groups. d, e) Bar plots showing the lesion effects on rise and decay time of mIPSCs.

Moreover no difference was found in rise time (WT SHAM: 0.67 ± 0.02 ms; WT LESION: 0.69 ± 0.05 ms; BDNF SHAM: 0.64 ± 0.02 ms; BDNF LESION: 0.7 ± 0.02 ms; $P > 0.05$) (Fig. 3.12D) and decay time constants of mIPSCs (WT SHAM: 6.7 ± 0.3 ms; WT LESION: 7 ± 0.5 ms; BDNF SHAM: 7.4 ± 0.5 ms; BDNF LESION: 6.5 ± 0.4 ms; $P > 0.05$) (Fig. 3.12E).

Taken together these data suggest that both the lesion and a lack of BDNF from birth weakened the GABAergic neurotransmission, most probably through alterations in the presynaptic terminals, whereas performing a lesion in BDNF (+/−) mice, which already have a functionally immature GABAergic system, rather triggered homeostatic compensatory mechanisms on the postsynaptic site.

3.3.4 Increased Paired Pulse ratio (PPR) post-lesion

To further explore the functional properties of the GABAergic synapses post-lesion and to gain more insights into the effects of the lesion on the presynaptic GABA release, a paired-pulse ratio protocol was applied (Fig. 3.13A). Two consecutive eIPSCs were elicited by two stimuli, delivered with the interevent interval (ISI) of 30 (Fig. 3.13B) and 50 ms (Fig. 3.13C). From these recordings the paired-pulse ratio (PPR) was calculated.

In the WT SHAM group the PPR was 0.68 ± 0.05 (n= 20 from 5 animals) at the ISI of 30 ms and 0.66 ± 0.04 (n=17 from 5 animals) at the ISI of 50 ms respectively.

In agreement with Abidin et al. (2008) the PPR was significantly increased at both ISI (30 ms: 0.85 ± 0.05, n= 15 from 5 animals; P= 0.016; 50 ms: 0.86 ± 0.07; n= 17 from 5 animals; P= 0.003) in BDNF SHAM animals in comparison with WT SHAM mice. The PPR was also significantly larger in WT LESION animals (30 ms: 0.81 ± 0.04, n=18 from 5 animals; P= 0.039; 50 ms: 0.84 ± 0.05, n= 19 from 5 animals; P= 0.017).

These findings substantiate the result of a decreased frequency of mIPSCs, as observed in the WT LESION and BDNF SHAM groups, supporting the hypothesis of a reduced GABA release.

The lesion further increased the PPR in BDNF LESION animals (30 ms: 0.95 ± 0.04, n=14 from 5 animals; 50 ms: 0.97 ± 0.04, n=14 from 5 animals). Despite this increment, the PPR in the BDNF LESION group was not significantly different from the PPR in the BDNF SHAM group (P= 0.384).

Taken together these data suggest that both lesion and BDNF deficiency reduced GABA release probability.

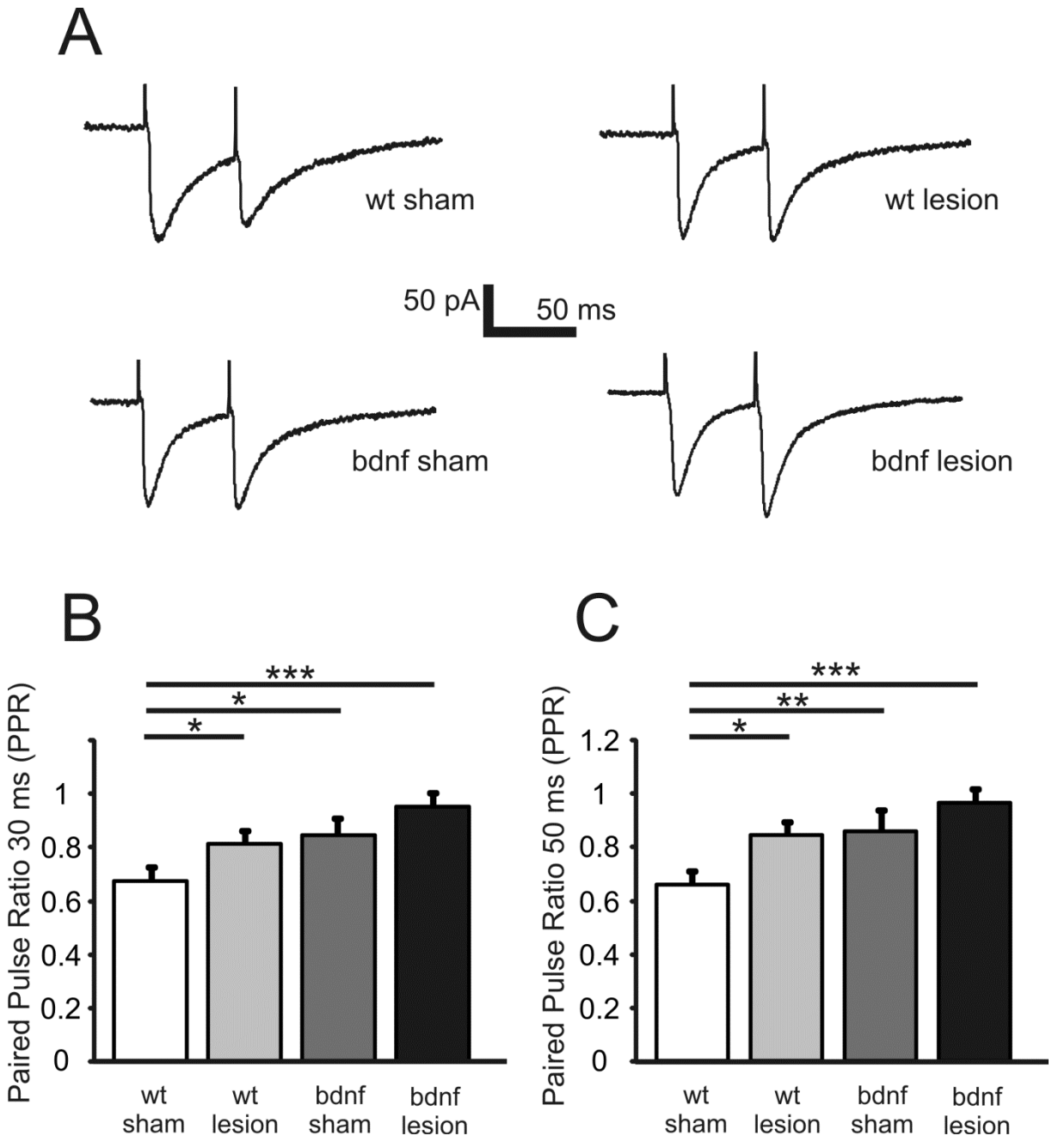

Fig. 3.13 Increased PPR of eIPSCs post-lesion
a) Examples traces of evoked pairs of eIPSCs at the interstimulus interval of 30 and 50 ms. b, c) Bar plots of the mean PPR.

3.3.5 Effects of the lesion on eIPSCs

The maximal amplitude of evoked PSCs is a very reliable criterion to estimate the synaptic strength in an *in vitro* brain slice preparation. To assess the overall strength of the GABAergic inhibition in the visual cortex, an input-output relationship of eIPSCs was determined (Fig. 3.14A). Ascending afferent fibers in layer IV were electrically stimulated. The stimulation intensity was adjusted to elicit a maximal response, and the eIPSCs amplitude was plotted against the gradually decreasing stimulus duration expressed (in μs) (Fig. 3.14B).

The maximal eIPSCs amplitude in the WT SHAM group was 529.7 ± 27.8 pA (n=12 from 4 animals). The lesion significantly reduced the eIPSCs amplitude in WT animals. In the WT LESION group eIPSCs amplitude was 384.5 ± 27.3 pA (n= 15 from 5 animals; P= 0.003). Furthermore, the eIPSCs amplitude recorded in the BDNF SHAM group was significantly lower than in the WT SHAM group (362.9 ± 33 pA, n=15 from 5 animals; P= 0.001). Moreover the strength of inhibition was further diminished in the BDNF LESION group (306 ± 32.2 pA, n= 17 from 6 animals; $P < 0.001$). There was, however, no significant difference between the BDNF SHAM and BDNF LESION groups (Fig. 3.14C).

