THE IMPACT OF PHARMACOLOGICAL MANIPULATIONS OF BRAIN ENERGY METABOLISM AND GLYMPHATIC SYSTEM ON TRKB NEUROTROPHIN SIGNALLING

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Tiivistelmä – Referat – Abstract

Since the discovery of ketamine's antidepressant response, numerous of studies have been observed it to alleviate depressive symptoms rapidly and effectively within hours. This is a significant advantage compared to traditional antidepressants, which take weeks to show treatment efficacy. Ketamine is a N-methyl-D-aspartate receptor (NMDA) antagonist and its underlying mechanism of is proposed to be in its ability to increase synaptic plasticity and this is ultimately believed to improve mood. On a molecular level, the antidepressant effects have been observed to be dependent on the activation of tropomyosin receptor kinase B (TrkB) signalling pathway. However, the antidepressant mechanism of ketamine remains still poorly understood as no new NMDA-antagonist or other rapid-acting antidepressants have been successfully developed for clinical use despite many years of effort. Therefore, some have proposed that the missing pieces of understanding its antidepressant effects might be linked to ketamine's ability to modify sleep patterns and circadian-related molecules. Ketamine has especially been demonstrated to increase slow-wave activity during the following night of treatment and these changes have been shown to predict the clinical outcome in patients with major depressive disorder (MDD). Slow-wave activity is a low-frequency and high-amplitude wave seen in electroencephalography, which is highly expressed during the deepest stage of sleep, and this has been prominently found to be reduced in MDD patients. Even more intriguing, there are indications that ketamine might increase slow-wave activity also immediately after its administration. During this time, TrkB signalling is observed to became active.

Following these molecular findings, we sought to investigate the link between the TrkB signalling pathway and two prominent processes occurring during slow-wave sleep. During slow-wave sleep processes such as (1) reduction of brain's energy expenditure and (2) the activation of glymphatic system is known to occur. The glymphatic system is as lymphatic-perivascular network, which is responsible for clearing the brain from the metabolic waste. Thus, in this study, our objective was to investigate whether by causing an acute decline in adenosine-triphosphate (ATP) production or by stimulating the glymphatic network, we could activate the same plasticity-related pathways as ketamine is capable of activating in mice prefrontal cortex.

The results of this study suggest that acute metabolic reduction can trigger pathways regarding synaptic plasticity. The metabolic inhibitor, 2-deoxy-D-glucose and mercaptoacetate (2DG+MA), was found to phosphorylate the TrkB receptor and its downstream signalling molecules GSK3β and p70S6K, while MAPK was dephosphorylated. These results correlate with the previous findings of ketamine's effect after its administration. We also found a plasticity-related marker, MAP2, to be heavily phosphorylated by 2DG+MA, indicating 2DG+MA having a surprising role on neuroplasticity. These results are promising indication of understanding the rapid effects of ketamine and might even give important insight to developing novel antidepressants. However, these findings are only preliminary, and more research is needed to directly link antidepressant effects and energy metabolic inhibition together, as our study did not directly investigate antidepressants and depression-like behaviour in mice.

Avainsanat – Nyckelord – Keywords
2-deoxy-D-glucose, mercaptoacetate, brain energy metabolism, glymphatic system, tropomyosin receptor kinase B signalling

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Tiivistelmä – Referat – Abstract

Ketamiinin masennusta lievittävät vaikutukset havaittiin ensimmäisen kerran vuonna 2000, jonka jälkeen lukuisat tutkimukset ovat todistaneet sen nopean tehon lievittää masennusoireita jopa tuntien sisällä hoidon aloittamisesta. Tämän on todettu olevan suuri etu verrattuna yleisesti käytössä oleviin perinteisiin masennuslääkkeisiin, joiden kliinisen tehon tiedetään alkavan vasta viikkojen kuluttua lääkehoidon aloituksesta. Ketamiini on N-metyyli-D-asparagiinihappo reseptorin antagonisti ja sen päävaikutusmekanismin on ajateltu olevan sen kyvyssä vahvistaa ja lisätä synapsien määrää aivoissa (synaptinen muovautuvuus) sekä täten vaikuttavan mielialan paranemiseen. Molekulaarisella tasolla, ketamiinin antidepressiiviset vaikutukset on osoitettu olevan riippuvaisia erityisesti tropomyosin reseptori kinaasi B (TrkB) signalointi reitin aktivoitumisesta. Ketamiinin masennusta lievittävää vaikutusmekanismia tosin todennäköisesti ei vielä täysin ymmärretä, sillä uusia masennuslääkkeitä ei olla pystytty kehittämään viimeisten kahdenkymmenen vuoden aikana kliiniseen käyttöön lukuisista yrityksistä huolimatta. Tämän vuoksi onkin esitetty, että ketamiinin teho saattaisikin perusta niiden kykyyn muokata unen rakenteita ja sirkadiseen rytmiin vaikuttavia molekyylejä. Ketamiinin on etenkin havaittu lisäävän hidasaaltoaktiivisuuden määrää seuraavana yönä sen annostelusta ja tämä näyttäisi vahvasti korreloivan lääkehoidon onnistumisen kanssa vakavasti masentuneilla potilailla. Hidasaaltoaktiivisuus on aivosähkökäyrällä havaittavaa matala taajuista ja korkea amplitudista aaltoa, jota esiintyy erityisesti syvän unen aikana. Hidasaaltoaktiivisuuden määrä on usein huomattu vähentyneen vakavasti masentuneilla potilailla. Sen lisäksi että hidasaaltoaktiivisuuden on havaittu lisääntyneen seuraavana yönä hoidon aloituksesta, ketamiini näyttäisi lisäävän hidasaaltoaktiivisuutta myös heti lääkkeen annon lopettamisen jälkeen. Erityisen kiinnostavaa onkin, että ketamiini näyttäisi tänä aikana aktivoivan spesifisesti TrkB reseptorin signalointireittiä.

Perustuen jälkimmäiseen havaintoon, tutkimuksemme tavoitteena oli tutkia TrkB reseptorin mekanismia ja sen yhteyttä kahteen syvän unen aikana tapahtuvaan prosessiin. Syvä uni on aivojen aktiivisuuden hitain tila, jonka aikana tiedetään (1) aivojen energiametabolian laskevan ja (2) glymfaattisen järjestelmän aktivoituvan. Glymfaattinen järjestelmä on imu- ja verisuoniverkosto, joka vastaa aivojen siivoamisesta aineenvaihdunta tuotteista. Täten, tässä tutkimuksessa päämääränämme oli tutkia aktivoiko adenosiini-trifosfaatin (ATP) äkillinen lasku tai glymfaattinen kierto samoja hermoston muovautuvuuteen liittyviä signalointireittejä kuin ketamiinin tiedetään aktivoivan hiirien etuaivokuorella.

Tämän tutkimuksen perusteella äkillisen energia-aineenvaihdunnan lasku näyttäisi stimuloivan hermoston muovautuvuuteen liittyviä signalointireittejä. 2-deoksy-D-glukoosi ja merkaptoasetaatti (2DG+MA), jotka estävät energia aineenvaihdunnan toiminnan, vaikuttaisivat fosforyloivan TrkB reseptoria ja sen signalointiketjun molekyylejä, GSK3β ja p70S6K sekä vähentävän MAPK2 fosforylaatiota tutkimuksemme mukaan. Nämä tulokset korreloivat ketamiinilla havaittuihin vaikutuksiin hidasaaltoaktiivisuuden aikaan (lääkkeen annon jälkeen). Lisäksi havaitsimme MAP2 proteiinin aktivoituneen voimakkaasti 2DG+MA vaikutuksesta, mikä viittaisi, että 2DG+MA:lla on yllättävää vaikutus hermoston muovautuvuuteen. Nämä tulokset antavat lupaavia viitteitä siihen, että ketamiinin molekulaarisilla mekanismeilla on yhteisiä signalointireittejä energia aineenvaihdunnan kanssa. Tulokset voisivat mahdollisesti auttaa tulevaisuudessa uusien parempien masennuslääkkeiden kehittämisessä. Nämä tulokset ovat kuitenkin alustavia, sillä tutkimuksessamme emme selvittäneet suoraa nopeavaikutteisten masennuslääkkeiden tai masennuksen kaltaisten käyttäytymismallien vaikutusta hiiriin.

Avainsanat – Nyckelord – Keywords
2-deoksiy-D-glukoosi, merkaptoasetaatti, aivojen energia metabolia, glymfaattinen järjestelmä, tropomyosin reseptori kinaasi B signalointi

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ABBREVIATIONS

ATP	Adenosine triphosphate
AMPK	AMP-activated protein kinase
BDNF	Brain derived neurotrophic factor
CTRL	Control group
C57BL/6	Inbred mice strain
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HRP	Horseradish Peroxidase
HTS	Hypertonic saline
MA	Mercaptoacetate
MAPK	Mitogen-activated protein kinase
MAP2	Microtubule-associated protein 2
MDD	Major depressive disorder
mTOR	Mammalian target of rapamycin
NMDAR	N-methyl-D-aspartate receptor
mPFC	Medial prefrontal cortex
p70S6K	P70 S6 kinase
S.E.M.	Standard error of the mean
Src	Proto-oncogene tyrosine-protein kinase Src
TrkB	Tropomyosin receptor kinase B
2DG	2-deoxy-D-glucose

1 INTRODUCTION

Major depressive disorder (MDD) is a debilitating mental disorder, which has a significant impact on the person's quality of life and also presents a considerable economic burden on society (Gustavsson et al. 2011). It has been estimated that nearly 270 million people are affected by the disorder worldwide and this number has consistently continued to rise (James et al. 2018). MDD is known to alter mood, cognition, behaviour, and even essential functions such as sleep. In fact, most MDD patients have a comorbid sleep disorder, most commonly insomnia (Breslau et al. 1996; Riemann et al. 2001). Despite decades of research, the pathophysiology of MDD remains poorly understood. However, it has been associated with reduced neuronal function and connectivity in brain regions related to mood and cognition (Liu et al. 2017). Particularly, synaptic plasticity has been observed to be impaired in these areas, including the prefrontal cortex and hippocampus (Duman and Aghajanian 2012). Synaptic plasticity refers to changes, where synapses' strength or number is modified (Citri and Malenka 2008). The current treatments of MDD, monoaminergic agents such as serotonin reuptake inhibitors (SSRIs), are proposed to enhance these plasticity changes and thus, explain their clinical effect (Castrén and Hen 2013). Unfortunately, these treatments also have some major limitations. For example, they take weeks to show full clinical efficacy after treatment initiation, which can be crucial for patients with increased risk of suicidal ideation (Gaynes et al. 2009). Moreover, up to 70% of patients do not achieve full remission with traditional antidepressants (Papakostas et al. 2008; Trivedi et al. 2006). Thus, the discovery of ketamine's rapid-acting antidepressant effects in 2000 was a major breakthrough in research of MDD treatment (Berman et al. 2000). A low subanaesthetic dose of ketamine was found to cause an acute and fast antidepressant effect in MDD patients only after a single administration. Since then, numerous of studies have demonstrated ketamine to alleviate depressive symptoms rapidly and effectively even in patients resistant to traditional antidepressants (Gerhard and Duman 2018; Machado-Vieira et al. 2010).

Ketamine is an N-methyl-D-aspartate (NMDA) receptor antagonist, and its main antidepressant effect is thought to occur through glutaminergic signalling (Zanos and Gould 2018). Ketamine is known to rapidly increase the levels of extracellular glutamate, which further leads to the release of brain-derived neurotrophic factor (BDNF) and the activation of its receptor, tropomyosin receptor kinase B (TrkB) (Fig. 1). In rodent models, ketamine's antidepressant effects have been observed to be dependent on the activation of TrkB

receptor's downstream molecules: mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) (Li et al. 2010; Réus et al. 2014). Ketamine's effects are also found to correlate with the inhibition of glycogen synthase kinase 3β (GSK3β) in mice (Beurel et al. 2011). These alterations in proteins are believed to lead to the synaptic changes seen after ketamine administration such as increases in synaptic strength and number in rodents (Gerhard and Duman 2018; Zanos and Gould 2018). However, the underlying mechanism of the antidepressant effects of ketamine is likely more complex as new NMDA-receptor antagonists have not been successfully developed for clinical use (Garay et al. 2018). The need to develop new antidepressants is high since ketamine's dissociative effects and abuse risk limit its use for a long-term treatment.

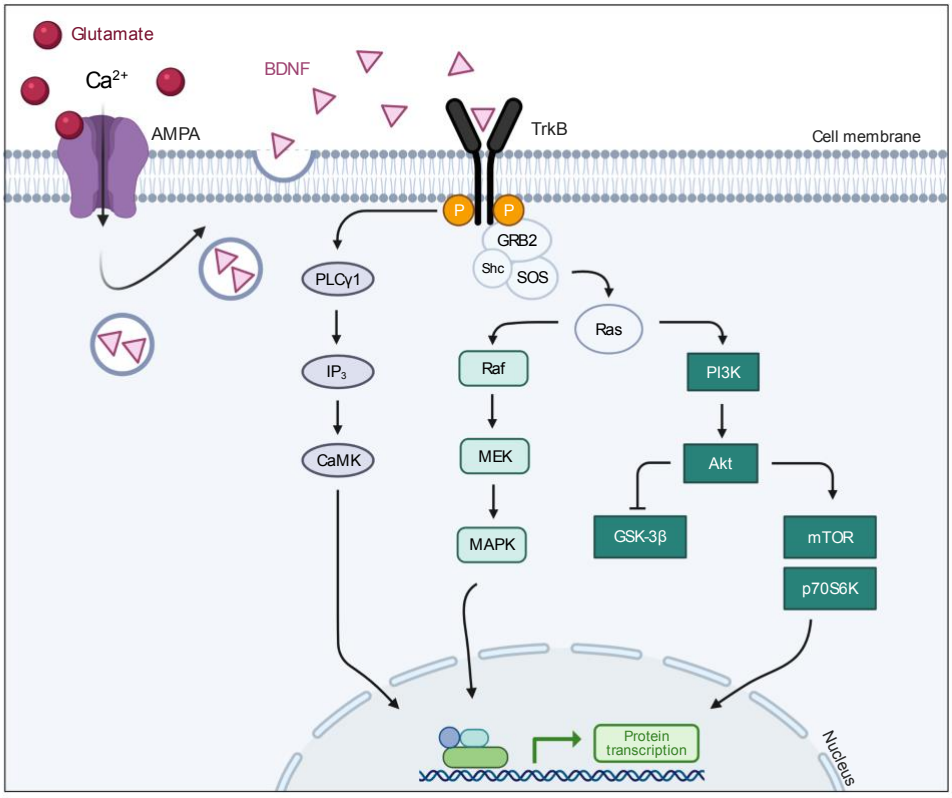

Figure 1. BDNF-TrkB signalling pathway. Ketamine has been reported to activate tropomyosin-receptor-kinase B (TrkB) downstream signalling pathway through its ability to increase extracellular levels of glutamate. Glutamate activates α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptors at the postsynaptic membrane, which leads to the release of the brain-derived neurotrophic factor (BDNF). From there, BDNF binds to its receptor, tropomyosin receptor kinase B (TrkB), which promotes neuronal growth, survival and synaptic plasticity via three intracellular cascade: (1) the Ras–mitogen-activated protein kinase (MAPK) cascade, (2) the phosphatidylinositol 3-kinase (PI3K) cascade, (3) the phospholipase Cγ1 (PLCγ1) cascade. (Minichiello 2009; Zanos and Gould 2018).

