The role of Src kinase in the TrkB signaling pathway induced by sleep deprivation and sedative-anesthetic drugs

Master's thesis

Piotr Michalowski

Master's Program in Genetics and Molecular Biosciences

University of Helsinki

2019

Table of Contents

ABBREVIATIONS

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

CREB: cyclic AMP response element binding protein

DMSO: dimethyl sulfoxide

ECL: enhanced chemiluminescence

EEG: electroencephalography

ERK: extracellular signal-regulated kinase

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GSK3β: glycogen synthase kinase-3β

HC: hippocampus

NFDM: non-fat dry milk

NMDA: *N*-methyl-D-aspartate

PBS: phosphate-buffered saline;

PFC: prefrontal cortex

PLCγ1: phospholipase Cγ1

PP2: pyrazolopyrimidine 2

PSD: postsynaptic density

SD: sleep deprivation

SWA: slow wave activity

TBS-T: Tris-buffered saline + Tween 20

TrkB: tropomyosin-related kinase B

Y: tyrosine

Tiedekunta – Fakultet – Faculty	Koulutusohjelma– Utbildingsprogram– Degree Programme	
Faculty of Biological and Environmental Sciences	Master's Program in Genetics and Molecular Biosciences	
Tekijä – Författare – Author Piotr Michalowski		
Työn nimi – Arbetets titel – Title The role of Src kinase in the TrkB signaling pathway induced by sleep deprivation and sedative-anesthetic drugs		
Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track Cell and Developmental Biology		
Työn laji – Arbetets art – Level Master's thesis	Aika – Datum – Month and year August 2019	Sivumäärä – Sidoantal – Number of pages 36

Tiivistelmä – Referat – Abstract

The TrkB signaling pathway plays an important role in synaptic transmission and plasticity. Synaptic plasticity is disrupted in many neurological disorders, such as major depression and dementia. A number of studies indicate that TrkB (tropomyosin-related kinase B) signaling is required for the therapeutic effects of antidepressants. Both conventional and rapid-acting antidepressants encompass the TrkB pathway but the underlying mechanism of this remains unknown. Recent studies have, however, revealed an intriguing link between emergence of slow wave EEG activity (SWA) or sedation and the TrkB pathway. Notably, various anesthetics and sedatives (e.g. isoflurane and medetomidine) that increase SWA concomitantly induce TrkB signalling, and this seems to happen independently of BDNF (brain-derived neurotrophic factor), the primary ligand of TrkB.

Given the ability of Src kinase to transactivate TrkB *in vitro*, we have examined the acute effects of medetomidine and isoflurane on Src^{Y416} and $TrkB^{Y816}$ phosphorylation in the adult rodent cortex and hippocampus by using Western blotting. Pyrazolopyrimidine 2 (PP2), a Src kinase inhibitor, was implemented in order to inhibit TrkB signalling pathway induced by medetomidine. The study was further extended to sleep deprivation experiments to investigate the effects of deep sleep on the Src and TrkB protein phosphorylation. Phosphorylation of $GSK3\beta^{S9}$, another important molecular event coupled with antidepressant effects, was also investigated.

The results indicate that both isoflurane and medetomidine activate Src kinase and TrkB signalling pathway. Such an effect was not, however, seen in the PP2 study and thus we failed to confirm the mechanistic connection between Src and TrkB. A trend in the phosphorylation of TrkB, Src and GSK3β was found in the brain samples collected after 15 minutes of recovery sleep, suggesting that TrkB signalling is also facilitated during physiological SWA. In conclusion, these results reinforce the hypothesis that SWA occurs simultaneously with TrkB signaling. Future studies are required to test the involvement of Src kinase in this phenomenon.

Avainsanat – Nyckelord – Keywords
Src kinase, TrkB signaling pathway, sleep deprivation, slow wave activity

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
Tomi Rantamäki and Marko Rosenholm

Säilytyspaikka – Förvaringställe – Where deposited

Muita tietoja – Övriga uppgifter – Additional information

1. Introduction

Recently, the tropomyosin-related kinase B (TrkB) signaling pathway has attracted much attention, as it has been proven to be involved in the mechanism of action of antidepressant drugs (Antila et al., 2017; Autry & Monteggia, 2012; Castrén & Rantamäki, 2010; Duman et al., 2012; Kohtala et al., 2019b; Rantamäki et al., 2011). This was a surprising finding, since antidepressant action had been previously linked chiefly to the brain monoamine (e.g. serotonin) levels. In addition, the TrkB signaling pathway has been observed to promote synaptic plasticity, which putatively underlies the positive antidepressant effects. According to previous research, the TrkB receptor is activated by its primary ligand i.e. brain-derived neurotrophic factor (BDNF). However, it has been determined that the TrkB receptor can be also activated independently of BDNF, with Src kinase being involved in the process.

Several sedative-anesthetic drugs have been shown to alleviate depressive symptoms and concomitantly induce the TrkB signaling pathway. The most prominent example of these drugs is ketamine, which already produces its positive effects within hours of drug administration. The sedative-anesthetic drugs that activate the TrkB signaling pathway have been also shown to simultaneously induce brain slow wave EEG activity (SWA). Given that the TrkB pathway and SWA co-occur, it can be inferred that eliciting SWA would induce TrkB signaling. SWA mainly takes place during sedated state and deep sleep. Sedative-anesthetic drugs cause sedation, thus inducing SWA. Alternatively, SWA occurs during deep sleep which is a physiological process. Interestingly, deep sleep can be intensified by implementing sleep deprivation. Sleep deprivation increases homeostatic pressure, thus causing the following sleep period to be more intense than normally. Therefore, it could be hypothesized that sleep deprivation with subsequent recovery sleep will elicit particularly intensive SWA.

In this study, we attempt to disclose the role of Src kinase in the TrkB signaling pathway induced by different sedative-anesthetic treatments. As a novel means to induce TrkB signaling, we used sleep deprivation with subsequent recovery sleep. The results were obtained by examining the phospho-protein levels in the mouse hippocampus and prefrontal cortex.

2. Review of the literature

The following literature review first provides an overview of the BDNF-TrkB signaling pathway. It then discloses Src kinase and its possible involvement in the activation of the TrkB receptor. After this, the literature review discusses the concurrence SWA and enhanced TrkB signaling. Finally, it evaluates the possibility of inducing the TrkB signaling pathway by implementing sleep deprivation with consequent recovery sleep.

2.1. An introduction to BDNF-TrkB signaling

BDNF belongs to the family of neurotrophins. Neurotrophins control growth, maintenance and function of vertebrate nervous system. The first neurotrophin, nerve growth factor (NGF), was discovered in 1950s by Rita Levi-Montalcini, Victor Hamburger and Stanley Cohen. In addition to NGF and BDNF, neurotrophin family in mammals comprises neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Hallböök et al., 1991; Maisonpierre et al., 1990). The members of the family structurally resemble each other, so much so that BDNF has the half of its amino acid sequence identical with NGF.

BDNF protein was first isolated from the pig brain (Barde et al., 1982). It is produced in endoplasmic reticulum in a form of pro-BDNF (molecular weight 30-35 kDa), that is later cleaved to its mature form. Mature BDNF is much smaller than pro-BDNF, its molecular weight being only 13 kDa (Barde et al., 1982; Bathina & Das, 2015). BDNF is the most abundant neurotrophin in the brain. While BDNF is widely expressed in the brain, most abundant expression is seen in the cerebral cortex and hippocampus regions (Katoh-Semba et al., 1997). Even the areas that do not produce BDNF can contain the transported form of it (Antila, 2016). BDNF is present mostly in neurons, but to smaller extent also in astrocytes and microglia (Wetmore et al., 1990). In addition, BDNF is also present in the bloodstream, preferentially in the platelets, although the correlation between the brain and blood BDNF concentration remains unclear (Fujimura et al., 2002).

BDNF is important for the early development of the central nervous system (CNS) in mammals (Huang et al., 1999; Huang & Reichardt, 2001; Thoenen et al., 1995). BDNF is also a chief factor in promoting the differentiation and survival of subpopulations of neurons in peripheral nervous system (PNS). Additionally, it is a significant promoter of maturation of the inhibitory interneurons. All the above-mentioned positive effects on the nervous system are produced upon the interaction of BDNF with its receptor TrkB.

TrkB serves as a receptor for BDNF and NT-4, although it has higher affinity to BDNF (Huang & Reichardt, 2003). It belongs to the family of Trk receptors, which includes also TrkA, a receptor of NGF, as well as TrkC, a receptor of NT-3. TrkB was first isolated by cloning from mouse brain tissue (Klein et al., 1989; Klein et al., 1990). The molecular weight of the full-length TrkB is 145 kDa (Saarelainen et al, 2003). The TrkB receptor has two ends: N-terminus facing the extracellular side and C-terminus inside the cell. The full-length receptor encompasses the extracellular domains, a short transmembrane domain and an intracellular domain, which contains tyrosine kinase domain with adjacent tyrosine (Y) motifs (Figure 1). BDNF binds to the extracellular domains of TrkB, namely immunoglobulin-like domains and leucine-rich repeats (Reichardt, 2006). Upon BDNF binding, phosphorylation of these cytoplasmic tyrosines takes place. Such modification adjusts tyrosine kinase activity and provides docking sites for adaptor proteins and enzymes that facilitate the initiation of intracellular pathways. In addition to the full-length receptor, also a truncated form of TrkB can be expressed (Antila, 2016). The truncated form resembles the full-length isoform, but it lacks the tyrosine kinase intracellular domain.

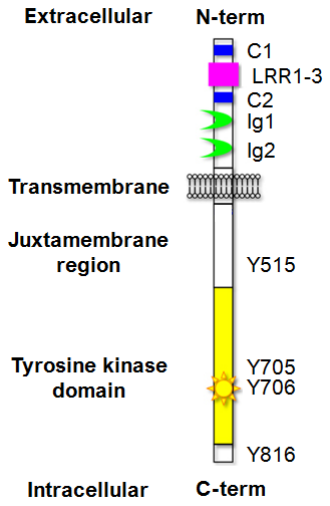

Figure 1. The full-length TrkB receptor. The extracellular domain includes two cysteine clusters (C), leucine-rich repeats (LRR) and two immunoglobulin-like domains (Ig). BDNF binds to the Ig and most probably to the LRR. As a result of BDNF binding, the TrkB receptor forms a homodimer. The catalytic domain (Y705/6) is marked with a symbol of the sun. During the homodimer formation, two TrkB receptors approach each other and phosphorylate each other's catalytic domains. Following that phosphorylation, the receptor is active and other intracellular tyrosine residues can be phosphorylated as well, thus initiating signaling cascades. Abbreviations: C1 and C2, cysteine clusters; LRR 1-3, leucine-rich repeats; Ig1 and Ig2, immunoglobulin-like domains (Figure based on that of Uren & Turnley, 2014).

Regarding the sites of expression, TrkB is widely expressed both in CNS and PNS. In the brain it is produced in cerebral cortex, hippocampus, corpus callosum, hypothalamus and spinal cord (Klein et al., 1993; Yan et al., 1997). Thus, the common expression sites for both BDNF and TrkB are chiefly hippocampus and prefrontal cortex (Wetmore et al., 1990; Klein et al., 1993).

BDNF-TrkB signaling is crucial for the formation and maintenance of CNS and PNS. This is confirmed by the fact that mice with complete deletion of BDNF/TrkB cannot survive after birth due to defects in the PNS (Klein et al., 1993). In addition to its recognized role in the

maintenance of the nervous system, the BDNF-TrkB signaling pathway was also proven to be intimately linked to the processes underlying neuronal plasticity and antidepressant drug effects (Thoenen, 1995; Saarelainen et al., 2003; Castrén & Rantamäki, 2010). As a result, much research in recent years has focused on the TrkB signaling pathway and its effects on synaptic plasticity and involvement in mood disorders and their treatment.

2.2. BDNF-TrkB signaling regulation in neuronal plasticity

By neuronal plasticity we understand the brain ability to adapt in response to environmental inputs or internal activity. Neuronal plasticity events depend on the activity of neurotrophins, as reviewed elsewhere (Castrén et al. 2007; McAllister et al. 1999; Thoenen, 1995). In order to neurotrophins underlie neuronal plasticity, they must meet the following requirements: neurotrophins and their receptors must be expressed in the appropriate brain areas; their secretion has to be activity-dependent, and they must regulate neuronal functions (McAllister et al., 1999). The plasticity of the brain is at its best during the critical period. The critical period can be defined as a "*well-defined time window during which environmental sensory stimulus is required for proper wiring and fine-tuning of neuronal networks in the brain*" (Antila, 2016). Only during the critical period, the brain adaptation is highly efficient, and once the period is over, some neural processes are irreversible.

This brain plasticity is mainly expressed in processes such as neuro- and synaptogenesis. Neurogenesis and the birth of new synaptic connections are clear signs of synaptic plasticity, while reduced volume of hippocampus and prefrontal cortex are hallmarks of depression (Castrén et al., 2007). Therefore, events like neuro- and synaptogenesis, as well as neuronal maturation should characterize an efficient antidepressant treatment. It was determined that antidepressants promote the maturation of newborn neurons and support synaptogenesis in hippocampus (Fujioka et al., 2004). According with these findings, it was tested whether an antidepressant drug fluoxetine can restore neuronal plasticity in the visual cortex of rat (Vetencourt et al., 2008). The experiments were performed on the visual cortex, since it is a widespread model system for studies of activity-dependent plasticity. A common method to study the formation of neuronal circuits in the visual cortex is monocular deprivation. Monocular deprivation consists in keeping one eye covered during the critical period of neuronal plasticity. As a result, only the neuronal circuits of the uncovered eye develop properly. Vetencourt et al. implemented monocular deprivation on young rats and later intended to restore the visual functions of the weaker eye, a change that is ordinarily possible only during the critical period. As a result of the monocular deprivation, a weaker eye and

stronger eye were constituted. In the first experiment, fluoxetine was being administered and the weaker eye was kept uncovered, while the stronger eye was closed for 1-week time. Afterwards, the visual acuity of both eyes was compared. Intriguingly, a shift in ocular dominance occurred in the favor of the weaker eye. The second test was performed on adult rats that had become amblyopic as a result of long-term monocular deprivation. The experiment lasted 2 weeks and during it fluoxetine was also administered. In the test, the weaker eye was kept uncovered, while the stronger eye was sutured shut. As a result, the weaker eye recovered from amblyopia. These above-described positive effects are attributed to BDNF activity, since infusion of BDNF into the visual cortex essentially recapitulated the effects of fluoxetine. Indeed, chronic administration of antidepressants causes the overexpression of BDNF and its receptor TrkB. Interestingly, mRNA of BDNF in the visual cortex is synthesized according to activity. It was determined that light exposure upregulates BDNF mRNA levels in rat visual cortex, whereas light deprivation lessened the synthesis of BDNF (Castrén et al., 1992). Therefore, the secretion of BDNF seems to be activity-dependent, what confirms its role as a regulator of neuronal plasticity. Interestingly, BDNF has been linked to the development of visual cortex already before the study of Vetencourt et al. (McAllister et al., 1999; Thoenen, 1995).

