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VLOGA ENDOGENEGA NEVROTROFIČNEGA DEJAVNIKA GLIALNEGA IZVORA PRI OD AMFETAMINA ODVISNEM POGOJEVANJU IZBIRE OKOLJA PRI MIŠIH

THE ROLE OF ENDOGENOUS GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR IN AMPHETAMINE-INDUCED PLACE CONDITIONING IN MICE

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Statement

Hereby, I declare, that I conducted the experiments and writing of the thesis independently and under the supervision and guidance of Assoc. Prof. Dr. T. Petteri Piepponen, Dr. Jaakko Kopra and Prof. Dr. Mojca Kržan.

Ljubljana, January 2017 Samo Weingerl

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Abstract

Drug addiction is a chronic brain disease, characterized by loss of control over drug use despite adverse consequences. It is a lifetime lasting condition, defined by drug cravings and repeated relapse. This implies the presence of long-lasting neuroadaptive changes in the brain. Since, neurotrophic factors are heavily involved in the neuroplasticity of central nervous system, this makes them an interesting subject for investigating how they affect or regulate long-term drug-induced neuroadaptive changes. Glial cell line-derived neurotrophic factor (GDNF) has been shown to be important for development, growth, survival and maintenance of midbrain dopaminergic neurons. It can also influence neurotransmission and regulate synaptic plasticity.

To better understand the role of GDNF in behavioral responses to repeated exposure to psychostimulants, we performed amphetamine-induced conditioned place preference (CPP) on two different genetically modified mouse strains. First, we tested heterozygous GDNF knockout mice, which have lower GDNF levels. We find that they experience similar amphetamine reward and relapse to drug-seeking as controls, while having lower amphetamine-induced locomotor activity. Second, we examined the newly developed heterozygous GDNF hypermorphic mice, which have increased GDNF levels. These mice experience higher, but not significantly different amphetamine reward as controls. Further, relapse to drug-seeking and amphetamine-induced locomotor activity is the same between heterozygous GDNF hypermorphic mice and controls.

In order to increase the credibility of observed results, our CPP system needs to be modified to offer unbiased preference for compartments of CPP boxes. Furthermore, in the near future the mice we used should be bred on a single genetic background to achieve better reliability and repeatability of CPP trials and better comparison with other studies.

Nevertheless, future research into addiction should make a great use of newly developed conditional GDNF knockout and heterozygous GDNF hypermorphic mice. These mice have not yet been thoroughly examined in a wide range of biochemical and behavioral responses to repeated drug exposure. Undoubtedly, they offer a great potential for better understating the role of endogenous GDNF and its therapeutic value.

Key words: addiction, amphetamine, conditioned place preference, genetically modified GDNF mice, glial cell line-derived neurotrophic factor

Razširjen povzetek

Zasvojenost z mamili in psihotropnimi snovmi (v nadaljevanju z drogami) je kronična možganska bolezen, ki jo zaznamuje izguba nadzora nad njihovim vnosom, kljub zavedanju o škodljivih posledicah. Vsebuje vidike impulzivnega in kompulzivnega vedenja, in pogosto za svoj nastanek potrebuje ponavljajočo izpostavljenost drogam. Nagnjenost k zasvojenosti je pogojena z razvojem živčevja ter z genskimi in socialnimi dejavniki. Posameznika tako spremlja skozi celo življenje z močnimi epizodami hlepenja po drogah in recidivih, ki nastopijo tudi po več desetletjih abstinence.

Praviloma vse droge z zasvojitvenimi zmožnostmi v možganih spodbujajo nagrajevalni sistem, kjer poglavitno vlogo igra mezolimbična pot. Ta se začne v ventralnem tegmentumu (srednji možgani), iz katerega dopaminergični nevroni projicirajo v nukleus akumbens (ventralni striatum). V slednjem poživila (psihostimulansi) kot sta npr. kokain in amfetamin, neposredno povzročijo sprostitev velikih količin dopamina, medtem ko ostale droge enak učinek dosežejo bolj posredno. Sprostitev dopamina v akumbensu sproži občutke ugodja in nagrade kar vodi v vrsto asociativnega učenja, imenovanega pogojevanje. Pri klasičnem pogojevanju dražljaj, ki je sočasen z uporabo drog, pridobi vlogo pogojenega dražljaja, in tako sam postane zmožen sprostiti velike količine dopamina. Na ta način zgolj pričakovanje nagrade po srečanju s pogojenim dražljajem vodi v močno motivacijo po zagotovitvi droge.

Droge za razliko od naravnih nagrad (hrana, spolna aktivnost), sproščajo dopamin tudi po njihovi zagotovitvi, kar še dodatno jača željo po njihovem uživanju. Vendar po dolgotrajnem uživanju tudi droge začnejo izgubljati svoj učinek in tako sčasoma pogojeni dražljaji postanejo zmožni sprostiti večje količine dopamina kot sama droga. Glavna spodbuda za uživanje drog tako ni več njen učinek, temveč z njo asociirani dražljaji (pogojeni dražljaji).

Zgodnjo odtegnitev od drog zaznamuje aktivacija averzivnih in stresnih možganskih sistemov (negativno pogojevanje). Pri dlje trajajoči abstinenci, pa vidnejšo vlogo odigra za zasvojenost značilna manjša funkcionalnost dopaminskega sistema, kjer posamezniki kljub izpostavljenosti prijetnim dražljajem ne zmorejo več občutiti zadovoljstva (anhedonija). Druga pomembna prilagoditev možganskih sistemov pri zasvojenosti pa je oslabljeno delovanje prefrontalne možganske skorje, kar še dodatno oteži preprečevanje ponovne in impulzivne zlorabe drog med abstinenco. Taka neusklajenost delovanja različnih možganskih sistemov je odraz nevroadaptacij tako pri živčnih prenašalcih in njihovih

receptorjih, kot pri možganski plastičnosti in sinaptični preureditvi. Posledično lahko ob prisotnosti stresa in pogojenih dražljajev živčne povezave iz prefrontalne možganske skorje (izvršilna funkcija), amigdale (stres) in hipokampusa (spomin) v akumbens in ventralni tegmentum lažje in močneje aktivirajo dopaminski sistem, kot pri zdravih možganih.

Obstoj tveganja za recidiv tudi po več desetletjih abstinence, nakazuje na zelo stabilne spremembe v možganskem tkivu. Odkar je znano, da so nevrotrofični dejavniki močno vpeti v proces možganske plastičnosti, so postali zanimivi kandidati za raziskovanje njihovega vpliva na vedenjske in biokemične odzive na droge. Za nevrotrofični dejavnik glialnega izvora (GDNF) velja, da ima pomembno vlogo pri razvoju, rasti, preživetju in ohranjanju srednjemožganskih dopaminergičnih nevronov, hkrati pa ima bistven vpliv na sinaptično plastičnost in nevrotransmisijo.

Glavna signalna pot GDNF poteka preko aktivacije receptorja RET (receptor tirozin kinaznega tipa) in vodi v zapleteno in drugim rastnim dejavnikom sorodno znotrajcelično signalizacijo. Temeljni možganski regiji delovanja GDNF sta ventralni tegmentum in substanca nigra. V ta področja je GDNF retrogradno prenesen iz striatuma. Zaradi svojega specifičnega delovanja na populacije dopaminergičnih nevronov je z leti postal predmet raziskav tako pri nevrodegenerativnih boleznih kot pri zasvojenosti. Raziskovanje njegovega vpliva na zasvojenost je na živalskih raziskavah do sedaj dalo mešane rezultate. Znižane vrednosti GDNF preko genske modifikacije ali eksogenih protiteles proti GDNF po nekaterih raziskavah povzročijo večjo psihostimulansno nagrado in večjo nagnjenost k recidivu, medtem ko eksogene metode za zviševanje GDNF dajo nasprotne rezultate. Zgolj ena raziskava, ki je uravnavala količino GDNF šele po tem, ko so si podgane več dni same injicirale kokain, je poročala, da zvišanje GDNF zveča vedenjsko iskanje droge, znižanje njegovih vrednosti pa ima nasprotne učinke.

Z namenom boljšega razumevanja vloge GDNF pri vedenjskih odzivih na večkratno izpostavljenost psihostimulansom, smo pri različnih gensko spremenjenih mišjih sevih izvedli z amfetaminom povzročeno pogojevanje izbire okolja (PIO). Ta metoda omogoča, da miši preko klasičnega pogojevanja povežejo drogo s prepoznavnimi znaki (npr. vrsta talne podlage) enega prostora, ne pa tudi z znaki drugega prostora. Tako lahko pri miših sami prepoznavni znaki enega prostora izzovejo podoben odziv kot droga. Po končanem pogojevanju se nagrajevalne lastnosti droge merijo preko povečanega zadrževanja miši v prostoru, ki ga povezujejo z drogo.

Prvi mišji sev, heterozigoti z izbitim genom za GDNF in nižjo vsebnostjo le tega, izkusijo podobno jakost amfetaminske nagrade in dovzetnost za recidiv kot kontrolne miši. Iz meritev o gibanju miši tekom PIO preizkusa, smo dobili tudi podatke o z amfetaminom povzročeni lokomotorični aktivnosti, ki je pri heterozigotih statistično značilno nižja tako pri enkratni kot ponavljajoči izpostavljenosti amfetaminu. Nato smo izvedli PIO pri na novo razvitih heterozigotnih miših, ki pretirano izražajo GDNF. Te sicer izkusijo večjo amfetaminsko nagrado kot kontrolne miši, a ta razlika ni statistično značilna, medtem ko je dovzetnost za recidiv bila podobna med obema skupinama. Primerjava obeh skupin glede na z amfetaminom povzročene lokomotorične aktivnosti, je pokazala da tudi tu ni razlik.

Naši rezultati ne sovpadajo povsem z ostalimi raziskavami na tem področju. Razloge za to je možno iskati v preveč pristranskih PIO aparatih in trojnem genskem ozadju testiranih miši. Povsem možno je tudi, da določeni rezultati so pravilni in ni večjih razlik med testiranimi skupinami med amfetaminsko nagrado. Namreč, določene meritve kažejo, da ima pretirano izražanje GDNF pri miših večji vpliv na dorzalni striatum, in ne na ventralni, kjer se nahaja center za nagrajevanje (nukleus akumbens). Lahko pa gre tudi za razliko med takojšnjimi in zakasnelimi učinki GDNF. Pri gensko spremenjenih živalih, kjer se gen manipulira že tekom razvoja zarodka, pa je tudi neznano če so razlike posledica spremembe normalne funkcije gena, prilagoditve, vpliva gena na razvoj, ali pa kombinacije teh dejavnikov. Težavna in vprašljiva pa je tudi neposredna primerjava med eksogeno in endogeno manipulacijo GDNF.

S ciljem izboljšanja verodostojnosti dobljenih rezultatov, moramo naš PIO sistem spremeniti tako, da bo omogočal manj pristransko izbiro prostora v PIO aparatih. Nadalje moramo v prihodnosti gojiti miši z enim genskim ozadjem, da dosežemo višjo zanesljivost in ponovljivost PIO poskusov, saj trenutno nimamo nadzora nad tem, kateri genotip se prevladujoče izrazi v danem fenotipu. S tema dvema korakoma pričakujemo bolj nepristransko izbiro prostorov v PIO aparatih, manjšo variabilnost med posameznimi živalmi in boljšo primerljivost z ostalimi raziskavami.

Ne glede na to lahko bodoče raziskave na področju zasvojenosti s pridom izkoristijo novo razvite miši s tkivno-specifičnim izbitim genom za GDNF in miši, ki ga pretirano izražajo. Te miši še niso bile obsežno raziskane glede širokega obsega biokemijskih in vedenjskih odzivov na droge in nedvomno, nudijo velik potencial za boljše razumevanje vloge endogenega GDNF in njegove terapevtske vrednosti.

List of used abbreviations

AMPA	**α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**
AMPAR	**AMPA receptor**
BDNF	**brain-derived neurotrophic factor**
CPP	**conditioned place preference**
DA	**dopamine**
DAT	**dopamine active transporter**
GABA	**γ-aminobutyric acid**
GFL	**GDNF family of ligands**
GDNF	**glial cell line-derived neurotrophic factor**
GFRα	**GDNF family receptor alpha**
NCAM	**neural cell adhesion molecule**
GLU	**glutamate**
KO	**knockout**
LTD	**long term depression**
LTP	**long term potentiation**
MSN	**medium spiny neuron**
NAcc	**nucleus accumbens**
NMDA	**N-methyl-D-aspartic acid**
NMDAR	**NMDA receptor**
PFC	**prefrontal cortex**
RET	**rearranged during transfection**
SN	**substantia nigra**
SNc	**substantia nigra pars compacta**
TH	**tyrosine hydroxylase**
VTA	**ventral tegmental area**

1 Introduction

Drug addiction is a chronic brain disease, characterized by a loss of control over drug use despite adverse consequences. It contains aspects of compulsive behavior through drug-seeking and drug-taking, and commonly requires repeated drug exposure to develop. This process is strongly determined by genetic vulnerability, neurodevelopmental factors, and social circumstances. Once developed, it is a lifetime lasting condition, defined by intense drug cravings and repeated relapse, which can occur even after decades of abstinence. This implies the presence of long-lasting and strong neuroadaptive changes in the brain. These adaptations occur on epigenetic, cellular and molecular levels, involving changes in already very complex neural circuitry participating in drug addiction. Transition on these levels from occasional and hedonic towards continuous drug-use, that progressively impairs certain brain functions, to the level when an individual can no longer self-control his or her urges to take drugs, is of the main interest of current drug abuse research. Progression to addiction involves different structures and neuronal circuits in the brain, and as viewed by some of the leading scientists of the field, can be divided into three stages for easier comprehension (1–4) (Figure 1).

1.1 Stages of addiction and underlying neurocircuitry

1.1.1 Binge and intoxication

Mainly all drugs of abuse exert their reinforcing effects is by activating reward systems in the brain, resulting in fast and large release of DA in ventral striatum, where NAcc is located (1, 2). Mesolimbic DA pathway, leading from VTA to NAcc, is activated by all drugs of abuse, but it is crucial target for psychostimulants to exert their acute rewarding effects (2). Reward signal in the brain leads to a type of associative learning called conditioning, and with repeated experience, reward becomes associated with the stimulus that precedes it. These conditioned stimuli or cues can on their own trigger firing of DA cells, which is believed to serve as an anticipation and prediction of reward. These surges of DA in the presence of conditioned stimuli (where, when and with whom the drug has been taken and in what mental state), can trigger cravings and drug-seeking behaviors long after the last drug exposure, which consequently leads to drug-binging (1).

NAcc, receiving information from frontal cortex, hippocampus and amygdala, can transfer this information into motivated behavior through its connections with extrapyramidal system. Moreover, central nucleus of amygdala and ventral pallidum also play a role in acute reinforcing effects of drugs and further reward processing (2).