In recent studies, ketamine has been found to modify sleep patterns and circadian-related molecules (Bellet et al. 2011; Bunney and Bunney 2013). Ketamine has especially been demonstrated to increase slow-wave activity during the following night of treatment and these changes have been shown to predict the clinical outcome in MDD patients (Duncan et al. 2013). Slow-wave activity is a low-frequency and high-amplitude wave seen in electroencephalography, which is highly expressed during the deepest stage of sleep: slow-wave sleep (Brown et al. 2012). Slow-wave sleep is often found reduced in depressed patients (Nutt et al. 2008; Riemann et al. 2001). It has been suggested that these findings might be key for developing novel antidepressants (Duncan et al. 2019). Moreover, there are some indications that ketamine might cause a slow-wave sleep-like-state also immediately after its administration (Kohtala et al. 2019b, 2019a). For instance, Kohtala et al. (2019b) showed with mice that ketamine could produce firstly an excitatory state in the brain, where neurons are known to be highly active, and subsequently, a rebound effect, where a slow-wave activity was detected. Intriguingly, during the first state, mitogen-activated protein kinase (MAPK) was found activated, while other detected TrkB signalling molecules stayed inactive. As the slow-wave activity emerged, the signalling of TrkB and its downstream pathways of GSK3β and p70 S6 kinase (p70S6K), which is a protein from the downstream cascade of mTOR, was found phosphorylated. Thus, they observed that during slow-wave activity, ketamine particularly regulated the TrkB signaling cascade, which is a critical pathway for ketamine's rapid antidepressant response.

Following the findings of Kohtala et al. (2019a), our aim in this study was to investigate the link between the TrkB signalling pathway and slow-wave activity by studying different processes occurring during slow-wave sleep. Slow-wave sleep is known to have a couple of prominent features that distinguishes it from other sleep phases. Firstly, the brain's energy expenditure and the cerebral metabolic rate are observed to decrease (Wisor et al. 2013). Secondly, the glymphatic system has been found to be predominantly active during slow-wave sleep (Reddy and van der Werf 2020). The glymphatic system is as lymphatic-perivascular network, which is responsible for clearing the brain of the metabolic waste accumulated throughout the day (Jessen et al. 2015). Thus, the objective was to investigate whether by reducing the brain's energy metabolism or by activating the glymphatic system the BDNF-TrkB signalling cascades are activated. From the TrkB receptor's downstream signalling cascade, GSK3β, MAPK, and p70S6K are detected. We also studied the molecular activation of a plasticity-related marker called microtubule associated protein 2 (MAP2).

Microtubule associated proteins (MAPs) are crucial regulators of cellular cytoskeleton microtubules (Marchisella et al. 2016). Alterations related to these proteins lead to disturbances in the neuronal cytoskeleton and these dysfunctions are often found in psychiatric disorders related to impaired neuroplasticity such as depression (Wong et al. 2013). Especially, microtubule associated protein 2 (MAP2) has been found to regulate pathways that promote synaptic plasticity (Harada et al. 2002).

2 MATERIALS AND METHODS

2.1 Animals

In this study, nine-week-old male mice from the C57BL/6JHsd strain (weighted 24.4 g ± 2.1) obtained from Envigo, the Netherlands, were used. The mice were kept under standard laboratory conditions (room temperature: 21°C, humidity: 50 %, 12h:12h light-dark cycle: lights on at 6.00) at the animal facility of the University of Helsinki, Finland. They were single-housed in individually ventilated cages, where bedding, nesting material, a play tunnel, and an Aspen brick were included. Mice had unlimited access to food and water at all times. The experiments conducted were approved by the County Administrative Board of Southern Finland (License: ESAVI/5844/2019) and carried out according to the guidelines of the Society for Neuroscience.

2.2 Pharmacological treatments

The brain's energy metabolism was suppressed by the combination of a glycolysis inhibitor, 2-deoxy-D-glucose (2DG) (#D8375-5G, Sigma-Aldrich, Saint Louis, MO, USA) (Wick et al. 1957), and a fatty acid oxidation inhibitor, mercaptoacetate (MA) (#125432500, Fisher Scientific, Leicestershire, UK) (Bauche et al. 1983). 2DG (1 g/kg) and MA (600 μmol/kg) were diluted into the same solution of isotonic saline (0.9 % NaCl). The doses were selected according to a previous study done by Stamper and Dark (1997). The combination was selected, because alone 2DG has been observed to cause fatal hypothermia in some studies. The glymphatic system was activated by a 1 M of 20 ml/kg hypertonic saline (HTS) (Plog et al., 2018). Isotonic saline was selected as a vehicle control to exclude the effects of the

injection itself. All treatments were administered by a single intraperitoneal injection between 10.00 a.m - 1.00 p.m with an injection volume of 20 ml/kg.

2.3 Experimental procedures

The precise experimental timeline is presented in Fig. 2. At the beginning, the mice were divided randomly into three groups (n = 8): control, HTS, and 2DG+MA and the investigator was unaware of which treatment each group received, while the experiments were conducted (blinded study). Prior to testing, the animals were handled (picked up by a tunnel or cupped, and immobilized) multiple times a week between 9.00 a.m - 14.00 p.m in order to reduce stress and anxiety of the mice during the experiments. Also, the animals' wellbeing was monitored during and after the experiments.

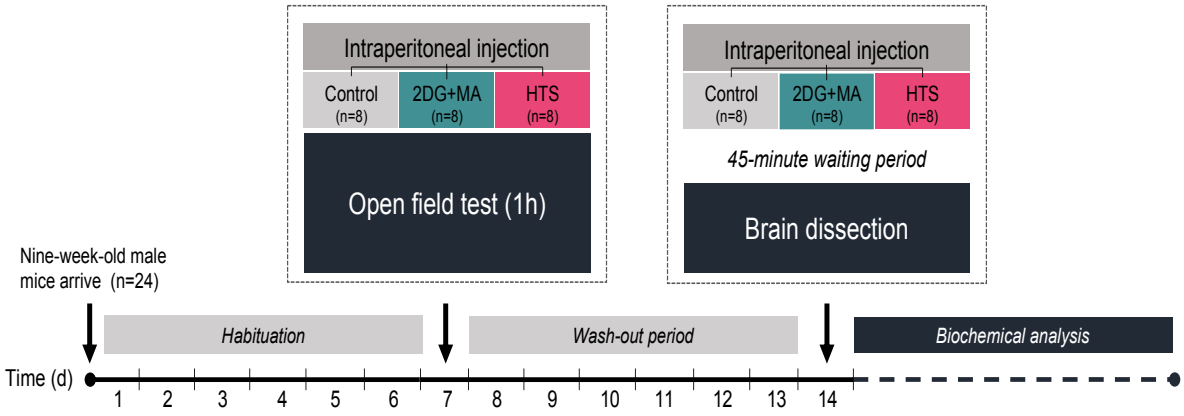

Figure 2. Experimental timeline. The mice were habituated for a week from arrival before any experiments. Subsequently, the locomotor activity of the mice was recorded for an hour immediately after i.p injection of control, 2DG+MA and HTS (n = 8/group). A week later, the mice were euthanized 45 minutes after the same i.p injection as previously. Brain samples were collected afterwards and stored at –80°C. 2DG+MA = 2-deoxy-D-glucose (1g/kg) and mercaptoacetate (600 µmol/kg), HTS = hypertonic saline (1 M), Control = 0.9 % NaCl, d = day, h = hour.

2.4 Spontaneous locomotor activity

One of the characteristic features of sleep is low muscle tone, which is seen as immobility (Chase 2013). This was detected by measuring the 1-hour locomotor activity of mice at 10.00 a.m - 1.00 p.m. The activity was recorded with an open field test using automated open field locomotor activity chambers (Activity monitor SOF-812, Med Associates Inc, Fairfax, VT,

USA). The mice were habituated to the testing room an hour before testing. Subsequently, they were placed in the activity chamber directly after i.p injection and horizontal and vertical activity was recorded for an hour.

2.5 Brain sample collection

The mice were euthanized 45 minutes after i.p injections by rapid cervical dislocation and decapitation without anaesthesia as most anaesthetics have been shown to affect the studied signalling pathways (Kohtala and Rantamäki 2019). Bilateral medial prefrontal cortex (mPFC), bilateral hippocampi, and cerebellum were dissected immediately after dislocation on a cooled dish and stored at -80°C (Wager-Miller et al. 2020). For this study, mPFC samples were used for the biochemical analyses.

2.6 Biochemical analyses

The mPFC were homogenized in lysis buffer (3 M Tris-HCl, pH 8.0, 5 M NaCl solution, 0.5 M NaF solution, NP-40, Glycerol in MilliQ-water) mixed with protease and phosphatase inhibitors (Complete inhibitor mix and PhosStop from Roche, Basel, Switzerland). Homogenization was done by an ultrasonicator (Rinco Ultrasonics, Romanshorn, Switzerland). The samples were incubation on ice for 15 min and centrifuged (16000g) for 15 minutes at 4 °C (Heraeus Fresco 17 centrifuge, Thermo Fisher Scientific, Waltam, MA, USA). Subsequently, supernatants were collected and the protein concentration of each sample was measured by using a DC Protein Assay Kit I (Bio-Rad Laboratories, Hercules, CA, USA) (ANNEX 1A).

2.6.1 Western blot

Western blot was used to detect protein phosphorylations in the relevant signalling pathways in the mPFC. The separation of the samples was conducted with a standard sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) method. NuPAGE Bis-Tris and Bolt Bis-Tris Plus Gel (Thermo Fisher Scientific, Waltam, MA, USA) was used in a XCell SureLock Blot module (Thermo Fisher Scientific, Waltam, MA, USA) and each well was loaded with samples consisting of 40 µg of protein mixed in 2xLaemmli buffer (heated prior

in 100 °C for 3 min). After an hour of running (180 V), the gels were blotted to a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, Waltam, MA, USA). The membrane was activated in 100% methanol and then transferred for an hour (300 mA) in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell chamber (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were first incubated with a 3% bovine serum albumin (BSA) mixed 0.1% Tween in tris-buffered saline (TBST) for an hour at room temperature and then with a primary antibody (1:1000 in 3% BSA in TBST) overnight at 4 °C. Primary antibodies used are listed in Table 1. After incubation, membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibodies (1:10000 in non-fat dry milk, #1721064 from Bio-Rad Laboratories, Hercules, CA, USA) for an hour at room temperature. After subsequent washes with TBS, secondary antibodies were detected by Bio-rad ChemiDoc MP camera (Bio-Rad Laboratories, Hercules, CA, USA) by using an enhanced chemiluminescence solution method (ECL Plus solution, Fisher Scientific, Leicestershire, UK). The optical densities of the bands were measured by ImageJ (ImageJ version 2.1.0).

Table 1. Primary antibodies used in Western blot analysis (from Cell Signaling Technology Danvers, MA, USA).

Detected protein	Form	Primary antibody	Product number
Tropomyosin receptor kinase B (TrkB)	total protein	anti-TrkB	#4603
	phosphorylated	anti-p-TrkB^{Y816}	#4168S
p70 S6 kinase (p70S6K)	total protein	anti-p70S6K	#2708
	phosphorylated	anti-p-p70S6K$^{T421/S424}$	#9204S
Mitogen-activated protein kinase (MAPK)	total protein	anti-p44/42-MAPK	#9102
	phosphorylated	anti-p44/42-MAPK$^{Thr202/Y204}$	#9106
Glycogen synthase kinase 3 beta (GSK3β)	total protein	anti-GSK3β	#9336
	phosphorylated	anti-p-GSK3β^{S9}	#9315
Microtubule associated protein 2 (MAP2)	total protein	anti-MAP2	#4542S
	phosphorylated	anti-p-MAP2$^{T1620/1623}$	#4544S
Proto-oncogene tyrosine-protein kinase Src (Src)	total protein	anti-Src	#2101S
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	total protein	anti-GAPDH	#2118

2.6.2 Enzyme-linked immunosorbent assay

The concentration of the proteins was measured as mentioned above. Sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the BDNF protein concentrations in the mPFC. Prior to the assay, samples were treated with HCl for 15 min in order to separate BDNF from its binding proteins and receptors and hence, increase the detectability of BDNF (Okragly and Haak-Frendscho 1997). Subsequently, samples were neutralized with NaOH. A commercial ELISA Kit (Human/Mouse BDNF DuoSet ELISA Development kit (DY248) and DuoSet ELISA Ancillary Reagent Kit 2 (DY008), R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. Firstly, a 96-well plate was coated with BDNF capture antibody overnight at 4 °C. Then samples were assayed in duplicates and incubated first for two hours in room temperature before biotinylated BDNF detection antibody was added. After another two-hour-incubation in room temperature, Streptavidin-HRP was added, and the plate placed in a shaker for 20 min protected from light. After every step, each well as washed with the kit's Wash Buffer three times. Absorbance (450 nm) was measured by ELx800 microplate reader (BioTek, Winooski, VT, USA) and concentrations calculated by using a comparison standard curve of human/mouse BDNF standard (ANNEX 1B).