In addition to neuro- and synaptogenesis, neuronal plasticity implicates the strengthening of synaptic connections. The process of strengthening of synaptic connection is called Long-Term Potentiation (LTP). LTP can be caused by either short and intensive electric impulse or simultaneous activation of pre- and postsynaptic neurons (Minichiello et al., 2002). It has been shown that LTP is a key process underlying learning and consolidation of memories in hippocampus (Bliss & Collingridge, 1993; Mizuno et al., 2003). Interestingly, it has been discovered that BDNF enhances LTP, whereas LTP in BDNF knockout mice is considerably reduced (Korte et al., 1995). All in all, the neuronal plasticity-related events occur as a result of the activation of the TrkB receptor by BDNF.

2.3. Activation of the TrkB receptor

As mentioned above, the TrkB receptor is tyrosine-phosphorylated upon BDNF binding. The phosphorylated i.e. active form of TrkB is frequently annotated as phospho-TrkB (pTrkB). BDNF binds to the membrane-proximal Ig2-domain of TrkB (Reichardt, 2006). In addition to Ig2-domain, it seems that also Ig-1 residue may be required for the ligand binding (Huang & Reichardt, 2003). Most probably also leucine-rich repeats (LRR) are crucial to the binding, since the truncated receptor that lacks LRR is unable to bind the ligand. BDNF compels

TrkB to undergo a conformational change, thus producing a TrkB homodimer. The new dimer conformation results in an active form of the receptor (Huang & Reichardt, 2001; Reichardt, 2006). The homodimer is formed, when two TrkB receptors approach each other and phosphorylate each other's tyrosine (Y) domains Y705/6. The Y705/6 site is the catalytic domain, since it is the first residue to be phosphorylated and it is required for the phosphorylation of additional residues.

The most studied additional tyrosine residues of TrkB are the Y515 and the Y816 cytoplasmic domains (Huang & Reichardt, 2001). Upon the consequent phosphorylation of above-mentioned tyrosine residues, new binding sites are created for adaptor proteins (Middlemas et al., 1994). The proteins that dock in the binding site must contain either phosphotyrosine-binding (PTB) or SH2 domain (Reichardt, 2006).

Following the initiation of several intracellular cascades, the receptor itself is endocytosed (Huang & Reichardt, 2003). Endocytosis of the TrkB receptor most probably modulates the duration of TrkB signaling. It was determined that prolonged administration of BDNF decreases the number of the TrkB receptors located on the cell surface, due to their endocytosis into cytosol. It seems that the endocytosis of TrkB is stimulated by the BDNF binding, as well as by the increase of the concentration of cytoplasmic Ca^{2+}, a result of the phosphorylation of the Y816 domain (Reichardt, 2006). In the cell, TrkB is localized to intracellular vesicles (Reichardt, 2006). Apparently, receptor internalization is required for the survival-promoting effects at least in sensory neurons (Antila, 2016).

Although BDNF is the key ligand for TrkB, the receptor can be also activated by additional factors. Activation in which the activating factor does not directly bind to the extracellular site of the receptor is called transactivation.

2.4. Transactivation of the TrkB receptor

Although BDNF is the primary ligand for the TrkB receptor, the TrkB signaling pathway can be also induced in the absence of the neurotrophin. Transactivation occurs directly through the activation of intracellular cascade (Lee & Chao, 2001). Most studies on the transactivation of TrkB have been performed *in vitro.* The first agents found to transactivate the TrkB receptor were adenosine and pituitary adenylate-cyclase-activating polypeptide (PACAP) (Lee & Chao, 2001; Lee et al., 2002). These factors stimulate the TrkB receptor respectively through adenosine A_{2A} receptor and PAC1 receptor, both G protein-coupled receptors (GPCR). Afterwards, it was shown that also adenosine agonist can transactivate

TrkB (Lee & Chao, 2001). In addition to adenosine and its agonists, also intracellular zinc can transactivate the TrkB receptor (Huang et al., 2008). In their study, Huang et al. tested the effects of zinc on mossy fiber-CA3 pyramid synapses. In order to do so, they applied high-frequency stimulation in order to elevate the intracellular zinc concentration. Apparently, upon high-frequency stimulation larger amounts of glutamate and zinc are released from the presynaptic end (Figure 2). Thus, zinc enters the postsynaptic end through *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and voltage-gated calcium channels. Inside the postsynaptic site Zn^{2+} inhibits C-end Src kinase (Csk). Inhibition of Csk disinhibits Src kinase, which in turn phosphorylates the TrkB receptor. Zinc works much faster than GPCR ligands, since it activates TrkB with few minutes (Huang et al., 2008). The above-mentioned agents transactivate the TrkB receptor thus leading to intracellular signaling cascades.

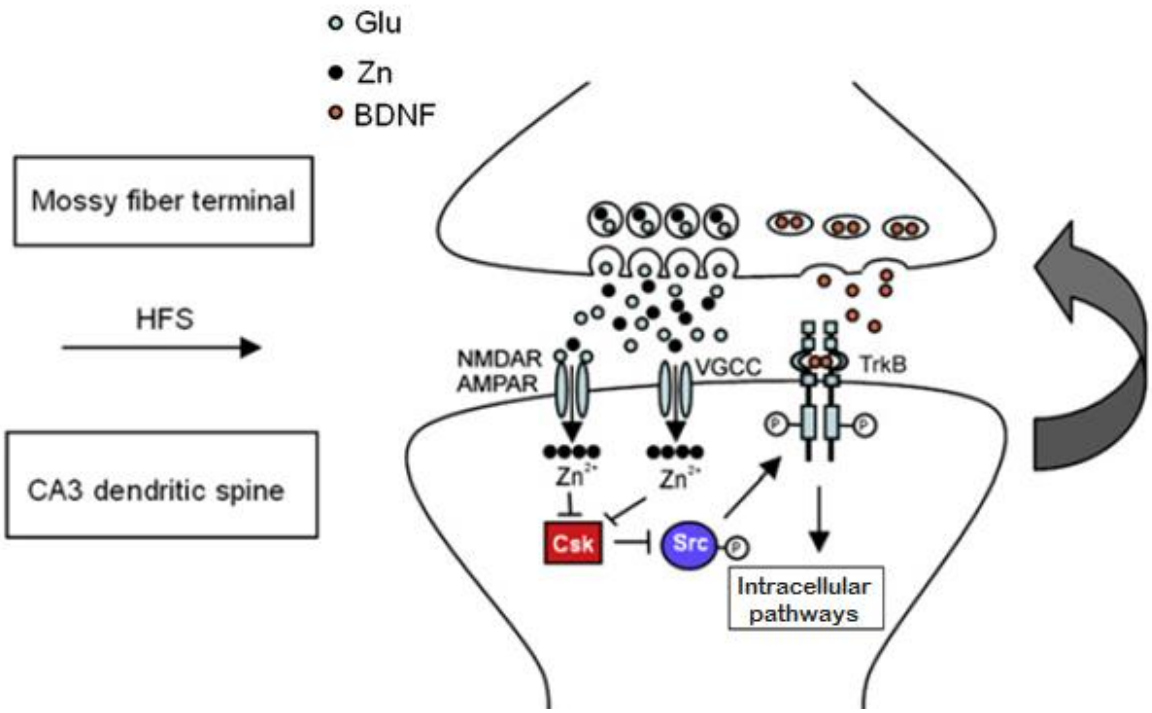

Figure 2. Transactivation of the TrkB receptor via Src kinase. Upon high-frequency stimulation, zinc is released from the presynaptic terminal to the synaptic cleft. From there it enters the postsynaptic site via NMDA and AMPA receptors as well as via voltage-gated calcium channels. In the postsynaptic density zinc inhibits Csk, thus activating Src kinase. Src kinase in turn activates the TrkB receptor, what results in intracellular signaling. Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Glu, glutamate; HFS, high-frequency stimulation; NMDAR, *N*-methyl-D-aspartate receptor; VGCC, voltage-gated calcium channel; TrkB, tropomyosin-related kinase B; Csk, C-terminal Src kinase; Zn, zinc. (Used by permission of Elsevier, figure from Huang et al., 2008).

2.5. Intracellular downstream cascades of TrkB signaling

Following the activation of the TrkB receptor, several intracellular cascades that promote cell survival and neuronal plasticity can be initiated. Which signaling cascade is initiated depends on the tyrosine residue that is phosphorylated (Figure 3). Once phosphorylated, the residue recruits an adaptor protein, which in turn communicates with the next signaling protein. In this fashion, the signal may finally arrive to the transcription factors that modulate gene expression in the cell nucleus.

The phosphorylated Y515 site (pY515) has been associated with neuronal survival and axon elongation (Huang & Reichardt, 2003). The domain serves as a docking site for both Shc and fibroblast growth factor receptor substrate 2 (Frs2). Both Shc and Frs2 promote cell survival and differentiation. Shc recruits phosphoinositide 3-kinase (PI3K) which phosphorylates Akt protein (Duman et al., 2012). Akt protein in turn phosphorylates glycogen synthase kinase-3β (GSK3β) and mammalian target of rapamycin (mTOR). The PI3K/Akt/mTOR signaling can be initiated for instance by adenosine and PACAP (Lee & Chao, 2001). The PI3K/Akt/mTOR intracellular cascade leads to cell survival (Takei et al., 2004). Alternatively, also Frs2 can bind to the pY515 domain (Lee et al., 2002). In this case, Frs2 forms a complex with SH2 domain-containing protein tyrosine phosphatase 2 (Shp2) and growth factor receptor binding protein 2 (Grb2) (Easton et al., 2006). The association of Grb2 with Frs2 probably leads to the activation of Ras protein. Then, Ras phosphorylates Raf protein, which in turn upregulates extracellular signal-regulated kinase (ERK). ERK can influence cyclic AMP response element binding protein (CREB). Ras/ERK pathway regulates spinogenesis and enhances the dendrite outgrowth (Alonso et al., 2004).

The phosphorylated Y816 domain (pY816) has been strongly linked to synaptic plasticity (e.g. LTP) (Korte et al., 2000; Minichiello et al., 2002). The mechanism of the cascade is the following: the pY816 recruits and activates phospholipase Cγ1 (PLCγ1), which hydrolyzes phosphatidyl(4,5)inositol bisphosphate (PIP2) to secondary messenger molecules diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Antila, 2016). Although DAG cannot penetrate the cytosol, it activates protein kinase C (PKC) which in turn enters the cytoplasm and there it activates ERK. IP_3, in turn, increases intracellular calcium concentration. The activation of PKC and the release of Ca^{2+} can further activate calcium/calmodulin-dependent protein kinase (CaMK) and CREB. CaMK is particularly responsible for LTP, whereas CREB is a transcription factor that regulates the protein synthesis in the cell nucleus (Minichiello et al., 2002). Both CaMK and CREB are linked to synaptic plasticity.

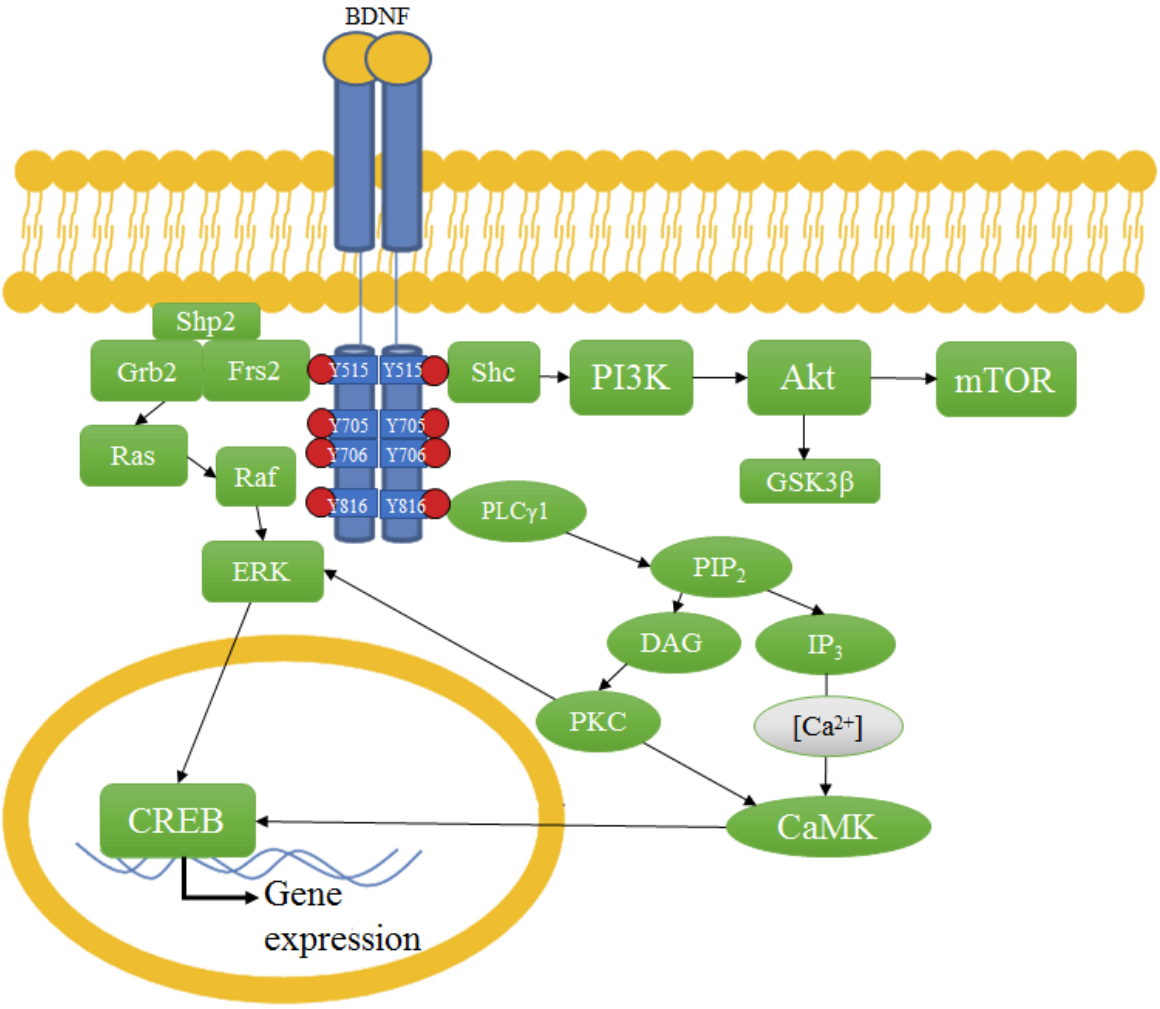

Figure 3. An overview of intracellular pathways induced by BDNF-TrkB interaction. The Y515 domain is the starting point for both PI3K/Akt/mTOR and Ras/ERK intracellular pathways that lead to cell survival and growth. The Y816 residue in turn activates the PLCγ1 signaling pathway that increases intracellular Ca^{2+} concentration and activates CaMK. All the pathways converge in CREB that directly influences gene expression and protein synthesis (Figure based on that of Autry & Monteggia, 2012).