These results indicate that the lesion impairs the GABAergic inhibition both in WT and (although to a lesser extent) in BDNF mice.

Next, in order to investigate in more detail the functional properties of the postsynaptic $GABA_A$ receptors, the kinetics of eIPSCs was analyzed (Fig. 3.15A).

The rise time was unaltered (WT SHAM: 3 ± 0.5 ms, n=16 from 5 animals; WT LESION: 2.7 ± 0.3 ms, n=18 from 6 animals; BDNF SHAM: 2.7 ± 0.3 ms, n=15 from 5 animals; BDNF LESION: 2.95 ± 0.5 ms, n=13 from 4 animals; $P > 0.05$) in all 4 groups (Fig. 3.15B). Conversely, the decay-time constant of eIPSCs revealed significantly prolonged in the BDNF SHAM group (24.5 ± 2.6 ms) in comparison with all other groups (WT SHAM: 17.8 ± 1.63 ms; P= 0.012; WT LESION: 15.9 ± 0.9 ms; P= 0.001; BDNF LESION: 17.3 ± 1.8 ms; P= 0.011) (Fig. 3.15C).

These data indicate that the lesion induced changes in the function of postsynaptic $GABA_A$ receptors in the BDNF (+/−) mice.

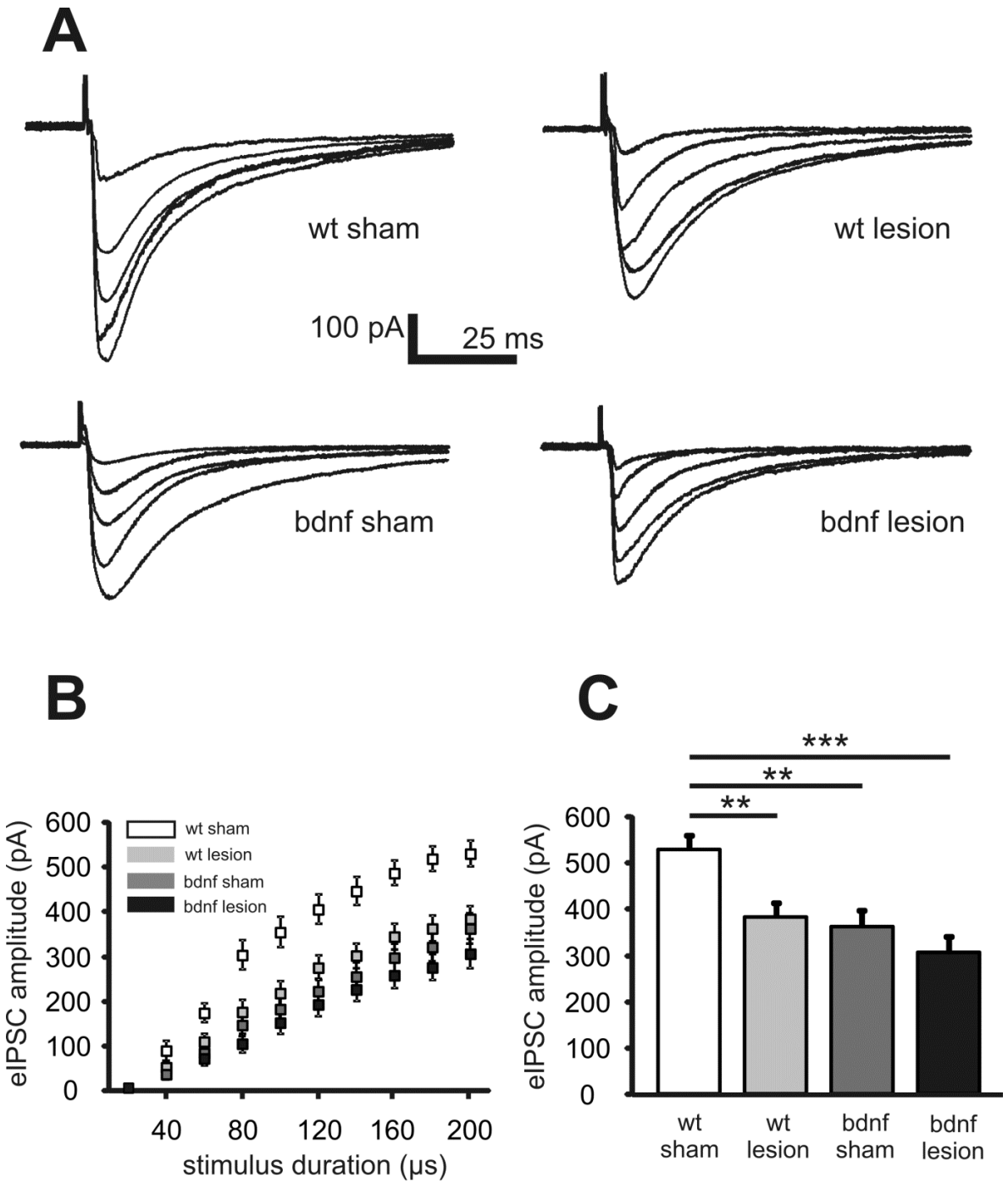

Fig. 3.14 The lesion reduced the overall strength of the GABAergic inhibition.
a) Representative traces of maximal eIPSCs recorded from WT SHAM, WT LESION, BDNF SHAM and BDNF LESION animals at different stimulus durations (40, 80, 120, 160, 200 µs). b) Input-output curves of eIPSCs c) Bar plot showing the the maximal amplitude of eIPSCs in the 4 experimental groups