2.7 Statistical analyses

Statistical analyses were conducted with either the one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test or the Kruskal-Wallis test followed by Dunn's *post-hoc* test depending on whether the data were normally distributed. For normally distributed data, outliers were examined with Grubb's test. All statistical analyses were performed with the Prism 7 software from GraphPad. p value of ≤ 0.05 was considered significant. Biochemical results were normalized to control group. Results are presented as mean ± standard error of the mean (S.E.M) unless otherwise mentioned.

3 RESULTS

3.1 2DG+MA and HTS decreased locomotor activity of mice

Locomotor activity of the mice was detected in the one-hour open field test. In both groups, 2DG+MA and HTS, mice were notably less active compared to the control group and the differences were seen within the first 5 minutes (from 0-5 minutes; p(2DG+MA) = 0.0002, p(HTS) = 0.0001, Fig. 3A-B). After 10 minutes, 2DG+MA group was practically immobile until testing ended at 60 minutes (Fig. 3A). 2DG+MA drastically reduced the overall locomotor activity in both horizontal ($p < 0.0001$, Fig. 3D) and vertical activity ($p < 0.0001$, Fig. 3C). A similar effect was also seen with hypertonic saline; however, these results (total distance travelled and total vertical count) were not statistically significant.

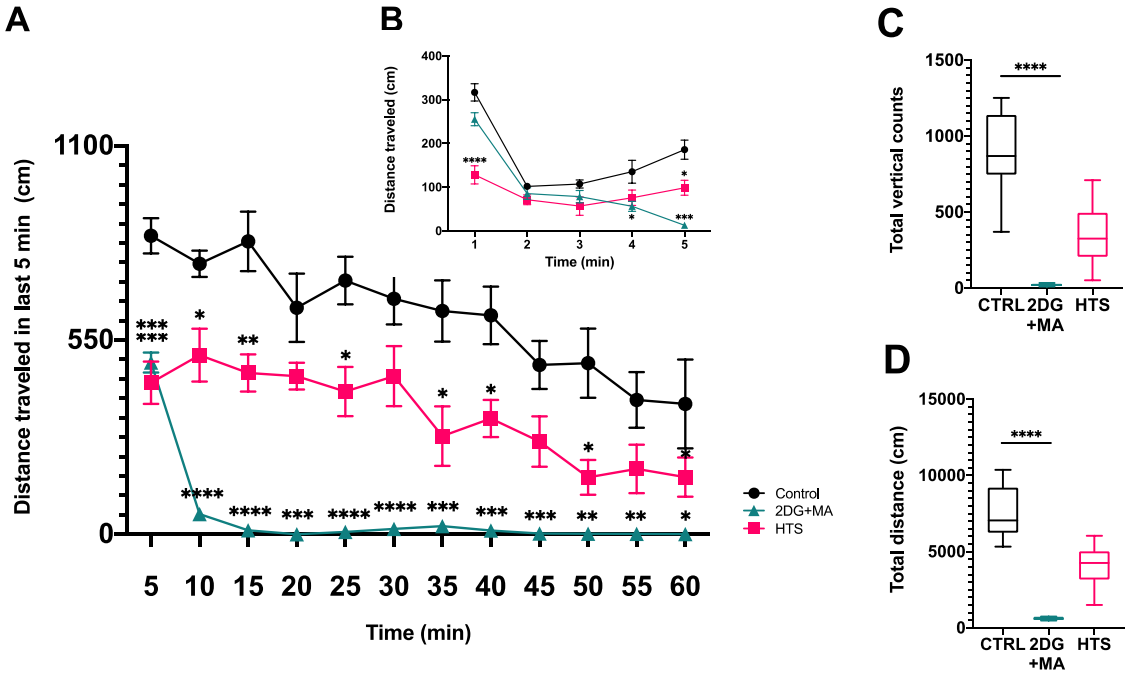

Figure 3. Changes in 1-hour locomotor activity of adult mice after i.p injection of CTRL (control, 0.9% NaCl), 2DG+MA (2-deoxy-D-glucose, 1g/kg and mercaptoacetate, 600 µmol/kg) or HTS (hypertonic saline, 1 M of 20ml/kg). (A-B) Mice treated with 2DG+MA or HTS were significantly less active compared to control group. In both treatment groups, the effects were seen early on in testing. (C-D) Overall, the mice in both testing groups were less active both in horizontal and vertical activity, however only 2DG+MA was statistically significant. Data are expressed as mean ± S.E.M in A-B and analyzed in 5 min (A) and one min (B) bins (n = 8/group). Data are expressed as median, interquartile range, and minimum and maximum values in C-D (n = 8/group). Significance was tested for by two-way ANOVA followed by Dunnett's post hoc test (A-B) or Kruskal-Wallis test followed by Dunn's post hoc test (C-D). For more detailed statistical analyses see ANNEX 1C. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

3.2 2DG+MA activates the TrkB signaling pathway and MAP2

The activation of the receptor TrkB and its downstream cascade were found to be altered by 2DG +MA. A strong increase was detected in the phosphorylation of $TrkB^{Y816}$ ($p = 0.0008$, Fig 4A) and its downstream signaling molecules $GSK3\beta^{S9}$ ($p < 0.0001$, Fig. 4B) and $p70S6K^{T421/S424}$ ($p = <0.0001$, Fig. 4D) and a decrease of $MAPK^{T202/Y204}$ phosphorylation ($p = 0.0002$, Fig. 4C) 45 minutes after 2DG+MA injection. Moreover, another relevant protein, $MAP2^{T1620/1623}$, which is also associated with synaptic plasticity, was found to be greatly induced by 2DG+MA ($p = 0.0094$, Fig. 5). HTS did not cause a statistically significant change in the phosphorylation of these proteins.

Levels of BDNF, a known ligand for TrkB receptor, were found to be slightly but significantly decreased by HTS ($p = 0.0209$, Fig. 6B). There was no statistical difference observed between 2DG+MA and control mice (Fig. 6B). Intriguingly though, the 2DG+MA treatment heavily increased the phosphorylation of Src^{Tyr416} protein ($p = 0.0075$, Fig. 6A), which has been suggested to also activate the TrkB receptor (Huang and McNamara 2010). HTS did not significant alter phosphorylation of Src^{Tyr416}.

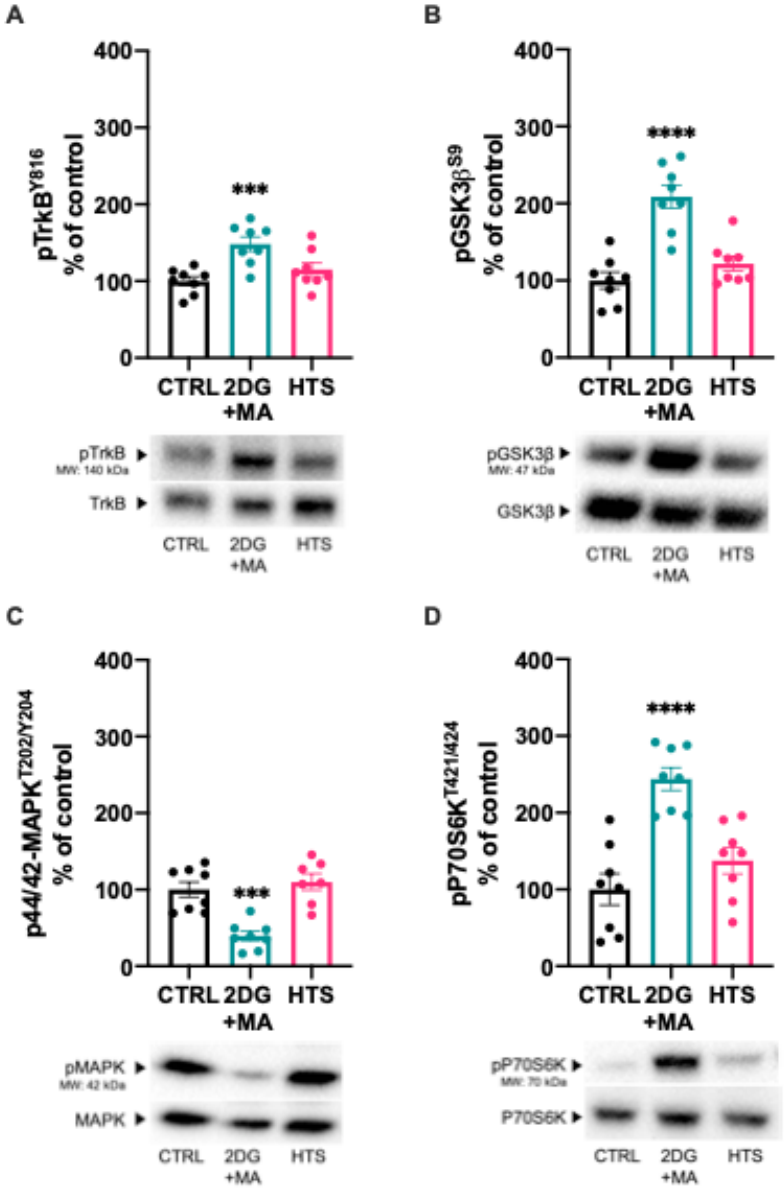

Figure 4. Phosphorylations of TrkB^{Y816}, GSK3β^{S9}, p70S6K$^{T421/S424}$ and MAPK$^{T202/Y204}$ in the adult mouse mPFC 45 min after i.p injection of CTRL (control, 0.9% NaCl), 2DG+MA (2-deoxy-D-glucose, 1g/kg and mercaptoacetate, 600 µmol/kg) or HTS (hypertonic saline, 1 M). 2DG+MA increased the phosphorylation of the proteins expect for MAPK$^{T202/Y204}$, which phosphorylation was decreased. HTS did not significantly alter the activity of the proteins. Data are expressed as mean ± S.E.M; n=7-8/group. Significance was tested for by one-way ANOVA followed by Dunnett's *post hoc* test. For more detailed statistical analyses see ANNEX 1C. *** < 0.001, **** < 0.0001. MW = molecular weight, kDa = kilodalton

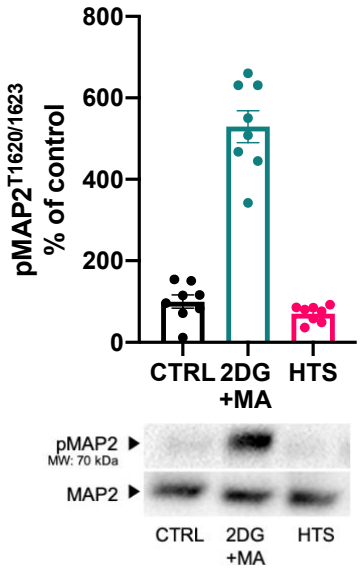

Figure 5. MAP2$^{T1620/1623}$ activity significantly increased in 2DG+MA treated mice in the mPFC 45 minutes after i.p injection. Data are expressed as mean ± S.E.M; n=8/group. Kruskal-Wallis test followed by Dunn's *post hoc* test. For more detailed statistical analyses see ANNEX 1C. ** < 0.01. CTRL = control, 2DG = 2-deoxy-D-glucose (1g/kg), MA = mercaptoacetate (600 µmol/kg) HTS = hypertonic saline (1 M), MW = molecular weight, kDa = kilodalton

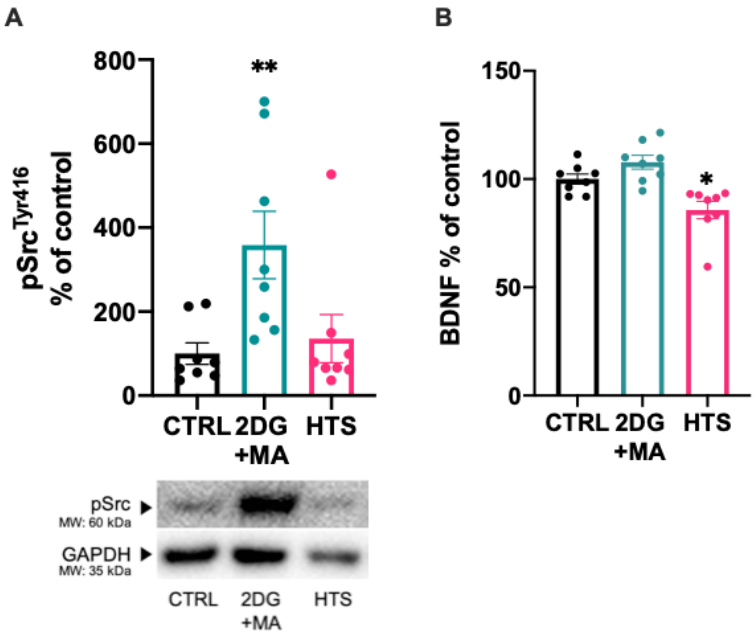

Figure 6. (A) 2DG+MA strongly increased phosphorylation of Src^{Tyr416} in adult mouse mPFC 45 min after i.p injection compared to control. (B) Hypertonic saline decreased the levels of BDNF, whereas 2DG+MA had no statistically significant effect in adult mice mPFC 45 min after i.p injections. Data are expressed as mean ± S.E.M; n=8/group. Significance was tested for by Kruskal-Wallis test followed by Dunn's *post hoc* test (A) and one-way ANOVA followed by Dunnett's *post hoc* test (B). For more detailed statistical analyses see ANNEX 1C. * < 0.05, ** < 0.01. CTRL = control, 2DG = 2-deoxy-D-glucose (1g/kg), MA = mercaptoacetate (600 µmol/kg), HTS = hypertonic saline (1 M), MW = molecular weight, kDa = kilodalton

4 DISCUSSION

Recent research has highlighted the importance of slow-wave activity for the antidepressant effects of ketamine (Duncan et al. 2013, 2019; Kohtala et al. 2019b). Ketamine's therapeutic effects have been shown to correlated in the changes of MDD patients' slow-wave activity during subsequent sleep (Duncan et al. 2013). Moreover, after ketamine's administration, a similar slow-wave activity is observed to emerge and TrkB pathway is observed to become activate (Kohtala et al. 2019b). In this study, our aim was to determine, which feature of slow-wave sleep could trigger this molecular pathway: metabolic reduction or glymphatic influx induction. According to our results, metabolic reduction, triggered by 2DG+MA, could phosphorylate the TrkB receptor and its downstream signalling molecules GSK3β and p70S6K, while MAPK was dephosphorylated (Fig. 7). These results correlated with Kohtala et al. (2019b) findings on ketamine. However, it is unlikely that 2DG+MA alone could cause an antidepressant effect as Kohtala et al. (2019b) also discovered that the antidepressant effects of ketamine were not only dependent on the emergence of slow-wave activity but also by the preceding cortical excitability. Nevertheless, ketamine has been in previous studies shown to affect the brain's energy metabolism (Ionescu et al. 2018; Långsjö et al. 2004) and even more, energy restriction has been observed to exhibit anxiolytic behaviour in rodents and improve mood in humans (Levay et al. 2007; Riddle et al. 2013). Therefore, it is intriguing that 2DG+MA phosphorylated the same proteins as ketamine after its withdrawal and these findings might give us new insight on how ketamine causes its rapid-acting antidepressant response.