2.6. Src kinase as a mediator of TrkB signaling

Src kinase is a ubiquitously expressed protein that is involved in important cellular processes, such as inflammation, cell proliferation and survival (Roskoski, 2015). It is a non-receptor protein-tyrosine kinase that belongs to the Src family kinases (SFKs). Src family kinases participate in cell signaling and are critical to diverse biological processes. There are eleven members of SFKs in humans, divided into 3 subgroups. From the point of view of this study only the first group is relevant. To this group belong Src, Fyn, Yes and Fgr proteins. Src was the first one of those molecules to be found. Src is a small molecule of 57

kDa. It contains Src homology 2 and 3 (SH2 and SH3) domains, tyrosine-kinase domain, as well as a regulatory tail (Figure 4). The most important residues of Src are Y527, located on the SH2 domain, and Y416, located in the tyrosine-kinase domain. Usually, Csk keeps the Y527 domain phosphorylated, thus making Src kinase inactive. In order to activate Src kinase, one option is to decrease the activity of Csk. When the Csk activity fades, then the Y527 domain is gradually dephosphorylated. This facilitates the phosphorylation of the Y416 residue which is linked to the activity of Src kinase. A phosphorylated (active) form of Src kinase can be marked as phospho-Src (pSrc). Src kinase is expressed in all tissues, but most abundantly in brain and osteoclasts, as well as in platelets. In the CNS, Src is abundantly present in the post-synaptic density (Huang & Reichardt, 2001).

So far, the investigation on Src kinase has been focused on its role in cancer (Frame, 2002). Src protein is overexpressed in epithelial and colon cancers. Oncogenic Src initiates the PI3K/Akt signaling pathway that leads to tumor cell survival. It is also probable that Src influences cell apoptosis. In addition to its role in cancer, Src is also a crucial agent in the regulation of NMDA, a subtype of glutamate receptors (Wang & Salter, 1994). In detail, Src enhances the current flow through NMDA receptors. This molecular event supports the hypothesis that Src kinase can modulate important physiological processes.

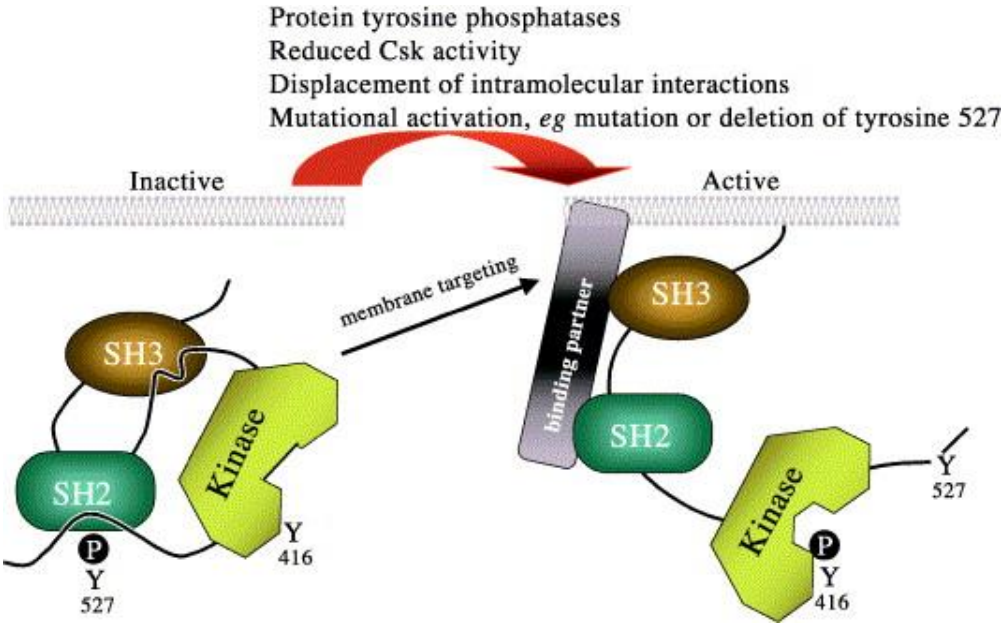

Figure 4. Src kinase in both inactive and active forms. Src kinase is inactive when Csk activity is high, and tyrosine-527 (Y527) is phosphorylated. Alternatively, when Csk activity is reduced, Src kinase becomes active. The activation is facilitated by the gradual dephosphorylation of the Y527 and the subsequent phosphorylation of tyrosine-416(Y416). (Used by permission of Elsevier, figure from Frame, 2002).

In addition to the above-mentioned functions, Src takes part in the transactivation of epidermal growth factor (EGF) (Roskoski, 2015). It is also involved in platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) signaling. Regarding Trk receptors, already in the 1990s it was hypothesized that there could be an interaction between Trk receptors and Src (Obermeier et al., 1994). In a research article, Obermeier et al. suggested: "*we see the possibility of a link between the Src signaling system and the pathway triggered by Shc/Trk, and possibly PLCγ1/Trk, interactions. Such a cooperative signal amplification scenario may, in fact, be critical for the generation of a more sustained activation of PLCγ1 and downstream signal transducers like Ras and ERK*". Later it was discovered that Src mediates the transactivation of TrkA receptor by adenosine in the absence of its typical ligand NGF (Lee et al., 2002). The same research group has found that SFKs inhibitors PP1 and PP2 block Trk transactivation (Rajagopal & Chao, 2006). In their study they focused on Fyn, a member of the same subgroup as Src. Interestingly, Fyn was activated in the same time pattern as Trk receptors. Therefore, an association was made that Fyn is closely connected to Trk receptor. Eventually, it was determined that Fyn interacts with juxtamembrane region of Trk. In addition, it was proposed that Src works as a mediator in the zinc-mediated transactivation of the TrkB receptor (Huang et al., 2008). Huang et al. observed that zinc activated TrkB independently of BDNF. However, zinc did not phosphorylate TrkB when Src kinase was inhibited with PP2. Thus, the researchers suggested a mechanism, in which Zn^{2+} inhibit Csk activity, what results in the increased activity of Src kinase (Figure 2). Src kinase then phosphorylates Y705/6 site of the TrkB receptor. In addition to the claims of Huang et al., there is more evidence that Src could be implicated in the activation of TrkB (Zhang et al., 2013). According to Zhang et al, Src kinase is a downstream of the BDNF-TrkB pathway. In their study, whenever TrkB was activated by BDNF, so was Src kinase. Once activated, Src kinase provided positive feedback, thus phosphorylating the TrkB receptor on the Y816 domain. This phosphorylation caused the PLCγ1 intracellular cascade, which promoted extracellular release of glutamate. Accordingly, inhibition of Src prevented the phosphorylation of PLCγ1 and subsequent glutamate release. However, the above-mentioned studies slightly differ regarding the mechanism of TrkB activation. Huang et al. reported transactivation, whereas Zhang et al. claimed BDNF being necessary in the process. What is more, the study by Huang et al. reported that Src kinase activates the Y705/6 domain. Zhang et al., in turn, claimed that Src only interacts with the Y816 domain. Notwithstanding the dissimilarities, both researchers agree that the phosphorylation of the TrkB receptor and Src kinase happens simultaneously.

In addition, both authors concluded that TrkB phosphorylation is the first molecular event, after which the TrkB receptor phosphorylates Src kinase which feeds back and further promotes phosphorylation of TrkB (Huang & McNamara, 2010; Zhang et al., 2013). Although there is evidence of the interrelation between TrkB and Src kinase, the detailed mechanism of this interaction is still debated. A putative mechanism of the TrkB-Src interaction based on the review of the literature is shown below (Figure 5).

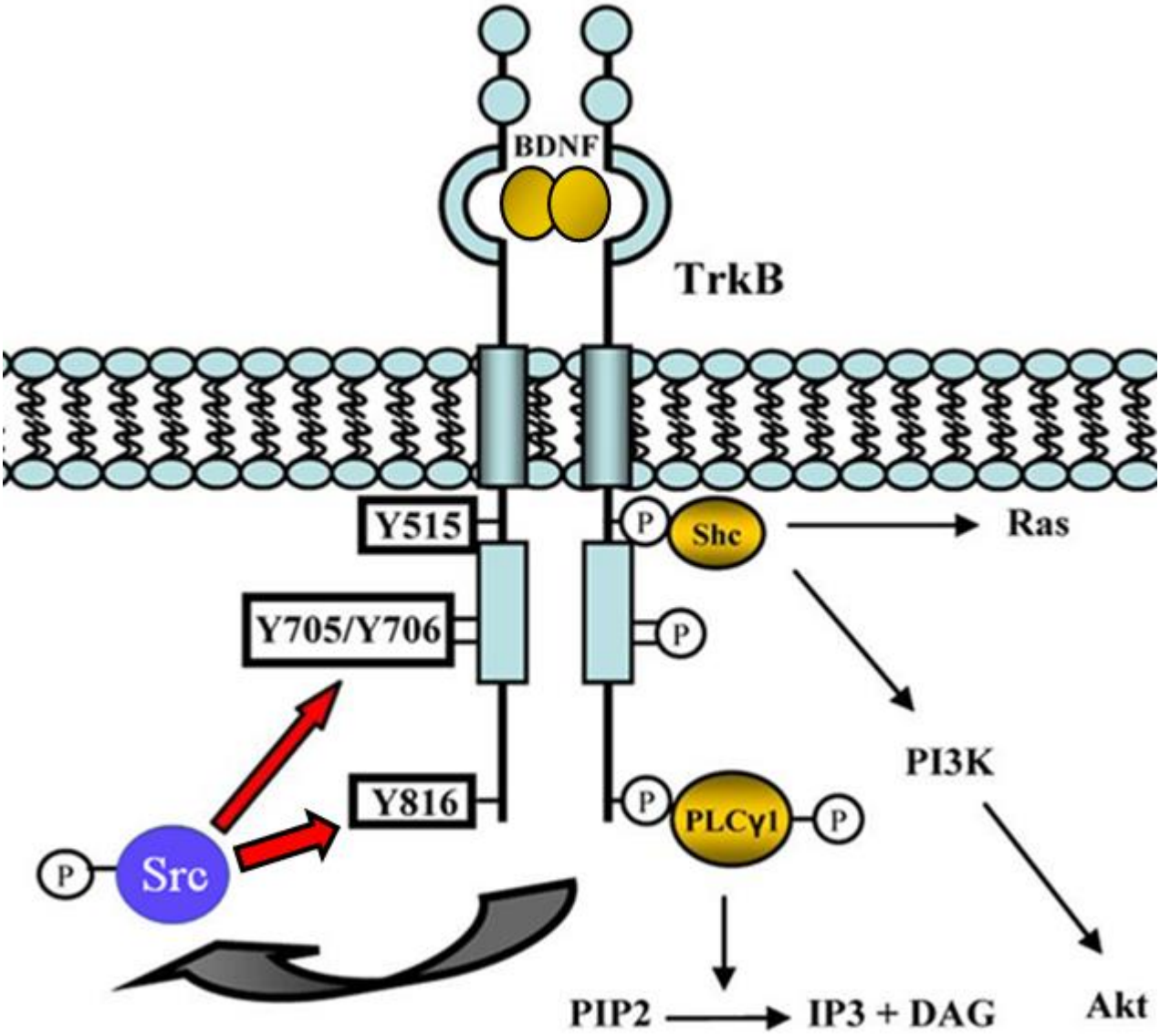

Figure 5. A putative mechanism of the function of Src kinase in TrkB signaling. Upon BDNF binding, TrkB is activated, thus phosphorylating Src kinase. Src kinase, in turn, further phosphorylates the domains Y705/6 and Y816 of the TrkB receptor, thus fully activating TrkB. Such receptor activation results in the intracellular signaling cascades PI3K/Akt, Ras/ERK and PLCγ1. Notably, BDNF is not the unique activator of TrkB, since the transactivation of the receptor can be induced by additional factors such as zinc, adenosine, PACAP or others (Figure based on an image from Huang & McNamara, 2010).

3. The TrkB receptor as a target for antidepressant treatments

3.1. Antidepressant treatments

The first antidepressants, iproniazid and imipramine, were discovered in the 1950s (Castrén, 2005). Iproniazid is a monoamine oxidase inhibitor, while imipramine is a type of tricyclic antidepressant (TCA). Both drugs were observed to increase the brain levels of monoamine neurotransmitters, namely norepinephrine and serotonin. Therefore, it was suggested that depression is caused by low concentration of mood-responsible monoamines (Schildkraut, 1965). This theory is named monoamine hypothesis. Later, classes of drugs were developed which were specifically designed to increase brain monoamine concentrations. They were: selective serotonin reuptake inhibitors (SSRI), serotonin–norepinephrine reuptake inhibitors (SNRIs) and norepinephrine reuptake inhibitors (NRI). SSRI were first launched in the late 1980s and they are widely used to this present day. As their name implies, the selective monoamine reuptake inhibitors prevent the neurotransmitter reuptake to the nerve endings, thus increasing its concentration. Although the described molecular events take place immediately after drug delivery, it takes several weeks until there are signs of improvement in patient mood (Nestler, 1998). Therefore, it appears that re-establishing serotonin levels does not immediately cure depression, but rather it causes its therapeutic effect by modulating an alternative mechanism (Ruhé et al., 2007).