1.1.2 Withdrawal and negative affect

With chronic drug use, earlier rewarding effects lose their motivational power. Both human and animal studies show, that drug-induced DA release is lower in the presence of addiction (1). Thus, decreased function of DA system (and other neurotransmitter systems implicated in addiction) results in lower motivation for every day and non-drug-related stimuli – a state called anhedonia (1, 2). Motivational circuits are therefore rewired so that only drug-cues can trigger sufficient motivation (1, 3). Dopaminergic neurons no longer fire subsequently to drug administration, but instead when exposed to drug-cues.

Concurrently, there is a shift in DA release from ventral striatum (NAcc) after initial drug exposure, to more dorsal striatum after chronic drug-use. Dorsal striatum, which is involved in habit formation, is therefore also implicated in development of compulsive drug-use (3).

Additional element that underlies drug withdrawal is an anti-reward system. This system is mediated through brain stress and aversive circuits, that try to restore normal function in the presence of chronic drug use (2). Brain regions such as extended amygdala, habenula, and amygdala CRF (corticotropin-releasing factor) stress system, together with increased levels of dynorphin all go into overdrive during acute withdrawal. Consequence of which is a highly aversive, stressful and discomforting state, that a person tries to escape from. After the discomfort of acute withdrawal is diminished, an addicted person in protracted withdrawal is left with higher sensitivity to stress, hypofunctional DA system (anhedonia) and enhanced reactivity to drug cues (1–3).

1.1.3 Preoccupation and anticipation (craving)

Besides, to dysregulation of reward and emotional systems, inhibition of drug-cue reactivity is also impaired. The latter is controlled by prefrontal cortical regions, which play a major role in inhibitory control, impulsivity and executive function (1, 2).

Therefore, it is believed that this stage represents addictions as a chronic relapse disorder, as PFC regions can no longer properly inhibit strong urges, resulting from exposure to drug-cues or stress (2, 3).

Studies reveal that reinstatement to drug-use involves glutamatergic projections from PFC and amygdala with its CRF system. Detoxified cocaine abusers have lower metabolic activity in PFC when they are not exposed to drug-cues, but their PFC becomes very active once exposed to them. Additionally, when cocaine-addicted subjects are presented with drug-cues and asked to consciously inhibit their cravings, PFC metabolic activity once again subsides (3).

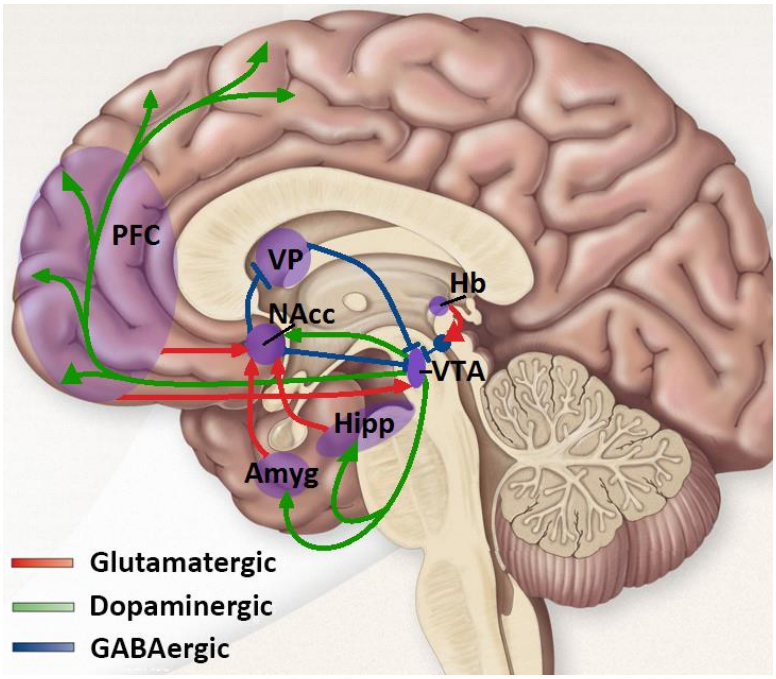

Figure 1: Neuronal circuitry involved in reward processing. This figure of sagittal section of human brain shows reward circuitry and brain regions that are heavily implicated in drug addiction. Mesolimbic DA pathway starts in the VTA (midbrain) and projects to NAcc (ventral striatum), while mesocortical pathway projects from VTA to PFC. VTA projects dopaminergic neurons to amygdala and hippocampus as well. These regions then feed back to NAcc and VTA via glutamatergic or GABAergic projections. Neuroadaptations within these circuits and regions on cellular, molecular and epigenetic levels are essential for development of drug addiction (1–4). **VTA** – ventral tegmental area, **NAcc** – nucleus accumbens, **PFC** – prefrontal cortex, **Amyg** – amygdala, **Hipp** – hippocampus, **VP** – ventral pallidum, **Hb** – habenula.

As a result of this complex imbalance, there is a shift from impulsive behavior that eventually becomes compulsive, chronic and relapsing without control over drug intake. These observations are a result of the processes taking place on a cellular and molecular level, where many complex mechanisms contribute to development and maintenance of addiction (1, 2).

1.2 Activation of mesolimbic pathway

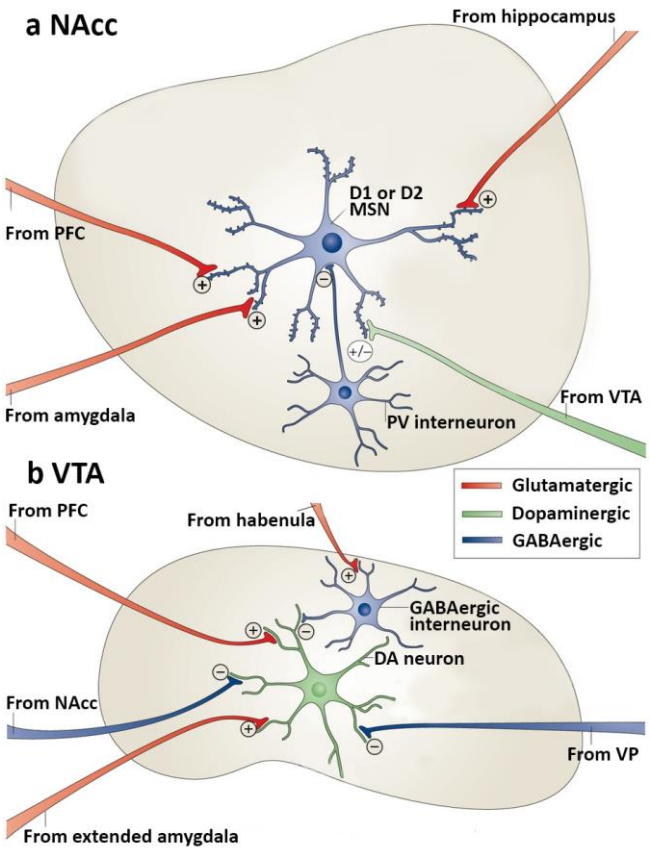

Figure 2: Neuronal input to NAcc and VTA. This figure shows, on the level of individual cells, the axonal input to NAcc (a) and VTA (b) (2–4). **NAcc** – nucleus accumbens, **VTA** – ventral tegmental area, **PFC** – prefrontal cortex, **VP** – ventral pallidum, **MSN** – medium spiny neuron, **PV** – parvalbumin interneuron. Modified from (5).

As previously mentioned, mesolimbic DA pathway plays a crucial role in reward processing. It begins with dopaminergic neurons of VTA that project to NAcc, which like the rest of striatum, is composed predominantly of GABAergic medium spiny neurons (MSNs). Two most relevant types are D1R expressing MSNs of 'direct pathway' and D2R expressing MSNs of 'indirect pathway' (4). D1 is a facilitatory DA receptor (stimulates cAMP signaling) and has lower affinity for DA, while D2 is an inhibitory DA receptor (inhibits cAMP signaling) and has higher affinity for DA. Therefore, tonic cell firing is sufficient for D2R stimulation, whereas phasic firing is needed to stimulate D1R. During phasic firing (which is produced by unexpected stimuli, novel reward or reward-cues) activation of D1R pathway facilitate reward and D2R pathway activation decreases aversion (3, 4).

All drugs of abuse, especially cocaine and amphetamine, increase DA release in the NAcc, that is both sharper and higher than dopamine release induced by natural stimuli and results in **activation of direct** and **inhibition of indirect pathway**. This sharp and large DA increase therefore stimulates both D1R and D2R, which is necessary for rewarding and reinforcing effects. Furthermore, the rate at which a drug increases DA levels correlates with intensity of high and euphoria, which also explains why faster routes of administration

(snorting, injecting, smoking), which enable higher initial DA concentrations, have larger rewarding effects than oral administration (2–4).

Box I: Behavioral animal models for studying drug addiction

Various behavioral animal models are being used throughout addiction research field, examining rewarding effects of drugs of abuse, relapse, and drug craving.

Conditioned place preference – This procedure is commonly used with rodents to measure rewarding properties of abused drugs through classical Pavlovian conditioning. During the experiment, drug reward becomes associated with distinctive environmental cues of the one compartment, but not with the cues of the other compartment. As a result, drug-paired cues can evoke a response similar to that of the drug itself. After conditioning, the drug rewarding properties are measured by animal's increased preference for drug-paired compartment.
Main advantages of CPP are methodological simplicity, high throughput, short duration of experiment, and adaptability of use for different genotypes, among others. The biggest drawback however, is that CPP might not be as relevant to study drug reinforcing effects as self-administration models.
CPP protocol usually consists of different phases – habituation, preconditioning, conditioning and postconditioning. Habituation is performed for mice to adapt to the novel environment, and preconditioning to determine their initial preference for each compartment. The following conditioning phase consists of sessions where drug administration is paired with one compartment and vehicle administration with the other. After the conditioning phase, a postconditioning test is carried out, to measure the time shift to the drug-paired compartment. Time shift to the drug-paired compartment in postconditioning test is a measure of drug's rewarding and reinforcing effects.
CPP boxes are typically divided into two or three compartments, with guillotine doors between them. Two compartment box has each compartment equipped with distinctive environmental cues (visual or tactile), while three compartment box is very similar, but has one smaller middle compartment for animal to be placed during habituation, preconditioning and postconditioning phases (6, 7).

Self-administration – In this procedure animals learn to voluntary self-administered the drug, by either nose-poke or lever-press. It is a type of operant conditioning, where positive reinforcement occurs if the drug's effects strengthen the likelihood of drug-taking behavior. Therefore, drug itself serves as a reward. It is considered to be one of the most accurate models to study drug-taking and drug-seeking behaviors (7).

Reinstatement models – It is a common model for relapse, where animals trained to self-administer the drug are extinguished from drug-seeking behavior, and then exposed to stress or drug associated cue or drug itself, which then reinstates their drug-seeking behavior (7). CPP paradigm can be used to study relapse as well. After animals are extinguished from preferring the drug-paired compartment, their preference is reinstated by re-exposure to abused drug (8).

Incubation of drug craving – This procedure is based on an observation that cue-induced drug-seeking increases with time after withdrawal (7).

In addition, D1R stimulation has the ability to induce conditioning, where stimuli that is repeatedly paired with drug-use, can on its own produce phasic DA cell firing. This firing leads to anticipation of reward and results in strong motivation to obtain and use the drug. Nevertheless, with regular procurement of natural reward, DA levels start to subside. In contrast, however, drugs due to their pharmacological effects maintain high DA levels even after their consumption, which further sustains motivation to use them. But even after chronic drug use, reactivity of VTA dopaminergic neurons to drugs attenuates as well (3, 4).

Henceforth, main motivational drive is no longer maintained by drugs, but by drug-cues, which by themselves at this stage might induce higher levels of DA release than drugs. These adaptations are therefore induced primarily by D1R activation, but are also notably mediated by GLU projections from PFC, amygdala and hippocampus into NAcc and VTA (2–4) (Figure 2).

1.3 Neuroadaptations in addiction

Repeated use of cocaine, amphetamine and other drugs, induce neuroplastic changes in dopaminergic neurons and in neurons to which they project. These changes involve adaptations on neurotransmitter release, neurotransmitter receptors and induction of LTP and LTD. Many of these adaptations of DA and GLU systems correlate with the development of behavioral sensitization in rats and mice (8).

1.3.1 Adaptations in the VTA

Long and short-term changes of VTA dopaminergic neurons have been observed in preclinical and clinical studies (for preclinical study designs see Box I). In animal studies, repeated cocaine administration increases excitability of VTA neurons, presumably via decreased sensitivity of D2 autoreceptors. Consequent potentiation of DA release stimulates D1R in VTA, which in turn increases GLU signaling, thus further facilitating the firing of DA projection neurons. Hence, the extracellular DA serves as a positive feedback loop to DA cells of VTA (8).

Moreover, single *in vivo* cocaine or amphetamine administration in rats can induce LTP in dopaminergic neurons. This LTP relies on activation of D1R, and lasts 3 months in cocaine self-administering rats, but recedes quickly in rats that undergo experiment-administered cocaine sessions. In addition, AMPARs inserted to the surface in the process of LTP, lack

GluR2 subunits, making them more permeable to Ca^{2+}. AMPARs in rats sensitized to cocaine or amphetamines are also more sensitive to GLU (2, 8).

However, sensitization is not observed in human cocaine abusers (they have decreased DA release from VTA) and monkeys that self-administer cocaine. Observed differences between species, might be explained by different dosing and administration models (2, 8).

1.3.2 Adaptations in the NAcc

Animals sensitized to cocaine and amphetamine show increased sensitivity of D1Rs (excitatory/direct pathway) and increased release of DA in NAcc when exposed to drug challenge (8). However, studies on effects of chronic drug use on D1Rs have been conflicting; some showing enhanced and others attenuated D1R signaling. Moreover, D1R availability after repeated cocaine exposure is lower in non-human primates, while this is not observed in humans (4).

On the other hand, reductions in the levels of the D2Rs in striatum (inhibitory/indirect pathway) after chronic drug exposure has been observed in both human beings (even after months of abstinence) and animals (3, 4). Furthermore, downregulation of D2Rs in animals augments sensitization to drugs (3).

Consequence of decreased D2R levels in striatum is attenuated inhibition of striatal indirect pathway, which enhances the inhibition of PFC. Henceforth, low D2R levels in animals are associated with impulsivity, compulsivity and escalation in drug-intake (4). Similarly, lower activity of PFC is indeed measured in drug abusers – as PFC plays an important role in salience attribution and inhibitory control, its attenuated activity is believed to increase motivational value of drugs and decrease the control over drug intake (3). Hindered DA modulation by increased D1R/D2R ratio therefore plays an important role in increasing impulsive and compulsive behaviors seen in addiction (3, 4).