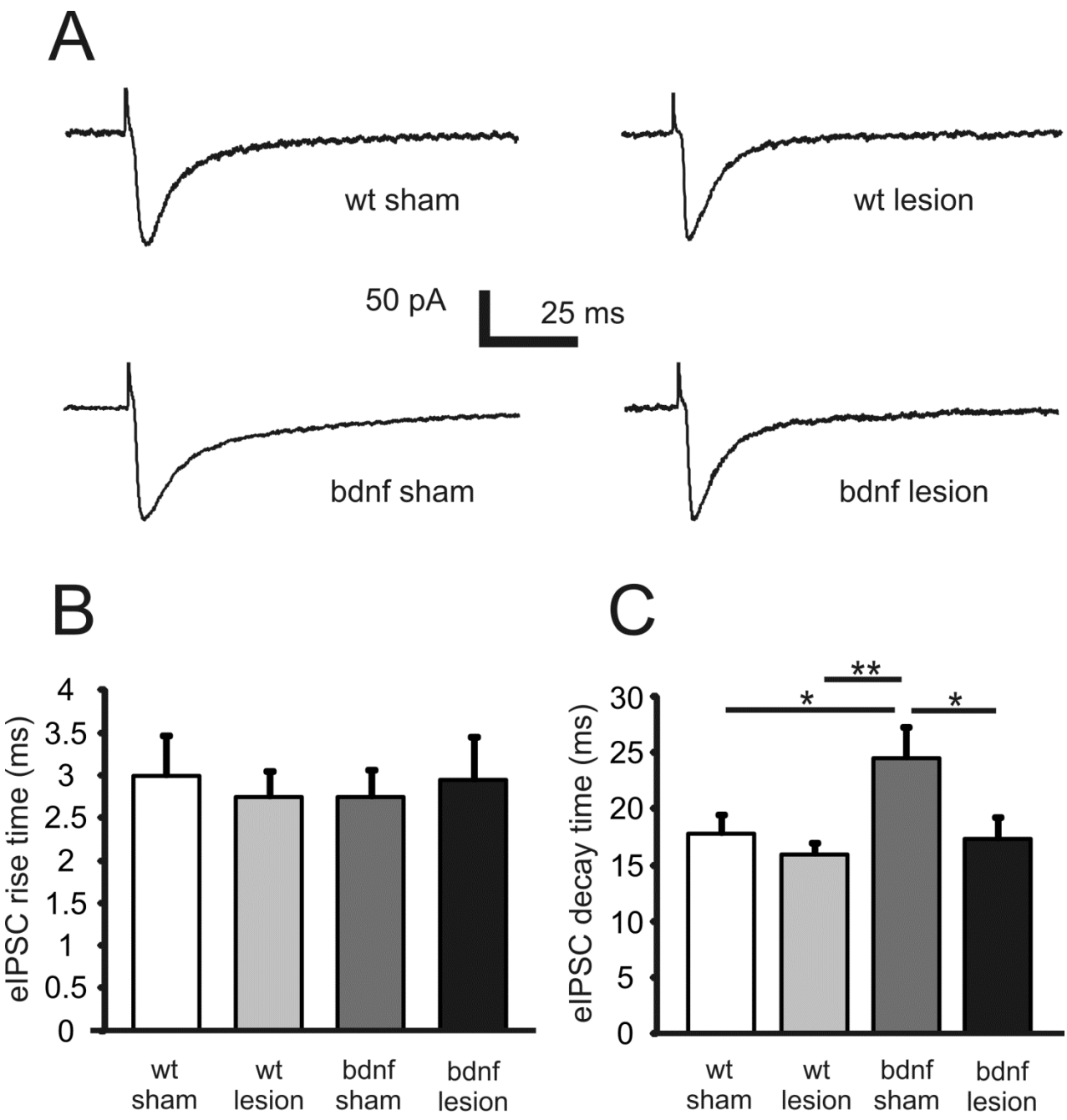

Fig. 3.15 Lesion-induced changes in the kinetics of eIPSCs
a) Representative traces of postsynaptic GABAAR-mediated currents b, c) bar plots showing the rise-time and the decay-time constant of eIPSCs from WT SHAM, WT LESION, BDNF SHAM and BDNF LESION animals

3.3.6 The effects of the lesion on the basal GABAergic neurotransmission are mimicked by an acute block of the TrkB receptor

The analysis of the mIPSCs revealed that the lesion caused a significant reduction in the mIPSCs frequency in WT LESION animals. A similar scenario was observed in the BDNF SHAM groups, in which a chronic absence of BDNF impaired the presynaptic GABA release. For these reasons, it is conceivable to hypothesize that the lesion might exert its effects on the GABAergic system through a direct modulation of the BDNF-TrkB signalling pathway.

The analysis of the mIPSCs also revealed a strong increase in the mIPSCs amplitude in the BDNF LESION group. This result was reported in a previous study (Hennenberger et al, 2002), in which it was documented that, in superior collicular slices deriving from P15 mice, an acute block of the TrkB receptor with the TrkB receptor antagonist K252a could mimic the observed increase in mIPSCs amplitude.

This is further confirmation of the hypothesis that BDNF-TrkB signalling might be involved in the lesion-induced alterations of the cortical inhibition.

To test this in our model the same protocol from Hennenberger et al (2002) was employed in my experiments.

Slices from all 4 experimental groups were preincubated for 1.5 h in 200 nM K252a. All the following electrophysiological recordings were performed in presence of K252a. Surprisingly, K252a completely reproduced the lesion effects on the mIPSCs amplitude.

The mIPSCs amplitude was significantly larger in the WT SHAM (control: 24.3 ± 1.9 pA, n= 14 from 5 animals; K252a: 34.3 ± 1.8 pA, n=15 from 5 animals; P= 0.001), WT LESION (control: 23.1 ± 2.1 pA, n= 15 from 5 animals; K252a: 30.8 ± 0.8 pA, n=16 from 5 animals; P= 0.003) and BDNF SHAM (control: 22.6 ± 1.2 pA, n= 14 from 5 animals; K252a: 34.4 ± 1.9 pA, n=14 from 5 animals; $P < 0.001$) groups. No additional effect on the amplitude of mIPSCs could be observed in the BDNF LESION group. The amplitude of mIPSCs recorded in control and K252a-treated neurons was indistinguishable (control: 31.3 ± 1.9 pA, n= 14 from 5 animals; K252a: 34.3 ± 1.8 pA, n=15 from 5 animals; P= 0.001) (Fig 3.16A).

Furthermore, incubation with K252a was able to significantly reduce the mIPSCs frequency in WT animals (control: 10.2 ± 0.6 Hz, n=14 from 5 animals; K252a: 7.2 ± 1.1 Hz, n=15 from 5 animals; P= 0.032) like in the lesion-treated group. Remarkably K252a did not induce any changes in the frequency of all other groups: WT LESION (control: 7.3 ± 0.6 Hz, n=15 from 5 animals; K252a: 7.4 ± 0.7 Hz, n=16 from 5 animals; $P > 0.05$), BDNF SHAM

(control: 4.9 ± 0.4 Hz, n=14 from 5 animals; K252a: 5.4 ± 0.7 Hz, n=14 from 5 animals; P > 0.05) and BDNF

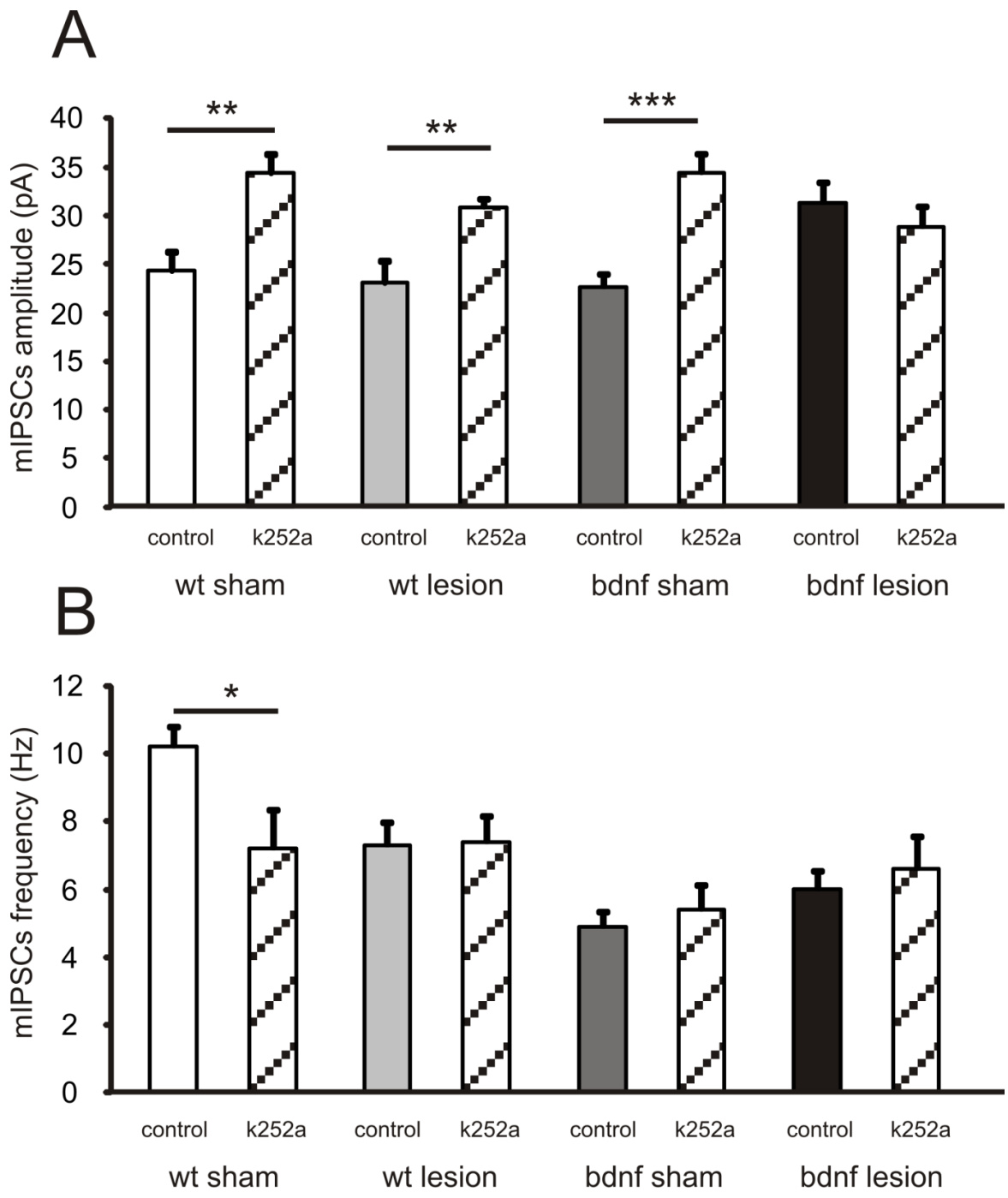

Fig. 3.16 Acute TrkB block with the anatagonist k252a mimicked the lesion-induced alterations of the basal GABAergic transmission
Diagram showing the mean a) amplitude and b) frequency of mIPSCs after K252a treatment.