Interestingly, it was determined that the positive effect of antidepressants correlates with the enhancement of BDNF-TrkB signaling (Castrén, 2005; Saarelainen et al., 2003; Rantamäki et al., 2011). In alignment with the above findings, rodent depression models revealed that the expression of BDNF was suppressed. In depressed patients lowered BDNF levels could be also observed in the blood. What is more, low brain BDNF concentration was posthumously detected in suicide victims (Dwivedi et al., 2003). Unsurprisingly, higher BDNF levels could be observed as a result of antidepressant treatment (Chen et al., 2001; Koponen et al., 2005). Regarding TrkB, the receptor of BDNF, antidepressants were also observed to rapidly activate TrkB signaling within one hour of drug administration (Castrén & Rantamäki, 2010). In addition to the activation of the TrkB receptor, also the levels of CREB, the downstream of the TrkB signaling pathway, were upregulated as a result of antidepressant treatment (Rantamäki et al., 2007). Hence, it seems that BDNF-TrkB signaling could underlie the efficacy of antidepressant action. Indeed, antidepressants did not produce positive results when tested in BDNF heterozygous knockout mice or mice with the truncated TrkB receptor, thus highlighting the importance of BDNF-TrkB signaling in the

remission from depression (Monteggia et al., 2004; Saarelainen et al., 2003). Therefore, it seems that the restoring of a proper functioning of the TrkB pathway reverses depression.

3.2. Antidepressant effects of sedative-anesthetics

Anesthetics are usually used to evoke a temporary loss of awareness during medical operations. They produce a sedated state of unconsciousness which quickly disappears after drug discontinuation. In addition to this conventional use, anesthetics such as ketamine and isoflurane may be used as antidepressants (Antila et al., 2017; Berman et al., 2000; Duman et al., 2012).

Ketamine is an NMDA receptor antagonist, which was observed to alleviate depression symptoms in depressed patients within hours (Berman et al., 2000; Duman et al., 2012). Because of the fast onset of its antidepressant effects, ketamine is also called a rapid-acting antidepressant (Kohtala et al., 2019b). The molecular mechanism of the action of ketamine is still unclear. Nevertheless, ketamine actively regulates the TrkB signaling pathway, thus activating transcription factors that promote synaptogenesis and synaptic plasticity. (Duman et al., 2012). Apparently, ketamine inhibits NMDA receptors, thus disinhibiting AMPA receptors and facilitating robust flow of glutamate into the nerve cell (Figure 6). In addition to glutamate, also Ca^{2+} enters the cell via voltage-gated calcium channels. The elevated intracellular concentration of glutamate and calcium enhances the expression and export of BDNF to the synaptic cleft. Consequently, extracellular BDNF binds to TrkB, thus activating the receptor and initiating its intracellular pathways. These pathways promote specific protein translation, importantly post-synaptic density protein 95 (PSD95) and glutamate-AMPA-receptor 1 (GluR1) which are further transported to the cell surface. They are recruited to the cell membrane, where they induce synaptogenesis and other neuronal plasticity-related events. Apparently, synaptogenesis and neuronal plasticity are the events that underlie the positive antidepressant effect of ketamine.

Furthermore, although not shown in the Figure 6, ketamine increases the phosphorylation of GSK3β (Beurel et al., 2011). This happens most probably through PI3K/Akt signaling (Figure 3), since it is known that Akt regulates GSK3β (Li et al., 2010). The phosphorylation of GSK3β results in its inhibition. GSK3β has been associated with important cellular processes such as cell metabolism and differentiation (Beurel et al., 2015). Interestingly, it was observed that ketamine does not produce its antidepressant behavioral effects in mice with a knock in mutation that blocks the phosphorylation of GSK3β (Beurel et al., 2011).

Therefore, the inhibition of GSK3β seems to be crucial event for the antidepressant action of ketamine.

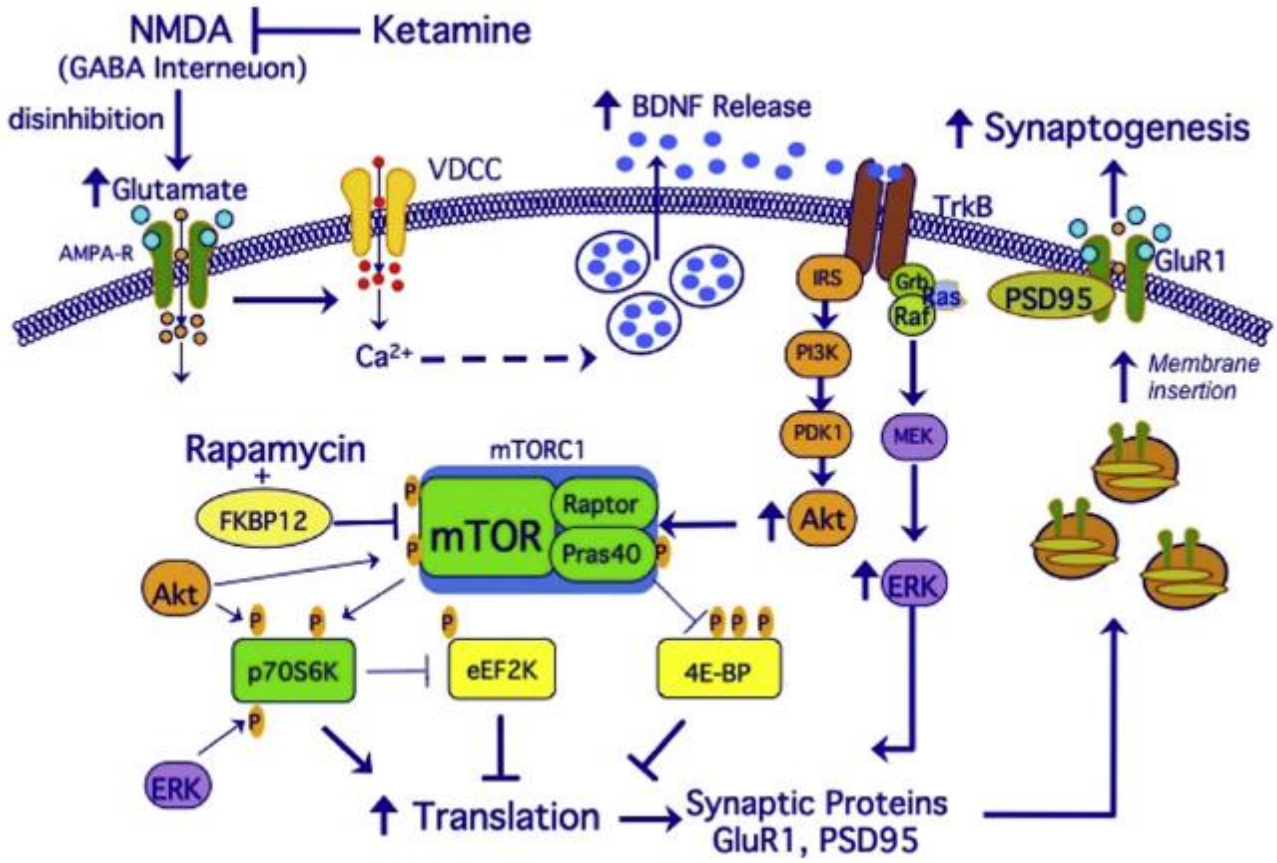

Figure 6. A putative molecular mechanism of the antidepressant actions of ketamine. Ketamine inhibits NMDA receptors and indirectly GABA inhibitory interneurons. This results in increased flow of glutamate into the cell through AMPA receptors. Additionally, also Ca^{2+} enters the cell through voltage-dependent calcium channels. As a result, BDNF is released and it binds to TrkB, thus initiating intracellular pathways such as PI3K/Akt and Ras/ERK that eventually lead to enhanced synaptic plasticity through synaptogenesis. Abbreviations: VDCC, voltage-dependent calcium channel; PSD95, post-synaptic density protein 95; GluR1, glutamate-AMPA-receptor 1 (Used by permission of Elsevier, figure from Duman et al. 2012).

Isoflurane, in a similar manner to ketamine, also activates TrkB signaling (Antila et al., 2017). Isoflurane is a volatile halogenated hydrocarbon. However, isoflurane promotes TrkB signaling independently of BDNF in conditional (CaMKII-Cre) BDNF knockout mice. This raises the question if BDNF required for eliciting antidepressant effects. In any case, isoflurane induces TrkB signaling and enhances synaptic plasticity. It was observed that as a result of the activation of the TrkB signaling pathway, also GSK3β and mTOR were activated, thus linking isoflurane activity to synaptic plasticity (Antila et al., 2017).

The TrkB pathway can be also activated by sedatives, such as medetomidine (Kohtala et al. 2019b). Medetomidine is a hypnotic-sedative agent, conventionally used for anesthesia of small domestic animals. It is an α_2-adrenoreceptor agonist. In Kohtala's research medetomidine promoted the phosphorylation of the TrkB signaling pathway-related proteins but failed to produce antidepressant-like response behavior in mice (Kohtala et al., 2019b). Intriguingly, Kohtala et al. observed a correlation between the TrkB pathway and slow brain waves. What is more, also ketamine was observed to induce a rebound emergence of slow brain EEG waves (Feinberg & Campbell, 1993). These findings suggest that the TrkB signaling pathway and slow brain waves are linked. Therefore, slow brain waves could be considered as an indicator of TrkB signaling.

3.3. Slow wave activity and deep sleep

By brain waves we understand the synchronized electrical activity of neuron populations. These waves can be measured with electroencephalogram (EEG) apparatus and are classified according to their frequency (Table 1). The slow brain waves or slow period consists in delta waves of low frequency (0.5–4 Hz) that are present through the whole brain. Slow brain waves take place mostly during deep sleep.

Sleep is a fascinating process. Despite it being a daily routine for all vertebrates, the mechanisms underlying sleep are still unclear. However, it is commonly accepted that there are two processes that regulate sleep: circadian rhythm (C) and homeostatic process (S). C process is associated with the biological clock of the internal organs and the expression of sleep genes. S process in turn is related to sleep pressure, which accumulates during wake hours. Both processes interact to initiate and maintain sleep phenomenon. The sleep process is divided into two major subphases: rapid eye movement (REM) and non-REM (NREM). The typical sleep in human adults consists of four stages: NREM stage 1 (α waves), NREM stage 2 (θ waves), NREM stage 3(δ waves) and REM phase. The above-mentioned phases form the sleep cycle. The sleep cycle lasts 90-120 minutes in humans

and repeats several times during the night time until waking. Different phases and the corresponding brain oscillations can be detected with the help of EEG apparatus.

Table 1. Brain waves classified according to their frequency.

Name	Frequency range	EEG	Associated state
Delta	0.5–4 Hz		Deep, dreamless sleep or sedation
Theta	5–8 Hz		Deep relaxation, meditation or sedation
Alpha	9–12 Hz		Relaxed, lucid, calm, not thinking
Beta	12–25 Hz		Awake, alert, consciousness

The first NREM stage is a transient state between wakefulness and sleep (Nutt et al., 2008). It is abundant in alpha waves (9–12 Hz). The second NREM phase is deeper and the brain oscillates with theta (5–8 Hz) frequencies. Theta frequencies also occur during meditation or deep relaxation. The third NREM stage is the period of the greatest muscle relaxation and consequently, of the most efficient recovery. It is also named slow wave sleep or deep sleep, since it is characterized by SWA i.e. low frequency delta brain waves (0.5–4 Hz).

3.4. Slow wave activity induced by sleep deprivation

The duration and intensity of SWA correlates with homeostatic sleep pressure, i.e. the longer the hours awake, the more intense slow wave sleep becomes. One of the physiological processes that increase homeostatic sleep pressure is sleep deprivation. Therefore, it could be assumed that sleep deprivation strengthens SWA, since it increases the homeostatic sleep pressure. Increased sleep pressure, in turn, causes more intense deep sleep. Interestingly, SWA does not occur only during deep sleep, but it may also emerge in the state of sedation (Table 1). Accordingly, SWA should be also induced by anesthetics, since they cause the state of sedation. Indeed, ketamine and medetomidine were observed to simultaneously potentiate SWA and TrkB signaling (Duncan et al., 2013; Kohtala et al., 2019b). SWA was even hypothesized to be a biomarker of ketamine-induced synaptic plasticity (Duncan et al., 2013). Therefore, it may be assumed that both SWA and TrkB signaling can be induced by sleep deprivation. In fact, sleep deprivation was repeatedly

reported to enhance the brain SWA in the recovery sleep period following sleep deprivation in humans as well as in rodents (Dijk et al., 1993; Farooqui et al. 1996; Huber et al., 2000).

To summarize, Src kinase seems to activate the TrkB receptor, thus inducing the TrkB signaling pathway. The TrkB signaling pathway is involved in synaptic plasticity, which is disrupted in major depression. Consistently, antidepressants have been observed to activate the TrkB receptor. In addition, it has been determined that some sedative-anesthetic antidepressants induce not only TrkB signaling, but also brain SWA. Thus, SWA may be considered as a biomarker of the active TrkB signaling pathway. There are several means to evoke SWA, one of them being sleep deprivation which intensifies the deep sleep.

4. Hypothesis and the aims of the study

The hypothesis of this study is linked to the interrelation between Src kinase and the TrkB signaling pathway. In this study, pharmacological agents, which were recently established as activators of the TrkB pathway, i.e. medetomidine and isoflurane, were used (Antila et al., 2017; Kohtala et al., 2019b). To our knowledge, this is the first study to investigate the role of Src kinase in the drug-induced TrkB signaling pathway.

Moreover, we hypothesized that Src kinase is an activator and a part of the TrkB signaling pathway. Accordingly, we theorized that pretreatment with Src kinase inhibitor (PP2) would prevent TrkB signaling despite the following treatment with medetomidine. In this way we could confirm that Src kinase mediates the activation of the TrkB pathway.

