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Negr1 geeni osalus sõltuvushäirete ja anhedooniaga seotud mesolimbilises dopamiini juhtetees

Bakalaureusetöö

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INFOLEHT

Negr1 geeni osalus sõltuvushäirete ja anhedooniaga seotud mesolimbilises dopamiini juhtetees

Mesolimbilist dopamiini rada peetakse seotuks motivatsiooni ja sõltuvuskäitumisega. Oma tõuke sõltuvushäirete kujunemisele annavad ka geneetilised tegurid. GWAS uuringutes on välja toodud, et *NEGR1* geen seostub mitmete sõltuvuse ja impulsikontrolliga seotud fenotüüpidega nagu tarbitava toidu kogus ja alkoholi tarbimine. Selle ajendil oli töö eesmärgiks mõõta türosiini hüdroksülaasi (*Th*), dopamiini retseptor 1 (*Dr1*), dopamiini retseptor 2 (*Dr2*), dopamiini transporteri (*Dat*), monoamiini oksüdaas A (*Maoa*), monoamiini oksüdaas B (*Maob*) ja katehhool-O-metüültransferaasi (*Comt*) geenide ekspressioonitasemeid *knockout* hiirte ventraalses tegmentaalses piirkonnas ja ventraalses striaatumis, kasutades reaal-aja PCR-i. *Negr1*-puudulikkusega hiirtel täheldati statistiliselt olulisi erinevusi *Dat*, *Comt, Th*, *Maoa* ja *Maob* mRNA ekspressioonitasemetes.

Märksõnad: Dopamiin, sõltuvuskäitumine, motivatsioon, *NEGR1/Negr1*

CERCS: B640 Neuroloogia, neuropsühholoogia, neurofüsioloogia

Involvement of the Negr1 gene in the mesolimbic dopamine pathway associated with addictive disorders and anhedonia

The mesolimbic dopamine pathway is thought to be linked to motivation and addictive behavior. Genetic factors also contribute to the development of addictive disorders. GWAS studies have shown that the *NEGR1* gene is associated with several phenotypes related to addiction and impulse control, such as food intake and alcohol consumption. The aim of this work was to measure the expression levels of the tyrosine hydroxylase (*Th*), dopamine receptor 1 (*Dr1*), dopamine receptor 2 (*Dr2*), dopamine transporter (*Dat*), monoamine oxidase A (*Maoa*), monoamine oxidase B (*Maob*) and catechol-O-methyltransferase (*Comt*) genes in the ventral tegmental area and ventral striatum of *knockout* mice using real-time PCR. Statistically significant differences in *Dat, Comt, Th, Maoa* and *Maob* mRNA expression levels were observed in *Negr1*-deficient mice.

Keywords: Dopamine, addiction behavior, motivation, *NEGR1/Negr1*

CERCS: B640 Neurology, neuropsychology, neurophysiology

SISUKORD

KASUTATUD LÜHENDID

AADC– aromaatne aminohapete dekarboksülaas (ingl. k. *aromatic aminoacid decarboxylase*)

ACT-B– beeta-aktiin (ingl. k. *beta actin*)

COMT– katehhool-O-metüültransferaas (ingl. k. *catechol-O-methyltransferase protein*)

COMT/Comt– katehhool-O-metüültransferaasi geen inimestel/hiirtel (ingl. k. *catechol-O-methyltransferase gene in humans/ in mice*)

DA– dopamiin (ingl. k. *dopamine*)

DAT– dopamiini transporter (ingl. k. *dopamine transporter*)

DAT/Dat– dopamiini transporteri geen inimestel/hiirtel (ingl. k. *dopamine transporter gene in humans/ in mice*)

DOPAC– dihüroksüfenüülalaniin (ingl. k. *dihydroxyphenylalanine*)

GABA– γ-võihappe (ingl. k. *γ-aminobutyric acid*)

GPI– glükosüülfosfatidüülinositool (ingl. k.*glycosylphosphatidylinositol)*

HVA– homovanilliin happe (ingl. k. *homovanillic acid*)

Ig– immunoglobuliin (ingl. k. *immunoglobulin*)

IgCAM– immunoglobuliinilaadne rakuadhesioonimolekul (ingl. k. *immunoglobuline-like cell-adhesion molecule*)

IgLON – immunoglobuliin LON (ingl. k. *immunoglobulin LON*)

L-DOPA– L-dihüdroksüfenüülalaniin (ingl. k. *L-dihyroxyphenylalanine*)

LSAMP– limbilise süsteemiga seotud membraanvalk (ingl. k. *limbic system-associated membrane protein*)

MAO– monoamiini oksüdaas (ingl. k. *monoamine oxidase*)