LESION (control: 6 ± 0.5 Hz, n=14 from 5 animals; K252a: 6.6 ± 0.9 Hz, n=15 from 5 animals; $P > 0.05$) (Fig 3.16B).

Taken together these results clearly show that an acute pharmacological downregulation of the activity of BDNF/TrkB pathway, through TrkB receptor block, can reproduce the lesion effects.

3.3.7 The lesion does not reduce BDNF protein levels

The observed decrease in the BDNF/TrkB signalling could be mediated by a lesion-induced reduction in the levels of the upstream generated BDNF protein.

In several studies it has been reported that different brain injury models led to a reduction of the BDNF post-lesion (for review see: Li et al, 2011; Schober et al, 2009, Fumagalli et al 2012).

To test whether the laser lesion can affect the expression of BDNF, the levels of BDNF were quantified by sandwich ELISA. The visual cortices were excised on ice and processed according to a standard protocol. Five animals per group were used for the statistical analysis. As expected, the ELISA revealed a ~50% BDNF reduction in the BDNF SHAM group in comparison with the WT SHAM group. In the WT sham group the concentration of BDNF was 228.2 ± 13.2 pg/mg, while in the BDNF SHAM group, the BDNF concentration was 125.6 ± 8.7 pg/mg ($P < 0.001$). Surprisingly, the lesion did not reduce the BDNF expression either in the WT LESION (243.4 ± 20.3 pg/mg; $P > 0.05$) or in the BDNF LESION (122.8 ± 8.1 pg/mg; $P > 0.05$) group (Fig 3.17).

These data indicate that a reduction in BDNF expression cannot account for the changes in the inhibitory transmission in the visual cortex post-lesion.

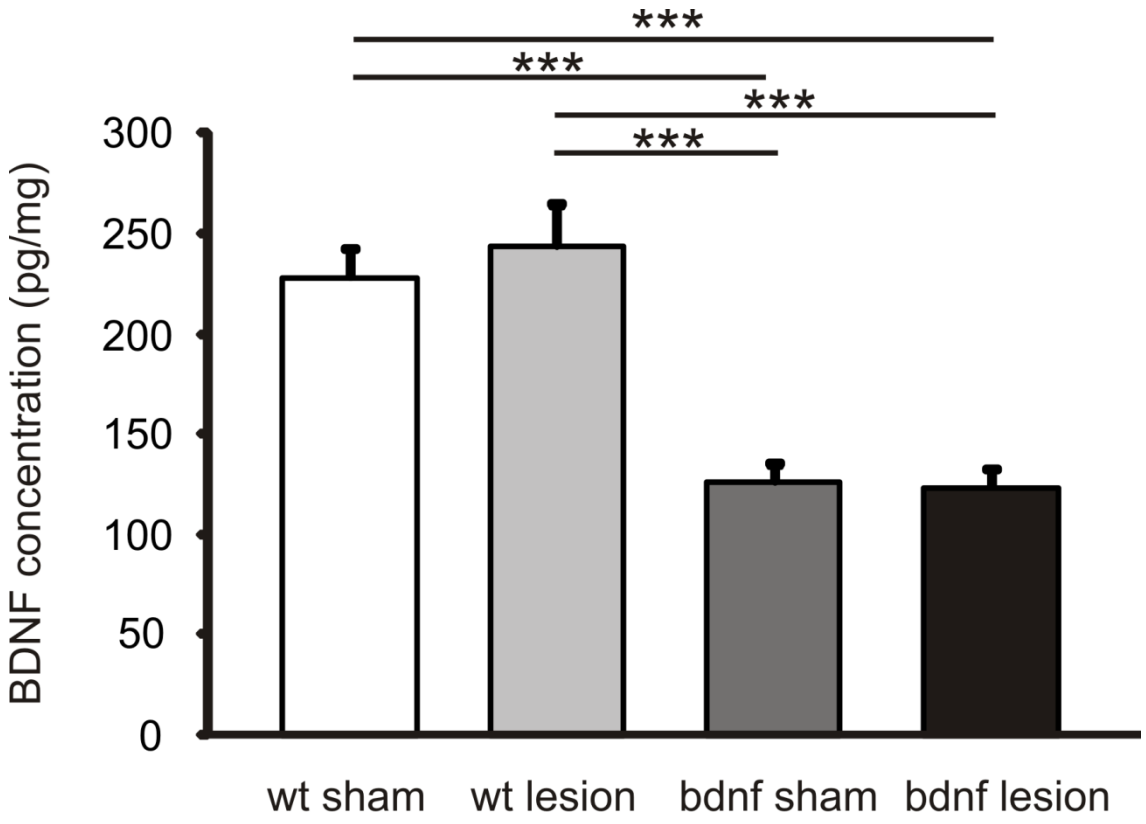

Fig. 3.17 Unaltered BDNF expression post-lesion
Bar plot showing the BDNF concentration estimated by ELISA sandwich in the 4 experimental conditions

4. Discussion

4.1 Characterization of the pharmacological profile of the new TrkB receptor agonist 7,8 Dihydroxyflavone

4.1.1 *In vitro* 7,8-DHF effects on synaptic transmission and intrinsic neuronal excitability

The lack of TrkB agonists and the fact that the BDNF is a relatively expensive pharmacological compound with a poor pharmacokinetic profile, prompted scientists to look for new molecules, which are able to reproduce BDNF effects. The identification of the flavonoid 7,8-DHF triggered a flurry of experimental activity to disclose its pharmacological profile.

In the initial part of this study the effects of 7,8-DHF on the electrophysiological properties of neocortical pyramidal neurons were investigated. Whole-cell patch-clamp recordings from layer 2/3 pyramidal neurons revealed that incubation of acute cortical slices for 30 min in 20 µM of 7,8-DHF caused a selective reduction in the strength of GABAergic inhibition, while the glutamatergic transmission was unaffected. Moreover, 7,8-DHF was able to increase both, input resistance and intrinsic excitability of neurons, leaving the resting membrane potential unaltered. Remarkably, all these effects were abolished in presence of K252a (a TrkB receptor antagonist), indicating a direct involvement of TrkB receptors in the action of 7,8-DHF (Marongiu et al., 2013).

There is an extensive number of evidence demonstrating that flavonoids interfere with the activity of receptors, ion channels or entire signaling pathways, inducing profound effects in the central nervous system (for review see: Jager and Saaby, 2011). In the most part of the *in vitro* studies flavonoids displayed significant physiological effects in the µM range (Losi et al, 2004, Ren et al, 2011, or review see: Hanrahan et al, 2011). Until now, 7,8-DHF was used on brain slices only in another study, where the authors reported that LTP in adult animals can be rescued by preincubation of the slices with 10 µM (Zeng et al, 2011). These findings are not much different from the concentration of 20 µM, which was employed in the present study.

Jang et al (2010) demonstrated that 7,8-DHF binds the same region of the TrkB receptor targeted by the BDNF, and like BDNF is able to induce the dimerization of the receptor and activation of the downstream signalling pathway.