Contrary to the VTA, animals behaviorally sensitized to cocaine, have lower basal levels of GLU in NAcc. However, when challenged with cocaine or amphetamine, sensitized rats experience higher GLU transmission, which is hypothesized to be a consequence of decreased function and expression of mGluR2/3 receptors (2, 8). These metabotropic GLU receptors function by inhibiting NMDAR activity, and are their function is decreased after repeated cocaine administration. On the other hand, lower GLU levels seem to be a consequence of decreased function of cysteine-glutamate exchangers and GLU transporters.

Both are located on glial cells and serve for regulation of GLU basal levels. Lower basal GLU levels and increased GLU release from PFC projections to NAcc after exposure to drug-cues, are hypothesized to manifest as strong drug-seeking behavior (2, 3, 8).

Table I: Neuroadaptations after repeated psychostimulant exposure and sensitization (2–4, 8)

Brain region		Effect
VTA		
	DA release	↑
	Autoreceptor function	↓
	GLU release	↑
	AMPA function	↑
	LTP	↑, but transient
NAcc		
	DA release	↑
	D2R levels	↓
	Basal GLU levels	↓
	GLU release	↑
	AMPA function	↑
	Cysteine-glutamate exchanger	↓
	mGluR2/3 function	↓
	LTP	Dynamic – ↑ after withdrawal, and ↓ after re-exposure to drug

Furthermore, the dysregulation of GLU transmission in NAcc is accompanied by rich neuroplasticity. Formation of silent synapses occurs shortly after the last cocaine administration, and they unsilence and strengthen themselves, after prolonged withdrawal from repeated cocaine exposure. However, this LTP-like state is reversed upon re-exposure to cocaine, which is indicative of the LTD (2, 3, 8).

Taken together – repeated exposure to psychostimulants induces LTP in VTA, then leads to LTP in NAcc, and in later stages of addiction shifts to more dorsal regions of the striatum (8) (Table I). The glutamatergic projections from cortical and limbic regions (PFC, amygdala, hippocampus) into the NAcc and VTA, can after exposure to drug cues, stress or drug itself, more thoroughly stimulate these two regions, through more sensitive and increased GLU transmission (4).

1.4 Molecular mechanisms underlying synaptic plasticity in addiction

Above-mentioned changes in synaptic structure obviously spur the interest in what molecular and cellular mechanism regulate and control these adaptations. Studies in different brain regions involved in reward, point to neurotrophic factors. They work together with certain transcription factors and have one of the main roles in dendritic remodeling, which occurs with drug exposure. Since neurotrophic factors are known to produce enduring

changes during brain development, they could play an important part in inducing stable changes and thus contributing to synaptic plasticity observed in addiction (Box II), (Figure 3).

1.5 GDNF

Glial cell line-derived neurotrophic factor (GDNF), is a secreted growth factor protein, which can influence neurotransmission and mechanisms involved in synaptic remodeling. It is a member of GDNF-family ligands (GFL) and was first purified in the early 90s from rat B49 glial cell line (9, 10).

1.5.1 GDNF signaling

All GDNF family of ligands (GFLs) signal through Rearranged during transfection (RET) receptor, a tyrosine kinase encoded by proto-oncogene *c-ret*. For GDNF to successfully activate RET, it first needs to bind GDNF family receptor alpha 1 (GFRα1), which is attached either to the cell membrane (cis signaling) or in soluble form (trans signaling). In total, four GFRs have been identified, one for each GFL, with minor cross talk between

Box II: Neurotrophic and transcription factors implicated in addiction related neuroplasticity

BDNF – brain derived neurotrophic factor is transiently upregulated after repeated psychostimulant exposure and is believed to be highly involved in process of neural remodeling (dendritic branching) after repeated exposure to psychostimulants. This is also supported by various animal studies with different approaches to manipulate BDNF levels, where BDNF positively affects psychostimulant reward, locomotor sensitization and drug-seeking in relapse (11–13).

ΔFosB – is a member of Fos family transcriptional factors. All Fos proteins are upregulated after acute drug exposure, however, repeated drug exposure leads to long-lasting accumulation of ΔFosB, primarily in D1R MSNs of NAcc. Studies have found that ΔFosB also positively affects locomotor response to drugs and drug reward, presumably through remodeling of dendritic spines (11, 13, 14).

CREB – is short for cAMP response element-binding protein. D1R stimulation, which increases cAMP activity, subsequently upregulates CREB in MSNs. However, CREB seems to decrease activity in reward circuitry through opioid peptide dynorphin, which acts on presynaptic dopaminergic neurons and attenuates DA release. Therefore, CREB plays a role in negative reinforcement, by contributing to aversive state and dysphoria during early drug withdrawal (11, 13, 14).

It is more than evident, that processes involved in regulating drug-induced synaptic plasticity in reward circuitry, share many similarities to mechanisms underlying learning and formation of memories.

them. In complex with GFRα1, GDNF gains high affinity for RET, leading to its activation by autophosphorylation of its intracellular tyrosine domain. Dimeric GDNF can ligate to monomeric or dimeric GFRα1, and together they interact with two RET molecules. Similarly, to other receptor tyrosine kinases, activation of RET leads to intracellular signaling through PI3/Akt, Ras/MEK/MAPK(ERK), and PCLγ pathways, which are involved in neuronal survival, neuritogenesis and enhancement of transmission (10, 15, 16).

GFRα1 is expressed together with RET in SNc and VTA, whereas their numbers in striatum are very low. However, in some regions (e.g. cortex and adult hippocampus), GFRα1 is present in the absence of RET, which suggests the existence of additional GDNF signaling. Indeed, GDNF can activate Neural Cell Adhesion Molecule (NCAM), but its binding affinity is much higher when NCAM is associated with GFRα1. Through NCAM, GDNF can activate Src-like kinases and MAPKs, which are involved in neurite outgrowth and synapse formation. Interestingly, this pathway seems to be present even in the brain regions, that express both GFRα1 and RET. Recently, another receptor was found to be activated by GDNF, named proteoglycan Syndecan-3, through which GDNF could mediate cell spreading and neurite outgrowth (15–17).

In addition, lipid rafts also play an important role in mediating GDNF signaling, enabling faster signal transduction when GFRα1 is attached to the cell membrane (cis signaling). GDNF also shows capability to potentiate excitatory neurotransmission in cultured midbrain dopaminergic neuron (10, 15, 16). All this demonstrates the importance of further work to properly understand GDNF-RET signaling. Currently known signaling pathways of GDNF are shown in Figure 3.

1.5.2 GDNF's role and function

Debate whether GDNF is crucial or not for long-term survival and maintenance of adult midbrain dopaminergic neurons is not yet settled. Furthermore, its role spreads to development and/or maintenance of sympathetic, parasympathetic and enteric nervous systems, kidneys and spermatogonial stem cell pool. Additionally, it is also important for survival of motor neurons and regrowth of dopaminergic neurons after brain injury (15, 16).

Nevertheless, prime brain regions of action for GDNF are dopaminergic neurons of VTA and SN. To affect these two regions, GDNF undergoes retrograde transportation from the striatum, where it is predominantly expressed by parvalbumin positive GABAergic

neostriatal interneurons. These neurons represent about 0.7% of all striatal neurons, so one DA axonal tree provides on average an input to 525 GDNF-expressing striatal neurons (16, 18).

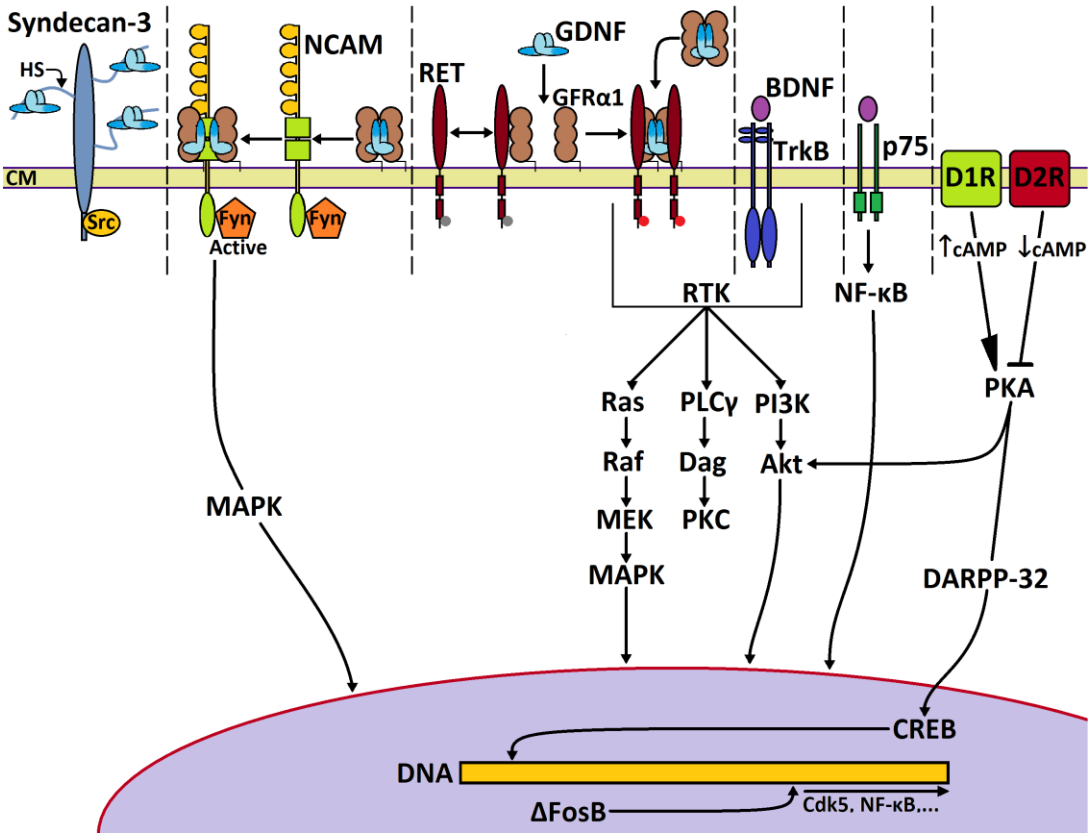

Figure 3: Overview of GDNF and BDNF signaling mechanisms. GDNF first binds to GFRα1 (soluble or attached to CM) and then as a GDNF-GFRα1 dimer interacts with two RET molecules, which leads to activation of several intracellular pathways. Both GDNF and BDNF activate PI3K-Akt, Ras-MAPK and PLCγ pathways, through activation of their corresponding RTKs (RET for GDNF and TrkB for BDNF). GDNF can also signal through non-RET mechanisms. GDNF-GFRα1 complex or GDNF alone (although with much lower affinity) can bind to NCAM, which leads to activation of Fyn (Src-like kinase) and MAPK. Furthermore, GDNF may induce Syndecan-3 signaling by binding to its HC chains, which involves Src kinase activation. Syndecan-3 could also locally concentrate GDNF and thus modulate RET and NCAM signaling. In addition, BDNF is able to signal through p75, which activates NF-κB. All these pathways are mostly involved in survival, growth, apoptosis, differentiation and cytoskeletal organization of neurons, and thus contributing to synapse formation, as well as to enhancement of neurotransmission, ultimately by regulating transcriptional activity (ΔFosB, CREB, NF-κB, …). D1R and D2R stimulation can also interfere into these pathways (10–13, 15, 19). **CM** – cell membrane, **RTK** – receptor tyrosine kinase **HS** – heparan sulfate, **MAPK (ERK)** – Mitogen-activated protein kinase.

Given its role, GDNF is still extensively studied for its therapeutic potential to treat Parkinson's disease (PD), and various other neurodegenerative diseases. So far, promising results in animal models of PD indicate that success is determined by delivery route and region of administration. On the other hand, clinical studies are still inconclusive, and likely in need of GDNF delivery optimization and further research into GDNF's mechanisms of action. Nevertheless, GDNF does in fact protect nigrostriatal neurons from neurotoxins (15, 16).

Research into GDNF's role during development and maintenance of dopaminergic neuron shows that GDNF, GFRα1 and *Ret* knockout mice die after birth due to undeveloped kidneys, but have intact DA systems (10, 15). However, conditional ablation of *Gdnf* or *Ret* expression results in conflicting observations. Mice with *Ret* deletion specifically in dopaminergic system have no observable effects on dopaminergic neurons of SNc and VTA up until 9 months of age (20). Another study with dopaminergic system specific and CNS-specific *Ret* ablation in mice reported progressive and late (12 and 24 months of age) loss of dopaminergic neurons of SNc and dopaminergic terminals in striatum, but without the loss of neurons in the VTA and locus coeruleus, suggesting that GDNF/RET signaling is modestly important for maintaining adult nigrostriatal dopaminergic neurons (21). Effects on development and maintenance of dopaminergic neurons by GDNF signaling through NCAM or even Syndecan-3, has not yet been thoroughly examined.

Ablation of *Gdnf*, has been studied in numerous ways. One of the first study reported that GDNF heterozygous knockouts (lacking about half of GDNF levels in striatum), experience only moderate age related deficits (lower activity and accelerated decrease in number of SN neurons) (22).

Next, ubiquitous *Gdnf* ablation in 2 months old mice, presumably results in substantial loss of neurons in the locus coeruleus, SN and VTA 7 months after GDNF inactivation, with subsequent motor decline. This finding determines that GDNF is absolutely needed for maintenance of adult catecholaminergic neurons (23). On the contrary, recent study, using the same *Gdnf* deletion technique in adult mice and novel mouse strain with *Gdnf* deletion from CNS during embryonic development, reported no such deficits in SN, VTA, and motor function (24). The striking difference between these two studies has yet to be settled with future research. Nevertheless, even if decrease in GDNF function does not affect survival of

dopaminergic neurons under normal conditions, it could still play a major protective role when exposed to neural challenge (neurotoxins, drugs of abuse, injury).

Overexpression of GDNF or GFRα1 results in increased SNc DA cell number and increased protection of DA cell fibers (but not cell bodies) in rodent PD model, while injection of recombinant GFRα1 into SNc shows similar increase in SNc dopaminergic neuron number and increased TH levels (15). MEN2B mice (having constitutively active RET receptor), show enhanced nigrostriatal system (increased number in dopaminergic neurons of SNc, increased levels of TH and DAT) and higher levels of DA in striatum (15). Similarly, newly developed *Gdnf* hypermorphic mouse strain, has increased GDNF and DA levels in striatum, with increased number of dopaminergic neurons in SNc (25). Furthermore, their DAT activity is enhanced, resulting in increased DA release and reuptake, whereas TH levels, unlike in MEN2B mice, stay the same (25). Furthermore, a study on *Ret* knockout heterozygote mice reported increased DAT surface localization and activity, producing enhanced DA reuptake, which is in contrast to study done on *Gdnf* hypermorphs (15).