MAO/Mao– monoamiini oksüdaasi geen inimestel/hiirtel (ingl. k. *monoamine oxidase gene in humans/ in mice*)

MDD– raske depressioon (ingl. k. *major depression dissorder*)

NEGR1/Negr1– neuronaalse kasvuregulaator 1 geen inimesel/hiirel (ingl. k. *neuronal growth regulator 1 gene in humans/ in mice*)

NEGR1– neuronaalse kasvuregulaatori 1 valk (ingl. k. *neuronal growth regulator 1*)

Negr$^{-/-}$–neuronaalse kasvuregulaatori 1 geenipuudulikkus hiirtel (ingl. k. *neuronal growth regulator 1 gene deficiency in mice)*

NTM– neurotrimin

OPCML– opioididega seonduv raku adhesioonimolekul (ingl. k. *opioid-binding cell adhesion molecule*)

TH– türosiini hüdroksülaas (ingl. k. *tyrosine hydroxylase*)

TH/Th– türosiini hüdroksülaasi geen inimestel/hiirtel (ingl. k. *tyrosine hydroxylase gene in humans/ in mice*)

VMAT– vesiikuli monoamiini transporter (ingl. k. *vesicle monoamine transporter*)

VST– ventraalne striatum (ingl. k. *ventral striatum*)

VTA– ventraalne tegmentaalne piirkond (ingl. k *ventral tegmental area*)

WT– metsik-tüüpi (ingl. k *wild type*)

SISSEJUHATUS

Dopamiin on ajus oluline neurotransmitter, mida sünteesitakse türosiinist (Schatzberg & Nemeroff, 2004). Dopaminergiliste neuronite aktivatsioonist põhjustatud elektrilaengute muutus rakumembraani mõlemal poolel toob endaga kaasa dopamiini vabanemise (Di Chiara, 1997). Peale presünaptilisest terminalist vabanemist on võimalik interakteeruda viie erineva retseptoriga (Schatzberg & Nemeroff, 2004). On täheldatud, et dopamiini retseptor D_2 mängib sõltuvuskäitumises olulist rolli, vahendades ainetest (alkoholist, suhkrust, kokaiinist, amfetamiinist jne…) saadud tugevat toimet (Volkow et al., 2002). Dopamiini neuroneid leidub keskajus asuvas ventraalses tegmentaalses piirkonnas. Mesolimbiline dopamiini süsteem vastutab ajus tasu ja stressi süsteemi töötlemise, aga ka mälu ning õppimise eest. Ühtlasi peetakse mesolimbilist dopamiini süsteemi seotuks motivatsiooni ja sõltuvuskäitumisega (Cai & Tong, 2022). Mitmed meelemürgid on võimelised alterneerima dopamiini transporterite tööd (Schatzberg & Nemeroff, 2004), Tiihonen jt. näitasid, et alkohoolikutel on dopamiini retseptorite esinemistihedus väiksem (Tiihonen et al., 1995). Lisaks on välja toodud, et etanool suurendab dopamiini neuronite langlemise määra, soodustades dopamiini vabanemist ventraalses striaatumis (Brodie et al., 1990; Zhu & Reith, 2008). Ühtlasi on täheldatud, et erinevad ravimid, mis blokeerivad D_2 retseptoreid, suurendavad isu ja põhjustavad kaalutõusu (Wang et al., 2001). Ka geneetilisi tegureid on seostatud depressiooni ja rasvumisega, üheks vastutavaks valguperekonnaks selles vallas on IgLON. Perekonda kuuluvad neurotrimin, *opioid-binding cell adhesion molecule*, *limbic system-associated membrane protein*, IgLON5 ja *neuronal growth regulator 1*. On täheldatud, et *NEGR1* puudulikkusega hiired söövad suuremaid koguseid ja kosuvad rohkem (Boender et al., 2014). Alkoholi ja *NEGR1* seoseid ei ole põhjalikult uuritud.

Negr1 geen seostub mitmete sõltuvuse ja impulsikontrolliga seotud fenotüüpidega, sellest tulenevalt on antud töö eesmärgiks uurida võimalikke kõrvalekaldeid Negr1 puudulikkusega hiirte dopaminergilises mesolimbilises süsteemis. Käesoleva töö eksperimentaalne osa on teostatud Tartu Ülikooli arstiteaduskonna bio- ja siirdemeditsiini instituudi füsioloogia õppetoolis.