Attenuation of the GABAergic transmission after acute administration of BDNF was observed and reproduced in several neuronal preparations (Bardoni et al., 2007; Cheng and Yeh, 2003; Frerking et al., 1998; Mizoguchi et al., 2003; Ohbuchi et al., 2009; Tanaka et al., 1997). However, due to the complexity of the downstream signaling pathways triggered by TrkB receptors activation and to the great number of targets subject to phosphorylation, the precise mechanism underlying 7,8-DHF effect is still a matter of debate. The effect was shown to depend most probably on BDNF/TrkB-dependent activation of PKC, which appears to specifically target the $GABA_A$ receptors (for review see: Gottmann et al, 2009). Nonetheless, the observed reduction in mIPSCs frequency together with the increase in PPR of eIPSCs strongly suggests that, 7,8-DHF is causing a reduction in presynaptic release of GABA.

A presynaptic effect of 7,8-DHF was also proposed in another study, in which 7,8 DHF enhanced neuromuscular transmission presynaptically (Mantilla, 2012).

Upon TrkB receptor activation, two mechanims might, at least in part, account for the reduced GABA release: a reduced GABA reuptake (Canas et al, 2004) and/or the production of the endocannabinoids (Lemtiri-Chlieh and Levine, 2010).

A reduced GABA transporters activity slows down the clearance of the neurotransmitter from the synaptic cleft. This usually increases ambient GABA levels, leading to activation of presynaptic $GABA_B$ receptors and to a consequent suppression of GABA release from the presynaptic terminals (Kirmse et al 2008).

Lemtiri-Clieh et al. (2010) have demonstrated that in layer 2/3 of the neocortex the effects of BDNF at inhibitory synapses are mediated by the release of endocannabinoids. These lipid compounds are synthesized by the postsynaptic pyramidal neurons following TrkB receptor activation, and, acting as retrograde messengers, suppress the GABA release via activation of CB1 cannabinoids receptors expressed at presynaptic inhibitory terminals.

In vitro experiments have also disclosed that 7,8-DHF can interfere with the kinetics of $GABA_A$ receptors, prolonging both rise time and decay time of the eIPSCs transients. Many factors contribute to the regulation of $GABA_A$ receptor kinetics (for review see: Farrant and Kaila, 2007). It can be influenced by the $GABA_A$ receptor subunits composition, as well as by GABA transporters.

It seems unlikely that it can originate from changes in the composition of $GABA_A$ receptor subunits. It is hard to believe that protein synthesis and the subunit exchange will be complete within 30 minutes, the incubation time used in the present study.

Another mechanism, which has to be taken in account, is the activity of the GABA transporters. The activity of these transporters determines the amount of neurotransmitter present in the synaptic cleft, controlling thus the timing of the GABA action. It is well known that Tyrosine Kinases regulate the function of GABA transporters (Law et al, 2000), and in particular, acute BDNF application has been shown to rapidly inhibit GABA uptake, leading to accumulation of GABA at the synapses (Vaz et al, 2008). This could, at least in part, explain the observed eIPSCs prolonged decay time, and it may represent a strategy to compensate for a reduction in the GABA release.

A change in the phosphorylation state of the receptors can also be responsible of the altered kinetics. Phosphorylation mechanisms have indeed been hypothesized to underlie the rapid modulation of the $GABA_A$ receptors function by BDNF. It has been reported that the administration of BDNF on a relatively short time scale leads to the activaction of several specific kinases and phosphatases, which target the $GABA_A$ receptors (Jovanovic et al, 2004). Phosphorylation and dephosphorylation of specific internal domains of the receptors concur to modulate their function, control receptor endocytosis and to shape the postsynaptic GABAergic responses (for review see: Brandon et al, 2002; Kittler and Moss, 2003).

Surprisingly, the glutamatergic transmission, mediated by AMPA and NMDA receptors, was unaltered. No significant changes following 7,8-DHF incubation were detected in either evoked EPSCs or mEPSCs. A similar scenario after acute application of BDNF was described by other groups in the CA1 region of the hippocampus (Frekling et al., 1998; Tanaka et al., 1997). They found that the BDNF exerts an effect exclusively on the GABAergic transmission, with no effects on the excitatory system.

Several other studies however, have reported that AMPA and NMDA receptors mediated currents are acutely potentiated by BDNF (Carmignoto et al., 1997; Lessmann et al., 1994; Levine et al., 1998; Madara and Levine, 2008; Taniguchi et al., 2000; for review see: Carvahlo et al, 2008; Gottmann et al, 2009; Poo, 2001).

The reason for this discrepancy is not clear. Many factors can have an impact on the pharmacological effects of 7,8-DHF. Crucial is for example the expression pattern and the synaptic location of TrkB receptors, which differ greatly among brain areas and during development and it is also strongly dependent on the state of neuronal activity (for review

see: McAllister et al, 1999). Another explanation for this apparent discrepancy might lie in the intracellular events, which are triggered by the binding of the 7,8-DHF to the TrKB receptors. This is followed by the activation of the three major downstream signalling cascades: the phosphatidylinositol 3-kinase (PI3K)/ Akt, phospholipase Cγ (PLCγ)/ protein kinase C (PKC) and mitogen-activated protein kinase (MAP kinase). Interestingly, each of these pathways is in charge of different effects of BDNF. This implies that a differential activation of these pathways might therefore mediate different physiological neuronal properties for example by altering the phosphorylation level of target molecules and by modulating ion channels and receptor function (for review see: Minichiello, 2009; Reichardt, 2006; Yoshii and Constantine-Paton, 2010).

Recently, Zeng et al. (2011) have shown that 7,8-DHF, like BDNF, is able to rescue Long Term Potentiation (LTP) in aged animals.

The observed reduction of the GABAergic tone, following 7,8-DHF application, might constitute one of the cellular mechanisms underlying the restoration of synaptic plasticity, typical of old animals. In support of this hypothesis, emerging evidence indicates that the strength of GABAergic inhibition is a crucial factor in determining the level of synaptic plasticity in many cortical and subcortical structures (for review see: Jiang et al, 2005). In this regard, it has been demonstrated that LTP can be pharmacologically enhanced *in vitro* (Kirkwood and Bear, 1994; Meredith et al., 2003; Steele and Mauk, 1999; Wigström and Gustafsson, 1983) as well as *in vivo* (Harauzov et al., 2010) by a partial $GABA_AR$ blockade.

In addition to the effects on synaptic transmission, incubation of cortical slices in 7,8-DHF induced an increase in neuronal excitability, most likely caused by an increased input resistance.

The intrinsic neuronal excitability is normally governed by the differential activation or distribution of some ion channels on the plasma membrane (for review see: Daoudal and Debanne, 2003).

In line with the findings of this study, it is well known that the BDNF-TrkB signaling plays an important role in regulating the excitability of cortical neurons (Desai et al., 1999). Recent reports pointed out that BDNF can exert a modulatory action on different types of ion channels. This modulation is mediated not only by activation of intracellular signalling cascades, but also by a direct and rapid (within milliseconds) TrkB-receptor dependent alteration of the gating properties of such channels (for review see: Rose et al., 2004).

Future studies should disclose, which channel subtypes are involved in the alterations induced by 7,8-DHF.

4.1.2 *In vivo* 7,8-DHF effects on GABAergic transmission in WT and BDNF (+/−) mice

The ability of 7,8-DHF to cross the BBB and to exert its pharmacological effects directly into the brain has been already exploited in several studies. Intraperitoneal injections of 7,8 DHF produced in mice and rats a great variety of effects. For example, 7,8 DHF turned out to be neuroprotective against Kainate-induced apoptosis and excitotoxicity (Jang et al, 2010). It also possesses strong antidepressant effects (Liu et al, 2010), the capability to modulate the emotional behaviour (Andero et al, 2011; 2012) and to enhance memory and cognition in aged animals (Zeng et al, 2012) as well as in animal models of Alzheimer disease (Devi and Ohno, 2011).