In addition, we sought to reinforce the association between TrkB signaling and SWA. Recent studies have indicated that various antidepressants concomitantly activate TrkB signaling and brain SWA (Kohtala et al., 2019a; Kohtala el al., 2019b). Therefore, we applied this finding and performed a sleep deprivation experiment in order to induce SWA. We hypothesized that as a result of sleep deprivation with deep recovery sleep, both Src kinase and TrkB signaling will be activated.

The general aim of this study was to assess how different treatments affect the phospho-Src (pSrc) levels. By analysis of the results obtained from Western blot assays we aimed to disclose the function of Src kinase in the TrkB signaling pathway. The experiments were performed in two stages (Figure 7).

The detailed aims of the investigation were the following:

1. To examine whether medetomidine and isoflurane activate the TrkB pathway and Src kinase;
2. To assess whether Src inhibition with PP2 results in the suppression of the TrkB pathway;
3. To determine whether sleep deprivation and subsequent recovery sleep can initiate the TrkB signaling cascade.

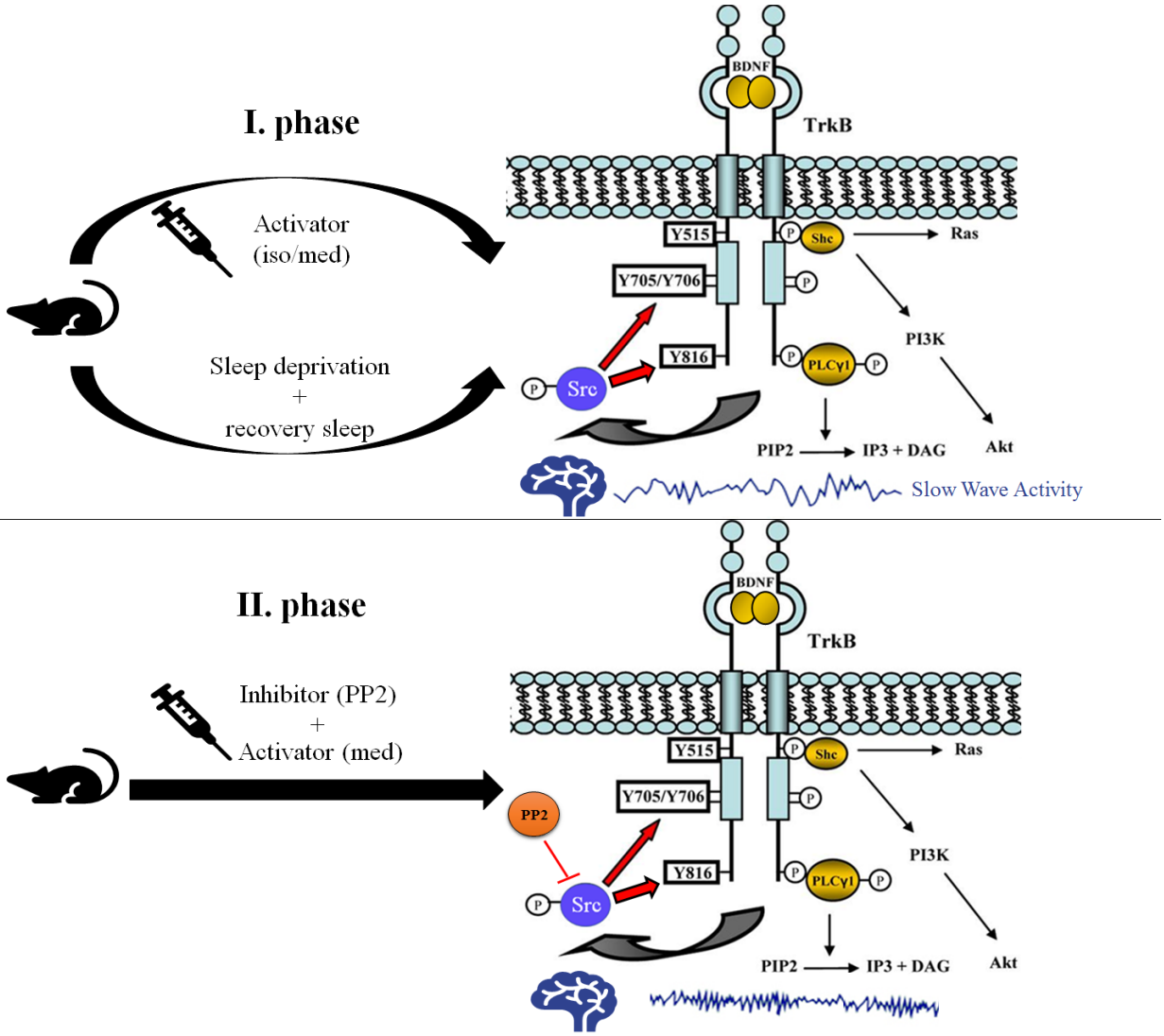

Figure 7. An overview of the experiments performed in this study. In the initial phase of the study, both pharmacological (isoflurane and medetomidine) and physiological (sleep deprivation with recovery sleep) means were used to promote the TrkB signaling pathway which is supposedly accompanied by brain SWA. In the second phase of the experiments, a Src kinase inhibitor (PP2), was used to suppress TrkB signaling. Abbreviations: Iso, isoflurane; med, medetomidine; SWA, slow wave activity.

5. Materials and Methods

5.1. Animals

We used adult male C57BL/6 mice (Envigo RMS, Netherlands) in the experiments. The mice were kept in standard conditions (21°C, 12h day-night cycle). The animals had unrestricted access to food and water. The mice were housed in pairs. The experiments were performed according to the guidelines of the Society of Neuroscience and were approved by the County Administrative Board of Southern Finland (License number: ESAVI/9793/04.10.07/2016).

5.2. Pharmacological treatment and the Src inhibitor experiment

Two sedative-anesthetic drugs were tested: medetomidine and isoflurane. In the first treatment medetomidine was administered (0.3 mg/kg, i.p., Domitor®, Orion Pharma), whereas the control group was given saline injection (Kohtala et al., 2019b). Isoflurane (Vetflurane®, Virbac) treatment was induced in a chamber with 4% isoflurane concentration and maintained with 2% concentration for 30 minutes (Antila et al., 2017). The control group was kept in the same conditions as the treatment group, but the inhaled gas was pure oxygen. Afterwards, we intended to inhibit TrkB signaling induced by medetomidine by using pyrazolopyrimidine 2 (PP2), a commonly implemented Src inhibitor (Figure 8).

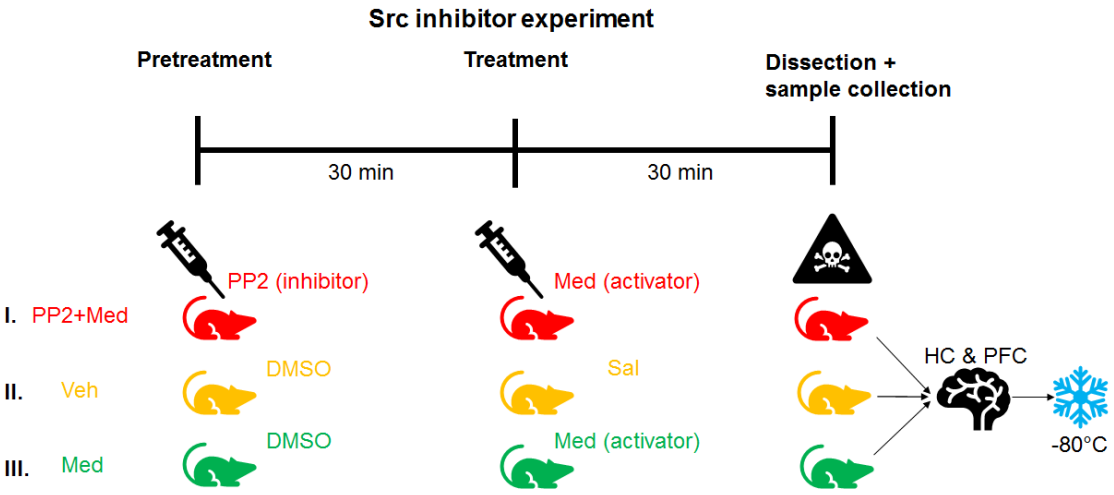

Figure 8. Second phase of the experiments (the Src inhibitor experiment). The mice were pretreated with either PP2 or DMSO. After 30 minutes, medetomidine or saline was administered. 30 minutes after the administration of the treatment, the mice were sacrificed, the brain areas were dissected and stored in -80°C. Abbreviations: PP2, pyrazolopyrimidine 2; Med, medetomidine; Sal, saline; DMSO, dimethyl sulfoxide; Veh, vehicle; HC, hippocampus; PFC, prefrontal cortex.

In the Src inhibitor experiment the mice were initially given either vehicle (dimethyl sulfoxide, DMSO, Sigma-Aldrich) or PP2 (5 mg/kg, Tocris) dissolved in DMSO (Table 2). 30 minutes after the first injection, medetomidine was administered to two groups, whereas the control animals were given saline injection. The mice were sacrificed 30 minutes after the second injection.

5.3. The sleep deprivation experiment

Since mice are nocturnal animals, they are mostly active during the night. Therefore, they were subjected to sleep deprivation during daytime, when their sleep pressure was extremely high. Immediately after the lights were turned on, sleep deprivation was initiated, and lasted six hours (Huber et al., 2000). The mice were kept awake by novel object introduction into the cage and by gentle handling (e.g. brushing) (Franken et al., 1991). After the established period of sleep deprivation, the mice of the first group (sleep deprivation; SD;) were euthanized (Figure 9). The second group of rodents (sleep deprivation and recovery sleep; SD+R;) had a short recovery sleep. As mentioned in the previous sections, the recovery sleep period following sleep deprivation abounds in SWA compared to a normal sleep without previous sleep deprivation. Indeed, it was observed that sleep deprivation causes an immediate increase in SWA, which lasts approximately 10 minutes (Huber et al., 2000). Therefore, we applied a recovery sleep which lasted 15 minutes, in order to be sure that SWA took place. The animals were monitored with video camera, and the duration of sleep was counted on from the moment of the movement cessation.

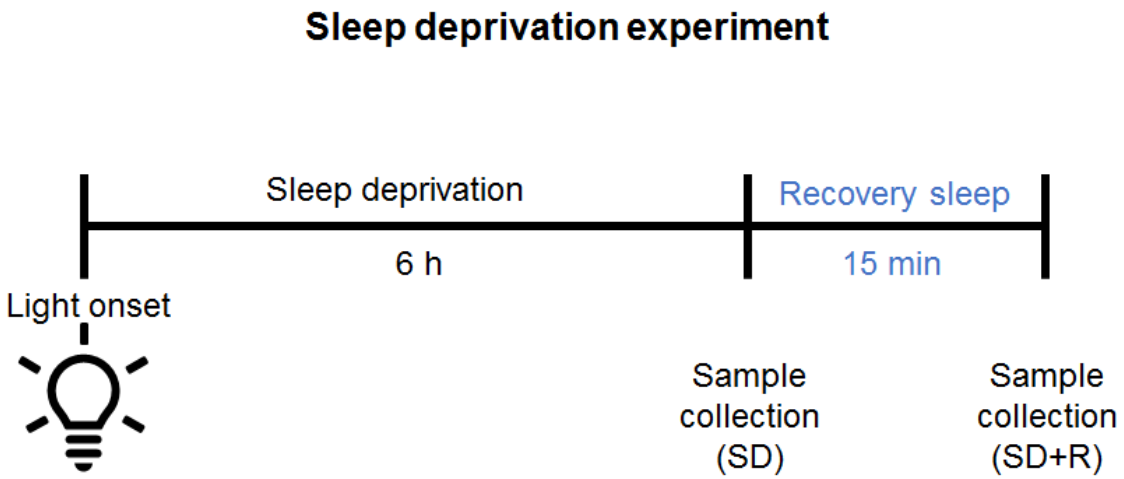

Figure 9. The design of the sleep deprivation experiment. The mice were either not sleep-deprived (control group, not shown here), sleep-deprived (SD) or sleep-deprived with recovery sleep (SD+R). Abbreviations: SD, sleep deprivation; SD+R, sleep deprivation and recovery sleep.

Table 2. The reagents used in the experiments.

Reagent	Source
Running Buffer NuPAGE™ MOPS SDS	Thermo Fisher
Transfer buffer NuPAGE™ (20x)	Thermo Fisher
Stripping buffer 3 M of Tris-HCl 41.6 ml; 20% SDS 200 ml; Milli-Q water 2000 ml; β-mercaptoethanol 350 µl;	Uni. Helsinki
Blocking buffer 3% BSA in TBS-T;	Uni. Helsinki
NP lysis buffer 3 M Tris-HCl 670 µl; 5 M NaCl 2.74 ml; 0,5 M NaF 9.6 ml; TritonX (0.5%) 1 ml; Milli-Q water ad. 100 ml;	Uni. Helsinki
NP ++ lysis buffer NP lysis buffer; Pierce™ Protease inhibitor tablet; Pierce™ Phosphatase inhibitor tablet;	Thermo Scientific
DC Protein assay reagents	BioRad
Laemmli sample buffer (2x)	BioRad
BSA protein standards	BioRad
WB detection substrate Pierce™ ECL Plus Western Blotting kit	Thermo Scientific
Isoflurane (Vetflurane)	Virbac
DMSO	Sigma-Aldrich
PP2	Tocris
Medetomidine (Domitor)	Orion Pharma
PVDF membrane (Amersham Hybond)	GE Healthcare
SDS-PAGE gel NuPAGE™ 4-12% Bis-Tris Gel 1.5 mm x 15 well	Thermo Scientific
TBS-T Tris-buffered saline; 0.1% Tween 20;	Uni. Helsinki MP Biomedicals

5.4. Sample Collection and Protein measurement

At the indicated times, the animals were euthanized by rapid cervical dislocation followed by decapitation. The brain was quickly removed and put on a plate with ice-cold phosphate-buffered saline (PBS). The hippocampus and the prefrontal cortex were rapidly dissected on the cold plate and stored at -80°C. Few days later, the brain samples were taken out of -80°C and placed on ice. They were covered with NP++ lysis buffer and then homogenized with a sonicator (Rinco Ultrasonics GM 35-400, Switzerland). The samples were incubated on ice for 15 minutes and centrifuged (16100g, 15 min, +4°C). Supernatant was carefully collected and transported to another set of Eppendorf tubes. From each lysate a volume of 5 µl was isolated for the further protein determination.