Recently, a very important study showed that GDNF can via GDNF/RET pathway directly regulate DAT function, through surface localization (26). Before that, another study of GDNF heterozygous knockouts, reported increased DAT function in these mice, presumably from increased surface expression of DAT (27). In addition to these findings, a new study using novel mouse strain with CNS specific *Gdnf* deletion during embryonic development demonstrated that these mice have higher levels of total and surface DAT levels, as well as reduced amphetamine-induced locomotor activity and striatal DA release (28).

1.5.3 GDNF's role in psychostimulant addiction

So far, studies of GDNF's role in chronic psychostimulant exposure show that it attenuates drug response. Heterozygous GDNF knockout mice, with about 60% of normal striatal GDNF levels, experience higher reward in cocaine and methamphetamine CPP (29, 30). The same mouse strain has also been reported to have increased methamphetamine self-administration and potentiated drug-primed and cue-induced reinstatement (31). Similar findings of increased cocaine CPP were reported with rats infused with GDNF antibodies into the VTA (29). However, rats infused with intra-VTA GDNF antibodies during withdrawal from cocaine self-administrating sessions, have potentiated cue-induced cocaine seeking (32). Additionally, locomotor sensitization to psychostimulants in heterozygous

GDNF knockout mice was found to be the same in two studies and higher in one comparing to wild types (29, 31, 33).

Comparatively, rats and mice with augmented GDNF function experience lower psychostimulant reward than controls. Rats with cocaine concomitant intra-VTA GDNF infusion show decreased CPP, and rats with intra striatal (including NAcc) GDNF infusion also show decreased cocaine self-administration (29, 34). Similarly, rats that undergo intra striatal transplantation of GDNF expressing cells or intra-striatal GDNF-conjugated nanoparticle infusion, also experience lower cocaine self-administration (34, 35). Likewise, mice treated with Leu-Ile (a GDNF and TNF-α inducer) before drug exposure, have decreased methamphetamine CPP and locomotor sensitization (30). Further, treatment with Leu-Ile after the development of CPP and locomotor sensitization was also found to have attenuating effects (30). Conversely, GDNF and GDNF cDNA-AAV injection in the beginning of withdrawal from cocaine self-administration increases cue-induced cocaine seeking (32).

In addition, studies into GDNF's role in other addictive substance gave mixed results on the subject of opioids, whereas GNDF seems to be a negative regulator of alcohol self-administration and intake (16, 19).

It is safe to say that GDNF modulates certain aspects of behavioral and biochemical responses to psychostimulants. It seems that GDNF negatively regulates psychostimulant reward, whereas its role in drug relapse shows mixing results (Table II). Diverse outcomes of GDNF's role in addiction could be attributed to several different factors; GDNF delivery method, time of GDNF administration (prior, during or after drug exposure, during/before drug withdrawal), and exogenous or endogenous increase and decrease of GDNF.

In order to avoid certain negative side effects of ectopic GDNF applications (axonal growth towards GNDF injection side, surpassed levels of endogenous GDNF, virally delivered GDNF causes decrease of TH, hyperactivity, reduction in food intake and bodyweight) and postnatal death of GDNF knockout mice, further research could make use of newly developed mouse strains (15). Mice with region specific GDNF ablation and with endogenous GDNF overexpression could prove very useful to addiction research field. In both of these cases, mice have altered endogenous levels of GDNF and could be a valuable asset to addiction research.

Table II: Psychostimulant induced behavioral effects of *in vivo* GDNF manipulation in rat and mouse models (29–35)

GDNF manipulation	Brain region	Animals	Drug	Behavioral effect	Reference
Drug concomitant GDNF infusion	VTA	Rats	Cocaine	↓ CPP	(29)
Anti-GDNF antibodies infusion	VTA	Rats	Cocaine	↑ CPP	
Het GDNF KO		Mice (C57BL6)	Cocaine	↑ CPP, ↑ Locomotor sensitization	
Transplantation of GDNF-expressing cells	Striatum and NAcc	Rats	Cocaine	↓ Cocaine SA	(34, 35)
Chronic micropump GDNF infusion					
GDNF-conjugated nanoparticle infusion					
Het GDNF KO		Mice (C57BL6)	Methamphetamine	↑ Methamphetamine SA ↑ Drug primed reinstatement ↑ Cue-induced reinstatement ↑ Prolonged cue-induced reinstatement − Locomotor sensitization	(31)
Het GDNF KO		Mice (C57BL6)	Cocaine	− Locomotor sensitization	(33)
Leu-Ile (GDNF and TNF-α inducer) i.p. administration 1h before drug s.c. injection		Mice (C57BL6)	Methamphetamine	↓ CPP ↓ Locomotor sensitization	(30)
Het GDNF KO		Mice (C57BL6)		1. CPP only in KOs, but not in Wts (using low dose of methamphetamine) 2. Leu-Ile administration 1h before drug administration did not decrease CPP in KOs	
Lei-Ile i.p. administration for 5-days after postconditioning				↓ CPP ↓ Locomotor sensitization (after their development)	
GDNF cDNA-AAV injection on withdrawal day 1	VTA	Rats	Cocaine	↑ Cue-induced cocaine seeking	(32)
GDNF injection immediately after last SA session				↑ Cue-induced cocaine seeking	
Chronic GDNF antibody infusion during first 14 days of withdrawal				↓ Cocaine craving	

CPP – conditioned place preference, SA – self administration, VTA – ventral tegmental area, NAcc – nucleus accumbens, ↓ - decreased, ↑ - increased, − - no difference.

2 Objectives

Most of the *in vivo* studies on GDNF have been done by applying exogenous GDNF or using heterozygous GDNF knockout mice. Since exogenous GDNF applications have certain drawbacks and mice with complete knockout of *Gdnf* die after birth, the host research team developed two new mouse strains with the goal to overcome these obstacles. In the first strain, *Gdnf* can be conditionally deleted in the CNS during embryonic development, while the second strain is overexpressing endogenous GDNF in a spatially unaltered manner.

The aim of this thesis is to unveil the role of GDNF in behavioral characteristics of drug addiction, due to its ability to regulate neuroplasticity and neurotransmission.

We intent to achieve this, by exposing above mentioned novel mouse strains to amphetamine, using conditioned place preference (CPP) paradigm, which allows us to get quantitative data on intensity of drug reward, development and duration of addiction, locomotor sensitization, and vulnerability to relapse.

With these new mice models, we will hopefully clarify the role of GDNF in drug addiction and further verify its therapeutic potential.

3 Materials and methods

3.1 Animals

The generation and genotyping of Nestin-Cre GDNF conditional knockout mice and of GDNF hypermorphic mice was previously described in details (24, 25). Both, heterozygous knockouts (*Gdnf*$^{wt/KO}$) and conditional knockouts (*Gdnf*$^{cKO/KO}$) carrying Nestin-Cre CNS-specific removal of *Gdnf*, are healthy. *Gdnf* removal in this mouse strain occurs already during embryonic development (*Gdnf*$^{wt/KO}$: aprox. 60% of Wt GDNF levels in striatum, *Gdnf*$^{cKO/KO}$: GDNF missing from the entire CNS) (24, 28, 30, 33).

Homozygous GDNF hypermorphs (*Gdnf*$^{hyper/hyper}$) die by postnatal day 18 due to kidney failure, whereas heterozygous GDNF hypermorphs (*Gdnf*$^{wt/hyper}$) are viable and show only occasional and mild reduction in kidney size, as a consequence of whole-body elevated levels of GDNF (two-fold increase of GDNF levels in the striatum) (25).

Littermate controls (Wt) were used in all experiments. All mice were males aged 7-10 weeks at the beginning of experiments. Mice were bred locally in the Laboratory Animal Center, University of Helsinki, Finland, and maintained on 129Ola/ICR/C57BL6 mixed background.

Animals were housed in cages, with plastic floor and metal bars, which included wooden mouse chew and houses. Cages were brought to the experiment room at the beginning of CPP trails and kept there throughout the experiment. The room where mice resided before CPP trail and experiment room were both temperature controlled (20-22°C) and set to 12-hour light/dark cycle beginning at 6 a.m. One cage contained 2-6 animals, which had free access to food and water, except during behavioral experiments. All animal experiments were authorized by the national Animal Experiment Board of Finland and were conducted in the accordance of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

3.2 Place Conditioning

Two separate CPP trials were conducted. The first trial (GDNF KO group) included *Gdnf*$^{cKO/KO}$ and *Gdnf*$^{wt/KO}$ mice and Wt controls, and the second trial (GDNFh group) included *Gdnf*$^{wt/hyper}$ mice and Wt controls.

The CPP sessions were conducted in transparent rectangular boxes (field activity monitor with place preference insert; MED Associates, St Albans, GA, USA). The boxes were divided into two equally sized compartments (21 x 42 x 41 cm^3) separated by black acrylic resin wall with integrated guillotine doors, with each compartment having different tactile floor cues. In the experiments with GDNF KOs, one compartment had metal wire grid floor, and the other compartment had black acrylic resin floor (Figure 4a). With GDNFh group, one compartment had metal wire grid floor, while the other had grey perforated PVC floor – each hole had diameter of 7.5 mm with 16 mm distance between them (Figure 4b). During the CPP sessions, white noise was present to mask the background sounds and box floors were sprayed with 70% alcohol solution and then wiped with cloth between each session. The CPP trial was composed of five phases: habituation and preconditioning (1), conditioning (2), post-conditioning (3), extinction (4) and reinstatement (5). Complete timetables of both trials can be seen in Table III.

3.2.1 Habituation and preconditioning

Mice from GDNF KO group were habituated for two consecutive days for 15 min with free access to both compartments (guillotine doors open). On the third day, a 15 min preconditioning session was carried out under the same conditions as habituation, while infrared sensors recorded their movements. For habituation and preconditioning sessions, each mouse received 0.1 mL of saline (i.p.) before being placed into the CPP box.

For the GDNFh group the protocol was otherwise the same except that they underwent only one habituation session, while the preconditioning session took place on the second day. They did not receive any injections either. After preconditioning of both groups, the preferred compartment of each mouse was assigned to be paired with saline administration, while the other was paired with amphetamine.

3.2.2 Conditioning

Conditioning started the next day after preconditioning. For 6 consecutive days, mice received saline in the mornings and were immediately placed into saline-paired compartment for 40 min (guillotine doors closed). Three to four hours later, they received amphetamine, and were immediately placed into amphetamine-paired compartment for 40 min (guillotine doors closed). Amphetamine was always used in the afternoons to prevent its effect interfering with saline session.

3.2.3 Postconditioning

Next day, after the last conditioning session, preference for compartments was tested in a similar way as in preconditioning. Mice from both groups were placed into the CPP boxes, with free access to both compartments for 15 min, and their movement during that time was recorded by infrared sensors. Again, GDNF KOs received 0.1 mL of saline before being placed into the CPP boxes, whereas GDNFhs were placed into the boxes without any substance administration.

Postconditioning time (a time shift to the amphetamine-paired compartment), was calculated as the difference in time spent in amphetamine-paired compartment during preconditioning and postconditioning session. Mice with a negative or minimal shift (less than 60 seconds) towards the amphetamine-paired compartment were excluded from the data analysis.

3.2.4 Extinction

During extinction, the conditions were generally the same as in pre- and postconditioning. However, there were slight differences in the protocol for each group.

With GDNF KOs first two extinctions included 0.1 mL administration of saline. All consequent extinctions were free of saline administration and lasted 40 min, while the recordings only lasted for 15 min.

All GDNFh extinctions lasted 40 min, with 15 min recordings and were free of substance administration.

Extinctions in both groups were conducted every 2 to 3 days, until time shift to amphetamine-paired compartment subsided.

3.2.5 Reinstatement

Once the animals did not show a preference toward the amphetamine-paired compartment anymore, they received amphetamine to measure the reinstatement of conditioned stimulus. The mice were placed into the CPP boxes for 15 min immediately after amphetamine administration and had free access to both compartments. The time spent in each side was recorded. Shift to amphetamine-paired compartment after drug-priming was used to measure the relapse to drug-seeking behavior.

3.3 Amphetamine-induced locomotor activity

Data for comparison of saline and amphetamine-induced locomotor activity and acute amphetamine-induced locomotor activity were obtained during first amphetamine conditioning session (40 min) in both CPP trials. Mice that covered a distance less than 1300 cm during amphetamine conditioning session were excluded from the data analysis. Afterwards, mice that covered distances higher or lower than 2 standard deviations from the group's mean were also excluded from data analysis.

Data for repeated amphetamine-induced locomotor activity were obtained in the same way as for acute amphetamine-induced locomotor activity, but cover all 6 conditioning sessions (6 days). Mice that covered a distance less than 7800 cm (6x1300 cm) in all 6 conditioning sessions together were excluded from the data analysis. Afterwards, mice that covered distance higher or lower than 2 standard deviations from the group's mean were also excluded from data analysis.

3.3 Drug treatments

D-amphetamine hydrochloride was produced and suplied by the Faculty of Pharmacy, University of Helsinki and dissolved in 0.9% NaCl solution. Amphetamine (2 mg/kg) and saline (0.9% NaCl) were injected intraperitoneally (i.p.).

3.4 Statistical analysis

All data are given as mean ± standard error of mean (SEM). The CPP data were analyzed by unpaired t-test with two tailed distribution and equal variances. Locomotor activity data were analyzed by paired t-test, unpaired t-test and one-way repeated measures (mixed-design) ANOVA. All results were considered significant at $P < 0.05$.