On the basis of these reports and of the *in vitro* data collected in this study, which disclosed a selective 7,8-DHF on the GABAergic transmission, the *in vivo* 7,8-DHF effects were investigated to answer the following questions: 1) are the *in vitro* 7,8-DHF effects reproducible in vivo? 2) what are the effects of chronic 7,8-DHF administration on GABAergic transmission? 3) is it possible to rescue the deficits in the GABAergic transmission in BDNF (+/−) mice through a chronic TrkB receptor activation?

The route of administration and concentration of the drug as well as duration of the treatment and interval between last injection and beginning of the in vitro recordings were chosen based upon previously published studies.

In the present study an intraperitoneal injection was used. This approach, which was employed also in almost all other studies, is the easiest way to administer drugs to small rodents. The only exception was presented in a recent study (Liu et al, 2013), in which an oral administration was preferred.

All mice received a dose of 5 mg/Kg once daily for 7 consecutive days and the last injection was performed 1h before preparation of acute slices.

Andero et al (2010) screened a range of differing dosages (1, 5, 25 mg/Kg) and demonstrated that 5 mg/Kg may be the optimal dose for animals treatment. Indeed the drug displayed its maximal effects at this concentration. In support of this finding another study (Liu et al, 2013) showed that chronic treatment with 7,8 DHF revealed no demonstrable toxicity at 5 mg/Kg.

The duration of the treatment in all other reports was longer than 1 week. It ranges from 10 days (Devi and Ohno, 2011) up to 5 weeks (Zeng et al, 2012). In this study the treatment was limited to 7 days in order to study specifically the modulatory effects of 7,8-DHF in the third-fourth postnatal week of young mice.

Furthermore the mice were sacrified 1h after the last injection, as 7,8-DHF exhibited its peaks of activity after 1-2 h, although its metabolites can be detected both in plasma and in the brain up to 8h after administration (Liu et al, 2013).

Seven consecutive days of treatment induced a slight, but not significant, reduction in the mIPSCs frequency. This result clearly shows that the effects of a 30 minutes incubation of 7,8 DHF on GABAergic inhibition *in vitro* incubation with 7,8-DHF are not comparable in WT mice after a chronical application in *in vivo*.

This is surprising, since it is well accepted that long-lasting TrkB activation enhances the formation and maturation of GABAergic synapses, whereas acute BDNF supply induces a fast reduction of GABA inhibition (for review see: Gottmann et al, 2009). In this regard one might expect different effect upon TrkB receptor activation.

So far mainly *in vitro* experimental strategies (e.g. cell cultures) have been employed to study the chronic effects of BDNF application (for review see: Gottmann et al, 2009). On the contrary, due to the challenging nature of this approach, there is only one study in which the consequences of an *in vivo* chronic exposure to BDNF on synaptic transmission have been studied. In this study the authors infused BDNF directly into the brain through a microcannulae connected with an osmotic pump (Celada et al, 1996).

In the present study the effects of a chronic systemic administration of a TrkB agonist on the GABAergic transmission were analyzed for the first time: 7,8-DHF failed to chronically potentiate the GABAergic transmission chronically.

Many parameters can influence the drug efficacy. Perhaps the duration of the treatment should be extended, or the number of daily injections increased. Another reason could be that, the compound does not reach an adequate concentration in the brain, after having passed the BBB, to significantly alter the GABA circuits.

Many finer details of information are still missing. For example it is unknown which transport mechanism 7,8-DHF uses to pass through the BBB after an *in vivo* injection. Furthermore no data are available on the drug half-life and on the activity of enzymes in charge of metabolizing 7,8-DHF. For these reasons additional pharmacokinetic investigations are necessary to address these points.

Another aim of this study was to investigate whether a chronic application of 7,8 DHF is able to rescue the deficits in GABAergic synaptic transmission in BDNF (+/−) mice.

Abidin et al (2008) documented that the GABAergic system in these animals is functionally impaired, most probably due to a chronic lack of BDNF during development, which prevents the emergence of mature properties of GABAergic synapses.

Rescue experiments in BDNF (+/−) mice have been successfully performed only *in vitro* by means of exogenous BDNF supply or adenoviral vectors in the attempt to reinstate the LTP, that in these animals turned out to be impaired (Korte et al,1996; Patterson et al, 1996). Rescue of deficits in the GABAergic system has been attempted only once *in vitro*, in the prefrontal cortex of mice carrying a genetic mutation, the BDNF Val66Met polymorphism, which leads to a reduction in BDNF release and ultimately to the suppression of the inhibition (Patwell et al., 2012). In this report, the deficits in the GABAergic transmission could not be reversed simply by activating the BDNF-TrkB pathway.

A similar scenario was disclosed in the visual cortex of BDNF (+/−) mice, where the GABAergic transmission was not restored subsequent to a chronic 7,8-DHF treatment to WT level.

It is plausible that morphological alterations, related to a delay in the maturation of the GABAergic circuitry, may contribute to the deficits in inhibitory system observed in BDNF (+/−) mice and to the impossibility of reversing them.

Most unexpectedly, in BDNF (+/−) mice a reduction of the mIPSCs frequency was observed. Surprisingly once again the results obtained *in vivo* perfectly matched the findings of the *in vitro* experiments. This fact, on one side corroborates and further validates the mechanism of action of the 7,8-DHF, and on the other side brings to the conclusion that most likely the exposure of the neurons to the compound in the brain after an *in vivo* injection is only transient and comparable to the *in vitro* incubation. One can speculate that this is due to a short 7,8-DHF half life as a consequence of a fast enzymatic degradation.

However, further studies will be needed to clarify the precise mechanisms of 7,8-DHF-induced suppression of GABA receptor transmission in the cortex, and to define the right protocol allowing a complete restoration of the GABAergic inhibition in BDNF (+/−) mice.

In several studies (for review see: Andero and Ressler, 2012; Choi et al, 2010; Andero et al., 2011; Choi et al, 2012; Zeng et al 2012) 7,8-DHF was successfully employed to reverse deficits in fear memory and emotional learning, resulting from BDNF depletion in both transgenic and aged animals. These studies highlighted the importance of the BDNF-TrkB

signalling pathway in the function of crucial brain areas such as amigdala, hippocampus, prefrontal cortex and nucleus accumbens, which are strongly involved in the regulation of the emotional behaviour. 7,8-DHF upon in vivo administration, not only rescued abnormalities in acquisition consolidation and extinction of fear memory, but also lowered the threshold of LTP induction and induced a long-lasting enhancement of synaptic plasticity.

Another interesting finding is that administration of $GABA_A$ receptor agonists disrupts the fear memory, whereas antagonists facilitate the formation of fear memories (for review see: Makkar et al., 2010).

In light of all these experimental evidence, it is conceivable that attenuation of the GABAergic inhibition is one of the mechanisms underlying 7,8-DHF effects, not only in the visual cortex but also in other brain areas.

4.2 Lesion-induced alterations of the GABAergic transmission in the visual cortex of WT and BDNF (+/−) mice

Brain injuries may lead to impairments and disabilities, which can vary greatly in severity. In the attempt to improve the prognosis of the patients post-lesion and to better understand pathophysiological dynamics of the damaged tissue at a cellular level, many different injury models have been established (for review see: Xiong et al. 2013).

In the second part of this thesis a well established and highly reproducible "ex vivo in vitro" lesion model was employed. So far, neocortical laser lesions have been performed only in cats and rats. In the present study this model was for the first time transferred into the visual cortex of the mouse (Marongiu et al, in preparation).

One of the most common and frequently observed alterations post-lesion is the attenuation of the GABAergic inhibition, which has profound consequences on network excitability and is presumed to cause epilepsy (for review see: Li et al, 2011).

It is well known that in physiological conditions the neurotrophin BDNF/TrkB signalling strongly regulates the function of the GABAergic system (Gottmann et al, 2009). Less is known about the role of BDNF in the functional reorganization of the GABAergic system in pathophysiological conditions such as after a brain injuries. To my knowledge, only one study addressing this issue have been published (Shulga et al. 2008). This study has highlighted the existence of a strong interaction between these two systems in injured

neurons: the post-injury changes in the GABAergic transmission make these neurons become dependent on BDNF/TrkB signalling for survival, and the BDNF itself promotes their recovery.