A plasticity-related marker, MAP2, was also observed to be highly phosphorylated by 2DG+MA. Thus, this could indicate that energy metabolism and neuroplasticity have parallel mechanisms. Energetic challenges such as energy restrictions (fasting) and heavy physical activity have been observed to promote neuroplasticity changes and improve the brain's resistance to damage (Mattson et al. 2018). It has been suggested that by switching energy balance from one extreme to another, such as high metabolic activity to low metabolic activity, multiple different plasticity-related pathways are triggered, one of which is the BDNF signalling (Marosi and Mattson 2014; Mattson et al. 2018). Our results in energy reduction did not, however, identify any significant changes in BDNF levels even though its receptor, TrkB, was found active. However, there are a couple of reasons, why this might be. Firstly, these results do not identify if the release of BDNF from the

postsynaptic neuron was increased. Instead, they examined the changes in the levels of mature BDNF and proBDNF in the samples. Secondly, our results were analysed in duplicates instead of triplicates, which could have given more reliable results. Lastly, TrkB receptor could have been activated BDNF-independently, as phosphorylation of Src was heavily increased in our result (Fig. 7) (Huang and McNamara 2010; Rantamäki 2019). However, there were large deviations in the data. This could be because Src was one of the last proteins detected in Western blot and prior stripping probably weakened the quality of the detection. Notably, Src was also the only protein that was normalized to GAPDH and variation between GAPDH bands was also detectable. GAPDH was the last protein detected. Thus, we cannot confirm the role BDNF or Src played in TrkB signalling, but yet, it is possible that BDNF could have triggered the TrkB signalling and further promoted neuroplasticity.

An important notion of these results is that there are several conflicting evidence regarding 2DG and its metabolic effects. For example, energy restriction studies have shown to suppress mTOR signalling via the AMP-activated protein kinase (AMPK), whereas 2DG alone has been found to both downregulate and upregulate mTOR signalling (Fig. 7) (Estañ et al. 2012; Garriga-Canut et al. 2006; Potter et al. 2010; Wang et al. 2020). 2DG has been also used in research as an inducer of torpor, which is a state animals enter to save energy upon demanding and harsh environmental conditions (Heldmaier et al. 2004; Kilduff et al. 1990). This leads to a decrease in the animal's physical activity, body temperature, and metabolic rate (Heldmaier et al. 2004). In our study, the locomotor activity of mice was drastically reduced by 2DG+MA within 10 minutes (Fig. 3), which indicates that the dose caused mice to enter a torpor-like state in time the brains were dissected. If indeed mice were in torpor, this might contradict with our plasticity-related findings as neuroplasticity is generally found inactive during torpor (Bullmann et al. 2019; Heldmaier et al. 2004; Horowitz and Horwitz 2019). Furthermore, torpor is also reported to suppress certain cellular mechanisms such as protein transcription and translation (Heldmaier et al. 2004), which yet again contradict with our result as mTOR signalling is known to activate protein translation. There is also evidence that MAPK signalling is upregulated in torpid primates (Biggar et al. 2015). Nevertheless, these findings are found to be dose, tissue, and even, species specific (Biggar et al. 2015; Estañ et al. 2012; Kilduff et al. 1990; Millesi et al. 2001; Ruczynski and Siemers 2011). For example, Biggar et al. (2015) detected upregulation of MAPK in tissues of skeletal muscle and kidney but not in heart muscle. 2DG has also been shown to affect

brain metabolism differently in different brain regions during torpid animals (Kilduff et al. 1990). Moreover, it is also possible that the combination of 2DG and MA imitated more closely of extremely severe fasting conditions, which cannot be compared to other milder energy restricting studies. Therefore, replicating and translating findings regarding 2DG, MA, and energy restriction can turn out to be difficult and even minor variation between study designs, protocols and techniques can have surprisingly large consequences in the results. Reproducibility is also known to be a general concern in animal research and also, with the semi-quantitative Western blot analysis (Gough 2015; Jilka 2016).

Lastly, a couple of notions about the results of HTS, which was used to induce the glymphatic influx. Although our results did not find HTS to have any significant effects on the plasticity-related molecules, there might be a couple of explanations for this. Firstly, 2DG+MA and HTS were loaded on the same membrane in Western blot. Because 2DG+MA caused an extremely high-intensity band in protein's it phosphorylated, the detection time was determined by its bands to avoid signal saturation. Therefore, timing of the detection had to be stopped perhaps too early to get a proper signal for the bands of HTS. Secondly, the timing of the brain dissection was not perhaps optimal as the locomotor activity did not differ significantly from the control group at the time of brain dissection. In fact in other studies, measurement of the glymphatic system activity have been conducted before or at 30 minutes after HTS administration (Plog et al. 2018), whereas we conducted at 45 minutes. Moreover, a baseline of locomotor activity was not measured, which may be emphasised in HTS results. Because HTS did not cause as an extreme behavioural change as 2DG+MA, the individual variations between the mice may have had an impact on the results, as some mice are naturally more active than others. Finally, an overall limitation of this study is that the levels of the loading control, GAPDH, varied between samples. This might indicate that either sample loading was not successful or, as mentioned above, stripping was conducted too many times on the membrane.

In conclusion, these results are the first to link 2DG+MA effects to the TrkB signalling pathway. Further research should be done to understand how these results compare to findings with ketamine's effects on energy metabolism and antidepressant effects. For example, by studying the combined effects of 2DG+MA and ketamine could further enhance understanding on how these two processes link together. Nevertheless, these results might

be the first steps towards understanding the rapid effects of ketamine and eventually developing novel antidepressants.

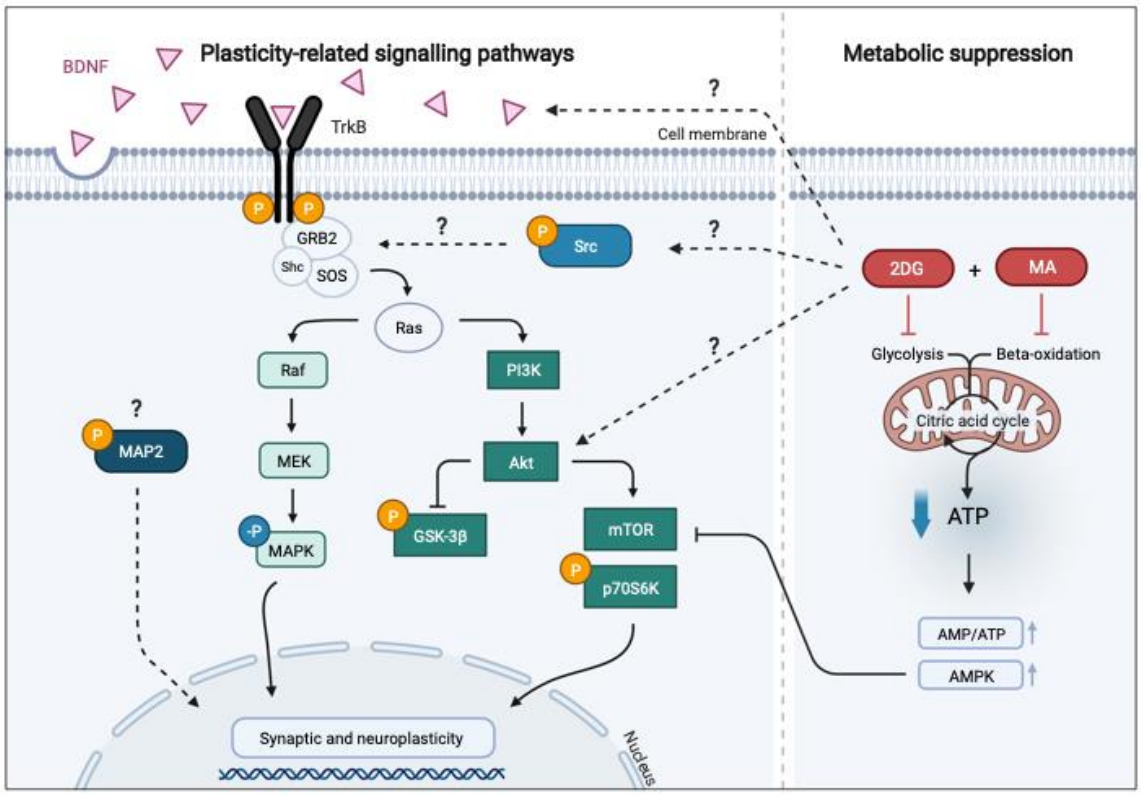

Figure 7. A schematic of 2DG+MA putative effects on plasticity-related signalling pathways. 2DG is a known glycolysis inhibitor and MA a known inhibitor of beta-oxidation. By blocking these pathways, ATP production is suppressed and the ratio of AMP/ATP increases. This activates AMPK, which is known to suppress mTOR activity and thus, further protein translation. According to our results however, mTOR downstream protein, P70S6K, is found phosphorylated, indicating that AMPK did not suppress mTOR activity. Our results also observed 2DG+MA to phosphorylate TrkB, Gsk3β, MAP2, Src and dephosphorylate MAPK. There are indications in previous studies that metabolic suppression might activate BDNF-dependent TrkB signalling. 2DG+MA = 2-deoxy-D-glucose+mercaptoacetate. P = activatory phosphorylation. -P = inhibitory phosphorylation. ATP = adenosine triphosphate, AMPK = AMP-activated protein kinase, BDNF = Brain derived neurotrophic factor, GSK3β = Glycogen synthase kinase 3 beta, MAPK = Mitogen-activated protein kinase, MAP2 = Microtubule-associated protein 2, mTOR = Mammalian target of rapamycin, p70S6K = P70 S6 kinase, Src = Proto-oncogene tyrosine-protein kinase Src, TrkB = Tropomyosin receptor kinase B

5 CONCLUSION

To our knowledge, this is the first study to demonstrate that 2DG+MA can activate the TrkB signalling pathway. 2DG+MA was found to phosphorylate TrkB and its downstream pathway molecules GSK3β and p70S6K while MAPK was found downregulated. Also, a plasticity-related marker MAP2 was observed heavily activated, indicating 2DG+MA having a surprising role on neuroplasticity. More research is needed to link these results to the similar findings of the mechanism of ketamine.

Acknowledgements

I would like to thank M.Sc. Roosa Saarreharju for helping me in the laboratory and on all issues at hand and for always giving a thorough answer to my questions. I would like to also thank Associate Professor Tomi Rantamäki for guidance and handling this interesting research topic that was a thrill to work on.

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ANNEX 1:
SUPPLEMENTARY MATERIAL: THE IMPACT OF PHARMACOLOGICAL MANIPULATIONS OF BRAIN ENERGY METABOLISM AND GLYMPHATIC SYSTEM ON TRKB NEUROTROPHIN SIGNALLING