Table III: Timetable of CPP trials

	a) GDNF KO trial		b) GDNFh trial
Days 1-2	Habituation	Day 1	Habituation
Day 3	Preconditioning	Day 2	Preconditioning
Days 4-9	Conditioning	Days 3-8	Conditioning
Day 10	Postconditioning	Day 9	Postconditioning
Day 12	Extinction	Day 11	Extinction
Day 14	Extinction	Day 13	Extinction
Day 18	Extinction	Day 15	Extinction
Day 20	Extinction	Day 18	Extinction
Day 22	Extinction	Day 20	Extinction
Day 25	Extinction	Day 22	Extinction
Day 27	Extinction	Day 25	Reinstatement
Day 29	Extinction		
Day 32	Extinction		
Day 34	Extinction		
Day 35	Extinction		
Day 36	Reinstatement		

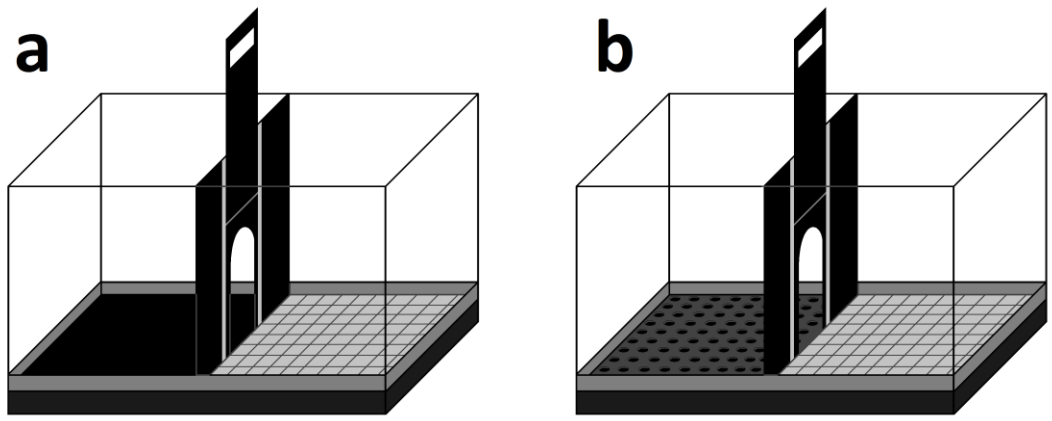

Figure 4: Visualization of CPP boxes used during experiments. In GDNF KO trial (a) a black acrylic resin floor on one side and a metal wire grid floor on the other were used, while in GDNFh trial (b) a grey perforated PVC floor was used instead of black acrylic resin. PVC – polyvinyl chloride.

4 Results

4.1 GDNF KO trial

Due to unfortunate occurrence of Cre recombinase not being functional in most mice, and thus unable to successfully remove GDNF in CNS-specific manner, what was discovered after the completion of the study, results here show only comparison between *Gdnf*$^{wt/KO}$ mice and their Wt littermates.

First, we examined the impact of endogenous GDNF ablation on behavioral responses to amphetamine using CPP, which can give us quantitative data related to drug reward, reinstatement to drug-seeking, locomotor activity and locomotor sensitization.

4.1.1 Amphetamine reward and reinstatement

CPP is primarily used to measure rewarding and motivational properties of drugs of abuse. Postconditioning test is considered a measure of drug reward, while reinstatement test, although not so often examined in CPP paradigm, is used to model a relapse to drug-seeking behaviors, by reinstating an extinguished CPP.

To reinstate extinguished CPP we used drug-priming, which is the most frequently used method for CPP reinstatement. Data from mice that did not form a place preference were excluded from experiment (shifts to amphetamine side lower than 60s). As seen in Figure 5, means of postconditioning times are similar between *Gdnf*$^{wt/KO}$ mice and Wt controls. Similarly, we observe no statistically significant difference between means of drug-primed reinstatement times (Figure 6).

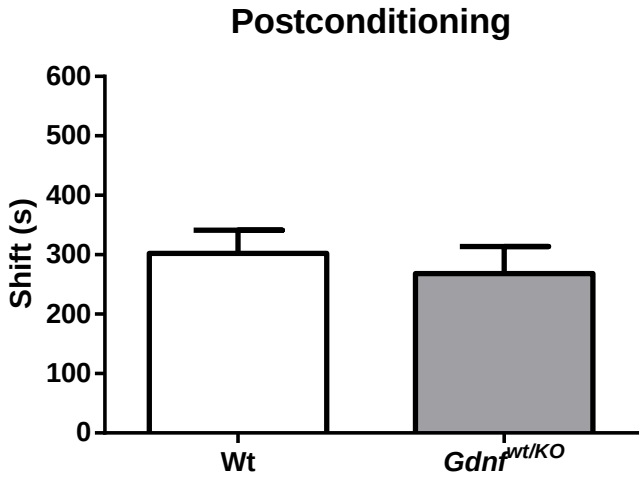

Figure 5: Postconditioning times of GDNF KO CPP trial. Bars show a comparison of shifts of both genotypes towards the amphetamine-paired compartment (y-axis, seconds ± SEM) in regard to preconditioning times. Shift towards the amphetamine-paired compartment serve as a measure for drug reward. Postconditioning session was conducted on day 10. Complete CPP trial protocol is described in the Table IIIa. In this session, mice received 0.1 mL of saline before being placed into the boxes for 15 min, with free access to both compartments. There is no statistically significant difference between the groups. P = 0.5988, unpaired t-test; n(Wt) = 9, n($Gdnf^{wt/KO}$) = 5. Wt – wild type controls; $Gdnf^{wt/KO}$ – GDNF heterozygous knockouts; bars – group's mean of time shifts to amphetamine-paired compartment; error bars – SEM.

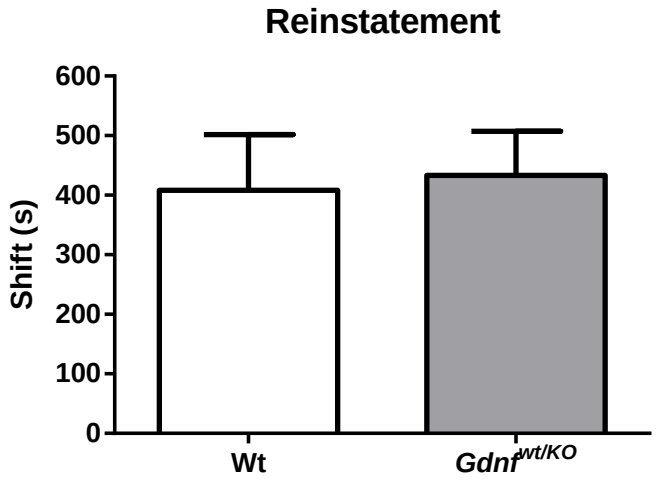

Figure 6: Reinstatement times of GDNF KO CPP trial. Bars show a comparison of shifts of both genotypes towards the amphetamine-paired compartment (y-axis, seconds ± SEM) in regard to preconditioning times. Shift towards the amphetamine-paired compartment indicates a vulnerability to relapse. Drug-primed reinstatement session was conducted on day 36, after a series of extinction sessions. Complete CPP trial protocol is described in the Table IIIa. In this session, mice received amphetamine before being placed into the boxes for 15 min, with free access to both compartments. There is no statistically significant difference between the groups. P = 0.8525, unpaired t-test, n(Wt) = 8, n($Gdnf^{wt/KO}$) = 5. Wt – wild type controls; $Gdnf^{wt/KO}$ – GDNF heterozygous knockouts; bars – group's mean of time shifts to amphetamine-paired compartment; error bars – SEM

4.1.2 Amphetamine-induced locomotor activity

As a side product from infrared measurements during CPP trial, we obtained data of locomotor activity. First, we compared saline and amphetamine-induced locomotor activity between first saline and first amphetamine conditioning sessions. Here, Wt mice have significantly increased locomotor activity during amphetamine exposure, while $Gdnf^{wt/KO}$ also have higher but not significantly different locomotor activity during amphetamine exposure (Figure 7). Afterwards, we compared the distance covered by each group during first amphetamine conditioning session to examine their acute response to the drug. We find that, $Gdnf^{wt/KO}$ mice have significantly lower amphetamine-induced locomotor activity than Wt littermates (Figure 8).

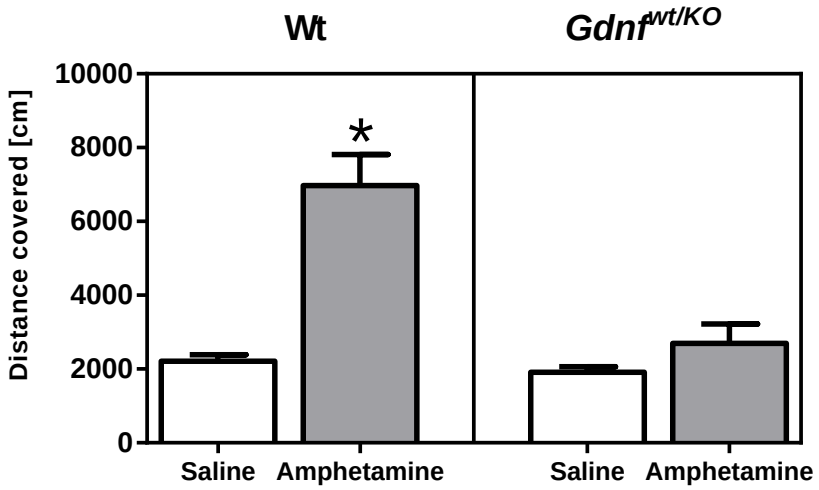

Figure 7: Comparison of locomotor activity between first saline and first amphetamine conditioning sessions of GDNF KO trial. Bars show the means of total distances covered by each group during first saline conditioning session and first amphetamine conditioning session. Wt mice cover significantly longer distances during amphetamine session compared to the saline session, while *Gdnf*$^{wt/KO}$ mice do not. Important to mention here is the fact, that each session was conducted in different compartment (all mice received saline on wire grid floor and amphetamine on acrylic resin floor) so comparison of saline and amphetamine distances is biased (see Discussion). Further, there is no difference in distances covered between Wt and *Gdnf*$^{wt/KO}$ mice during saline session. Saline and amphetamine sessions of the same group were compared by paired t-test (Wt; $P < 0.0001$, *Gdnf*$^{wt/KO}$; $P = 0.1555$, two tailed), and saline sessions between Wt and *Gdnf*$^{wt/KO}$ mice by unpaired t-test ($P = 0.3260$, two tailed, equal variances); n(Wt) = 13, n(*Gdnf*$^{wt/KO}$) = 5. Bars – group's mean of covered distance; error bars – SEM; * – statistical significance.

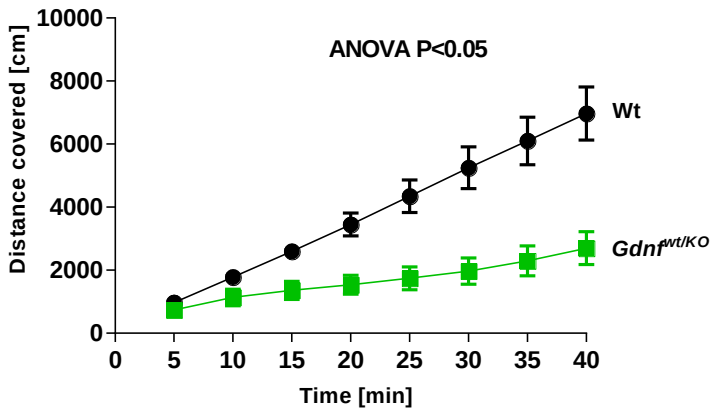

Figure 8: Acute amphetamine-induced locomotor activity of GDNF KO trial. Direction graph with error bars shows means of acute amphetamine-induced locomotor activity from the first amphetamine conditioning session. Distances covered by mice are cumulative and in 5 min intervals, meaning that at 40 min mark (the end of session) graph shows the mean of total covered distances during entire session. *Gdnf*$^{wt/KO}$ mice have significantly lower amphetamine-induced locomotor activity in comparison to Wt controls. P = 0.008, repeated measures ANOVA with between-subjects factor of genotype and within-subject factor of time; n(Wt) = 13, n(*Gdnf*$^{wt/KO}$) = 5. Wt – wild type controls; *Gdnf*$^{wt/KO}$ – GDNF heterozygous knockouts; error bars – SEM.

Similar to the measurements of locomotor activity after acute amphetamine injection, we examined locomotor activity after repeated amphetamine exposure. Again, *Gdnf*$^{wt/KO}$ mice have significantly lower amphetamine-induced locomotor activity than Wt controls during all 6 amphetamine conditioning sessions (Figure 9). The distances covered are gradually increasing with conditioning sessions, which could indicate a development of behavioral sensitization (enhanced locomotor response with repeated exposure to the drug). However, separate analysis of each group by repeated measures ANOVA failed to show a significant difference.

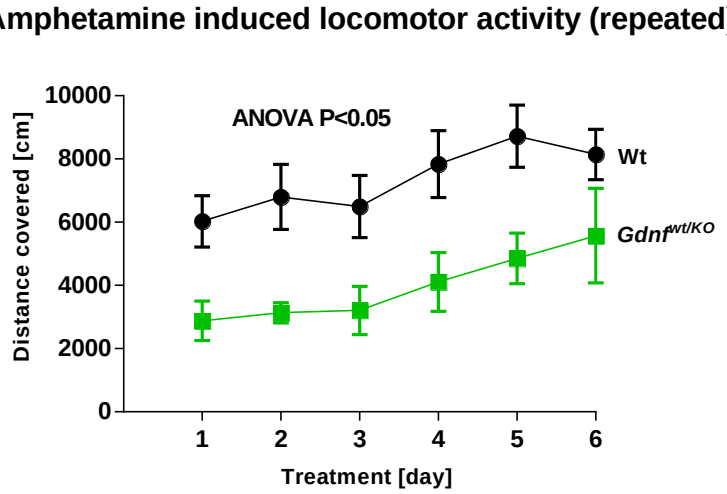

Figure 9: Repeated amphetamine-induced locomotor activity of GDNF KO trial. Direction graph with error bars shows means of amphetamine-induced locomotor activity from all 6 amphetamine conditioning sessions. During CPP trial, mice were treated with the same dosage of amphetamine every day. *Gdnf*$^{wt/KO}$ mice have significantly lower amphetamine-induced locomotor activity in comparison to Wt controls. P = 0.034, repeated measures ANOVA with between-subjects factor of genotype and within-subject factor of time; n(Wt) = 13, n(*Gdnf*$^{wt/KO}$) = 5. Wt – wild type controls; *Gdnf*$^{wt/KO}$ – GDNF heterozygous knockouts; error bars – SEM.

4.2 GDNFh trial

4.2.1 Amphetamine reward and reinstatement

Next, we explored the effects of elevated levels of endogenous GDNF under similar CPP paradigm as in GDNF KO trial, with minor modifications. Although *Gdnf*$^{wt/hyper}$ mice have notably higher mean of postconditioning times than Wt littermates, this difference is not statistically significant (Figure 10). Genotypes do not differ in drug-primed reinstatement times either (Figure 11).