Therefore a laser lesion was performed in the visual cortex of WT and BDNF (+/−) mice to address these points.

4.2.1 Lesion-induced effects on layer 2/3 pyramidal neurons intrinsic properties

In WT SHAM and LESION mice, as well as in BDNF SHAM and LESION mice, the intrinsic properties of the visual cortical layer 2/3 pyramidal neurons were not altered. This is in line with previous studies from our lab.

It has been reported by Abidin et al. (2006) that the genetic deletion of one BDNF gene does not alter the excitability of the neurons.

In another study, Imbrosci et al. (2010) documented that the laser lesion did not induce significant changes in the passive properties of the pyramidal neurons.

All the following experiments in the present study focused on possible changes in the GABAergic transmission in the visual cortical network.

4.2.2 Presynaptic functional properties of GABAergic synapses in sham and lesion-treated WT and BDNF (+/−) mice

Electrophysiological recordings from layer 2/3 pyramidal neurons in BDNF SHAM animals revealed a decreased mIPSCs frequency in comparison with WT SHAM animals. These findings are in agreement with previous studies (Abidin et al. 2008; Laudes et al, 2012), which documented that a chronic absence of BDNF impaired the GABAergic transmission in different brain areas.

In contrast to these reports, a reduction in mIPSCs amplitude could not be observed in the present experiments. This difference can be probably attributed to the experimental procedures (i.e animal housing, anaesthesia, fixation at the stereotaxic apparatus etc), that, the BDNF SHAM mice underwent before the recordings. These factors may strongly influence the neurotransmission (Biggio et al., 1987).

A reduction in the mIPSCs frequency normally indicates a decrease in presynaptic GABA release. Two potential mechanisms may account for this effect: reduction in the number of

functional GABAergic synapses, which contact the postsynaptic neurons, and/or decrease presynaptic release probability.

It is well known that BDNF regulates the maturation of the GABAergic system (Huang et al, 1999). One could expect that a long-term BDNF deficit would lead to a delay in the structural development of the GABAergic synapses. However, Korte et al (1995) reported that BDNF (+/−) mice did not display any remarkable morphological abnormality in the hippocampus. A similar observation was also made by Genoud et al (2004) which showed that, the synaptic structure of the barrel cortex was indistinguishable between WT and BDNF KO animals. These studies suggest that during circuit formation, the BDNF levels in the transgenic animals should be near a threshold and are apparently still sufficient to guarantee a normal brain development.

For these reasons a reduced anatomical arborization cannot be considered responsible of a impairment in GABA release, which most probably has a functional nature.

Reduction in the release probability may be a likely mechanism. This hypothesis is further supported by the observed increase in the paired pulse ratio, a parameter that is inversely correlated with the release probability.

The release probability at the GABAergic synapses is normally linked to the activity of both Ca^{2+} channels (Baldelli et al, 2002; 2005) and Glutamate Decarboxilase (GAD, the enzyme in charge of GABA synthesis), which are influenced by the BDNF concentrations (Henneberger et al, 2005). These two elements were not investigated in detail in this study.

According to Abidin et al (2008), BDNF KO animals have a slower vesicles turnover than WT animals. This can contribute to the reduction in GABA release.

The lesion caused similar effects in WT animals. Indeed, the mIPSCs frequency was significantly reduced in WT LESION animals in comparison with WT SHAM mice, although to a lesser extent, in respect to BDNF SHAM group, whereas the amplitude was unaffected. The reduction in the frequency was accompanied by a larger PPR. These two findings are once again a clear sign of impairments in the presynaptic neurotransmitter release.

Many lesion models are known to provoke alterations in the presynaptic terminals. In the “undercut cortex”, a well established model of posttraumatic epilepsy, for example, the presynaptic terminals are functionally abnormal (Faria and Prince, 2010). One reason for these abnormalities appears to be a dysfunction and a decrease density of perisomatic of N-type calcium channels. This in turn reduces the calcium influx in presynaptic GABAergic terminals contributing to decreased inhibitory input onto pyramidal cells (Faria et al, 2012).

Moreover structural abnormalities, such as reduction in axonal length, decreased axonal volume, were noted (for review see: Prince et al, 2009).

Using the same laser model lesion in rats, Imbrosci et al. (2012) failed to detect any structural deficit in the GABAergic interneurons, which are the major source of inhibition in the mammalian brain and are known to degenerate following a brain damage (for review see: Prince et al, 2009). The main outcome of this study was the finding that the laser lesion induces a downregulation of the phasic GABAergic inhibition due to a suppression of the GABA release, and induces a potentiation of the tonic component. Imbrosci et al proposed that this shift from phasic to tonic inhibition should preserve a basal level of inhibition post-injury to maintain the brain homeostasis.

The results of Imbrosci and al (2012) concerning the phasic GABAergic transmission obtained in the rat visual cortex, are perfectly in line with the findings of the present study, which have been obtained in mice. This speaks in favour of the reproducibility of the laser lesion model, which guarantees the same results in different animal species.

Impairments in the GABA release most probably account for the reduction in the overall strength of the GABAergic inhibition in the layer 2/3 of the visual cortex, as resulted in the decreased maximal eIPSCs amplitude in the Input-Output curve of WT LESION and BDNF SHAM animals.

A slight, but not significant, reduction in the I/O curve was detected also in the BDNF LESION group.

Interestingly, in this group the lesion did not affect mIPSCs frequency. No significant changes could be observed in the PPR either. These results indicate absence of presynaptic modulation by the lesion.

Surprisingly, the lesion caused a significant increase in mIPSCs amplitude. This is a strong evidence of a postsynaptic action of the lesion.

4.2.3 Postsynaptic properties of GABAergic synapses in sham and lesion-treated WT and BDNF (+/−) mice

The level of expression of BDNF strongly influence the GABAergic transmission: overexpression of BDNF enhances the inhibition, whereas a depletion of BDNF downregulates it (Huang et al, 1999; Abidin et al, 2008).

The BDNF concentration in the brain seems also to be important in the context of an injury.

Indeed, according to the electrophysiological data collected in the present thesis, the impact of the lesion appears different in WT and BDNF (+/−) mice.

In WT animals, as described above, the lesion acted presynaptically by reducing the release of GABA.

Conversely, in BDNF KO animals, which contain ~50% less BDNF, the lesion seems to exert a postsynaptic action.

This assumption is corroborated by the fact that, in BDNF LESION animals (but not in WT animals) the injury induced a significant increase in mIPSCs amplitude and altered the kinetics of eIPSCs, which rely on postsynaptic mechanisms, with no alteration in the frequency and PPR, and which are normally related to presynaptic changes.

The study by Abidin et al. (2008) disclosed a prolonged decay time of eIPSCs in BDNF (+/−) mice, which was confirmed in the present work. Moreover, I have also shown that the lesion in BDNF LESION animals shortened the decay time constants of eIPSCs.

A similar, not significant, trend was also noted in the kinetics of mIPSCs. The decay time was slightly prolonged in BDNF SHAM animals in comparison with all other 3 experimental groups, and appeared shorter postlesion. On the contrary the rise time of both mIPSCs and eIPSCs was unaffected.

Many factors can contribute to the regulation of the kinetics of the GABAergic currents.

Barberis et al. (2011) have shown that the GABA concentration in the synaptic cleft may have an impact on the IPSCs kinetics. At the synapses, the levels of GABA are kept constant by the activity of the GABA transporters, GAT1 and GAT 2/3, which re-uptake the neurotransmitter into nerve terminals. It has been demonstrated that the GABA transporters function is impaired post-lesion (Clarkson et al., 2010). However, according to the study by Imbrosci et al. (2012), in the present lesion model variations in eIPSCs kinetics are unlikely to be dependent on dysfunctional GABA transporters.