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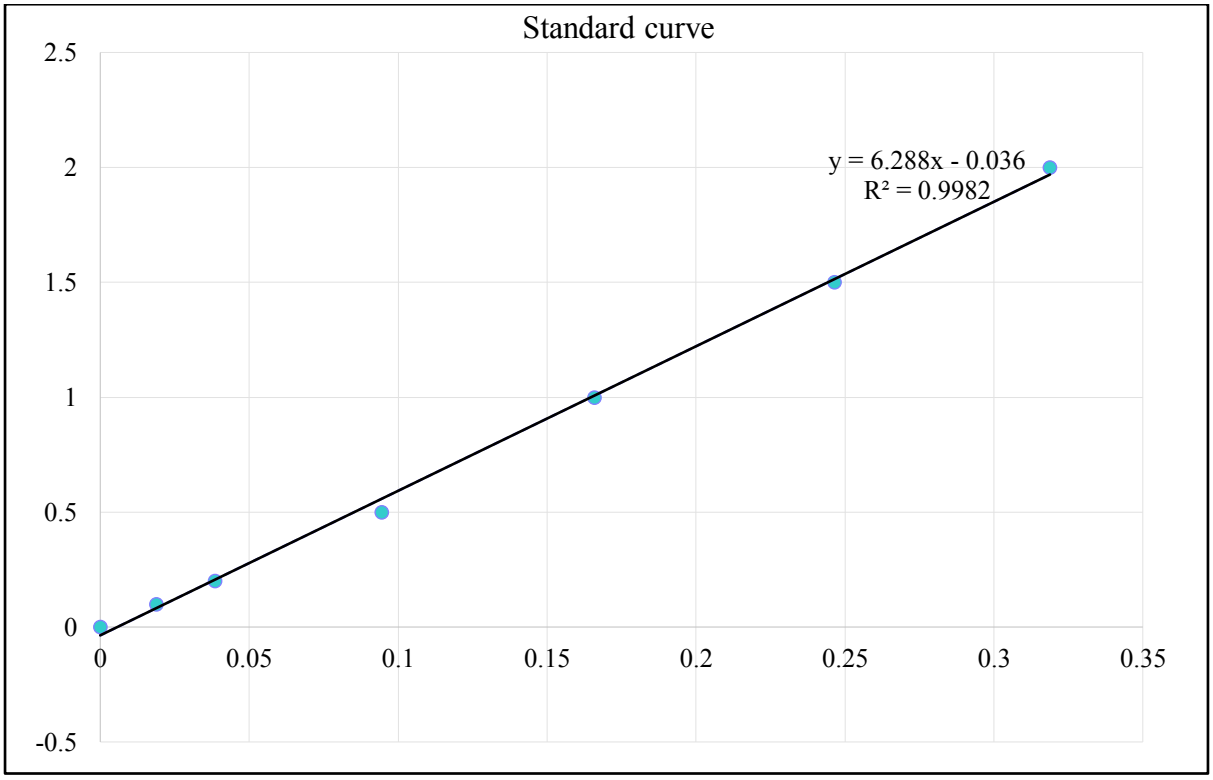
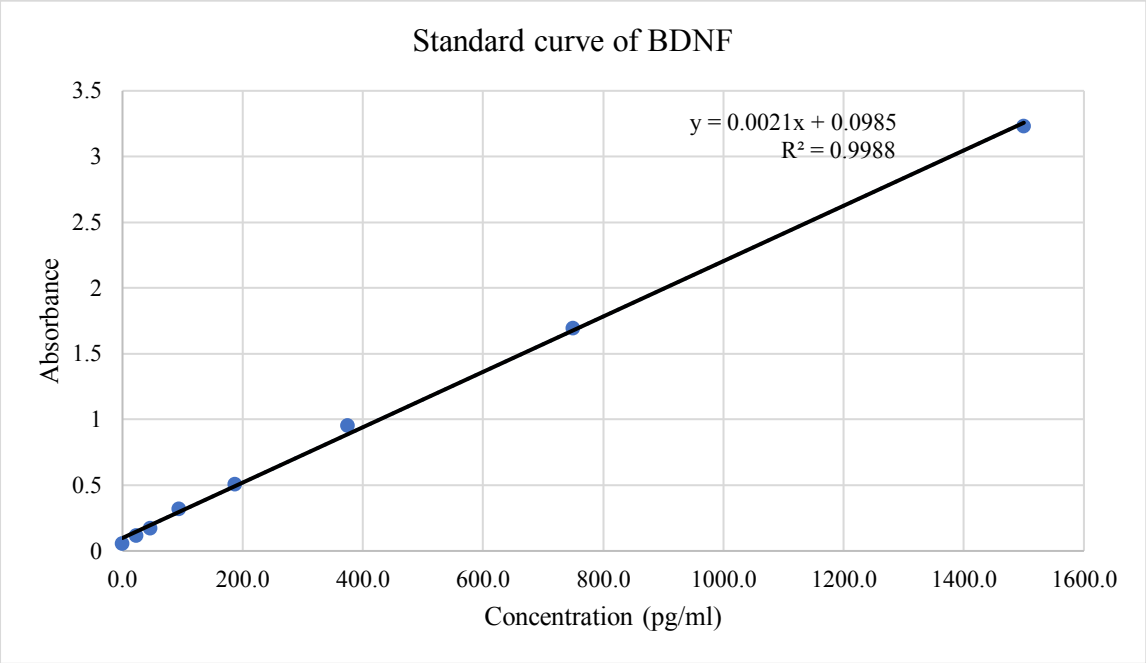
Supplementary A. Western blot and ELISA protein measurements



Supplementary B. ELISA BDNF standard curve



Supplementary C. Statistical analyses: Western Blot, ELISA, Locomotor activity

Western Blot

Protein detected	Treatment groups	n	Statistical test			Significance	
pTrkB		n = 8, 8, 8	One-way ANOVA			F (2, 21) = 9.262	p = 0.0013
	Control vs HTS	n = 8, 8		Dunnett's post hoc test	ns	p = 0.3340	
	Control vs 2DG+MA	n = 8, 8		Dunnett's post hoc test	***	p = 0.0008	
pGSK3B		n = 8, 8, 8	One-way ANOVA			F (2, 21) = 22.87	p < 0.0001
	Control vs HTS	n = 8, 8		Dunnett's post hoc test	ns	p = 0.3465	
	Control vs 2DG+MA	n = 8, 8		Dunnett's post hoc test	****	p < 0.0001	
pP70S6K		n = 8, 8, 8	One-way ANOVA			F (2, 21) = 17.85	p < 0.0001
	Control vs HTS	n = 8, 8		Dunnett's post hoc test	ns	p = 0.2525	
	Control vs 2DG+MA	n = 8, 8		Dunnett's post hoc test	****	p = <0.0001	
pMAPK		n = 8, 8, 8	One-way ANOVA			F (2, 20) = 17.86	p < 0.0001
	Control vs HTS	n = 8, 7		Dunnett's post hoc test	ns	p = 0.6685	
	Control vs 2DG+MA	n = 8, 8		Dunnett's post hoc test	***	p = 0.0002	
pMAP2		n = 8, 8, 8	Kruskal-Wallis test			H = 16.64	p = 0.0002
	Control vs HTS	n = 8, 8		Dunn's post hoc test	ns	p = 0.5158	
	Control vs 2DG+MA	n = 8, 8		Dunn's post hoc test	**	p = 0.0094	
pSrc		n = 8, 8, 8	Kruskal-Wallis test			H = 9.905	p = 0.0071
	Control vs HTS	n = 8, 8		Dunn's post hoc test	ns	p > 0.9999	
	Control vs 2DG+MA	n = 8, 8		Dunn's post hoc test	**	p = 0.0075	

ELISA

Protein detected	Treatment groups	n	Statistical test			Significance	
BDNF		n = 8, 8, 8	One-way ANOVA			F (2, 21) = 11.60	p = 0.0004
	Control vs HTS	n = 8, 8		Dunnett's post hoc test	*	p = 0.0109	
	Control vs 2DG+MA	n = 8, 8		Dunnett's post hoc test	ns	p = 0.1871	

Locomotor activity

Function	Treatment groups	Time	n	Statistical test		Significance	
Traveled distance x time			n = 8, 8, 8	Two-way ANOVA		F (22, 231) = 3.702	p < 0.0001
	Control vs HTS		n = 8, 8	Dunnett's post hoc test			
		0 - 5			***	p = 0.0001	
		5 - 10			****	p < 0.0001	
		10 - 15			****	p < 0.0001	
		15 - 20			***	p = 0.0006	
		20 - 25			****	p < 0.0001	
		25 - 30			****	p < 0.0001	
		30 - 35			***	p = 0.0004	
		35 - 40			***	p = 0.0003	
		40 - 45			***	p = 0.0004	
		45 - 50			**	p = 0.0031	
		50 - 55			**	p = 0.0035	
		55 - 60			*	p = 0.039	
	Control vs 2DG+MA		n = 8, 8	Dunnett's post hoc test			
		0 - 5			***	p = 0.0002	
		5 - 10			*	p = 0.0201	
		10 - 15			**	p = 0.0056	
		15 - 20			ns	p = 0.1629	
		20 - 25			*	p = 0.0113	
		25 - 30			ns	p = 0.1245	
		30 - 35			*	p = 0.0202	
		35 - 40			*	p = 0.02	
		40 - 45			ns	p = 0.0803	
		45 - 50			*	p = 0.0256	
		50 - 55			ns	p = 0.1439	
		55 - 60			ns	p = 0.2721	
Total distance traveled		0 - 60	n = 8, 8, 8	Kruskal-Wallis one-way ANOVA		H = 20.17	p < 0.0001
	Control vs HTS		n = 8, 8	Dunn's post hoc test	ns	p = 0.0568	
	Control vs 2DG+MA		n = 8, 8	Dunn's post hoc test	****	p < 0.0001	
Total vertical count		0 - 60	n = 8, 8, 8	Kruskal-Wallis one-way ANOVA		H = 19.30	p < 0.0001
	Control vs HTS		n = 8, 8	Dunn's post hoc test	ns	p = 0.0952	
	Control vs 2DG+MA		n = 8, 8	Dunn's post hoc test	****	p < 0.0001	

ANNEX 2:
PROV-002A LITERATURE REVIEW: THE RELATIONSHIP BETWEEN RAPID-ACTING ANTIDEPRESSANTS AND SLEEP

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1 INTRODUCTION

Major depressive disorder (MDD) is a devastating psychiatric disease, where patients suffer from persistent feelings of hopelessness, despair, and loss of interest. Patients often show signs of impaired cognitive function affecting memory and the ability to concentrate (Hasler et al. 2004; Pittenger and Duman 2008). Additionally, a core symptom of MDD is insomnia, which can often be disregarded in the aims of treatment (Nutt et al. 2008; Riemann et al. 2001). Almost 90% of depressed patients have some type of sleeping disorder, and sleep disturbances are more likely to occur in patients with the most severe symptoms (Breslau et al. 1996; Hinkelmann et al. 2012; Riemann et al. 2001). Sleep continuation is typically found to be fragmented in depressed patients, and the total time of sleep is reduced (Nutt et al. 2008; Riemann et al. 2001). Notably, sleep architecture is characteristically found altered with decreased latency of rapid eye movement (REM) sleep, and decreased time of non-REM (NREM) sleep (Benca et al. 1987; Nutt et al. 2008; Tsuno et al. 2005). In the electroencephalogram (EEG) studies, subjects with mood disorders show reduction in slow-wave activity (SWA), which is normally found to be particularly prominent in the first cycles of NREM sleep (Nutt et al. 2008; Tsuno et al. 2005). Several clinical studies have also reported that continued sleep problems after remission lead to a significant risk of relapse (Giles et al. 1987; Hinkelmann et al. 2012; Steiger and Holsboer 1997). Overall, sleep disturbances are known to be major risk of developing MDD (Kaneita et al. 2006; Ohayon et al. 2006; Taylor et al. 2005).

In the light of current research, neuronal atrophy and dysfunctions in neuronal networks seem to play a role in the development of major depression (Liu et al. 2017). Findings such as volumetric reduction at the prefrontal cortex and the hippocampus (Lorenzetti et al. 2009; Schmaal et al. 2017) and the loss of synapses and neurons discovered in postmortem brains of depressed patients have given support to these theories (Kang et al. 2012; Rajkowska et al. 1999). Moreover, antidepressants have shown to activate plasticity-related pathways such as increasing synaptic strength, dendritic spine density and the number of hippocampal cells

(Castrén and Hen 2013; Miller and Hen 2015). There are also indications that most antidepressants can normalize the altered structures of sleep cycles in depression (Wilson and Argyropoulos 2005). Clinical studies also show that this normalization correlates with improvements of depressive symptoms, which could indicate that the mechanism of action with antidepressants could be more related to mechanisms of sleep than has been initially thought (Bunney and Bunney 2013; Hasler et al. 2010).

There is a crucial need for more effective antidepressants as the prevalence rate of MDD continues to rise worldwide (Liu et al. 2020). Even though current conventional antidepressants (such as selective serotonin reuptake inhibitors) do have some efficacy in alleviating depressive symptoms, their major problems are in their prolonged therapeutic onset-time and low remission rate (Gaynes et al. 2009). Conventional antidepressants take weeks to achieve their antidepressant effects, which introduces a high risk especially to patients with suicidal ideations. In addition, over half of the treated patients relapse and up to 70% do not respond to the treatment sufficiently (Fawcett and Barkin 1997; Papakostas et al. 2008; Trivedi et al. 2006). Intriguingly, a new putative category of antidepressants, rapid-acting antidepressants (RAADs) such as ketamine, have demonstrated to be effective in suicidal behaviour and with treatment resistant depression (Gerhard and Duman 2018; Machado-Vieira et al. 2010). Moreover, RAADs, as implied, rapidly improve depressive symptoms. Thus, RAADs offer a promising treatment option in solving the unmet need in MDD treatment. One disadvantage in developing new RAADs is that the mechanisms of their antidepressant effects are still poorly understood. In this review, this is examined by focusing on the relationship between sleep and RAADs, as both sleep and depression seem to share a lot of features and a growing number of studies have linked sleep and RAADs to each other.

2 SLEEP

2.1 The regulation of sleep

When falling asleep, responsiveness to environmental stimuli gradually weakens and muscle tone decreases. Sleep pressure reaches its peak and the brain's master clock, suprachiasmatic nucleus (SCN) at the hypothalamus, sends signals to activate its nearby nucleus, the ventrolateral preoptic nucleus (VLPO) (Saper et al. 2005). Thus, the VLPO suppresses wake-promoting pathways such as monoaminergic pathways and orexin neurons, and sleep emerges. Sleep consists of multiple sleep cycles, each lasting around 1.5 hours, where REM and NREM sleep alternates (Brown et al. 2012). REM sleep is characterized by high-frequency and low-amplitude EEG waves that closely resemble EEG activity during wakefulness (7-9 hertz). NREM sleep, on the contrary, shows low-frequency and high-amplitude EEG activity, where wave frequency alters from 14 to 0.5 hertz. At 0.5-4 hertz, which is referred as SWA, slow-wave sleep (SWS) is the most prominent. Normally, the first cycles of NREM sleep consists of the most SWS and then, gradually the amount of SWS decreases throughout the night. Prolonged wakefulness is known to increase SWS on the following night and, thus, SWA is viewed as a marker for sleep pressure.

Overall, sleep is proposed to be regulated by two processes: the homeostatic (referred to as process S) and circadian (referred to as process C) process (Borbély 1982). The homeostatic process is thought to promote sleep, when wakefulness is prolonged, and the body signals its need for sleep. This pressure to fall asleep is thought to be caused by an unknown substance, of which levels accumulate during wakefulness and after reaching its threshold, drives the body to sleep. One possible substance is suggested to be adenosine, which is a known by-product of energy expenditure (Benington and Craig Heller 1995; Radulovacki et al. 1984; Strecker et al. 2000). Injections of adenosine or its receptor's agonist into the brain drive animals to sleep (Scammell et al. 2001; Strecker et al. 2000).