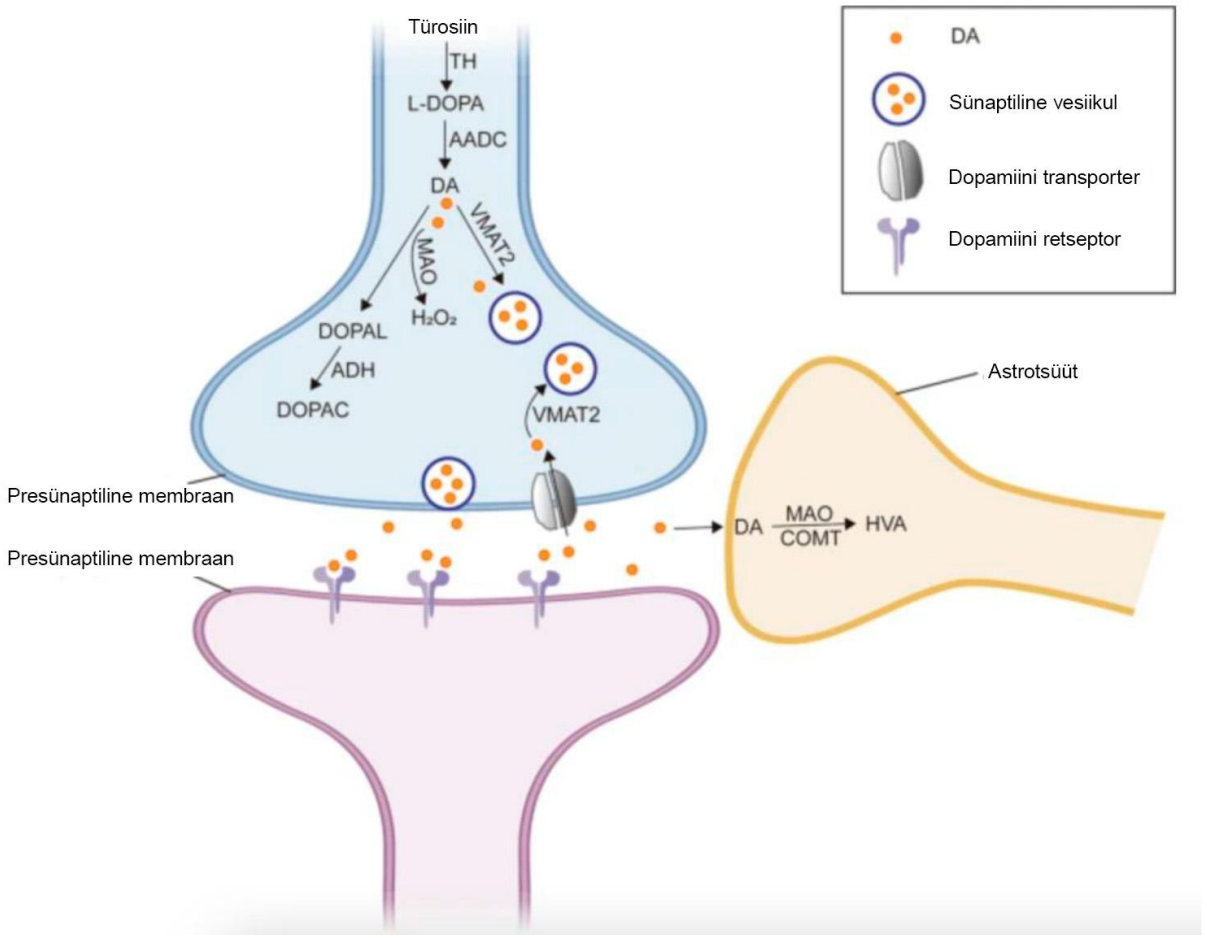
1. KIRJANDUSLIK ÜLEVAADE

1.1. Neurotransmitter dopamiin

Neurotransmitteriks peetakse neuroni poolt vabastatavat molekuli, millel on võime mõjutada kindlat sihtmärki mingil kindlal viisil. Dopamiini (DA) peeti pikalt pelgalt noradrenaliini sünteesi tarvilikuks osaks. 1957. näitas A. Carlsson jäneskatses, et dopamiin funktsioneerib ka kui neurotransmitter. (Kandel et.al., 2021)