Another possible explanation may lie in the subunits composition of the $GABA_A$ receptors, which is known to strongly affect the rise and decay time of the IPSCs transients (Eyre et al, 2012). It is well established for example that, $GABA_A$ receptors containing $\alpha 1$ and $\alpha 6$ subunits give rise to IPSCs characterized by faster kinetics, than the kinetics of currents generated by the activation of receptors containing $\alpha 2$ and $\alpha 3$ subunits (Eyre et al, 2012).

It has been demonstrated that cortical lesions profoundly modify the pattern of expression of the $GABA_A$ receptor subunits (Redecker et al, 2000). Therefore, this mechanism must be taken into account.

Additional cellular mechanisms, which can affect IPSCs kinetics, are for example the differential phosphorilation state of the $GABA_A$ receptors (Jones and Westbrook, 1997) or a rearrangement in the location of the receptors inside the synapses (which is in part determined by the receptor subunits composition), in relation to the neurotransmitter release site (Farrant and Kaila, 2007).

As mentioned before, besides the kinetics of eIPSCs, also the mIPSCs amplitude turned out to be altered post-lesion in BDNF (+/−) mice.

Generally amplitude of mIPSCs primarily depends on the number and activity of postsynaptic receptors (Perrais and Ropert 2000).

The selective increase in mean mIPSC amplitude without effects on mIPSC kinetics observed in the BDNF LESION group, supports the idea that the lesion induced an increase in the total number of cell functional $GABA_A$ receptors on the surface of visual cortical pyramidal neurons.

Another factor, which can influence the mIPSCs amplitude, might be the amount of neurotransmitter in individual vesicles (Rannals and Kapur, 2011). The production of GABA is strictly regulated by the enzyme glutamic acid decarboxylase (GAD), which exists in two distinct isoforms: GAD65 and GAD67.

In a recent study (Imbrosci et al., 2012) employing the same lesion model, the Western blot analysis did not reveal changes in the expression of these two enzymes. Therefore it is unlikely that these enzymes play a role in the increase of mIPSCs amplitude.

It is noteworthy to mention that a larger mIPSCs amplitude did not correspond to an increase in eIPSCs amplitude post-lesion.

This indicates that in BDNF animals the lesion only modulated the basal synaptic GABAergic transmission at a single synapse level, rather than the action potential-dependent transmission in absence of TTX.

The maximal amplitude of eIPSCs in BDNF LESION mice was not further suppressed by the lesion in comparison with BDNF SHAM mice, which already have a reduced efficiency of the inhibitory system from birth (Abidin et al, 2008).

It could be speculated that this is a result of a series of compensatory homeostatic mechanisms triggered by the lesion, which take place in the already impaired GABAergic system.

The aim of these compensatory adjustments in the inhibitory synaptic strength may be to keep the activity of the affected neurons within a physiological range, in order to guarantee a

minimal level of inhibition for the normal brain function in response to external perturbations.

The increase in mIPSCs amplitude, also called "up-scaling", might indeed be one of the above-mentioned compensatory mechanisms taking place post-lesion. A recent study reported the occurrence of a similar lesion-induced homeostatic synaptic scaling at the excitatory synapses in the entorhinal cortex (Vlachos et al, 2012).

Homeostatic changes of mIPSCs amplitude are mostly mediated by changes in the number of synaptic $GABA_A$ receptors (Rannals and Kapur, 2011). This was shown by quantitative immunohistochemistry. This issue was not addressed in detail in the present thesis.

Several factors are implicated in the homeostatic regulation of inhibition. BDNF and TrkB receptors seem to play a pivotal role in this phenomenon (for review see: Wenner, 2011).

For these reasons the contribution of the BDNF-TrkB signalling in the lesion-induced alteration in the GABAergic transmission were investigated. The results are discussed in the next paragraph.

4.2.4 Implication of the BDNF-TrkB signalling in the lesion-induced alterations of GABAergic transmission

A pioneering study by Rutherford et al. (1997) has demonstrated that pharmacological manipulation of the BDNF/TrkB signalling can modify the quantal amplitude of pyramidal neurons.

Moreover, an up-scaling of IPSCs, similar to the one observed in the present work, was described in another study, performed in the superior colliculus of BDNF (-/-) mice (Henneberger et al., 2002). Interestingly this effect could be mimicked by acute blocking of the TrkB receptors, by pre-incubating the slices with the TrkB antagonists K252a.

Surprisingly, the same experimental protocol was able to reproduce the lesion effects in the visual cortex: in the WT SHAM group K252a reduces the presynaptic GABA release. In WT SHAM, WT LESION, BDNF SHAM animals the amplitude was significantly larger. Remarkably no further increment in the already increased mIPSCs amplitude could be observed in BDNF lesion animals.

This may be an occlusion effect due to an overlap in the lesion and K252a action: most probably, both the pharmacological block of the TrkB receptor and the lesion activate the same signalling pathways and no cumulative effects can be observed.

The pre- and postsynaptic action of K252a can be explained by the ubiquitous location of the TrkB receptors. A study by Drake et al (1999) pointed out that they can be found both in the presynaptic terminals and in the postsynaptic densities of every synapse in the central nervous system.

The physiological significance of a downregulation of the BDNF/TrkB signalling post-lesion is unknown.

A recent review (Boulle et al, 2012) showed that in many pathophysiological conditions, such as brain injury, epilepsy, addiction, eating disorders etc., a prolonged TrkB receptors activation may underlie the course and the development of clinical symptoms. For this reason, decreasing the TrkB activation appears to be a promising therapeutic landscape in the treatment of various diseases.

In our preparation this could be a neuroprotective strategy, put in practice by the brain, to dampen the deleterious consequences of the injury.

Downregulation of the BDNF /TrkB signalling can be the result of a reduction in the levels of BDNF, which selectively binds and activates the TrkB receptors.

Several studies have reported that the BDNF expression is reduced post-lesion (Fumagalli et al, 2009; Prince et al, 2009; Schober et al, 2012).

However, the ELISA test revealed that, the BDNF levels after a laser lesion were unaffected.

The tissues of lesioned animals have been collected 3 days post-lesion. Possibly, the BDNF downregulation occurs at a different time point.

BDNF is not the only endogenous ligand of TrkB receptors: the neurotrophin NT4 is also able to activate the TrkB receptors with comparable efficacy (for review see: Chao, 2003). The expression levels of NT4 mRNA post-lesion was investigated. It turned out that NT4 mRNA level were not affected by this laser lesion model (Mittmann and Wahle, unpublished observations).

The p75 receptor represents another important key player in pathophysiological conditions. This receptor is normally upregulated after an injury. It mediates opposite effects to the TrkB receptor, although it has been demonstrated that there is a mutual interaction between the TrkB and the p75 signalling.

Moreover a recent study (Shulga et al., 2012) reported that, the p75 receptor is strongly implicated in the post-traumatic changes in the GABAergic transmission.

Further investigations are necessary to address these issues.

5. Concluding remarks

The data of the present PhD-thesis indicate, that the impact of a laser lesion on GABAergic transmission in the mouse visual cortex is dependent on the initial concentration of BDNF. Indeed, in WT mice the lesion acts presynaptically, by reducing the GABA release from the presynaptic terminals, whereas in BDNF (+/−) mice the lesion affected the function of the postsynaptic $GABA_A$ receptors. Furthermore, a block of the TrkB receptors completely reproduced the lesion effects both in WT and BDNF (+/−) animals. In light of these results, the activity of the BDNF/TrkB signalling cascade seems to play a fundamental role in the functional reorganization of the GABAergic circuits post-lesion, and the possibility to pharmacologically modulate this pathway could be important for the development of future therapeutic strategies.

The new TrkB receptor agonist 7,8-DHF represents a pharmacological tool, which might be used to this purpose. In the present study, its pharmacological profile at the cellular level has been characterized. 7,8-DHF acts selectively on the GABAergic system, by attenuating the strength of the inhibition. These findings could be a starting point for the development and test of other new compounds "BDNF mimetics", which might be employed to treat human patients in the future.

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