While the homeostatic process keeps time on when the body needs sleep, the circadian process controls the 24-hour cycle (Borbély 1982). At the core is the SCN, of which cells are known to synchronize a 24-hour rhythm (Jin et al. 1999; Reppert and Weaver 2002). Simplified, the cycle is generated by the production and degradation of two proteins in the SCN: period (Per) and cryptochrome (Cry) (Colwell 2011). The expression of both proteins is promoted by a heterodimer of a circadian locomotor output cycles kaput (CLOCK) and a brain and muscle ARNT-like 1 (Bmal1). When CLOCK and Bmal1 are active, the transcription of Per and Cry is triggered, and their levels gradually increase. At midday, they reach their peak levels, from where the two proteins begin to degrade. The interaction of Per and Cry suppresses the ability of CLOCK and Bmal1 to activate their transcription, which eventually leads to a reduction of Per and Cry levels. Thus, as Per and Cry concentrations are low, CLOCK and Bmal1 are available again to stimulate their production. This loop creates the 24-hour cycle, which is adjusted by the external and internal cues the SCN receives (Cassone et al. 1986; Colwell 2011; Johnson et al. 1988). For examples, light is an external cue to signal daytime and rising melatonin levels an internal cue to signal nighttime. When both processes, the homeostatic and circadian, drive for sleep at the same time, sleep is the most likely to occur (Borbély 1982).

2.2 The function of sleep

Humans spend one third of their life sleeping. This alone suggests that sleep is essential for survival even though its primary function still remains unknown (Cirelli and Tononi 2008). Sleep appears to participate in maintaining homeostasis in various functions. During sleep, energy is restored, synaptic plasticity pathways are active, inflammatory processes are reduced, and metabolic byproducts are removed (Benington and Craig Heller 1995; Bryant et al. 2004; Tononi and Cirelli 2014a; Xie et al. 2013). Prolonged wakefulness is known to be detrimental for cognitive function, have negative effects on the function of the metabolism and immune system, and can even be fatal in the long run (Benington and Craig Heller 1995; Bryant et al. 2004; Fortier-Brochu et al. 2012; Montagna 2005; Montagna and Lugaresi

2002). Thus, some vital processes occur during sleep, of which synaptic plasticity and energy metabolism are discussed next in more detail.

Synaptic plasticity changes are an important part of memory formation and sleep loss is known to impair memory and learning processes (Diekelmann and Born 2010). Thus, one of the main functions of sleep has been suggested to be the consolidation and reorganization of memory traces (Diekelmann and Born 2010). Consolidation is a process, where neuronal networks reprocess memories that have been encoded or recalled during wakefulness. This has been suggested to lead to forming new long-term memories and, thus, storing information. In fact, numerous studies have shown that sleep indeed is beneficial for memory and learning (Maquet 2001; Rasch and Born 2007; Stickgold 2005). After a night of sleep or even a daytime nap, subjects recall better learned information or show improvements in their motor skills compared to sleep deprived subjects (Robertson et al. 2004; Smith 2001). Sleep even improves task solving, which has been thought to be the result of the reorganization of memory traces (Miller 2000). As the brain is offline during sleep and does not receive new stimuli from the environment, sleep seems to provide optimal conditions for storing memories.

The mechanism of how sleep enhances memory is still, however, under investigation. Multiple studies have previously found that the same neuronal pathways, which have been active during wakefulness, are reactivated during sleep (Maquet et al. 2000; Peigneux et al. 2004). In fact, rats have been shown to activate the same neurons in the same order in the hippocampus and cortex during subsequent sleep, as when performing new spatial tasks previously during the day (Ji and Wilson 2007; Nádasdy et al. 1999; Pavlides and Winson 1989). A similar pattern has also been found in humans with neuroimaging studies (Maquet et al. 2000; Peigneux et al. 2004). It appears that during sleep, newly encoded memory traces are reactivated and thus, strengthened.

On a neural level, memory formation and consolidation are suggested to be based on a process referred to as long-term potentiation (LTP) (Dudai 2004; Frankland and Bontempi 2005). LTP appears to be a way by which the brain modifies its neuronal connections by strengthening its synapses and spines (synaptic plasticity) (Bliss and Collingridge 1993). LTP most likely occurs in active and frequently firing neurons, in which a burst of glutamate is released to the synapse cleft (Figure 1) (Bliss and Collingridge 1993). From there, glutamate mainly binds and activates the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at the postsynaptic membrane, which causes the neuron to depolarize. As the depolarization continues, the magnesium-ions blocking the N-methyl-D-aspartate (NMDA) receptors nearby are removed. This activates NMDA receptors and calcium ions are then free to flow through the receptor. This large calcium influx activates a cascade of events, which extend the activation of AMPA receptors by phosphorylating them and by inserting more receptors to the membrane by re-cycling. As the number of AMPA receptors on the membrane is increased and they are more likely to be activated, the postsynaptic neuron becomes more sensitive to glutamate and thus, is more likely to be depolarised in the future. Activation of NMDA and AMPA receptors also stimulate transcription factors and protein syntheses related to synaptic plasticity, which is referred as the late phase of LTP (L-LTP). This activation of the synaptic plasticity pathway seems to be important for the long-lasting effect of memory consolidation (Dudai 2004). Indeed, by blocking the LTP process, learning is found to be impaired (Morris et al. 1986). A similar effect is found in sleep deprived mice, where plasticity-mediated pathways were altered resulting in the loss of learning (Vecsey et al. 2009).

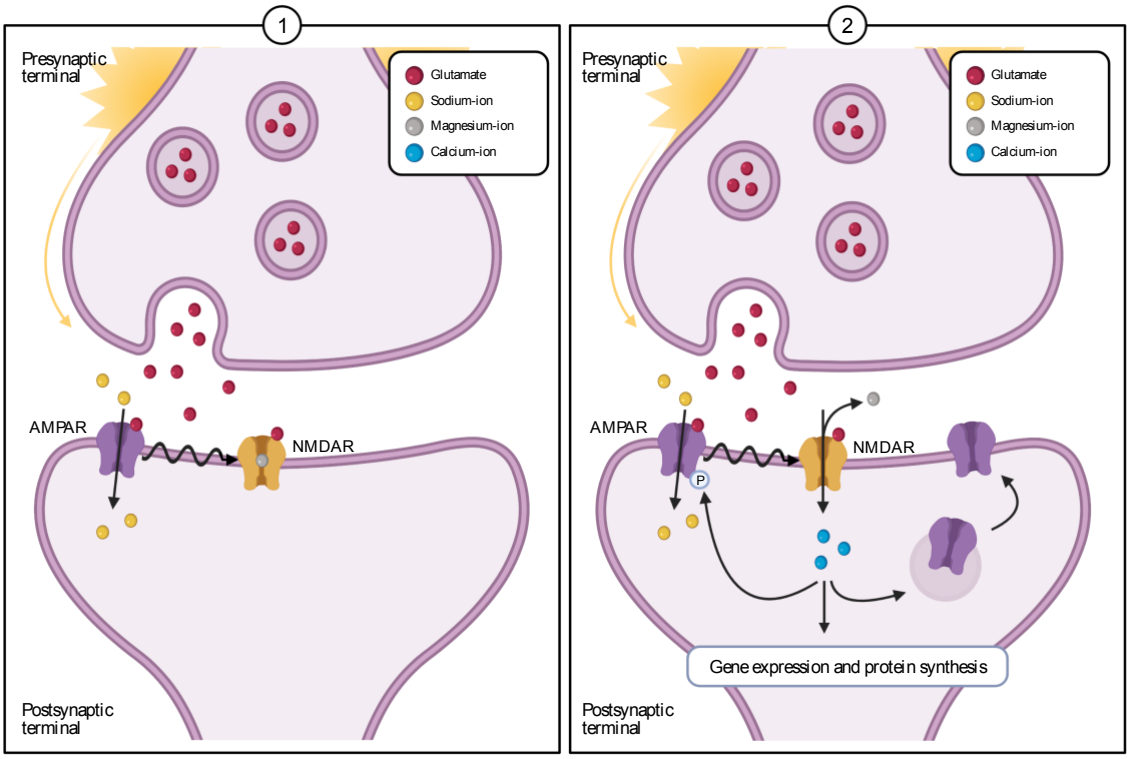

Figure 1. NMDAR-dependent long-term potentiation. 1. Firing at the presynaptic neurons causes a burst of glutamate, which activates AMPA receptors at the post-synapse. Continued activation of AMPA receptors ultimately activates NMDA receptors. 2. An influx of calcium through the NMDA receptor enhances AMPA receptors activation and translation to the membrane. Ultimately, this triggers protein transcriptions, which can lead to changes in gene expression and protein synthesis and ultimately, to the formation of new synapses. Modified from (Kauer and Malenka 2007).

There are several hypotheses that model the mechanism of sleep-dependent memory consolidation, of which two have remained the most relevant. The first one is the active system consolidation hypothesis, which argues that re-activations of memory traces, which closely resemble traces activated during learning that occur during sleep result in memories being transferred into long-term memory (Born and Wilhelm 2012; Diekelmann and Born 2010; Marshall and Born 2007). These neuronal replays are most consistently found to occur during SWS sleep (Euston et al. 2007; Ji and Wilson 2007; Peigneux et al. 2004; Wilson and McNaughton 1994). Thus, the consolidation process is thought to rely on SWS, where,

according to the model, re-activated memory traces are selected through synaptic potentiation (Born and Wilhelm 2012; Diekelmann and Born 2010). The importance of SWS for memory was demonstrated in a study, where subjects memorised card location on a computer game in the presence of an odour (Rasch et al. 2007). After a night of sleep, the subjects recalled better the card locations if they had been exposed to the same odour during subsequent SWS. Those, who were not exposed to the odour or were only exposed to it during REM sleep, did not show improvements in memorising the locations. Moreover, the increase of SWS is found to correlate with consequent learning in numerous of studies (Bramham and Srebro 1989; Diekelmann and Born 2010; Feldman 2009). In some versions of the model, SWS is specially suggested to select memories that are then, during REM sleep, strengthened, as REM sleep naturally follows NREM sleep (Born and Wilhelm 2012). However, there are some contradictory findings regarding this model. For example, replays during sleep differ remarkably from their original ones in frequency, speed, and duration and similar replays have also been found to occur during wakefulness (Davidson et al. 2009; Foster and Wilson 2006; Ji and Wilson 2007; Kudrimoti et al. 1999). This raises the question, whether sleep is then essential for memory consolidation, if it can also occur during wakefulness. The risk for an animal to sleep in a dangerous environment would, therefore, be relatively high and seem unnecessary.

The second model, synaptic homeostasis hypothesis, offers an altogether different solution for the function of sleep as it suggests memory consolidation to only be a secondary outcome of a larger process (Tononi and Cirelli 2014a). The hypothesis states that during sleep, the brain aims to optimize its energy function and space by pruning its neuronal connections. The brain alone requires almost a fifth of an adult's total energy consumption, as upholding a neuronal network is highly energy-consuming (Attwell and Gibb 2005; Sokoloff 1960). Thus, this process would prevent a situation, where no new memories could be encoded, and where the global network would consume energy over its limits (Tononi and Cirelli 2014a). The hypothesis assumes that during wakefulness (and learning), LTP activates synaptic plasticity processes, and the number and strength of synapses increases. This increase reaches

its peak at the end of wakefulness and from there, sleep acts to maintain the balance of synaptic homeostasis by strengthening stronger synapses and downscaling weaker ones. Similar to the active system consolidation model, the model indicates that during SWS, neurons that were activated during wake are selected to be strengthened. However, this selection is primarily done to protect reactivated neuronal connections from the downscaling process and, thus, from forgetting newly learned information. At the end of sleep, synaptic strength is renormalized to its baseline and space for encoding new information has been cleared. Perhaps the strongest evidence supporting this theory was provided by De Vivo et al. (2017). By using a three-dimensional electron microscopy in mice, they found that stronger synapses, referred as ones with bigger size, were protected from sleep-dependent renormalization compared to weaker ones, which were classified as small or medium size synapses.

The function of sleep and energy expenditure have been traditionally linked together as it has been previously presumed that the brain saves energy by decreasing its activity (Benington and Craig Heller 1995; Brown et al. 2012; Maquet 1995). This has however been long overruled as the brain is known to be, on the contrary, relatively active during sleep. Metabolism rates are shown to drop during the induction of sleep and NREM sleep, but during REM sleep, they increase back towards the levels at which they were during wakefulness (Maquet 1995). Therefore, the synaptic homeostasis hypothesis offers an alternative explanation for how energy is conserved during sleep and presents a putative function for sleep (Tononi and Cirelli 2014a). Moreover, the regulation of both processes have been shown to have similar neuronal pathways as both are influenced by the SCN, which links these two, perhaps, even closer together (Northeast et al. 2020).

3 PUTATIVE RAPID-ACTING ANTIDEPRESSANTS

Ketamine was the first drug discovered to alleviating depressive symptoms rapidly (Berman et al. 2000). Thereafter, the search of finding new rapid-acting antidepressants has been an on-going mission. In the next chapters, antidepressant treatment, which have shown have a faster clinical effect compared to traditional antidepressants, are discussed.

3.1 Nonpharmacological antidepressant treatments

3.1.1 Electroconvulsive therapy

ECT is one of the oldest antidepressant therapies used to date (Bini 1938). Its effect has been consistently found to be superior to conventional antidepressants in several meta-analyses (Kho et al. 2003; Pagnin et al. 2004; UK ECT Reviews Group 2003). ECT is especially effective with treatment resistant patients and severely depressed patients, who often have an increased risk of relapse (UK ECT Reviews Group 2003). ECT is usually conducted multiple times a week in a period of three weeks for patients to achieve full remission (Husain et al. 2004; Kho et al. 2003). For example, Husain et al. (2004) found in their study that after a week of treatment, only half of the patients had achieved remission, whereas after three weeks almost 90% of patients achieved remission. Without other concurrent treatment, the effect of ECT wears off in a couple of weeks (Kellner et al. 2006). Currently, the most effective and safest form of ECT is brief pulse waveform, where electrical pulses of 0.5-2 milliseconds are conducted under anesthesia (Tor et al. 2015). The most common adverse effects are nausea, confusion, and stiffness, which are largely the cause of anesthesia and muscle relaxants used alongside the treatment (Merkl et al. 2009). Follow-up studies have also reported that while the quality of life remains improved after treatment, occurred side effects do diminish (Giacobbe et al. 2018).