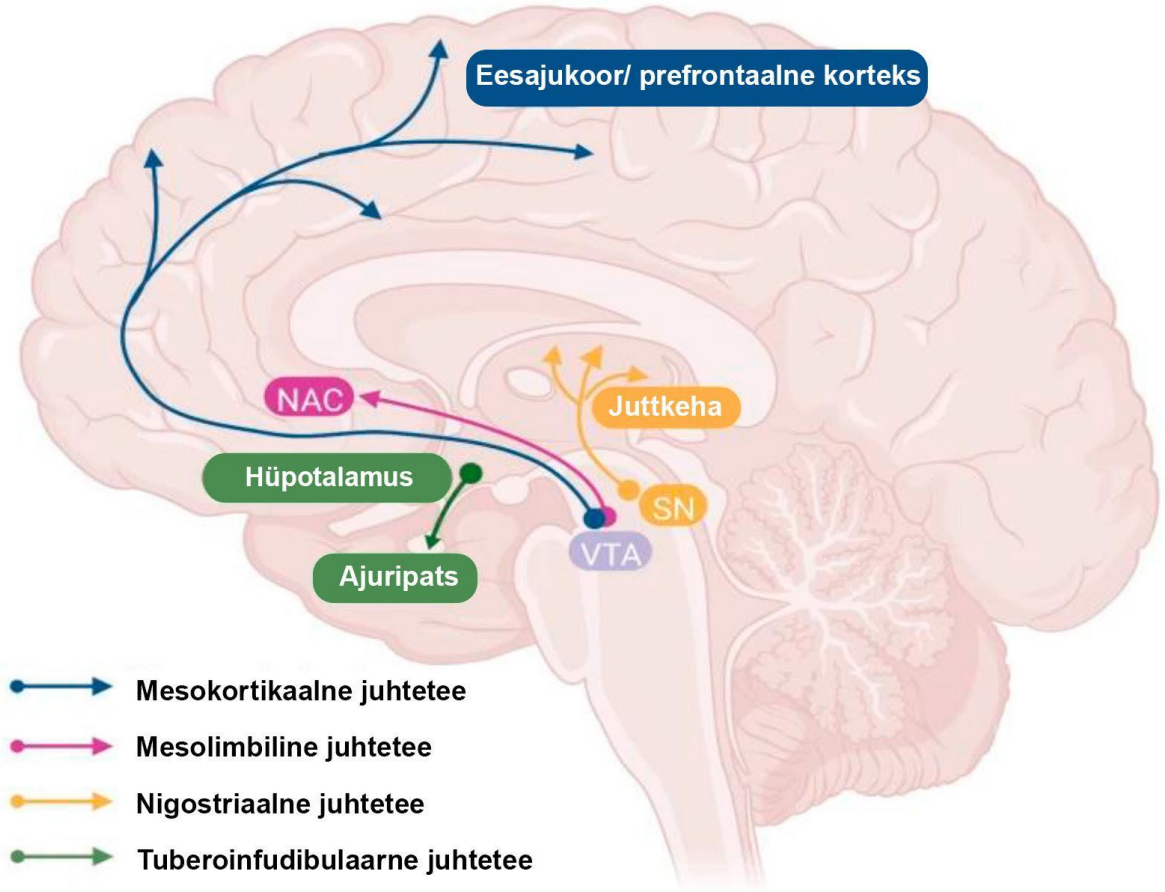
Dopamiin on ajus oluline neurotransmitter, mille sünteesiks (Joonis 1) on tarvis L-türosiini (Schatzberg & Nemeroff, 2004), mida kodeerib Türosiini hüdroksülaasi (*TH*) geen (*NCBI*, s.a.). Türosiin muudetakse türosiini hüdroksülaasi abil L-dihüdroksüfenüülalaniiniks (L-DOPA), seejärel peab toimet avaldama L-aromaatne aminohapete dekarboksülaas (AADC) (Schatzberg & Nemeroff, 2004). Dopamiini transporteri (*DAT*) geen kodeerib DA transporterit, mille tandemkorduste arvu erinevusi seostatakse muuseas ka alkoholi- ja kokaiinisõltuvusega (NCBI, s.a.). DA tootmistegevus lõppeb, kui dopamiini transporterite (DAT) abil suunatakse dopamiin sünapsist presünaptilisse neuronisse. DAT töötab nagu naatrium-kaaliumpump, eemaldades DA sünaptilisest pilust. Monoamiini oksüdaasi A (*MAO-A*) ja monoamiini oksüdaasi B (*MAO-B*) geenid kodeerivad monoamiini oksüdaasi A või B (MAO-A/B), mille toimel metaboliseeritakse dopamiin dihüroksüfenüülalaniiniks (DOPAC). Vastasel juhul ladustatakse sekretoorsetesse vesiikulitesse vesiikuli monoamiini transporterite (VMAT) abil. (Schatzberg & Nemeroff, 2004) Metüülrühma üle kandmist dopamiinile katalüüsib katehhool-O-metüültransferaasi geeni (*COMT*) poolt kodeeritud COMT valk (NCBI, s.a.). Neuronites ja gliiarakkudes võib DA laguneda katehhool-O-metüültransferaasi (COMT) või MAO toimel homovanilliinhappeks (HVA) (Xu & Yang, 2022). Dopaminergiliste neuronite aktivatsioonist põhjustatud elektrilaengute muutus rakumembraani mõlemal poolel toob endaga kaasa dopamiini vabanemise (Di Chiara, 1997). Peale presünaptilisest terminalist vabanenemist on võimalik interakteeruda viie erineva retseptoriga D_1-D_5 (Schatzberg & Nemeroff, 2004). Dopamiini retseptorid jagatakse kahte rühma: D_1 tüüpi retseptorid, kuhu alla kuuluvad D_1 ja D_5, ning D_2 tüüpi retseptorid, kuhu kuuluvad D_2, D_3 ja D_4 retseptor (Xu & Yang, 2022). D_1 tüüpi retseptorid seonduvad G-valku stimuleerivate kohtadega ja D_2 tüüpi retseptorit G-valku inhibeerivate kohtadega. D_2, D_3 ja D_4 retseptoreid peetakse oluliseks inimese dopamiini neuronite ellujäämise ja neuronite arengu signaliseerimises (Bhatia et al., 2023). Dopamiini retseptori geenid *DR1* ja *DR2* kuuluvad seitsmetransmembraani domeeniga perekonda. Seega transkribeeritud valgud DR1 ja DR2