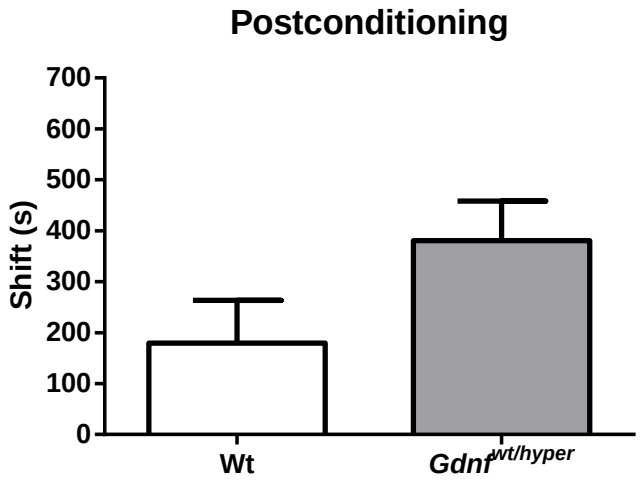

Figure 10: Postconditioning times of GDNFh CPP trial. Bars show a comparison of shifts of both genotypes towards the amphetamine-paired compartment (y-axis, seconds ± SEM) in regard to preconditioning times. Shift towards the amphetamine-paired compartment serve as a measure for drug reward. Postconditioning session was conducted on day 9. Complete CPP trial protocol is described in the Table IIIb. In postconditioning session, mice were placed into the boxes for 15 min, with free access to both compartments. There is no statistically significant difference between the groups. P = 0.1239, unpaired t-test; n(Wt) = 4, n(*Gdnf*$^{wt/hyper}$) = 6. Wt – wild type controls; *Gdnf*$^{wt/hyper}$ – GDNF heterozygous hypermorphs; bars – group's mean of time shifts to amphetamine-paired compartment; error bars – SEM.

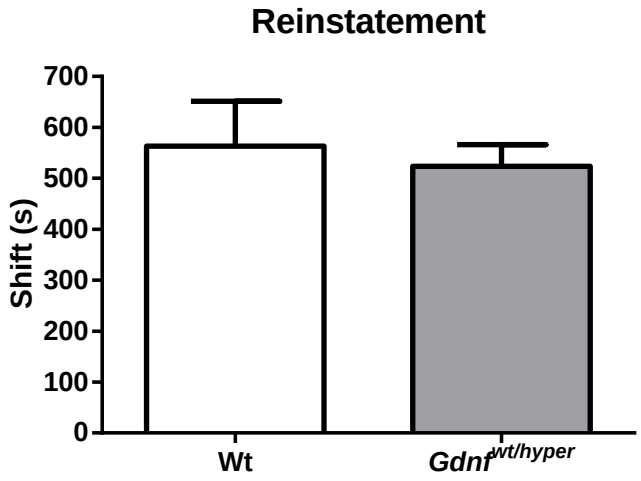

Figure 11: Reinstatement times of GDNFh CPP trial. Bars show a comparison of shifts of both genotypes towards the amphetamine-paired compartment (y-axis, seconds ± SEM) in regard to preconditioning times. Shift towards the amphetamine-paired compartment indicates a vulnerability to relapse. Drug-primed reinstatement session was conducted on day 25, after a series of extinction sessions. Complete CPP trial protocol is described in the Table IIIb. In this session, mice received amphetamine before being placed into the boxes for 15 min, with free access to both compartments. There is no statistically significant difference between the groups. P = 0.6615, unpaired t-test, n(Wt) = 8, n($Gdnf^{wt/KO}$) = 5. Wt – wild type controls; $Gdnf^{wt/hyper}$ – GDNF heterozygous hypermorphs; bars – group's mean of time shifts to amphetamine-paired compartment; error bars – SEM.

4.2.2 Amphetamine-induced locomotor activity

Locomotor activity of saline and amphetamine condition session and acute and repeated amphetamine-induced locomotor activity were measured and analyzed in the same way as in GDNF KO trial. Both Wt and $Gdnf^{wt/hyper}$ mice have significantly increased locomotor activity in first amphetamine conditioning session in comparison to first saline conditioning session, while at the same time do not differ in the distances covered in first saline conditioning (Figure 12).

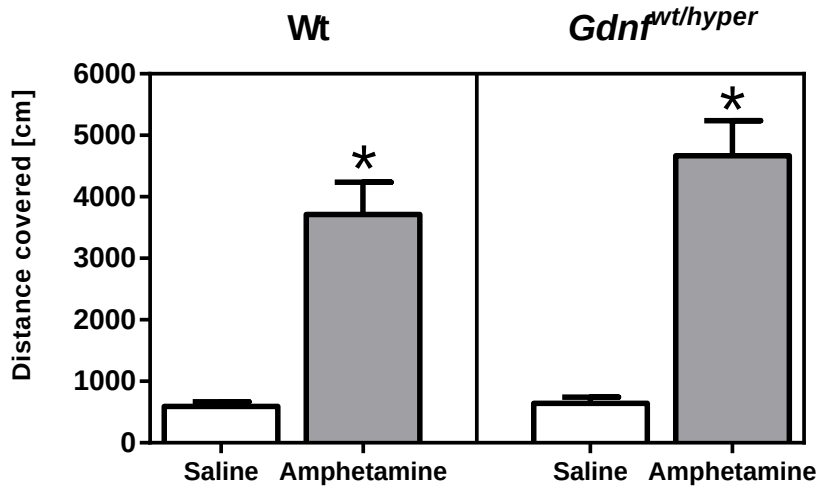

Figure 12: Comparison of locomotor activity between first saline and first amphetamine conditioning sessions of GDNFh trial. Bars show the means of total distances covered by each group during first saline conditioning session and first amphetamine conditioning session. Wt and *Gdnf*$^{wt/hyper}$ both cover significantly longer distances during amphetamine session compared to the saline session. Important to mention here is the fact, that each session was conducted in different compartment (some mice received saline on wire grid floor and other on acrylic resin floor, while amphetamine in opposite compartment) so comparison of distances is biased (see Discussion). Further, there is no difference in distances covered between Wt and *Gdnf*$^{wt/hyper}$ mice during saline session. Saline and amphetamine sessions of the same group were compared by paired t-test (Wt; P = 0.0002, *Gdnf*$^{wt/hyper}$; P < 0.0001, two tailed), and saline sessions between Wt and *Gdnf*$^{wt/KO}$ mice by unpaired t-test (P = 0.6580, two tailed, equal variances); n(Wt) = 10, n(*Gdnf*$^{wt/hyper}$) = 9. Bars – group's mean of covered distance; error bars – SEM; * – statistical significance.

Next, *Gdnf*$^{wt/hyper}$ mice have slightly higher acute amphetamine-induced locomotor activity, but the difference is not statistically significant (Figure 13). The same stands for repeated amphetamine-induced locomotor activity, where *Gdnf*$^{wt/hyper}$ mice do show higher but not significantly higher amphetamine-induced locomotor activity (Figure 14). Like in GDNF KO trial, the distances covered are gradually increasing with repeated amphetamine exposure, but separate analysis of each group by repeated measures ANOVA failed to show a significant difference.

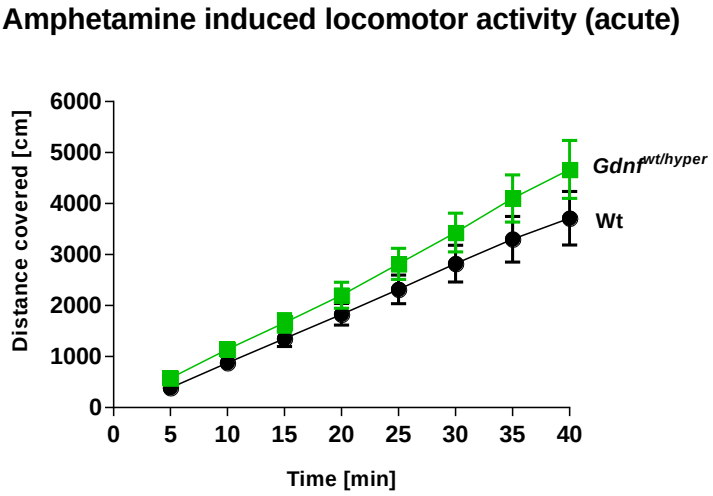

Figure 14: Acute amphetamine-induced locomotor activity of GDNFh trial. Direction graph with error bars shows means of acute amphetamine-induced locomotor activity from the first amphetamine conditioning session. Distances covered by mice are cumulative and in 5 min intervals, meaning that at 40 min mark (the end of session) graph shows the mean of total covered distances during entire session. *Gdnf*$^{wt/hyper}$ mice do not have significantly higher locomotor activity in comparison to Wt controls. P = 0.195, repeated measures ANOVA with between-subjects factor of genotype and within-subject factor of time; n(Wt) = 10, n(*Gdnf*$^{wt/hyper}$) = 9. Wt – wild type controls; *Gdnf*$^{wt/hyper}$ – GDNF heterozygous hypermorphs; error bars – SEM.

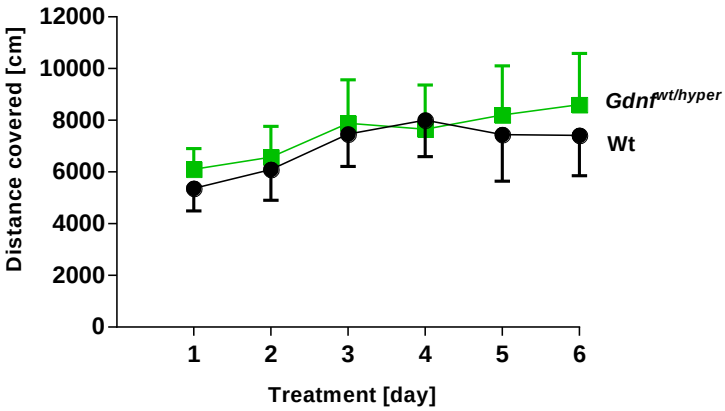

Figure 13: Repeated amphetamine-induced locomotor activity of GDNFh trial. Direction graph with error bars shows means of amphetamine-induced locomotor activity from all 6 amphetamine conditioning sessions. During CPP trial, mice were treated with the same dosage of amphetamine every day. *Gdnf*$^{wt/hyper}$ mice do not have significantly higher locomotor activity in comparison to Wt controls. P = 0.768, repeated measures ANOVA with between-subjects factor of genotype and within-subject factor of time; n(Wt) = 7, n(*Gdnf*$^{wt/hyper}$) = 7. Wt – wild type controls; *Gdnf*$^{wt/hyper}$ – GDNF heterozygous hypermorphs; error bars – SEM.

5 Discussion

The main goal of this study was to clarify the role of endogenous GDNF in behavioral responses to psychostimulants. We intended to pursue this goal with two novel mouse strains (*Gdnf*$^{cKO/KO}$ and *Gdnf*$^{wt/hyper}$) for the first time, using CPP paradigm to measure amphetamine reward and relapse to drug-seeking, with concurrent measurements of amphetamine-induced locomotor activity. The first CPP trial, which involved mice with three different genotypes; *Gdnf*$^{wt/KO}$, *Gdnf*$^{cKO/KO}$ and Wt littermates, unfortunately ran into certain troubles, which resulted in only two mice with successful CNS-specific GDNF ablation. Therefore, we could not appropriately examine the effects of CNS-specific ablation of GDNF when challenged with amphetamine under CPP paradigm. This left us with *Gdnf*$^{wt/KO}$ and Wt mice only, a comparison of genotypes, which has already been explored in numerous studies of the GDNF's role in addiction to various drugs of abuse. Second CPP trial, that compared *Gdnf*$^{wt/hyper}$ with Wt littermates, ran according to the plan.

5.1 Amphetamine-induced CPP in heterozygous GDNF knockout mice

5.1.1 Amphetamine reward and relapse

Our findings demonstrate no difference in amphetamine reward as well as in reinstatement to drug seeking behavior (relapse) between *Gdnf*$^{wt/KO}$ and Wt controls under CPP paradigm. This is in contrast to two other studies on this KO mouse model using psychostimulants under CPP paradigm. Messer et al. reported an increased cocaine induced CPP in *Gdnf*$^{wt/KO}$ mice comparing to Wts (29). Similar observation was reported by Niwa et al., where a small methamphetamine dose induces CPP in *Gdnf*$^{wt/KO}$ but not in Wt controls (30). Further, Yan et al. explored *Gdnf*$^{wt/KO}$ mice by training them to self-administer methamphetamine and found that KOs have potentiated methamphetamine self-administration and increased drug-primed, cue-induced and prolonged cue-induced reinstatement compared to controls (31).

Another way of decreasing endogenous GDNF levels is by anti-GDNF antibodies infusion, an option that was already explored with rats in two studies, one with cocaine CPP and other with cocaine self-administration. Rats infused with GDNF antibodies into the VTA have higher CPP than controls (29). Moreover, rats under low dose chronic cocaine treatment do not experience cocaine-induced increase in TH, while rats under the same treatment infused with GDNF antibodies, have significantly increased TH levels (29). TH is an enzyme and rate-limiting factor in DA synthesis, and its levels are decreased by virally delivered GDNF,

so GDNF's potential regulation of TH levels could underlie effects observed in this experiment (15, 29). Contrary, Lu et al. reported that chronic GDNF antibody infusion into VTA during first 14 days of withdrawal in rats trained to self-administer cocaine, actually decreases cocaine craving, showing that GDNF in this study design potentiates cocaine craving, which is in contrast to what Yan et al. observed in *Gdnf*$^{wt/KO}$ mice (mentioned above in discussion) (31, 32). However, studies where GDNF levels are manipulated within specific brain region (like VTA) and at specific time (before, during or after) drug exposure are not easily comparable with studies where gene manipulation occurs in the whole brain and body.

Furthermore, another important concern should be raised when using *Gdnf*$^{wt/KO}$ mice – as they lack GDNF levels already during embryonic development, it is unknown whether observed differences in biochemical and behavioral responses to drugs, reflect a change in normal gene function, compensatory changes, gene's influence on development or a combination of all.

One important possible compensatory change in *Gdnf*$^{wt/KO}$ mice is that they possibly have increased extracellular DA levels in NAcc and caudate putamen (dorsal striatum) and increased FosB/ΔFosB expression in NAcc, while having lower GDNF concentrations in these regions as was reported in certain studies (31, 33). Given the importance of DA release in rewarding effects of psychostimulants and FosB/ΔFosB increase after initial and repeated drug exposure, both could underlie increase in reward and relapse to drug seeking observed in *Gdnf*$^{wt/KO}$ mice. Furthermore, the exogenous GDNF application *in vitro* and *in vivo* potentiates release of DA by dopaminergic neurons, which only further complicates the complexity of GDNF signaling (9, 10, 15, 33, 36).

It is also possible, that *Gdnf*$^{wt/KO}$ experience some sort of reward deficit, but Yan et al. in their study show that there is no difference in food-reinforced behavior and motivation, as well as in reinstatement of food-seeking behavior in regard to controls (31). Still, there could be a decrease in experience of reward when *Gdnf*$^{wt/KO}$ mice are exposed to drugs and thus exerting increased drug self-administration in order to obtain the same level of reward as Wts. However, this seems unlikely, as under CPP paradigm, where mice are exposed to the same amount of daily drug intake, *Gdnf*$^{wt/KO}$ mice experience higher CPP than Wt controls (29, 30).