Protein concentrations from the brain samples were measured using a revised version of Lowry protein measurement method i.e. DC protein assay (Lowry et al., 1951). In this method, protein concentration can be deduced from a standard curve which is constructed from bovine serum albumin (BSA) protein standards. In this way an equation is obtained and based on this, the protein concentration of a sample can be determined. The samples and protein standards were pipetted into a 96-well SpectraPlate. Then the plate was placed on a platform rocker for 10-15 minutes. Afterwards, the absorbance of each well at 750 nm was measured with absorbance reader (Biotek ELx800). The higher the absorbance of a lysate, the higher its protein concentration. Once determined the protein amount in the lysate, the volume needed for SDS-PAGE was calculated. The amount of total protein in each sample was 40 µg.

5.5. SDS–PAGE and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate proteins according to their molecular weight. The volumes corresponding to 40 µg of total protein amount were mixed with 2x Laemmli buffer in 1 to 1 ratio. Laemmli buffer contains SDS, an agent that denatures the proteins and gives them a negative charge. The samples were then kept in 100°C for 3 minutes in order to ensure the denaturation of the proteins. Then the samples were cooled on ice and loaded onto a polyacrylamide gel, each sample into a separated well. In addition to the samples, a protein standard (BioRad Precision Plus Protein™ Dual Color) solution was loaded into the gel, so that in the detection step the molecular weight of each protein could be easily identified by comparing with the standard. The gel was put into a chamber with running buffer. The gel electrophoresis was performed at 180 V for 1 hour. During electrophoresis, the electric current transports the

proteins according to their molecular weight across the gel. One end of the gel has negative charge, while the other end has a positive charge. The positive end attracts the negatively charged proteins. The smaller the protein, the further it will migrate through the gel pores. Electrophoresis completed, a wide spectrum of separated protein bands is obtained.

Electrophoresis was followed by a protein transfer onto a polyvinylidene difluoride (PVDF) membrane. The principle of the protein transfer is the same as that of the electrophoresis: the negatively charged proteins are migrating towards the positive electrode. Thus, the proteins bands from the gel are transferred onto a membrane. The transfer is performed by inserting the gel on top of the PVDF membrane. This system is then secured and put into a chamber filled with transfer buffer. Since plenty of heat is produced during the protein transfer, a small ice box was placed inside the chamber and the whole system was put inside a box filled with ice. Then the proteins were transferred at 300 mA for 1 hour. Once transferred, specific phosphorylated protein domains can be indirectly detected from the membrane.

In this study we implemented indirect detection in which two types of antibodies are used: a primary antibody that is specific to the protein of interest, and a secondary antibody, which detects the presence of the primary antibody as well as the target protein. The secondary antibody used in this study was coupled to horseradish peroxidase (HRP), which is an enzyme and a reporter molecule commonly used in immunoblotting. HRP catalyzes a chemiluminescent reaction that produces visible light, which can be detected by a digital imager. The substrate (Pierce™ ECL Plus) used in the chemiluminescent reaction was converted into acridinium ester intermediates by HRP enzyme. Then, acridinium ester intermediates reacted with peroxide, thus producing chemiluminescence. The amount of luminosity obtained from a protein band correlates with the amount of the protein. The membranes were placed in a small box and equilibrated in Tris-buffered saline (TBS). Then they were blocked with the 3% BSA in TBS-Tween 20 (TBS-T) solution for 1 hour to prevent nonspecific binding of the antibodies. Then the membranes were incubated overnight in the primary antibody solution (Table 3) on a platform rocker at -4°C. Next morning the membranes were washed in TBS-T for 40 minutes for the purpose of minimizing background signal. The washing step was followed by incubation in HRP-coupled secondary antibody (room temperature, 1 h). After the incubation, the washing step in TBS-T was repeated. Finally, the membranes were incubated in ECL Plus Western Blotting Substrate for 3 minutes. In order to measure the intensity of the emitted light, images were taken using the

digital imager (BioRad ChemiDoc™ MP System), immediately after the incubation in ECL substrate. Finally, the protein bands from the images were quantified using Image J Software (ImageJ 1.46v, National Institute of Health, USA).

Table 3. Antibodies used in the experiments.

Antibody	Host	Solution	Dilution	Producer
pGSK3β ^{S9}	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	Cell Signaling Technology (CST)
pSrc ^{Y416}	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	CST
pTrkB ^{Y816}	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	CST
TrkB	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	CST
GAPDH	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	CST
GSK3β	Rabbit	3% BSA in TBS-T + NaN_3	1:1000	CST
Anti-rabbit HRP conjugate	Goat	5% NFDM in TBS-T	1:10000	BioRad

In this study, the level of phosphorylation of the proteins involved in the TrkB pathway (pTrkB^{Y816} and pGSK3β^{S9}) was determined. These proteins were chosen on account of their phosphorylation being a hallmark of a positive antidepressant effect (Duman et al., 2012; Kohtala et al., 2019a; Rantamäki et al., 2007). In addition, we checked the levels of pSrc^{Y416}, in order to assess whether there is a positive correlation between the TrkB-related proteins and Src activation. The same, above-mentioned procedure was repeated, and the luminosity value was calculated for the corresponding total protein amounts i.e. TrkB and GSK3β. GAPDH housekeeping protein was used as a loading control for pSrc^{Y416}. The levels of the proteins of interest were normalized to the total amount of corresponding proteins.

5.6. Statistical analysis

Statistical analysis and drawing of the graphs were performed using Prism 8.1.1 (GraphPad Software Inc). For the majority of the analyses Student's unpaired t-test was used (for experiments with two treatment groups). The Mann-Whitney U-test was implemented for pSrc analysis in isoflurane treatment, since the variation of results was higher compared to the results of other experiments. In the experiments that involved more than two treatment groups i.e. sleep deprivation and the Src inhibitor experiment, the ordinary one-way analysis of variance test was used for the analysis. The results are presented as mean ± SEM. P-values equal to or lower than 0.05 were considered statistically significant.

6. Results

6.1. Medetomidine upregulates pSrc and pTrkB levels

Medetomidine (0.3 mg/kg; n = 4) was administered to the mice by an intraperitoneal injection (i.p.). Control group (n = 4) was given a saline injection. After 30 minutes, the mice were sacrificed, and the prefrontal cortex samples were carefully collected and cooled on the ice. TrkB and Src phosphorylation were examined in a Western blotting assay as described in the Materials and Methods section. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for pSrc in all the performed Western blotting assays, since it is a ubiquitously and stably expressed protein. Regarding pTrkB, it was normalized against the corresponding total protein amount. Compared to the control group, medetomidine produced clear phosphorylation effects on Src kinase ($p = 0.0034$) and the TrkB receptor ($p = 0.0351$) (Figure 10).

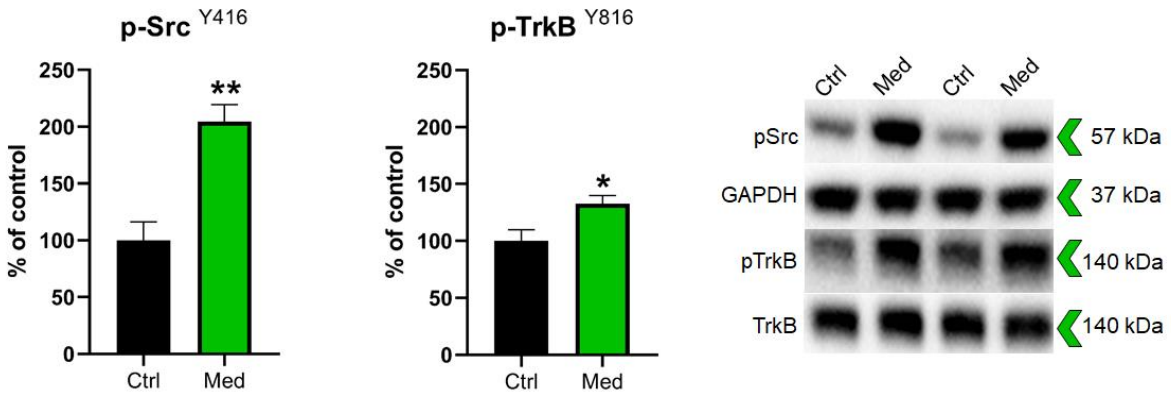

Figure 10. Src and TrkB phosphorylation in the prefrontal cortex 30 minutes after the intraperitoneal injection of medetomidine (n = 4; 0.3 mg/kg) in mice. Control group (n = 4) was given a saline injection. The graph shows high phosphorylation levels in the Y416 domain of Src kinase ($p = 0.0034$) and the Y816 domain of the TrkB receptor ($p = 0.0351$). Western blotting results can be seen on the right. pSrc was normalized against GAPDH, whereas pTrkB was normalized against the corresponding total protein amount. Unpaired Student's t-test was used in both cases; asterisk indicates statistical significance $*p < 0.05$, $**p < 0.01$. The data are represented as a group mean + SEM. Abbreviations: Ctrl, control treatment; Med, medetomidine treatment.

6.2. Isoflurane enhances the phosphorylation of Src and TrkB proteins

The mice were exposed to volatile isoflurane (4% during the induction and 2% during the maintenance; n = 6) for 30 minutes. The control group (n = 5) was also kept in the anesthesia chamber for 30 minutes, but the animals inhaled pure oxygen. Compared to the control mice, isoflurane robustly activated (p = 0.0303) the Y416 domain of Src kinase after a 30-minute isoflurane anesthesia (Figure 11). The Y816 domain of TrkB was also significantly phosphorylated (p = 0.0040), even more than in the case of medetomidine treatment.

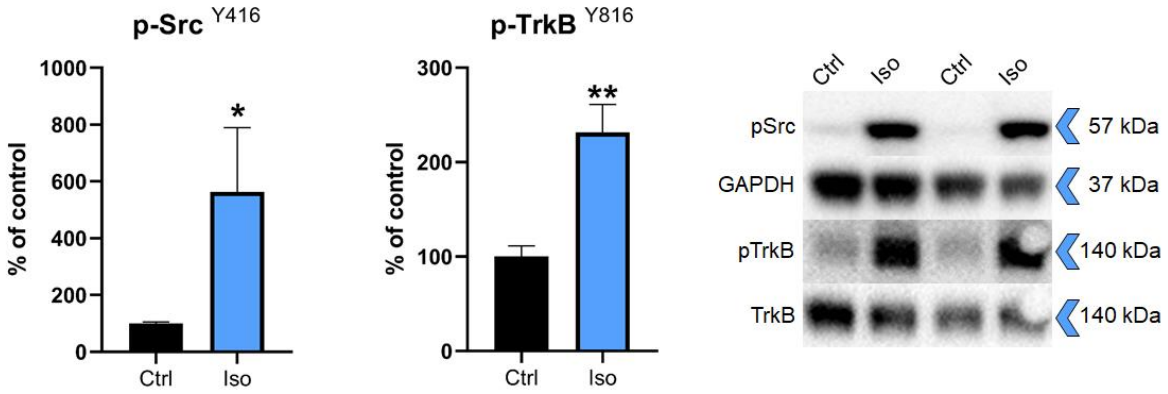

Figure 11. Isoflurane anesthesia (4% -> 2%, 30 min, n = 6) induces Src and TrkB phosphorylation in the mouse hippocampus. The graph shows high phosphorylation levels of the Y416 domain of Src kinase (p = 0.0303) and of the Y816 domain of the TrkB receptor (p = 0.0040). Control group (n = 5) was also kept in the anesthesia chamber but inhaled pure oxygen. pSrc was normalized against GAPDH, whereas pTrkB was normalized against the corresponding total protein amount. Unpaired Student's t-test was used for pSrc, while the Mann-Whitney U-test was implemented for pTrkB; asterisk indicates statistical significance *p < 0.05, **p < 0.01. The data are represented as a group mean + SEM of protein band intensities. Abbreviations: Ctrl, control treatment; Iso, isoflurane treatment.

6.3. The Src inhibitor did not suppress TrkB signaling

The experiment had three treatment groups in total: control group which received saline (Veh; 0.3 mg/kg, n = 4), medetomidine group (Med; 0.3 mg/kg, n = 4) and the inhibitor group that received both PP2 and medetomidine (PP2 + Med, n = 4). Medetomidine was chosen to the experiment due to its confirmed ability to activate Src kinase. The groups were pretreated either with DMSO (Veh and Med) or PP2 (PP2 + Med). The levels of pSrc were slightly elevated for the PP2 + Med mice both in the prefrontal cortex and the hippocampus, compared to the control animals (Figure 12). The same statistically non-significant results can be seen in the levels of pSrc for the group only treated with medetomidine.

Regarding pTrkB and pGSK3β, there were no major differences between different treatment groups. Results of this study were not consistent with those of previous research, where PP2 attenuated the phosphorylation of Src. In this study, PP2 did not inhibit Src kinase.