läbivad plasmamembraani seitse korda ja on ühendatud G-valkudega (Schatzberg & Nemeroff, 2004).



Joonis 1. Dopamiini neuron ja närvi ülekanne (Modifitseeritud Xu & Yang, 2022 järgi). Türosiin muutub türosiini hüdroksülaasi abil L-dihüdroksüfenüülalaniiniks, millest omakorda L-aromaatne aminohapete dekarboksülaasi toimel moodustub dopamiin. Seejärel suunatakse dopamiin dopamiini transporterite abil sünapsist presünaptilisse neuronisse või ladustatakse sekretoorsetesse vesiikulitesse vesiikuli monoamiini transporterite kaasabil. Neuronites ja gliiarakkudes võib dopamiini katehhool-O-metüültransferaasi või monoamiini oksüdaasi toimel lagundada homovanilliinhappeks. Peale vabanemist võib dopamiin interakteeruda ka dopamiini retseptoritega.

1.2. Sõltuvuskäitumise ning meeleoluga seotud ajupiirkonnad



Joonis 2. Dopamiini rajad ajus (Modifitseeritud Xu & Yang, 2022 järgi). Nigostriaalne juhtetee on märgitud kollasega ja kulgeb *substantia nigrast* (SN) juttkehasse. Sinisega on välja toodud mesokortikaalne juhtetee, mis saab alguse ventraalsest tegmentaalsest piirkonnast (VTA) ja suubub frontaalkoorde. Rada, mis näidatud rohelisega saab alguse hüpotalamusest ja projetseerub hüpofüüsi eessagarasse, kannab nime tuberoinfundibulaarne juhtetee. Roosaga on välja toodud mesolimbiline juhtetee, mis kulgeb VTA-st ventraalsesse striaatumisse.

Nigostriaalne süsteem (Joonis 2, märgitud kollasega) saab alguse *substantia nigrast* (SN) ja kulgeb *striatum'*isse ning taalamusse (Schatzberg & Nemeroff, 2004). Nigostriaalne süsteem vastutab õppimise ja motoorse kontrolli eest kehas (Xu & Yang, 2022). Tuberoinfundibulaarne dopamiini süsteem (Joonis 2, märgitud rohelisega) suundub hüpotalamusest hüpofüüsi eessagarasse (Schatzberg & Nemeroff, 2004). Üksus reguleerib prolaktiini sekretsiooni (Xu & Yang, 2022).

Töös keskendutakse peamiselt mesolimbilise raja uurimisele (Joonis 2, märgitud roosaga). Mesolimbilise dopaminergilise juhtetee moodustavad ventraalses keskajus asuv ventraalne tegmentaalne piirkond (VTA), mis projetseerub ventraalsesse striaatumi (VST), eelkõige

*nucleus accumbens'*isse (Ikemoto, 2010; Schatzberg & Nemeroff, 2004). VTA asub mediaalselt *substantia nigra* suhtes (Schatzberg & Nemeroff, 2004) ja koosneb dopaminergilistest neuronitest, GABAergilistest (γ-võihappe) neuronitest ning glutamaatneuronitest (Cai & Tong, 2022).

VTA vastutab inimestel tasu ja stressi süsteemi töötlemise, aga ka mälu ja õppimise eest. Lisaks on tihedalt seotud ka uimastisõltuvus käitumisega (Cai & Tong, 2022), vahendades nikotiini, opiaatide, kannabinoidide ja etanooli rahuldust pakkuvat toimet. On leitud, et kui selektiivselt kahjustada VTA dopamiini neuroneid, siis nikotiini intravenoosne manustamine rottidel väheneb (Ikemoto, 2007). VST-d on seostatud ka motivatsiooniga (Di Chiara, 1997).