Nevertheless, most of the studies done on rodents that have decreased GDNF levels suggest that lower GDNF levels increase psychostimulant's rewarding and reinforcing effects. Since we did not observe similar effects in amphetamine-induced CPP with *Gdnf*$^{wt/KO}$ and littermate controls as Messer et al. and Niwa et al., next explanations should be considered (29, 30).

First, our CPP protocol and study design differs from theirs (29, 30). The major issue we had was a very strong bias towards the compartment with metal wire grid floor in habituation and preconditioning phase of the CPP trial (data not shown). This suggests that mice experienced a certain amount of aversion towards black acrylic resin floor, possibly because its smooth slippery surface gives them poor grasp. Therefore, they naturally spend more time on the side with metal wire, which offers sufficient grasp. We then paired amphetamine with less preferred compartment (in this case the one with black acrylic resin floor), as it is a very well established fact, that amphetamine produces preference to its paired compartment. This would be much bigger issue if we were to test a new drug, not yet knowing whether it produces pleasurable/rewarding or aversive effects – in this case, so biased CPP box would render the trial useless.

However, aversive/fearful sensation of one of the compartment can reduce the motivational response elicited by the rewarding drug (e.g. amphetamine) when paired together (6). Having an unbiased CPP box and randomly assigning which compartment is paired with the tested drug in each animal, is therefore one of the most critical steps in avoiding the complications when interpreting results (6). Thus, the encountered problem emphasizes the importance of choosing the proper tactile cue for each compartment of CPP box in order to achieve an unbiased system.

In study by Niwa et al., they did not mention how they established the initial preference for each compartment, but from their citations they seemed to use a similar protocol to ours and pairing methamphetamine with less preferred and saline with more preferred compartment (30). However, Messer et al. excluded animals that showed preference to any compartment and thus achieving an unbiased CPP system for tested animals (29). Next difference between CPP trials was in conditioning phase, where we injected mice with saline in the morning and amphetamine in the afternoon, while Niwa et al. and Messer et al. both injected animals with saline and drug on alternating days (29, 30). We chose everyday amphetamine conditioning in order to achieve stronger conditioning in mice. Moreover, the main difference is in how

data were excluded from our statistical analysis. We excluded mice that developed no or weak conditioning – those with postconditioning shifts under 60 seconds. Consequently, most of excluded data were from Wt group, while all mice from *Gdnf*$^{wt/KO}$ group had positive shift to amphetamine compartment. By doing this, we potentially excluded important data that could influence the results and their interpretation. Another issue was high variability of data within groups, which further decreases the chances of finding difference between genotypes.

Next possible reason for different results is in the mouse strains. Niwa et al. and Messer et al. used C57BL6 mouse strain. Mice for our study were maintained on a triple-mixed genetic background (129Ola/ICR/C57BL6), which gives us no control over which genetic background is predominantly expressed in the phenotype. This could be an important issue, as several studies demonstrated differences between mouse strains in spontaneous locomotor activity, and in behavioral and biochemical responses to psychostimulants. C57BL6 inbred strain has higher spontaneous locomotor activity and cocaine induced CPP than 129SvJ, while cocaine effects on locomotor activity are the same in both strains, indicating that 129SvJ is very sensitive to cocaine effects on locomotor activity (37). Interestingly, 129SvJ's locomotor response to cocaine is greatly augmented by the act of injecting, as saline injection alone produces high increase in locomotor activity (37). On the other hand, C57BL6 and 129SvHsd inbred strains have the same basal locomotor activity and basal striatal DA levels, but C57BL6 experience higher amphetamine-induced locomotor activity and higher striatal DA release (38). Furthermore, a study by Isles et al., compared four mouse strains and found C57BL6 to have higher spontaneous locomotor activity and higher impulsivity than 129Sv strain (39). Authors also demonstrated that more active animals tend to be more impulsive (39). Therefore, these differences between mouse strains or even substrains, might express itself in the phenotype, when mice are bred on triple-mixed genetic background and thus influencing behavioral and biochemical responses to drugs of abuse, and possibly causing high variability among individual animals.

Interestingly, *Gdnf*$^{wt/hyper}$ mice and Wt littermates, that we used in second CPP experiment, and were also bred on the same genetic background (129Ola/ICR/C57BL6), were (subjectively) less aggressive and active, easier to handle, moved and ran with different technique, and were larger and softer to touch.

Nevertheless, it is possible that there is in fact no difference in CPP between tested genotypes when exposed to amphetamine, but is when exposed to cocaine and methamphetamine. However, this seems unlikely, as amphetamine's mechanism of action is similar to methamphetamine's, although different to cocaine's, but this option should not be completely excluded (40). All three psychostimulants rapidly increase DA levels in NAcc. Cocaine blocks DAT by competitive inhibition, which decreases DA uptake, and consequently raises synaptic DA levels (40). Amphetamine and methamphetamine on the other hand, enter the neuron through DAT, and consequently also cause competitive reuptake inhibition. Furthermore, they are both also able to diffuse through cell membrane due to their lipophilic properties. Once inside the cell cytoplasm, they can enter secretory vesicles, as well as reverse DAT function, which even further potentiates DA release (36). Outcome in both cases is however the same – potent stimulation of D1 and D2 receptors of NAcc neurons, with consequent activation of various intracellular downstream mechanisms, leading to a broad range of neuroadaptations (40). However, *Gdnf*$^{wt/KO}$ have shown to have increased DAT function, which might produce a difference in how they response to cocaine or amphetamines (27). As cocaine cannot enter the cells, higher DAT surface expression might attenuate its efficacy. On the other hand, amphetamine could accumulate in the cell faster, because of higher surface DAT expression, and then reverse DAT's function, which would produce more reversed DATs than in Wt mice, resulting in higher DA release. However, DAT reversal time might play a role here as well (see discussion in next chapter). But this is all highly speculative, as understating of how each of these two drugs work is not yet fully understood.

In addition, amphetamine CPP study with *Gdnf*$^{wt/KO}$ and Wt littermates, was previously already performed in our lab (data not shown nor published) using very similar study design. They also observed no difference between genotypes in postconditioning and reinstatement times, which suggests that both results (ours and theirs) are either accurate or suffer from the same flaws in study design. However, current CPP experiments in our lab show that handling the mice for 5 days prior to the experiments reduces data variability, presumably due to lower stress levels. Furthermore, decrease in data variability was also achieved by crossing the mouse strain once again with C57BL6, in order to achieve strain that is more isogenic.

It seems that results obtained from various heterozygous GDNF knockout studies can partially be attributed to compensatory adaptations of increased extracellular DA levels in

dorsal striatum and NAcc and enhanced accumulation of ΔFosB in NAcc. In contrast, we do not observe similar behavioral effects as previous studies of increased psychostimulant reward in heterozygous GDNF knockouts using amphetamine. However, an option where other neurotrophic factors in the CNS are upregulated to compensate for the reduced GDNF levels should most certainly not be excluded.

Additionally, effects of GDNF antibody infusion can perhaps be explained by weakened ability of GDNF to oppose TH induction (29). On the other hand, TH levels are normal in heterozygous GDNF knockout mice (29). Undoubtedly, further investigation of effects of decreased GDNF levels needs to be conducted.

5.1.2 Amphetamine-induced locomotor activity

Our results clearly demonstrate that *Gdnf*$^{wt/KO}$ mice have much lower locomotor response to both acute and repeated amphetamine exposure than Wt controls. This is in contrast with study by Gerlai et al., where both genotypes show the same response to acute amphetamine in doses of 2mg/kg and 4mg/kg (41). Further research into psychostimulant induced locomotor activity of the *Gdnf*$^{wt/KO}$ shows that basal activity and cocaine or methamphetamine-induced locomotor activity do not differ in regard to Wts either (29, 31, 33). However, higher cocaine locomotor sensitization of *Gdnf*$^{wt/KO}$ mice was observed by Messer et al., but not by Airavaara et al., under the same protocol (29, 31). Yan et al. did also not observe difference in methamphetamine-induced locomotor sensitization between *Gdnf*$^{wt/KO}$ and Wts (33).

Explaining such a difference in results might prove difficult, and could also involve previously mentioned issues with mixed genetic background. Additionally, all of our mice received amphetamine in the same compartment of CPP boxes (acrylic resin floor), so this should not pose a problem in data interpretation. However, results in a new study from Kopra et al., which compared Wts, *Gdnf*$^{wt/KO}$ mice and *Gdnf*$^{cKO/KO}$ mice (Nestin-Cre conditional KOs, with CNS specific GDNF ablation), might offer explanations to some extent (28). According to their study, *Gdnf*$^{cKO/KO}$ mice experience even lower amphetamine-induced locomotor activity than *Gdnf*$^{wt/KO}$. In their previous study by Kopra et al., they found that TH+ cell number in SNc and VTA do not differ among all three genotypes (24). Their data now in some way expectedly show that all three genotypes have the same DA levels in dorsal and ventral striatum, VTA and SN. However, there are differences in striatal DAT levels and

in amphetamine effects on striatal DAT function between the genotypes. As mentioned in previous chapter, amphetamine, once inside the cell, reverses DAT function (instead of pumping DA inside the cell, DAT pumps it out, which even further increases DA release) (40). Interestingly, their study shows that, when exposed to amphetamine, $Gdnf^{cKO/KO}$ mice have the longest DAT reversal time (time needed for DAT to reverse the direction of DA transport), $Gdnf^{wt/KO}$ in-between and Wts the shortest DAT reversal time (28). Expectedly, Wts have the highest amphetamine-induced striatal DA release, followed by $Gdnf^{wt/KO}$ and then $Gdnf^{cKO/KO}$ (28). Furthermore, when they measured striatal DA release and uptake after single-pulse stimulation, DA release is the same in all three genotypes, however reuptake is fastest in $Gdnf^{cKO/KO}$, followed by $Gdnf^{wt/KO}$ and then Wts (28). Indeed, $Gdnf^{cKO/KO}$ have the highest level of DAT in dorsal striatum, followed by $Gdnf^{wt/KO}$ and then Wts. Similar finding was observed regarding DAT surface expression, which is highest in the $Gdnf^{cKO/KO}$ mice, while $Gdnf^{wt/KO}$ and Wts show no difference (28). However, it is important to mention that results and data described above is not always statistically significant between genotypes, although it points so certain differences between them, which could explain some of the behavioral responses to psychostimulants. Taking all this together, it seems that DAT function is somewhat proportional to the GDNF levels (less GDNF less DAT, and less GDNF longer amphetamine-induced DAT reversal time).

Therefore, it could also make sense why either of KOs mouse strains have lower response to amphetamine, as longer DAT reversal times render amphetamine mechanism of action much less effective. However, higher DAT tissue levels allow amphetamine to enter the cells faster, but at the same time increases cell's ability to pump DA from the synaptic cleft. Net effect of these processes seems to be attenuated response to amphetamine, at least in $Gdnf^{cKO/KO}$ mice, while $Gdnf^{wt/KO}$ mice show different result across studies.

To some extent, these findings are also supported by study of Letrell et al. (27). They found that $Gdnf^{wt/KO}$ mice have enhanced DAT function (presumably though increased surface expression), and more recently a very important finding by Zhu et al., when they showed on a cell line that GDNF can, through RET signaling, directly modulate DAT function by changing its surface expression (26, 27). They also observed, that $Ret^{wt/KO}$ mice have enhanced DAT function and DAT surface expression in NAcc, and interestingly also higher DA tissue levels in the same bran region (26). In addition, some other not yet measured

characteristics can underlie observed differences between genotypes, as neurotrophic mechanisms of action are very complex.

Results of the Kopra et al. study on DA levels are therefore also in contrast with the study by Airavaara et al., which reported increased extracellular DA levels in NAcc and dorsal striatum in *Gdnf*$^{wt/KO}$ mice, so further investigation needs to be carried out to address these differences (28, 33). Our results on amphetamine-induced locomotor activity are also in contrast with other studies on cocaine and methamphetamine-induced locomotor activity, which predominantly show no differences between genotypes (29, 31, 33).

Finally, we observed that acute locomotor response to amphetamine in *Gdnf*$^{wt/KO}$ mice is not significantly higher than locomotor activity induced by saline. However, it is important to mention that all mice received amphetamine in the compartment with acrylic resin floor, while saline in the compartment with metal wire grid floor, which gives better grip and presumably makes moving easier. This renders direct comparison very hard and biased.

5.2 Amphetamine-induced CPP in heterozygous GDNF hypermorphic mice

5.2.1 Amphetamine reward and relapse

Our second amphetamine CPP trial compared *Gdnf*$^{wt/hyper}$ mice with Wt littermates. Again, we do not observe a difference in amphetamine reward as well as in relapse to drug-seeking between the two genotypes. Although that *Gdnf*$^{wt/hyper}$ mice experience larger amphetamine reward, the difference is not statistically significant.

Kumar et al., the team that developed *Gdnf*$^{wt/hyper}$ mice and extensively tested them, reported that adult mice (2.5-3 months of age) have increased and spatially unaltered levels of GDNF (approx. two folds in dorsal striatum), 25% higher dorsal striatal DA levels, 15% increased number of dopaminergic terminals in dorsal striatum, 35-34% higher DA release and steeper rising curve after electrical stimulation and increased DAT activity compared to Wt controls (25). More importantly, *Gdnf*$^{wt/hyper}$ mice have increased amphetamine-induced DA release (observed as extracellular striatal DA levels) (25). Given amphetamine's mechanism of action (described earlier in Discussion), authors hypothesize that increased response to amphetamine is a result of faster amphetamine uptake by (increased activity of) DAT, 15% higher number of dopaminergic terminals, resulting in higher number of striatal DA release sites, and overall increased DA release from dopaminergic terminals (25). Therefore, it is not completely unsubstantiated to expect *Gdnf*$^{wt/hyper}$ mice to have higher amphetamine-

induced CPP. However, their measurements of DA levels and release and number of dopaminergic terminals were conducted mostly in dorsal striatum, not ventral striatum, where NAcc is located. Interestingly, in *Gdnf*$^{wt/hyper}$ mice GDNF levels are increased only in early postnatal days, but not when they are adult compared to Wt controls (25). Since ventral striatum plays a crucial role in drug reward, this observation might explain why we do not observe statistically significant difference in amphetamine reward between genotypes. Needless to say, to determine whether *Gdnf*$^{wt/hyper}$ mice experience increased amphetamine reward certainly needs further investigation.

Even if we did not observe increased reward and relapse in *Gdnf*$^{wt/hyper}$ mice, there are again several issues to consider in regard to our findings. As we used the same data exclusion criteria as with the GDNF KO trial, many animals were excluded from data analysis, but this time the number was more equally distributed between genotypes (i.e. similar number of mice were excluded from both groups). Still, this means that important data were potentially excluded. High variability was again present within both groups of mice, which lowers the chances of observing a difference.