After decades of research, the underlying mechanism of ECT still remains unclear. ECT is known to alter processes regarding neurotransmitters, neuroplasticity, neuronal connections, and energy metabolism (Merkl et al. 2009; Singh and Kar 2017). During ECT, electrical pulses are believed to stimulate neurons, which begin to spontaneously fire simultaneously (Singh and Kar 2017). These groups of neurons induce a generalized seizure affecting brain regions all away from the limbic system to the cortex. After the induction of the seizure, a reduction in the brain activity appears, where prefrontal EEG activity slows to 1-4 hertz (Sackeim et al. 1996). This slowing of the EEG wave is found to correlate with symptom improvements in depressed patients and its intensity on the on-set time of the treatment (Folkerts 1996; Nobler et al. 1993; Sackeim et al. 1996; Suppes et al. 1996).

One of the most consistent finding with ECT is its ability to induce neuroplastic changes (Enomoto et al. 2017; Hellsten et al. 2002; Madsen et al. 2000; Malberg et al. 2000; Perera et al. 2007). A course of electroconvulsive shock (ECT for animals) treatment has been observed to trigger the formation of new neurons (neurogenesis) and to enhance neuronal survival (Hellsten et al. 2002; Madsen et al. 2000; Perera et al. 2007). Some studies have also found ECS to induce plasticity through brain-derived neurotrophic factor (BDNF) signaling (Basar et al. 2013; Enomoto et al. 2017; Kang et al. 1994). Changes in BDNF levels have largely been linked to the mechanism of antidepressants and studied profoundly in depression previously (Castrén and Rantamäki 2010). BDNF levels are reported to increase after treatment in both clinical and preclinical studies (Basar et al. 2013; Brunoni et al. 2014; Enomoto et al. 2017; Kang et al. 1994; Polyakova et al. 2015; Rocha et al. 2016). This said, increases of BDNF have not been found to correlated with the clinical outcome in several meta-analyses of MDD patients, who have received ECT multiple times (Brunoni et al. 2014; Polyakova et al. 2015; Rocha et al. 2016). One reason for the negative correlation might be that clinical studies measure BDNF levels from plasma instead of the brain. However, a similar contradiction is found in another presumed marker of plasticity: a volumetric increase in the hippocampus does not seem to be linked to treatment responses with MDD patients according to a recent study (Oltedal et al. 2018). Intriguingly, Abbott et al. (2014) found that

the therapeutic outcome was more connected to the normalization of hippocampal functional connectivity than in the expansion of its volume. Indeed, neuroimaging studies of MDD patients have previously demonstrated abnormalities in neuronal connections, where some brain regions are hypoactivate and other hyperactivate (Kaiser et al. 2015; Wang et al. 2012). Clinical studies have shown improvements in several neuronal networks related to depressive symptoms such as areas suggested to affect regulations in attention (cognitive control network), emotions and mood (default mode and affective attention network) (Nissen et al. 2010; Sheline et al. 1999). This normalization of mood regulation may explain how patients recover from depressive symptoms and enhance their ability to process new external stimuli after treatment. Therefore, the therapeutic mechanism of ECT may be in its ability to normalize altered networks in MDD patients rather than simply activate neuroplasticity related pathways (Farzan et al. 2014).

3.1.2 Sleep deprivation

Another old nonpharmaceutical antidepressant treatment is sleep deprivation (SD), in which patients are kept awake either the whole night (total SD) or partially (partial SD) (Wirz-Justice et al. 2004, 2005). Depending on the method, 40-60 % of depressed patients have been found to respond to treatment within hours (Giedke and Schwärzler 2002; Wu and Bunney 1990). The treatment is surprisingly effective and has only mild adverse effects such as headache and nausea (Bhanji and Roy 1975; Pflug 1976). On the downside, as immediate as the antidepressant effect is, on average 80% of responders relapse after recovery night (Riemann et al. 1993; Wu and Bunney 1990). Even short naps or ultrashort sleep episodes during the day, of which subjects are often not aware of themselves, effect the treatment respond negatively (Kerkhofs et al. 1991; Wiegand et al. 1987, 1993). Therefore, SD treatment is often combined with conventional antidepressants to maintain its effect (Bunney and Bunney 2012; Wu and Bunney 1990).

The neuronal mechanism of SD is largely unknown, but like ECT, SD is found to modify neuronal connectivity and function (Bosch et al. 2013). Bosch et al. (2013) discovered an increase in the functional activity within the default mode network, in the area of medial prefrontal cortex, after patients were partially sleep deprived. There are also some indications that SD may alter glutaminergic circuits and increase levels of AMPA receptors in the cortex (Benedetti and Smeraldi 2009; Havekes et al. 2012). Moreover, SD is suggested to play a role in stabilizing the circadian rhythm (Bunney and Bunney 2013). SD synchronizes the SCN and normalizes abnormal sleep phases associated with depression (Bunney and Bunney 2013; Nutt et al. 2008). In fact, diurnal variation in mood, fatigue and nighttime awakenings seem to predict patient's response to SD (Reinink et al. 1990; Van den Hoofdakker and Beersma 1988). Another chronobiological treatment, bright light therapy, has also been demonstrated to be effective for depression, particularly in seasonal depression (Westrin and Lam 2007).

3.2 Pharmacological antidepressant treatments

3.2.1 Ketamine

At the beginning of 2000, Berman et al. 2000 were the firsts to discover the antidepressant effects of ketamine, where a low dose of ketamine was intravenously administered to seven MDD patients. Today, a wealth of studies have demonstrated that a low dose ketamine provides an acute antidepressant effect that appears within hours of treatment and can lasts up to weeks (Berman et al. 2000; Duncan et al. 2019; Machado-Vieira et al. 2010). Treatment is usually conducted at the beginning twice a week either by a slow 40-minute intravenous infusion or by a single intranasal dose (EMA 2019; Hashimoto 2019). After treatment initiation, ketamine administration is continued once a week up to three months with infusion or up to six months with nasal spray. Similar to ECT, ketamine is found to be the most effective for sever and treatment resistant depression (Price et al. 2009; Wilkinson et al. 2018). Ketamine's efficacy is even indicated to be dependent on the severity of the disease

(Nugent et al. 2019a). Nugent's research group (2019a) discovered that patients with milder version of depression did not respond to treatment as strongly as MDD patients. Noticeably, healthy volunteers showed even a reverse therapeutic effect. However, despite ketamine's high response rate with MDD patients, its dissociative effects and abuse risk has limited its larger clinical use (Liu et al. 2016; Yang and Hashimoto 2014).

The mechanism on how ketamine alleviates depressive symptoms is not completely understood, though it is thought to lie in findings related to changes in neuronal structure and function (Duman and Aghajanian 2012; Yang et al. 2019). Functionally, ketamine has been reported to normalize neuronal networks related to depression in human neuroimage studies (Abdallah et al. 2017b, 2017a). Evans et al. (2018) found the hyperactivity of DMN to be normalized in MDD patients treated with ketamine. This effect was found to withhold even after a week (Evans et al. 2018). Therefore, ketamine, like ECT, may play an important role in the normalization of neuronal networks in depression. In rodent studies, ketamine has shown repeatedly to activate synaptogenesis and other pathways regarding synaptic plasticity (Duman and Aghajanian 2012; Kohtala et al. 2019a; Li et al. 2010). For example, ketamine has been demonstrated to upregulate *bdnf* transcription and activate its protein's translocation and also its receptor, tropomyosin receptor kinase B (TrkB), in rodent's prefrontal cortex and hippocampus (Yang et al. 2013; Zhang et al. 2016). Moreover, *bdnf* gene knockout mice have shown to prevent ketamine's antidepressant effects, which indicates that the protein has a critical role for the drug's action (Autry et al. 2011). Conventional antidepressants are also suggested to act through BDNF-TrkB receptor signaling (Zanos and Gould 2018).

Ketamine has a broad affinity to several receptors such as opioid, serotonin, dopamine, and sigma (Zanos and Gould 2018). The main antidepressant action, though, is suggested to occur through glutamatergic transmission via the inhibition of NMDA receptors in the prefrontal cortex (Duman and Aghajanian 2012; Zanos and Gould 2018). This is assumed to trigger the neuroplasticity-related changes seen during and after ketamine exposure. There are several

hypotheses, however, on how these mechanisms are activated through the NMDA receptors (Figure 2) (Zanos and Gould 2018). One hypothesis suggests ketamine to directly block selectively extrasynaptic NMDA receptors. Another hypothesis claims it inhibits the spontaneous activation of the receptor and its transmission. The perhaps most prevalent theory is the disinhibition hypothesis, where ketamine is proposed to bind to NMDA receptors at the gamma-aminobutyric acid (GABA) interneurons. This inhibition is suggested to lead to an enhanced activation of pyramidal neurons, which conversely evokes glutamate release into the synapse. Glutamate, thereafter, activates postsynaptic AMPA receptors, which ultimately triggers the BDNF-TrkB signaling pathway. As a proof of concept, AMPA receptor antagonists have been reported to blocked ketamine's antidepressant effects in rodent, alluding to its importance in ketamine's mechanism (Yang et al. 2019: Maeng et al. 2008).

However, translating new NMDA-antagonist to clinical use has not been successful and several clinical trials have failed to demonstrate a sufficient antidepressant effects in humans (Garay et al. 2018). In addition to the NMDA-mediated pathways, some studies have found that ketamine's metabolite, hydroxynorketamine (HNK), may mediated the antidepressant effects (Zanos et al. 2016). HNK itself binds poorly to NMDA receptors but seems to have a stronger affinity towards AMPA receptors. HNK is suggested to directly activate AMPA receptors and results this way on the therapeutic outcome (Figure 2) (Zanos and Gould 2018). However, contradictory results were found by Kohtala et al. (2019a), who observed no changes in the downstream pathway of TrkB with HNK. This study additionally raised an issue regarding the high doses of HNK used in most studies. Antidepressant-like outcome is most consistently found with a high dose of HNK, contrary to that it is a low dose of ketamine that accomplishes antidepressant-like effects. Therefore, it seems unlikely that HNK alone could cause an antidepressant effect.

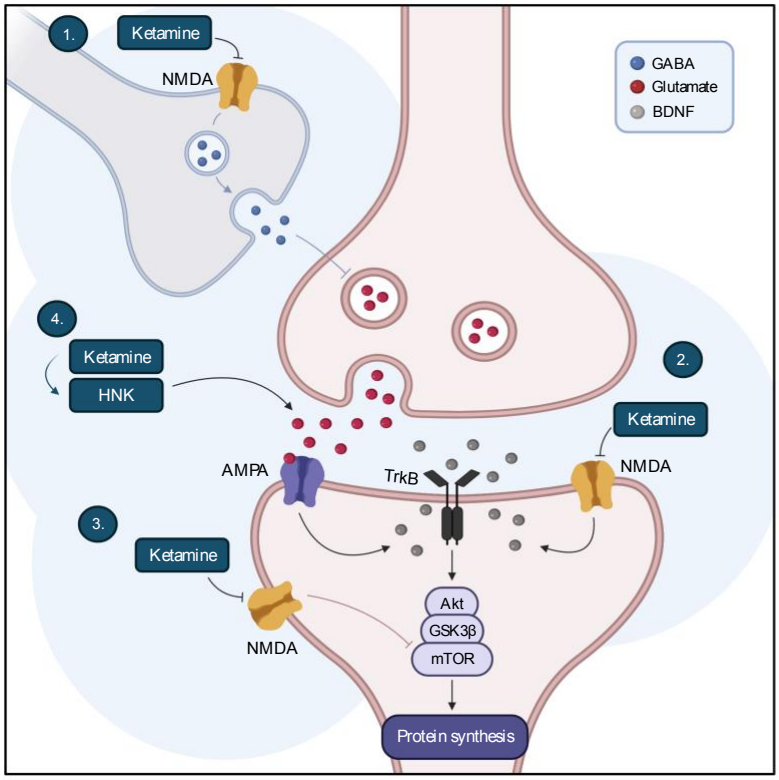

Figure 2. Putative mechanisms of ketamine, which eventually lead to the activation of BDNF-TrkB signaling pathway and enhanced synaptic plasticity. 1. Disinhibition of glutamate release. Ketamine blocks the NMDA receptor at the gamma-aminobutyric acid (GABA) interneuron, which suppresses its inhibitory effect on glutamate release on pyramidal neurons. 2. Blockade of spontaneous NMDA receptor activation directly activates BDNF release and thus, TrkB signaling. 3. Blockage of extra-synaptic NMDA receptor directly suppresses the receptor's inhibition on the mechanistic target of rapamycin (mTOR) activation, which is known to directly enhance neuronal survival and protein synthesis. 4. Ketamine metabolites into hydroxynorketamine, which is thought to activate AMPA receptors at the post-synaptic neuron. Modified from (Zanos and Gould 2018).

3.2.2 Nitrous oxide and other anesthetics

Besides ketamine, other anesthetics have also shown promise of having antidepressant effects. Isoflurane, propofol and, nitrous oxide have been observed to cause an antidepressant effect in clinical studies (Mickey et al. 2018; Nagele et al. 2015; Weeks et al. 2013). For example, nitrous oxide has been found to alleviate depressive symptoms in treatment-

resistant depression in a preliminary clinical study (Nagele et al. 2015). There, patients inhaled nitrous oxide for an hour and measurements of the severity of depressive symptoms were evaluated before treatment, two hours, and 24 hours after treatment (Nagele et al. 2015). At both time points (2h and 24h), eight out of ten patients responded to treatment whereas placebo group showed no signs of clinical improvements. Three patients, who received nitrous oxide, even achieved full remission, even though the treatment was conducted only once. Nitrous oxide is a known NMDA receptor antagonism, which molecular mechanism has not been fully studied under the relationship of an antidepressant (Yamakura and Harris 2000). However, a recent study conducted to mice found nitrous oxide to cause a similar cortical excitatory tone during treatment and EEG slowing after drug withdrawal as ECS previously mentioned (Kohtala et al. 2019b). Ketamine has also been demonstrated to cause parallel patterns in the EEG (Kohtala et al. 2019b). The implications of these similarities are further discussed in the next chapter (Chapter 4.).