1.3. Sõltuvushäired, motivatsioon ja nende seos NEGR1-ga

Inimesed on üritanud oma meeleolu ja selle tekkepõhjuseid juba ammusest ajast uurida. Vana-Kreekas õpetas Hippocrates oma järgijatele, et meeleolu on sõltuv vere, lima, kollase- ja must sapi tasakaalust kehas. Tema õpetuse kohaselt peeti musta sappi meeleheite, hirmu ja masenduse allikaks, mis arvati olevat ühe inimkonna vanima haiguse ehk depressiooni põhjus (Kandel et al., 2021).

1.3.1. Toitumis- ja alkoholi tarbimiskäitumine

Dopamiin mõjutab tasusüsteemi mesolimblises rajas ja sellega ka tarbimiskäitumist. Mitmed meelemürgid on võimelised alterneerima dopamiini transporterite tööd (Schatzberg & Nemeroff, 2004). D_2 retseptorid mängivad olulist rolli narkootikumide ja alkoholi sõltuvuse kujunemises, vahendades meelemürkidest saadud tugevat toimet (nauding, eufooria) otsimissüsteemi rahuldamise ja tasusüsteemi aktiveerimise näol (Volkow et al., 2002). Etanool suurendab DA neuronite langlemise määra, mis omakorda soodustab dopamiini vabanemist VST-s (Brodie et al., 1990; Zhu & Reith, 2008). Tiihonen jt. (1995) on näidanud, et vägivaldsetel alkohoolikutel on DR tihedus suurem kui kontrollgrupil. Seevastu alkoholi kuritarvitajatel, kes ei ole vägivaldsed, on kontrollgrupiga võrreldes DR paiknemistihedus oluliselt madalam, mis viitab ka vähenenud dopamiini aktiivsusele (Tiihonen et al., 1995; Volkow et al., 2002). Lisaks on näidatud, et ravimid, mis suurendavad dopamiini taset ajus, soodustavad kaalulangust ja vastupidiselt dopamiini D_2 retseptoreid blokeerivad ravimid suurendavad isu ning põhjustavad kaalutõusu (Wang et al., 2001).

Di Chiara (1997) on välja pakkunud, et alkoholil on VST-s kaks dopamiini vabanemist soodustavat süsteemi, millest esimene on seotud alkoholi maitsega ja teine alkoholi mõjuga ajule. Teooria kohaselt aktiveerides alkoholist põhjustatud dopamiinergilise signaali ülekannet võib tugevneda ka maitsmisstiimulite motiveerivad omadused. Maitsmisstiimulid muutuvad selle käigus motivatsioonilisteks ja panevad tarbija alkoholi veel enam otsima. Kui enamasti tavapärase stiimuli poolt esile kutsutud suurenenud dopamiini vabanemine tekitab lõpuks mingisuguse harjumuse, misjärel stiimulite korduv esitamine ei päädi enam dopamiini vabanemisega, siis alkoholiga sellist harjumust ei teki. Alkoholiga seotud stiimulid omandavad aja jooksul emotsionaalse ja motivatsioonilise tähtsuse, mis moodustavadki oma olumuselt sõltuvuse. (Di Chiara, 1997)

Anhedoonia väljendub võimetuses tunda rõõmu asjadest, mis peaksid pakkuma rahuldust. Anhedooniat peetakse depressiooni üheks peamiseks sümptomiks koos alanenud meeleolu ja energia vähenemisega (Höflich et al., 2018; Liu et al., 2018; RHK-10, s.a.). Liu ja teised näitasid sahharoosi eelistuskatses, et hiirtel, kes on pikemaajaliselt puutunud kokku kerge kroonilise stressiga, esineb vähenenud reageerimine tasule (Liu et al., 2018). Lisaks on ka näidatud, et kõrge rasvasisaldusega dieet päädib hiirtel VTA aktiivsuse madaldumise ja anhedooniaga (Altherr et al., 2021). Depressioon on levinud häire, mille all umbes 5% maailma populatsioonist kannatab (WHO, s.a.). Ka alkoholi tarvitamisest tingitud kahjud võivad olla psüühilised ja sõltlastel võib välja kujuneda depressioon (RHK-10). Howe jt. (2021) näitasid, et alkoholi sõltuvusega inimestel olid kõrgenenud ärevuse ja depressiooni sümptomid ning inimestel, kes tarvitasid mitut meelemürki korraga, olid tulemused veelgi kõrgemad (Howe et al., 2021).