However, this time we switched out black acrylic resin floor for perforated (drilled) PVC, with the goal of improving mice's grasp and making CPP boxes less biased. Although this approach made CPP boxes less biased, some bias still remained (i.e. approx. 60:40 bias towards wire grid floor, with approx. 20% of mice preferring perforated PVC – data not shown). Same issues should be considered here regarding biased CPP systems and pairing rewarding drug with less-preferred compartment as in previous chapter of Discussion.

Since these novel *Gdnf*$^{wt/hyper}$ mice were as of yet not explored in any other published study, except from Kumar et al., direct comparison to other experiments of similar design is currently impossible (25). However, multiple studies that augmented GDNF function, via different approaches using mice or rats, were conducted in previous years.

Messer et al., used continuous drug-concomitant GDNF intra-VTA infusion in rats, and reported decreased cocaine CPP (29). Furthermore, they examined biochemical effects of continuous intra-VTA GDNF infusion and found that it blocked chronic cocaine induced elevation of TH, and NMDAR1 levels in the VTA and ΔFosB, PKA levels in the NAcc (these effects were not observed in intra-SN infusion) (29). Changes in levels of these proteins were not observed in control animals that received only intra-VTA GDNF

administration, so they can very well at least partially explain GDNF attenuating effects of chronic cocaine exposure, observed in this study (29). Additionally, Green-Sadan et al. in their first study examined rats that underwent transplantation of GDNF-expressing cells into the striatum and NAcc prior to cocaine exposure, as well as rats that were chronically infused with GDNF (the same dose as Messer et al. (29)), also into the striatum and NAcc (34). In both cases, they observed a decrease in cocaine self-administration. They also reported decreased GDNF mRNA levels in striatum in rats that underwent cocaine self-administration – this is in accordance with previous report by Messer et al. that chronic cocaine causes decrease in RET phosphorylation (the main GDNF signaling receptor) in rat's VTA (29, 34). In addition, Green-Sadan et al. in their later study used GDNF-conjugated nanoparticle infusion, which also resulted in decreased cocaine self-administration, however they did report increased DA levels in the NAcc, which consequently enhances synaptic DA activity (35).

In comparison, Salvatore et al., reported that rats after intracranial GDNF injection into the striatum have lowered TH levels (in striatum, but not SN) (36). However, they also have increased TH phosphorylation (striatum and SN), and enhanced amphetamine-induced DA release in striatum (36). Therefore, GDNF's capability of increasing DA synthesis and release could be a consequence of TH phosphorylation, which increases its capacity to synthesize DA, thus negating effects of lowered TH levels. Under this hypothesis, it would be expected that GDNF augments responses to various psychostimulants, not decrease them. Unfortunately, they did not measure TH levels and phosphorylation in the VTA. Nevertheless, these results even further demonstrate the complexity of GDNF signaling and its effects.

Additionally, an interesting study of GDNF and TNF-α inducer, Leu-Ile, demonstrated that it decreases methamphetamine CPP and locomotor sensitization in mice, if used during conditioning, but had no effect on attenuating methamphetamine CPP in GDNF KO mice comparing to Wts (30). Leu-Ile also attenuates CPP and locomotor sensitization if administered after their acquisition (30). Contrary to the previous reports from Green-Sadan et al. and Messer et al. on chronic cocaine decreasing GDNF levels, methamphetamine in their study induced GDNF levels, even more so if paired with Leu-Ile administration (29, 30, 35). However, even if they clearly demonstrated GDNF and TNF-α induction after Leu-Ile administration, and that certain cellular signaling pathways of how Leu-Ile achieve their

expression are known, it is still very speculative that their observation is in fact a direct consequence of elevated GDNF and TNF-α levels.

Indeed, in contrast, Lu et al., studied GDNF's role in cue-induced cocaine seeking, after withdrawal from cocaine self-administration (32). They injected VTAs of rats either with GDNF cDNA-AAV or GDNF itself after the last self-administration session, and in both cases they observed increased cue-induced cocaine seeking (which was not observed in intra-SN GDNF injection) (32). On the other hand, chronic GDNF antibody infusion during first two weeks of withdrawal had the opposite effect (32). Moreover, they argued that Green-Sadan's et al. observations of increased cocaine self-administration in GDNF augmented rats, can due to their study design, actually be a result of increased cocaine reward, which attenuates the need for cocaine self-administration (32, 34, 35). As they previously demonstrated a very similar role of BDNF in potentiating cue-induced cocaine seeking, they assumed that MAPK (ERK) inhibitor can, as it did with BDNF, reverse GDNF increased cue-induced cocaine seeking (32). Their assumption is based on the observation that GDNF enhances neurotransmission in cultured TH+ DA cells through MAPK mechanism (10, 16, 32). Indeed, they observed that MAPK inhibitor blocks GDNF's potentiation of cue-induced cocaine seeking, just as it does with BDNF (32). Given that both BDNF and GDNF partially signal through the same MAPK mechanism, these findings suggest that both BDNF and GDNF function through similar cellular mechanism in development of cue-induced cocaine seeking (32).

Returning to $Gdnf^{wt/hyper}$ mice used in our CPP experiment, an interesting observation occurred, when they were examined in amphetamine CPP for the first time in our lab (data not shown nor published). CPP experiment design was similar to ours, (although it used different compartment floor options) and postconditioning times look comparable to what we report here (higher in $Gdnf^{wt/hyper}$ mice, but not significantly different). However, $Gdnf^{wt/hyper}$ were immune to extinction, as conditioning persisted for over 30 days after postconditioning. Therefore, they were unable to conduct a reinstatement session. This could indicate that $Gdnf^{wt/hyper}$ mice develop very strong and persistent conditioning, perhaps through enhanced dopaminergic system and consequent potentiation of DA-dependent learning/memory formation (2–4). Likewise, we came across a similar issue, although in lesser extent. Still, we decided to perform a reinstatement session anyway.

This observation together with Lu's et al., that GDNF augmentation increases cue-induced cocaine seeking, suggests that elevated GDNF levels can indeed strengthen conditioning and/or drug associated cues, either by pairing cocaine self-administration with certain environmental cues or CPP floor cues with amphetamine exposure (32).

Nevertheless, comparing so different approaches to GDNF manipulation and different animal models (CPP vs. incubation of drug seeking) should always be done carefully. It is important to note, that GDNF manipulation via injection and viral overexpression result in very high GDNF levels (even over 100 fold higher than normal), which might lead to unspecific and secondary effects. On the other hand, adult *Gdnf*$^{wt/hyper}$ mice have only two folds increase of GDNF levels in dorsal striatum. Furthermore, as discussed in previous chapter, when manipulating GDNF levels already during embryonic development, it is unknown whether observed differences in behavioral and biochemical responses to drugs, reflect a change in normal gene function, compensatory changes, gene's influence on development or a combination of all. Next, in CPP daily drug intake is much lower, than in extended access self-administration model used in Lu et al. study (32). Also, Lu et al. raised GDNF levels specifically in the rat's VTA, while *Gdnf*$^{wt/hyper}$ mice have increased but spatially unaltered levels of GDNF (32).

Moreover, there are also differences in drug-induced plasticity in MSNs of NAcc in animals self-administering the drug and animals receiving experimenter administered drug (8, 42). The former experience greater drug-induced neuroplastic changes, which could indicate that an act of willing to take the drug plays a role in drug-induced neuroadaptations (42). Additionally, distinct rodents (rats or mice) used for research could respond differently to manipulations of GDNF levels.

Nonetheless, current research in this field suggest that there are great differences in the outcome of studies regarding how and when GDNF is manipulated. It seems that initial psychostimulant's rewarding effects can be attenuated by GDNF augmentation prior or during this phase, which can at least partially be explained by blocking cocaine induced increase in TH levels in the VTA, thus decreasing DA production and release and decreasing ΔFosB accumulation (29, 34, 35). However, other studies show that GDNF increases TH phosphorylation and DA production and release (36). Moreover, GDNF augmentation after drug exposure seems to induce drug seeking behaviors in the following days or weeks and

thus increasing vulnerability to relapse, through MAPK mediated mechanism (32). We could be looking here at acute versus delayed effect of GDNF augmentation.

Finally, increasing GDNF levels through gene editing, gives a phenotype with enhanced function of dopaminergic neurons, which in our case experience slightly higher but not significantly different amphetamine reward, enduring conditioning and similar reinstatement to amphetamine seeking behavior.

5.2.2 Amphetamine-induced locomotor activity

Our result show that there are no differences in acute and repeated amphetamine-induced locomotor activity between *Gdnf*$^{wt/hyper}$ mice and their Wt littermates. This is in contrast to so far the only reported study on these mice, where *Gdnf*$^{wt/hyper}$ mice have higher acute amphetamine-induced locomotor activity, hypothesized to be a consequence of increased amphetamine-induced DA release (25). However, there are some differences between our and their methods. They measured locomotor activity for 90 min and 1 mg/kg dose, while we for 40 min and 2 mg/kg dose. Incidentally, in our case *Gdnf*$^{wt/hyper}$ mice also have higher, but not significantly higher locomotor activity than Wt controls. Furthermore, *Gdnf*$^{wt/hyper}$ mice seem to be gaining the difference as time passed – just as in their study. It could be that if our study design allowed for 90 min measurements, we would measure a statistically significant difference as well. In addition, some mice received amphetamine in compartment with metal wire grid floor, while other on perforated PVC floor. Moreover, the floor in the CPP boxes might also affect behavior of mice differently than floor of the locomotor activity boxes.

Nevertheless, both Wts and *Gdnf*$^{wt/hyper}$ mice have statistically significant higher locomotor activity when exposed to amphetamine compared to saline. But as before, saline was received in different compartment than amphetamine, so these measurements are not fully comparable.

5.3 Concluding remarks

Discussion whether GDNF is a negative modulator for psychostimulant reward, is far from over. The same goes for its effect on relapse to drug taking and induction of locomotor activity. As we did not observe differences in reward and relapse in both CPP experiments, one with heterozygous GDNF knockouts and other with heterozygous GDNF hypermorphs, this additionally complicates better understanding of GDNF's role in behavioral responses

to psychostimulants. The same goes for heterozygous GDNF knockouts having lower amphetamine-induced locomotor activity, while heterozygous GDNF hypermorphs showing no difference in comparison to Wts. Realization, that our CPP study might very well be flawed by biased CPP boxes, high data variability, to strict data exclusion criterion and use of mice with triple genetic background, even further impedes interpretation of results.

Accordingly, mechanisms by which GDNF exerts its anti- or pro-addictive properties still remain unknown. It has been suggested that GDNF works by opposing drug induction of TH, or protecting neurons from drug's toxicity or from drug induced adaptations and changes (GDNF's action on neuronal morphology) (34, 35). On the other hand, GDNF augmentation increases DA neurotransmission presumably through TH phosphorylation, thus strengthening function of dopaminergic system, so it is somewhat expected to increase drug reward, conditioning and drug induced locomotor activity. Moreover, its similar signaling pathway to BDNF also suggests that it has more of a pro-addictive role. Next, its ability to modulate drug response appears to be region-specific – behavioral and biochemical effects of drug exposure are observed by increasing its levels in the VTA, but not in the SN (29, 32).

Then there are gene editing techniques that have proven to be very useful in the past, but when they change gene expression during embryonic development they come with certain drawbacks. With both of our mutant mice used, changes in DAT function seems to be particularly important. Both, conditional GDNF KOs and heterozygous GDNF KOs presumably have elevated DAT levels, and suggestion of increased amphetamine-induced DAT reversal time, which could explain their attenuated locomotor response to amphetamine. Heterozygous GDNF hypermorphs on the other hand, have increased DAT function, which accumulates amphetamine faster, and increases DA release, once amphetamine reverses it function. Thus, genetic manipulation in both cases apparently changes DAT function, albeit in a completely different manner.

Nevertheless, GDNF's role in the behavioral and biochemical responses to psychostimulant drugs seems to be very complex, and current observations suggests that many factors can in fact influence the final results (in which phase of study and in what brain region GDNF levels are changed, what GDNF manipulation technique and which animal model is being used, etc.). This also means that any future therapeutic potential of GDNF to treat drug

addiction, whether by inducing or decreasing its function is currently unlikely and still far away.

6 Conclusion

In both of our CPP trials we observed no significant differences between mutant mice and their wild type littermates in amphetamine reward and relapse to drug seeking behavior. Heterozygous GDNF KOs however experience lower amphetamine-induced locomotor activity, while for heterozygous GDNF hypermorphs no such difference was found.

Nevertheless, each of those CPP trials came across certain issues, which made our interpretation of results challenging. Our main concerns lie with unbiased CPP boxes, high data variability, data exclusion criterion and triple-mixed genetic background of mice used in the experiments.

In order to overcome these known issues, we need to develop unbiased CPP boxes for tested mouse strains. This should give us less variability and more accurate, credible and reliable results. It would most likely also reduce the need for the exclusion criterion we used prior to statistical analysis for CPP data. Furthermore, in the future, mouse strains used for the experiments should be bred on a single genetic background, thus giving us control over their phenotypes. Isogenic mice can also deliver more repeatable results between litters and lower variability among individuals, while at the same time giving us more robust CPP compartment preference (i.e. CPP box would be unbiased for all future CPP trials with specific isogenic mouse strain). Isogenic mice also offer better comparison to other studies in which the same mouse strain was used.

Afterwards, many possible study designs and approaches should be considered. Since $Gdnf^{wt/hyper}$ are relatively new mouse strain, they can be further examined with different drugs of abuse, with drug self-administration models, relapse models, incubation of drug craving models and relapse after prolonged (few months or more) withdrawal. Furthermore, TH phosphorylation, TH, CREB and ΔFosB levels (and perhaps of some other neurotrophic factors) should be measured prior and after drug exposure in VTA and striatum, all of which would give us an indication of how these mutant mice respond to drugs on biochemical levels. Same goes for another new mouse strain – $Gdnf^{cKO/KO}$, which has CNS specific GDNF deletion, that occurs during embryonic development. This mouse strain should already be examined in this study, but unfortunately this was not accomplished. Another not yet

thoroughly tested mutant mouse strains on the subject of addiction are MEN2B (having constitutively signaling RET receptor), conditional *Ret* KOs (RET not present in dopaminergic system) and heterozygous *Ret* KOs.
And perhaps in the future, when gene editing techniques become even more advanced a conditional (homozygous and heterozygous) GDNF hypermorphs could be developed, or GDNF conditional KOs, that have wild type levels of GDNF in the whole body except in the CNS or specific CNS systems (e.g. dopaminergic). Current conditional KOs are still heterozygous in the body, but complete KOs in specific regions/tissues. Furthermore, studies where gene deletion is induced during adulthood and thus avoiding compensational development, should be considered for addiction research.

Either way, the future holds many possibilities to enrich our knowledge on the subject of drug addiction, which over and over proves to be a very complex and extremely challenging brain disease.

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