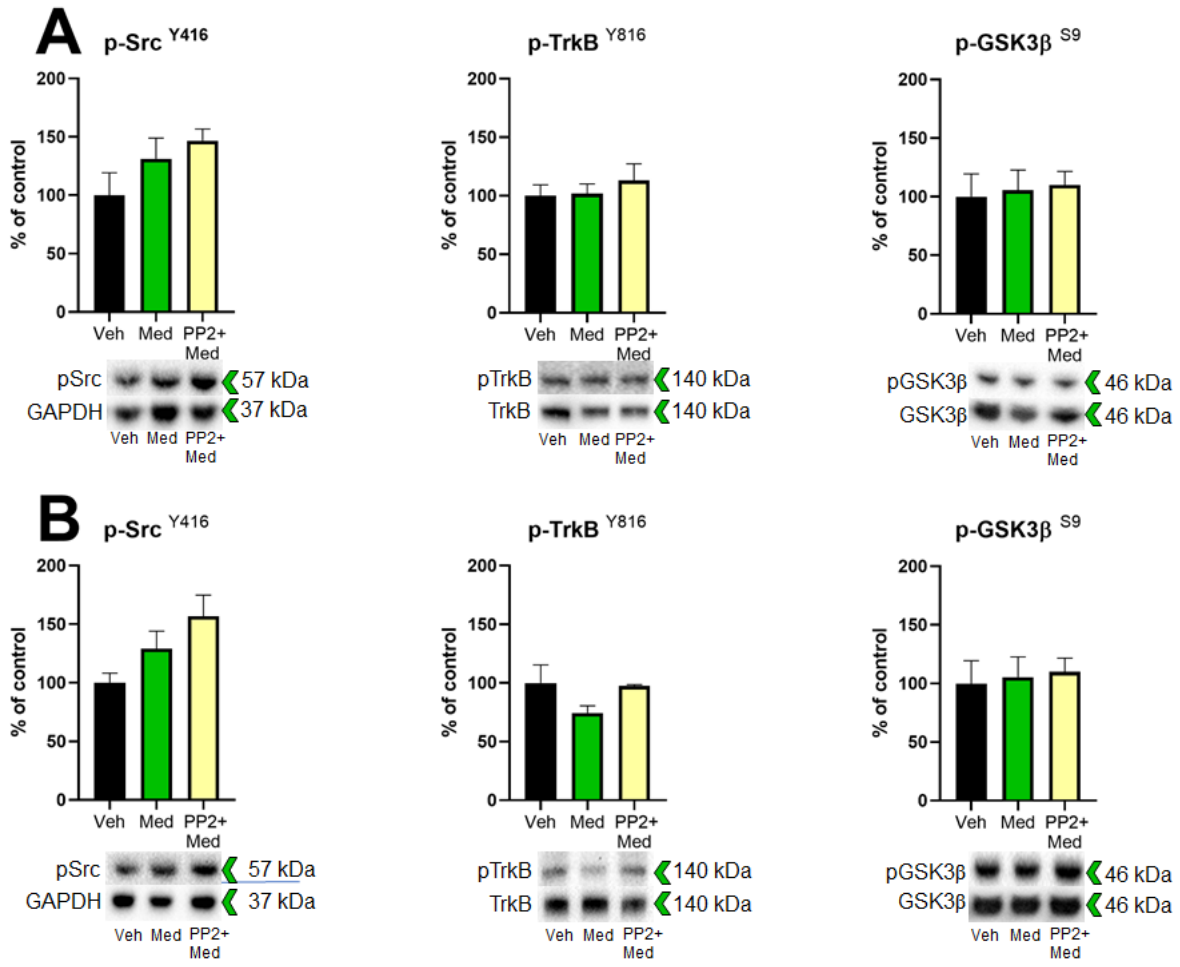

Figure 12. The Src inhibitor (PP2) did not inhibit the TrkB signaling pathway. (A) Effects on the phosphorylation of Src, TrkB and GSK3β proteins in the adult mouse prefrontal cortex after the Src inhibitor experiment. (B) Phosphorylation of Src, TrkB and GSK3β in the adult mouse hippocampus after the Src inhibitor experiment. The animals were divided into 3 treatment groups: saline group (Veh; 0.3 mg/kg; n = 4), medetomidine treatment (Med; 0.3 mg/kg) and the Src inhibitor treatment (PP2 + Med; 5 mg/kg + 0.3 mg/kg; n = 4). Groups Veh and Med received DMSO (5 mg/kg, i.p.; n = 4) instead of PP2. The representative Western blot protein bands can be seen below each corresponding graph. Band intensities were normalized against GAPDH (pSrc) or against their correspondent total protein values (pTrkB and pGSK3β). One-way analysis of variance was implemented for the analysis of the results. Abbreviations: Veh, vehicle group; Med, medetomidine group; PP2 + Med, Src inhibitor PP2 + medetomidine group.

6.4. Effects of sleep deprivation on Src, TrkB and GSKβ

The sleep deprivation experiment involved two trial groups and one control group. The first trial group (sleep deprivation; SD; n = 10) was sleep deprived for 6 hours. The second group (sleep deprivation and recovery sleep; SD+R, n = 10) was sleep deprived for 6 hours and then allowed to sleep for 15 minutes. The control group (Ctrl; n = 10) was kept in a different room and could sleep without restrictions during the corresponding time. Western blotting results showed that the levels of all specific phospho-proteins (pSrc, pTrkB and pGSK3β) were slightly upregulated in both the prefrontal cortex and hippocampus in the group that had recovery sleep (Figure 13).

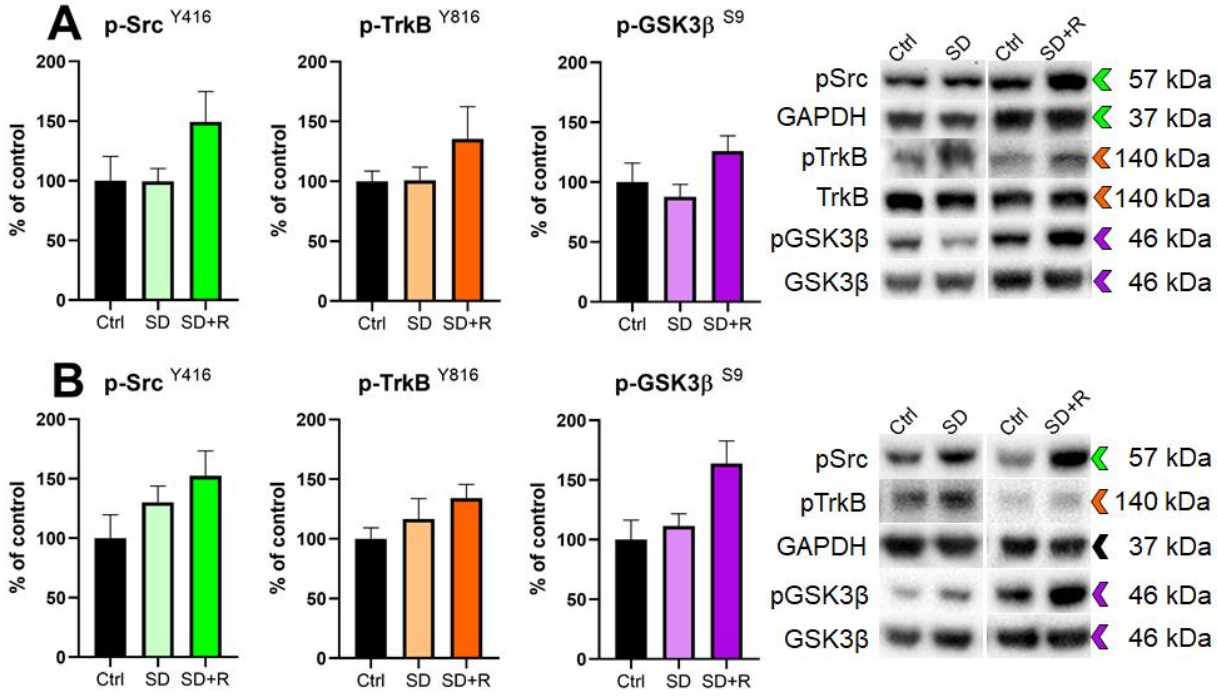

Figure 13. Sleep deprivation slightly upregulates the TrkB signaling pathway-related phosphoproteins. (A) The levels of pSrc, pTrkB and pGSK3β are upregulated in the mouse prefrontal cortex. The results have been normalized against GAPDH (pSrc) or corresponding total protein value (pTrkB, pGSK3β). (B) The results of sleep deprivation in the mouse hippocampus. All the phospho-protein levels were higher in SD+R mice compared to the control or SD group. Band intensities of pSrc and pTrkB were both normalized against GAPDH, whereas pGSK3β band was normalized against its corresponding total protein value. Results are presented as mean + SEM of protein band intensities. (Unpaired Student's t-test) Abbreviations: PFC, prefrontal cortex; Ctrl, control group; SD, sleep deprivation; SD+R, sleep deprivation and recovery sleep.

7. Discussion and Conclusions

The general aim of this study was to assess how different treatments affect pSrc and the TrkB pathway-related phospho-protein levels. Different treatments were applied i.e. isoflurane and medetomidine, Src kinase inhibitor (PP2) as well as sleep deprivation with recovery sleep. Afterwards, the mouse brain samples were examined with Western blotting and checked for the presence of the proteins of interest.

The results from the first experiment, the pharmacologically induced TrkB pathway, were consistent with those of previous research (Antila et al., 2017; Kohtala et al., 2019b). A notable upregulation of pSrc and pTrkB was observed. In particular, isoflurane treatment enhanced the phospho-protein levels in the mouse hippocampus, whereas medetomidine caused a strong response of the same phospho-proteins in the mouse prefrontal cortex. Both medetomidine and isoflurane simultaneously activated Src kinase and the TrkB receptor. No increase in phospho-protein levels was observed in the control groups. Our results support the hypothesis that Src kinase is closely linked to the TrkB activation, since both proteins were phosphorylated in the experiment.

Earlier research has confirmed PP2 as a powerful Src kinase inhibitor (Huang et al., 2008; Huang & McNamara, 2010; Zhang et al., 2013), but the results obtained in this study were slightly disappointing in this regard. Namely, a minor, non-significant phosphorylation of Src occurred despite the pretreatment with PP2. Furthermore, the levels of pTrkB and pGSK3β proteins in the treatment group resembled those of the control group, i.e. there was no visible effect of PP2 pretreatment. Surprisingly, even the medetomidine-treated group exhibited only a small, non-significant upregulation of pSrc, although in the medetomidine experiment it was much more robust. Therefore, the interpretation of the results is slightly challenging. The failed inhibition of Src kinase might have been due to the effects of DMSO that could be observed during the experiment. Interestingly, we observed that mice that received both DMSO and medetomidine treatment became sleepy and motionless after the injection. Thus, it seemed like DMSO caused a state of sedation in animals. This state of sedation could interfere with TrkB signaling. One way to avoid this in the future could be by implementing a different Src kinase inhibitor that would not need dissolution in DMSO. Alternatively, future experiments could be performed with PP2, but in this case the doses of DMSO should be much lower in order not to sedate the animal. Another possibility would be to implement an inert vehicle. Future research should include an improved version of the

Src inhibitor experiment in order to determine, whether Src kinase is essential for the TrkB pathway.

In this study, we hypothesized that by inducing SWA we would also activate TrkB signaling (Kohtala et al., 2019b). According to the literature, SWA appears to be particularly intensive during the immediate sleep period after sleep deprivation (Franken et al., 1991; Huber et al., 2000). Therefore, in this study we implemented sleep deprivation combined with recovery sleep as a means to enhance SWA and, in consequence, TrkB signaling. Our results from the sleep deprivation experiment showed a clear trend: the levels of the TrkB pathway-related phospho-proteins in the sleep-deprived and then -recovered mice were upregulated, without the statistical significance, compared to the other groups. In addition to a positive trend in Src and TrkB, the phosphorylation levels of GSK3β were also elevated. As stated in the literature, high phosphorylation levels of TrkB and GSK3β are associated with the action of rapid-acting antidepressants (Duman et al., 2012; Kohtala et al., 2019a). In this study, we saw a clear, although statistically non-significant trend in the activation of TrkB both in the prefrontal cortex and hippocampus after the recovery sleep. Therefore, it could be inferred that sleep deprivation with recovery sleep induces TrkB signaling. Regarding the group of sleep-deprived mice without recovery sleep, the phosphorylation levels of the TrkB-related proteins were equal to those of the control group. Hence, it seems that the TrkB signaling pathway is only activated during slow wave sleep and not during waking hours. Although our results were statistically non-significant, it must be observed that sleep deprivation is a highly variable physiological phenomenon. Such phenomenon depends on the individual sleep differences between animals, such as duration and intensity of deep sleep. Conversely, the pharmacological treatment (medetomidine and isoflurane) implemented in the first experiment is more predictable, and thus it produced statistically significant results. It is because the same drug doses reliably produce similar effects in each animal. Thus, the results obtained from pharmacological treatment were naturally less variable than those attained in the sleep deprivation experiment. Intriguingly, we have recently reproduced the Western blotting analysis and observed that the pGSK3β levels were significantly higher in the brain samples of sleep-deprived mice with recovery sleep compared to the control group (Rosenholm et al., unpublished data). Therefore, it is possible to obtain statistically significant results from the sleep deprivation experiment.

Despite the good results we obtained, the sleep deprivation experiment could be further improved. Namely, a video camera used for surveillance was not enough to ensure the equal

amount of sleep for each animal. Each mouse did not necessarily fall asleep immediately. It was challenging to determine the exact moment of falling asleep. In this study, the duration of recovery sleep was measured from the cessation of mouse movement, but the lack of movement did not undeniably mean that the animal had fallen asleep. Therefore, the surveillance method in future experiments could involve EEG recordings. Such recordings can detect SWA and thus the duration of deep sleep would be precisely measured. Another way to improve the sleep deprivation experiment could be the extension of recovery sleep duration to 30 minutes. Recovery sleep of 15 minutes duration might not be sufficient for animals to experience SWA, due to the delay in falling asleep for some animals. Our results encourage further sleep deprivation experiments in order to elucidate the TrkB pathway-linked molecular mechanisms behind this phenomenon.

Huang & McNamara suggested in 2010 that there are two types of TrkB activation. The first one is transactivation, in the absence of BDNF, where Src kinase is upstream in this molecular cascade. In this activation, the phosphorylation of the Y705/6 TrkB domain is the initial event and it is mediated by Src kinase. Once the catalytic site of TrkB is activated, the Y515 domain is also phosphorylated by the TrkB receptor itself. The second option for TrkB activation is the one mediated by prototypic ligand BDNF. In this model, BDNF phosphorylates the Y416 domain of Src through the initial activation of TrkB, demonstrating Src to be downstream of TrkB. However, in this case, Src kinase is also upstream of TrkB since once activated, it further phosphorylates TrkB, thus fully activating it. Interestingly, Zhang et al. later confirmed the second model of Huang & McNamara, namely the BDNF-induced TrkB pathway. In this model, Src kinase is a downstream molecule for TrkB. Src kinase phosphorylates the Y816 domain of the TrkB receptor, which results in further TrkB activation. In this study, the data we collected do not allow confirmation of the hypothesis of Zhang et al., since we did not check the BDNF levels. Nevertheless, our findings support the hypothesis that the activation of Src and TrkB is coupled, i.e. Src kinase and TrkB closely interact and mutually depend on each other. However, it is challenging to determine, which of the two molecules is upstream of the other. A study with conditional BDNF knockout mice could be the next step to confirm the mechanism of TrkB activation.