1.3.2. Sõltuvuskäitumine ja *NEGR1*

Lisaks keskkonnateguritele on suuresti meeleoluhäirete ja sõltuvushäirete mõjutajaks ka geneetika. Kaare jt. (2022) uurisid *Negr1*-puudulikkusega hiirte (*Negr1*$^{-/-}$) ja metsik-tüüpi kontroll hiirte (WT) kehakaalu erinevusi. Selgus, et WT hiirte kehakaal langes nädala jooksul keskmiselt 0,27g, aga *Negr1*$^{-/-}$ hiired kosusid keskmiselt 0,69g võrra (Kaare et al., 2022). Lisaks on näidatud, et hiired, kellel on madal *Negr1* ekspressioonitase, söövad vabalt kätte saadavat toitu suuremates kogustes. Selline käitumine viib kaalutõusuni (Boender et al., 2014).

NEGR1, toitumiskäitumise- ja mesolimbilise juhtetee vahelised seosed annavad alust arvata, et ka alkoholi tarbimiskäitumises on *NEGR1* valgul osalus. Seoseid alkoholi tarbimiskäitumise ja *NEGR1* vahel on uuritud võrdlemisi vähe. Siiski on näidatud, et polümorfismid *NEGR1* geenis on oluliselt ($p= 2 \times 10^{-16}$) seotud sellega, kui suure koguse alkoholi inimene päevas tarbib (Saunders et al., 2022).

1.4. IgLON superperekond

Antud töös vaadatakse lähemalt IgLON valguperekonda, mille liikmeid on seostatud ka ülekaalulisuse ja depressiooniga.

Immunoglobuliinilaadsed raku adhesioonimolekulid (IgCAM) sisaldavad ühte või mitut Ig-domeeni (Cavallaro & Christofori, 2004). Immunoglobuliinides esineb mitut varianti domeene: V-domeen ehk muutuv (*variable),* C-domeen ehk konstantne (*constant)* ja lisaks C2 domeen. C2 domeenid on üleüldiselt sarnasemad V-domeenile, aga suuruse poolest pigem C-domeenide moodi (Barclay, 2003). Raku adhesioonimolekulid mängivad olulist rolli nii immuunsüsteemis kui ka närvisüsteemi arengus (Walsh & Doherty, 1997).
IgLON on IgCAM alla kuuluv supervalgu perekond, kuhu kuuluvad: neurotrimin (edaspidi NTM), *opioid-binding cell adhesion molecule* (edaspidi OPCML), *limbic system-associated membrane protein* (edaspidi LSAMP), IgLON5 ja *neuronal growth regulator* 1 (edaspidi NEGR1)/Kilon (*Kindred of* IgLON) (Venkannagari et al., 2020; Funatsu et al., 1999). Molekulid koosnevad kolmest C2 tüüpi immunoglobuliini domeenist ja kinnituvad plasmamembraanile GPI (glükosüülfosfatidüülinositool) ankruga (Kubick et al., 2018; Venkannagari et al., 2020; Funatsu et al., 1999). Kõik IgLon perekonna adhesioonimolekulid, peale NTMi, on leitavad nii neuronite pinnalt kui ka oligodentrotsüütidelt. NTM on omane vaid neuronitele (Venkannagari et al., 2020). Kuigi NTM, LSAMP ja OBCAM rolle on vähem kirjeldatud kui Kiloni omasid, on siiski teada, et ka need suurendavad või vähendavad neuronite adhesiivsust, neuriidi väljakasvu ja sünapsite moodustumist (Venkannagari et al., 2020; Kubick et al., 2018), formeerides homo- või heterofiilseid komplekse nii rakupinnal kui ka kõrvalolevate rakkudega (Sanz et al., 2015).

1.4.1. NEGR1

Geneetika mängib sõltuvus- ja meeleoluhäirete juures suurt rolli. Antud töös võetakse vaatluse alla IgLON perekonna neuraalse adhesioonimolekuli NEGR1 (*neuronal growth*

regulator) valk ja proteiini kodeeriv geen *NEGR1*. Geen asub inimesel 1. kromosoomi pikas õlas (1p31.1) (*NCBI*, s.a.), kust toimub 46 kDa suuruse valgu NEGR1 transkribeerimine. Kilon ekspresseerub peamiselt hippokampuses, suurajukoores, aju tüves ja väikeajus (Funatsu et al., 1999; Miyata et al., 2003). NEGR1 on seotud paljude protsessidega ajus, valk osaleb neuriitide väljakasvus ja neuriitide rändel sihtpiirkonda. Lisaks ka neurogeneesis, kus paigutub postsünaptilistesse kohtadesse dentriitilistes ja somaatilistes sünapsides (Venkannagari et al., 2020). Need protsessid mõjutavad ka juhteteede võrgustike moodustamist.