4 SLEEP AND RAPID-ACTING ANTIDEPRESSANTS

4.1 Normalization of sleep regulation and its architecture

Accumulating evidence shows that most antidepressants modify the architecture of sleep in MDD patients (Doghramji and Jangro 2016; Wilson and Argyropoulos 2005). As previously stated, sleep duration is often reduced and, especially, the length of SWS diminished in MDD (Nutt et al. 2008; Tsuno et al. 2005). Antidepressants seem to normalize these changes, especially RAADs (Bunney and Bunney 2012; Wilson and Argyropoulos 2005). During recovery night, ketamine, ECT and, SD have demonstrated to increase the time of SWS and total time of sleep in preclinical and clinical studies (Duncan et al. 2013; Feinberg and Campbell 1993; Nissen et al. 2001; Sackeim et al. 1996). Intriguingly, Duncan et al. (2013) discovered a correlation between prolonged SWA and treatment response with ketamine

treated MDD patients. Patients, who showed increased SWA in the first and second NREM periods of sleep, were found to more likely respond to treatment and recover from depression (Duncan et al. 2013). SD and ECT have also showed similar correlations in SWA and clinical improvement (Nissen et al. 2001; Sackeim et al. 1996). These findings seem to highlight the importance of sleep regulation mechanisms in the rapid on-set time of RAADs.

As earlier described, sleep regulation consists of the homeostatic and circadian process (Borbély 1982). The homeostatic regulation is thought directly to be influenced by SD, since prolonged wakefulness increases sleep pressure and thus, effects subsequent sleep (Hemmeter et al. 2010). Therefore, SD modifies sleep structure and timing through the homeostatic process. Several studies support this theory as microsleep and short naps during treatment is reported to decrease response rates (Knowles et al. 1979; Roy-Byrne et al. 1984). Indeed, even brief sleep may alleviate sleep pressure enough to affect the clinical outcome of SD treatment (Knowles et al. 1979; Roy-Byrne et al. 1984). Moreover, SD is reported to not improve mood with healthy subjects, indicating that normalization of sleep architecture is a key function in the treatment of MDD patients with altered sleep structures (Wiegand et al. 1987, 1993). Ketamine's effects on sleep have also been claimed to be in its ability to alter sleep pressure as SWS is shown to increase in subsequent sleep (Duncan et al. 2019).

There are several indications that RAADs could additionally affect the circadian process. In depression, several cues regulating SCN such as hormone levels and body temperature are unsynchronized (Emens et al. 2009; Hasler et al. 2010; Robillard et al. 2014). Mutations in proteins associated to the circadian rhythm such as CLOCK and Per have been linked to the disease (Hampp et al. 2008; Hampp and Albrecht 2008; McClung 2011; Roybal et al. 2007). Interestingly, RAADs might particularly alter Per regulation (Bunney et al. 2015). For instance, a study found SD to increase the levels of Per1 and Per2 in the cerebral cortex of mice (Wisor et al. 2002). In sleep deprived patients, however, the expression of *per1* and *per2* was not found to rise even though a distinguished reduction in the regulation of Bmal1

amplitude was reported (Ackermann et al. 2013). Nevertheless, this study was only conducted to 12 subjects, and biomolecular measurements were done from leukocytes, so these findings indicate more about physiological timekeeping. Ketamine has also shown promising signs of altering clock molecules. A study conducted *in vitro*, found ketamine to suppress the transcriptions activated by CLOCK and Bmal1, which resulted in the downregulation of *Per2* (Bellet et al. 2011). Another recent study reported similar results using mice (Orozco-Solis et al. 2017). Ketamine treatment reduces the expression of several clock genes including *Per1* and *Per2* at the beginning of the mice's activity phase (nighttime). These results also correlated with the improvements seen in depression-like behavioral tests. The same study reported comparable results with SD treatment. Overall, these results suggest that ketamine and SD could shift the timing of the circadian phase via the circadian molecules. The first study examining ketamine's effect on circadian activity in depressed patients also supports these findings (Duncan et al. 2017). Duncan's group (2017) observed ketamine to affect, indeed, the timing of circadian activity rhythm in patients, who responded to the treatment. Another interesting notion is that the receptors and molecules, which ketamine has been reported to influence, follow circadian rhythm (Bunney et al. 2015). For example, NMDA receptor, AMPA receptor, glycogen synthase kinase 3 beta (GSK3β) and, mechanistic target of rapamycin (mTOR) all seem to modulate circadian clock genes (Benedetti et al. 2012; Cao et al. 2013; Mizoro et al. 2010). When AMPA is injected to the SCN during the active period of mice, *Per1* is upregulated (Mizoro et al. 2010). In conclusion, both SD and ketamine seem to interfere with both sleep regulating processes: the homeostatic and the circadian process. In 2019, Duncan research group (2019) in fact hypothesized that ketamine's core antidepressant effect is in its ability to alter both processes (Duncan et al. 2019).

4.2 Enhancing network functionality and synaptic homeostasis

One of the main functions of sleep has been suggested to be its impact on neuronal networks and memory consolidation (Diekelmann and Born 2010). The activation of neurons and

strengthening synapses has also been highlighted as a main mechanism for RAADs (Gerhard and Duman 2018; Pittenger and Duman 2008; Zanos and Gould 2018). Thus, sleep and RAADs share parallel pathways, especially, regarding neuroplasticity. Activation of neuroplasticity is typically observed either by as an increase in high-frequency waves called gamma power (30-80 hertz) in the EEG or as the activation of LTP-related pathways (Gilbert and Zarate 2020). High gamma power typically indicates of an increased neuronal activity, specially at the cortical area (Neske 2015).

Intriguingly, after ketamine administration, gamma power has been observed to rise acutely in both human and animal studies (Anderson et al. 2014; Pinault 2008; Rivolta et al. 2015; Shaw et al. 2015). These acute increases have been found to occur at the frontal, temporal, and parietal lobe and last up to six to nine hours (Muthukumaraswamy et al. 2015; Nugent et al. 2019a; Rivolta et al. 2015). Moreover, these changes have been observed to be dose-dependent (Caixeta et al. 2013). Caixeta et al. (2013) reported in mice that a distinguish increase in gamma power was detected only with a subanesthetic dose of ketamine and not with higher doses. These findings could explain, why only with a lower dose of ketamine, depressive symptoms are found to diminish. Thus, perhaps with only a subanesthetic dose, ketamine can trigger neurons so that the neuroplasticity-related pathways activate, functional connectivity enhances, and eventually mood is improved. Indeed, studies have observed neuronal excitation to be critical for the clinical outcome and gamma power to correlate with the result (Cornwell et al. 2012; Nugent et al. 2019b). Notably, after gamma power activity, EEG waves are found to decelerate and SWA to emerge in rodent studies (Campbell and Feinberg 1996; Kohtala et al. 2019b). This activity resembles closely to the same pattern detected during SWS. This rebound effect has also been observed with nitrous oxide in humans (Foster and Liley 2011; Henrie et al. 1961).

A strong stimulation, of which increased gamma power indicates of, can trigger neurons to initiate memory formation via the LTP process (Bliss and Collingridge 1993; Frey and Morris

1997). During LTP, similar changes in post-synaptic pathways seem to occur as what RAADs have shown to activate. For example, calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase B (Akt), and mitogen-activated protein kinase (MAPK) are observed to be active during the late phase of LTP (L-LTP), which can also be influenced by the TrkB signaling (Ma et al. 2015; Pen et al. 2016; Thomas and Huganir 2004; Zanos and Gould 2018). There is some direct evidence that LTP-mediated plasticity is impaired in MDD patients and that ketamine may enhance this deficiency (Kuhn et al. 2016; Machado-Vieira et al. 2010; Nugent et al. 2019b; Player et al. 2013; Sumner et al. 2020; Widman et al. 2018). Summer et al. 2020 observed an LTP-mediated activation after three hours from ketamine infusion in MDD patients. Moreover, Gilbert et al. 2018 found the antidepressant effect of ketamine to be mediated by the AMPA and NMDA glutamatergic signaling, which is a core mechanism of LTP (Gilbert et al. 2018).

To connect these findings, Rantamäki and Kohtala (2020) have proposed a hypothesis of RAADs putative mechanism referred to as Encoding, Consolidation, and Renormalization in Depression (ENCORE-D). The hypothesis is based on the theory of synaptic homeostasis, where according to ENCORE-D, memory function is normalized in depression in three steps in RAAD treatment (Rantamäki and Kohtala 2020). The first step is suggested to occur during administration, where the drug excites the brain, and this firing stimulates the encoding process, where glutamate is released rapidly to activate post-synaptic neurons (early phase of LTP). ECT is probably the best example of this step, as stimulating the neurons is its core function at the beginning of treatment. After a rapid withdrawal of the drug or treatment, SWA emerges as a response to the excitatory state the brain has undergone. This triggers the initiation of L-LTP, where more AMPA receptors are activated and transferred to the post-synaptic membrane, remarking the consolidation process of memory formation. Thus, excitatory effect leads to synaptic strength and activation of neuroplasticity pathways. The third, and final stage, of the hypothesis, is, as sleep homeostasis hypothesis proposes, the renormalization of synaptic strength and pruning its connections during subsequent sleep. As SWS (during subsequent sleep) is increased after RAAD

administration, synaptic downscaling occurs, and weaker connections are pruned and stronger one's strengthened. Thus, RAADs improves functionality of neuronal connections after subsequent sleep. Therefore, the hypothesis might explain why antidepressant response of ketamine is found to peak after a night of sleep and highlights the relevance of sleep in RAADs clinical effects.

5 DISCUSSION

Sleep disturbances are one of the most common symptoms found in depression (Nutt et al. 2008). Patients comorbid with sleep disorders are shown to suffer from severer depressive symptoms and be more likely resistant to treatment compared to patients without sleep disorders (Breslau et al. 1996; Hinkelmann et al. 2012; Riemann et al. 2001). The connection between these two disorders has been well known, but however only recently has it been paid more attention. Especially studies, where RAADs, such as ketamine, have been found to alter the circadian rhythm and sleep architecture, have shifted the focus to the prominent relationship of sleep and antidepressant treatment (Duncan et al. 2013, 2017; Nissen et al. 2001; Sackeim et al. 1996). Notably, ketamine, ECT and SD have been shown to be especially effective with patients with the severe forms of depression, who are also the most likely to suffer from disrupted sleep (Nugent et al. 2019a; UK ECT Reviews Group 2003; Wu and Bunney 1990). Indeed, improving sleep disturbances in depressed patients has been linked to predict the outcome of the treatment (Giles et al. 1987; Hinkelmann et al. 2012). Moreover, RAADs have been observed to increase slow-wave activity and total sleep during the recovery night, which has been shown to correlated with treatment response and success (Duncan et al. 2013; Foster and Liley 2011; Nissen et al. 2001; Sackeim et al. 1996).

The main mechanism of RAADs has been suggested to be in their capacity to trigger synaptic plasticity and thus, improve neuronal function (Gerhard and Duman 2018; Zanos and Gould

2018). This being said, accumulating evidence suggest that their influence in sleep and the circadian system seem to be critical (Bellet et al. 2011; Bunney and Bunney 2013; Duncan et al. 2019). This, however, does not contradict their importance in neuroplasticity as sleep also is a critical factor for the normal function of neuronal network (Tononi and Cirelli 2014b). RAADs and sleep regulation share several molecular pathways, which all result in structural changes in neurons like formation of new synapses and strengthening them (Gerhard and Duman 2018; Tononi and Cirelli 2014b). According to several sleep hypotheses, sleep is thought to be key on information processing and thus, enhancing neuronal function and connectivity (Born and Wilhelm 2012; Tononi and Cirelli 2014b). As the sleep homeostasis hypothesis states that sleep is for balancing the neuronal network and memory function, RAADs may indeed be an important factor of enhancing these systems during sleep resulting in its therapeutic outcome.

Although RAADs effects on sleep regulation seems probable, more research is needed on a molecular level to understand, how RAADs influence these processes. Ketamine and SD appear to both play a role in several sleep-related activities. They influence circadian molecules and restore sleep homeostasis (Bellet et al. 2011; Bunney and Bunney 2013; Duncan et al. 2017). Findings of SD's and ketamine's effects on SCN specific molecules may be key as SCN is the brain's main regulator of homeostasis (Saper et al. 2005). Little research has been done to fully understanding these effects. In the future, it would be interesting to investigate especially, what happens during SWA that effects the treatment outcome of RAADs. It is also important to mention, that not all MDD patients suffer from sleep disturbance as well as not all respond to treatment. However, perhaps it is because RAADs actually work better for patients with sleep problems, if their antidepressant effect relays in restoring and normalizing sleep. This could further help predict treatment response and to choose the right type of treatment of MDD patients. Furthermore, this may explain why RAADs do not improve mood in healthy subjects. With healthy subjects, whose circadian rhythm and sleep architecture is normal, RAADs may swift these structures and rhythm into abnormality, which may paradoxically manifest in depressive symptoms.

Finally, if sleep-related pathways are the most important mechanism of RAADs, one important question is in need to discuss: how studies consider circadian timing in their studies (Alitalo et al. 2020). Most animal studies are conducted with rodents, which are nocturnal animals. Thus, results might differ largely, if studies are done at the same time of the day with nocturnal animals as with humans. For example, ketamine has been demonstrated to affect timekeeping and swift circadian timing in rodents (Orozco-Solis et al. 2017). In this study, ketamine was administered in the evening, which is the early phases of the mice's activity period. If this would have been done at the beginning of the mice's inactivity period, which would be the activity period for humans, results could have been drastically different in behavioral studies. As researcher maybe prefer to conduct their studies during the day instead of night, might this be why new RAADs have not been successfully developed into clinical use.

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