In summary, our findings support the hypothesis that Src works as a mediator of the TrkB signaling pathway, although further investigations are needed in order to confirm the detailed role of Src kinase. Our study indicates that medetomidine and isoflurane treatment initiates coupled activation of Src kinase and the TrkB pathway. In addition, the results obtained in

the sleep deprivation experiment provide support for the TrkB signaling pathway being induced during slow wave sleep. It would be beneficial to continue the research into sleep deprivation and its implications for TrkB signaling. Although the role of Src kinase in the TrkB pathway is still debated, if Src kinase is necessary for the TrkB pathway, it could be applied in future antidepressant treatment. Future research in this field should eventually lead to the discovery of more effective antidepressants.

Acknowledgements

I would like to thank Associate Professor Tomi Rantamäki for supervising me and giving me the opportunity to familiarize myself with an extremely interesting research topic in his Neurotherapeutics lab. I would like to express my deepest gratitude to Marko Rosenholm, M.Sc., who was the second supervisor of my thesis. I am very grateful for his guidance and admirable patience and all the advice he has provided me with along the Master's thesis' journey. I thank other members of Rantamäki lab for their practical advice, which was extremely important during the experimental stage of this study. Finally, I also thank Markus Korpela for support during the writing process.

List of references

1. Alonso, M., Medina, J.H., and Pozzo-Miller, L. (2004). ERK1/2 Activation Is Necessary for BDNF to Increase Dendritic Spine Density in Hippocampal CA1 Pyramidal Neurons. Learn. Mem. 11, 172-178.
2. Antila, H. (2016). Activation of the TrkB Neurotrophin Receptor by Antidepressant Drugs. (Doctoral dissertation). Retrieved from: https://helda.helsinki.fi/bitstream/handle/10138/166226/activati.pdf?sequence=1&isAllowed=y.
3. Antila, H., Ryazantseva, M., Popova, D., Sipilä, P., Guirado, R., Kohtala, S., Yalcin, I., Lindholm, J., Vesa, L., Sato, V.*, et al.* (2017). Isoflurane produces antidepressant effects and induces TrkB signaling in rodents. Sci. Rep. 7, 7811.
4. Autry, A.E., and Monteggia, L.M. (2012). Brain-derived neurotrophic factor and neuropsychiatric disorders. Pharmacol. Rev. 64, 238-258.
5. Barde, Y.A., Edgar, D., and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. EMBO J. 1, 549-553.
6. Bathina, S., and Das, U.N. (2015). Brain-derived neurotrophic factor and its clinical Implications. Arch. Med. Sci. 11, 1164-1178.
7. Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., and Krystal, J.H. (2000). Antidepressant effects of ketamine in depressed patients. Biol. Psychiatry 47, 351-354.
8. Beurel, E., Song, L., and Jope, R.S. (2011). Inhibition of glycogen synthase kinase-3 is necessary for the rapid antidepressant effect of ketamine in mice. Mol. Psychiatry 16, 1068-1070.
9. Beurel, E., Grieco, S.F., and Jope, R.S. (2015). Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. Pharmacol. Ther. 148, 114-131.
10. Bliss, T.V.P., and Collingridge, G.L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. Nature 361, 31-39.
11. Castrén, E. (2005). Is mood chemistry? Nat. Rev. Neurosci. 6, 241-246.
12. Castrén, E., and Rantamäki, T. (2010). The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. Dev. Neurobiol. 70, 289-297.
13. Castrén, E., Võikar, V., and Rantamäki, T. (2007). Role of neurotrophic factors in depression. Curr. Opin. Pharmacol. 7, 18-21.

14. Castrén, E., Zafra, F., Thoenen, H., and Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc. Natl. Acad. Sci. U. S. A. 89, 9444-9448.
15. Chen, B., Dowlatshahi, D., MacQueen, G.M., Wang, J.-., and Young, L.T. (2001). Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. Biol. Psychiatry 50, 260-265.
16. Duman, R.S., Li, N., Liu, R.-., Duric, V., and Aghajanian, G. (2012). Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology 62, 35-41.
17. Duncan Jr., W.C., Sarasso, S., Ferrarelli, F., Selter, J., Riedner, B.A., Hejazi, N.S., Yuan, P., Brutsche, N., Manji, H.K., Tononi, G., and Zarate, C.A. (2013). Concomitant BDNF and sleep slow wave changes indicate ketamine-induced plasticity in major depressive disorder. Int. J. Neuropsychopharmacol. 16, 301-311.
18. Dijk, D.-., Hayes, B., and Czeisler, C.A. (1993). Dynamics of electroencephalographic sleep spindles and slow wave activity in men: effect of sleep deprivation. Brain Res. 626, 190-199.
19. Dwivedi, Y., Rizavi, H.S., Conley, R.R., Roberts, R.C., Tamminga, C.A., and Pandey, G.N. (2003). Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. Arch. Gen. Psychiatry 60, 804-815.
20. Easton, J.B., Royer, A.R., and Middlemas, D.S. (2006). The protein tyrosine phosphatase, Shp2, is required for the complete activation of the RAS/MAPK pathway by brain-derived neurotrophic factor. J. Neurochem. 97, 834-845.
21. Farooqui, S.M., Brock, J.W., and Zhou, J. (1996). Changes in monoamines and their metabolite concentrations in REM sleep-deprived rat forebrain nuclei. Pharmacol. Biochem. Behav. 54, 385-391.
22. Feinberg, I., and Campbell, I.G. (1993). Ketamine administration during waking increases delta eeg intensity in rat sleep. Neuropsychopharmacology 9, 41-48.
23. Frame, M.C. (2002). Src in cancer: Deregulation and consequences for cell behaviour. Biochim. Biophys. Acta Rev. Cancer 1602, 114-130.
24. Franken, P., Dijk, D.-., Tobler, I., and Borbely, A.A. (1991). Sleep deprivation in rats: Effects on EEG power spectra, vigilance states, and cortical temperature. Am. J. Physiol. Regul. Integr. Comp. Physiol. 261, R198-R208.
25. Fujimura, H., Altar, C.A., Chen, R., Nakamura, T., Nakahashi, T., Kambayashi, J.-., Sun, B., and Tandon, N.N. (2002). Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. Thromb. Haemost. 87, 728-734.
26. Fujioka, T., Fujioka, A., and Duman, R.S. (2004). Activation of cAMP Signaling Facilitates the Morphological Maturation of Newborn Neurons in Adult Hippocampus. J. Neurosci. 24, 319-328.
27. Hallböök, F., Ibañez, C.F., and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in xenopus ovary. Neuron 6, 845-858.
28. Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: Roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677-736.
29. Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: Roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609-642.
30. Huang, Y.Z., Pan, E., Xiong, Z.-., and McNamara, J.O. (2008). Zinc-Mediated Transactivation of TrkB Potentiates the Hippocampal Mossy Fiber-CA3 Pyramid Synapse. Neuron 57, 546-558.
31. Huang, Y.Z., and McNamara, J.O. (2010). Mutual regulation of Src family kinases and the neurotrophin receptor TrkB. J. Biol. Chem. 285, 8207-8217.
32. Huber, R., Deboer, T., and Tobler, I. (2000). Effects of sleep deprivation on sleep and sleep EEG in three mouse strains: Empirical data and simulations. Brain Res. 857, 8-19.
33. Kaplan, D.R., and Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. 10, 381-391.
34. Katoh-Semba, R., Takeuchi, I.K., Semba, R., and Kato, K. (1997). Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. J. Neurochem. 69, 34-42.
35. Klein, R., Conway, D., Parada, L.F., and Barbacid, M. (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61, 647-656.
36. Klein, R., Parada, L.F., Coulier, F., and Barbacid, M. (1989). TrkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J. 8, 3701-3709.
37. Klein, R., Smeyne, R.J., Wurst, W., Long, L.K., Auerbach, B.A., Joyner, A.L., and Barbacid, M. (1993). Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell 75, 113-122.
38. Kohtala, S., Theilmann, W., Rosenholm, M., Müller, H.K., Kiuru, P., Wegener, G., Yli-Kauhaluoma, J., and Rantamäki, T. (2019a). Ketamine-induced regulation of TrkB-GSK3ß signaling is accompanied by slow EEG oscillations and sedation but is independent of hydroxynorketamine metabolites. Neuropharmacology 157, 107684.

39. Kohtala, S., Theilmann, W., Rosenholm, M., Penna, L., Karabulut, G., Uusitalo, S., Järventausta, K., Yli-Hankala, A., Yalcin, I., Matsui, N., Wigren, H.-., and Rantamäki, T. (2019b). Cortical Excitability and Activation of TrkB Signaling During Rebound Slow Oscillations Are Critical for Rapid Antidepressant Responses. Mol. Neurobiol. 56, 4163-4174.
40. Koponen, E., Rantamäki, T., Voikar, V., Saarelainen, T., MacDonald, E., and Castrén, E. (2005). Enhanced BDNF signaling is associated with an antidepressant-like behavioral response and changes in brain monoamines. Cell. Mol. Neurobiol. 25, 973-980.

41. Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., and Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. U. S. A. 92, 8856-8860.
42. Korte, M., Minichiello, L., Klein, R., and Bonhoeffer, T. (2000). Shc-binding site in the TrkB receptor is not required for hippocampal long-term potentiation. Neuropharmacology 39, 717-724.
43. Lee, F.S., and Chao, M.V. (2001). Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. U. S. A. 98, 3555-3560.
44. Lee, F.S., Rajagopal, R., Kim, A.H., Chang, P.C., and Chao, M.V. (2002). Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J. Biol. Chem. 277, 9096-9102.
45. Li, X., and Jope, R.S. (2010). Is glycogen synthase kinase-3 a central modulator in mood regulation. Neuropsychopharmacology 35, 2143-2154.
46. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
47. Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M., and Yancopoulos, G.D. (1990). Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. Science 247, 1446-1451.
48. McAllister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. 22, 295-318.
49. Middlemas, D.S., Meisenhelder, J., and Hunter, T. (1994). Identification of TrkB autophosphorylation sites and evidence that phospholipase C-γ1 is a substrate of the TrkB receptor. J. Biol. Chem. 269, 5458-5466.

50. Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36, 121-137.
51. Mizuno, M., Yamada, K., He, J., Nakajima, A., and Nabeshima, T. (2003). Involvement of BDNF receptor TrkB in spatial memory formation. Learn. Mem. 10, 108-115.
52. Monteggia, L.M., Barrot, M., Powell, C.M., Berton, O., Galanis, V., Gemelli, T., Meuth, S., Nagy, A., Greene, R.W., and Nestler, E.J. (2004). Essential role of brain-derived neurotrophic factor in adult hippocampal function. Proc. Natl. Acad. Sci. U. S. A. 101, 10827-10832.
53. Nestler, E.J. (1998). Antidepressant treatments in the 21st century. Biol. Psychiatry 44, 526-533.
54. Nutt, D.J., Wilson, S., and Paterson, L. (2008). Sleep disorders as core symptoms of depression. Dialogues Clin. Neurosci. 10, 329-336.
55. Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J., & Ullrich, A. (1994). Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. The EMBO Journal, 13, 1585-1590.

56. Rajagopal, R., and Chao, M.V. (2006). A role for Fyn in Trk receptor transactivation by G-protein-coupled receptor signaling. Mol. Cell. Neurosci. 33, 36-46.
57. Rantamäki, T., Hendolin, P., Kankaanpää, A., Mijatovic, J., Piepponen, P., Domenici, E., Chao, M.V., Männistö, P.T., and Castrén, E. (2007). Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cγ signaling pathways in mouse brain. Neuropsychopharmacology 32, 2152-2162.
58. Rantamäki, T., Vesa, L., Antila, H., Lieto, A., Tammela, P., Schmitt, A., Lesch, K.-., Rios, M., and Castrén, E. (2011). Antidepressant drugs transactivate trkb neurotrophin receptors in the adult rodent brain independently of bdnf and monoamine transporter blockade. PLoS ONE 6, e20567.
59. Reichardt, L.F. (2006). Neurotrophin-regulated signaling pathways. Philos. Trans. R. Soc. B Biol. Sci. 361, 1545-1564.
60. Roskoski, R. (2015). Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors This paper is dedicated to the memory of Prof. Donald F. Steiner (1930-2014) - Advisor, mentor, and discoverer of proinsulin. Pharmacol. Res. 94, 9-25.

61. Ruhé, H.G., Mason, N.S., and Schene, A.H. (2007). Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: A meta-analysis of monoamine depletion studies. Mol. Psychiatry 12, 331-359.

62. Saarelainen, T., Hendolin, P., Lucas, G., Koponen, E., Sairanen, M., MacDonald, E., Agerman, K., Haapasalo, A., Nawa, H., Aloyz, R., Ernfors, P., and Castrén, E. (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. J. Neurosci. 23, 349-357.
63. Schildkraut, J.J. (1965). The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am. J. Psychiatry 122, 509-522.
64. Takei, N., Inamura, N., Kawamura, M., Namba, H., Hara, K., Yonezawa, K., and Nawa, H. (2004). Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J. Neurosci. 24, 9760-9769.
65. Thoenen, H. (1995). Neurotrophins and neuronal plasticity. Science 270, 593-598.
66. Uren, R.T., and Turnley, A.M. (2014). Regulation of neurotrophin receptor (Trk) signaling: Suppressor of cytokine signaling 2 (SOCS2) is a new player. Front. Mol. Neurosci. 7, 39.
67. Vetencourt, J.F.M., Sale, A., Viegi, A., Baroncelli, L., De Pasquale, R., O'Leary, O.F., Castrén, E., and Maffei, L. (2008). The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science 320, 385-388.
68. Wang, Y.T., and Salter, M.W. (1994). Regulation of NMDA receptors by tyrosine kinases and phosphatases. Nature 369, 233-235.

69. Wetmore, C., Ernfors, P., Persson, H., and Olson, L. (1990). Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp. Neurol. 109, 141-152.
70. Yan, Q., Radeke, M.J., Matheson, C.R., Talvenheimo, J., Welcher, A.A., and Feinstein, S.C. (1997). Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J. Comp. Neurol. 378, 135-157.
71. Zhang, Z., Fan, J., Ren, Y., Zhou, W., and Yin, G. (2013). The release of glutamate from cortical neurons regulated by BDNF via the TrkB/Src/PLCγ1 pathway. J. Cell. Biochem. 114, 144-151.