NEGR1 geen seostub inimgeneetika (GWAS) uuringutes tugevalt mitmete haiguste ja häiretega, näiteks ülekaalulisuse, depressiooni (MDD), skisofreenia ja autismispektri häiretega (Venkannagari et al., 2020). Samuti seostub NEGR1 geen GWAS uuringutes mitmete sõltuvuse ja impulsikontrolliga seotud fenotüüpidega nagu tarbitava toidu kogus ja alkoholi tarbimine (Merino et al., 2022; Zhou et al., 2020). Füsioloogia osakonnas on varem näidatud, et Negr1 puudulikkusega hiired söövad väiksemaid toidukoguseid (Kaare et al., 2021).

2. EKSPERIMENTAALOSA

2.1. Töö eesmärgid

Kuna Negr1 geen seostub mitmete sõltuvuse ja impulsikontrolliga seotud fenotüüpidega ning Negr1 puudulikkusega hiired söövad vähem, oli käesoleva töö üldisemaks eesmärgiks uurida võimalikke kõrvalekaldeid *Negr1*-puudulikkusega hiirte dopaminergilises mesolimbilises süsteemis. Täpsemalt, dopamiinisüsteemiga seotud geenide ekspressiooni muutusi *Negr1*-puudulikkusega hiirte aju VTA ning VST piirkondades.

Spetsiifilisemad eesmärgid olid:

- mõõta qRT-PCR-iga geenieksрessiooni tasemeid signaaliülekandega seotud geenides, dopamiini retseptor 1, dopamiini retseptor 2, dopamiini transporteri, nii *Negr1*-puudulikkusega kui ka metsik-tüüpi isastel hiirtel;
- uurida erinevusi mRNA ekspressioonis $Negr^{-/-}$ ja WT isastel hiirtel dopamiini sünteesis ja lammutamises osalevates geenides nagu türosiini hüdroksülaasi, monoamiini oksüdaas A, monoamiini oksüdaas B ja katehhool-O-metüültransferaasi.

2.2. Materjalid ja metoodika

2.2.1. Katseloomad ja koeproovid

Töös kasutatud katseloomade paljundamine ja hooldus viidi läbi Tartu Ülikooli bio- ja siirdemeditsiini katseloomakeskuses. Katseloomadeks olid isased metsik-tüüpi kontrollhiired (WT) ja pesakonnakaaslastest homosügootsed *Negr1*-puudulikud hiired ($Negr1^{-/-}$). Hiired olid paigutatud rühmiti standardsetesse laboratoorsetesse puuridesse, kus olemas pesamaterjal, toidugraanulid ja vesi. Ajukudede kogumisel olid hiired nelja kuu vanused ($Negr^{-/-}$ n=6-10 ja $Negr^{+/+}$ n=6-10). Katseloomadel uuriti VTA ja VST kudesid. Hiirte dekapitatsioon ja ajuosade dissektsioon peaajust viidi läbi vastavat FELASA sertifikaati omava spetsialist poolt.

2.2.2. RNA eraldamine

RNA eraldamiseks kasutas autor Trizol® reagenti (Invitrogen) tootja juhendi järgi. Esmalt lisati koetükile 50-100 µl Trizol reagenti ja homogeniseeriti plastikuhmriga jääl. Seejärel lisati ülejäänud reagent, et maht oleks kokku 750 µl ja proovid litsuti umbes 1 mm paksuseks. Proove inkubeeriti toatemperatuuril viis minutit. Seejärel lisati 150 µl kloroformi ja raputati eppendorfe (Axygen®) umbes 15 sekundit. Tuube inkubeeriti veel kaks minutit

toatemperatuuril ja seejärel tsentrifuugiti proove eeljahutatud tsentrifuugis (Thermo Scientific™ Fresco™ 17 Microcentrifuge) 15 minutit 12 000 xg 4℃ juures. Fuugimise tulemuseks oli kolm kihti: roosa, mis sisaldab valke, suhkruid ja kromosomaalset DNAd; valge, mis sisaldab lipiide, ja ülemine vesifaas, mis sisaldab RNAd. Vesifaas tõsteti uude tuubi ja RNA sadestamiseks lisati isopropanooli pool Trizoli mahust. Seejärel segati proovi tuubi käes pöörates ning inkubeeriti kümme minutit toatemperatuuril. Pärast seda tsentrifuugiti proove taas eeljahutatud tsentrifuugis kümme minutit 12 000 xg 4℃ juures. Eemaldati supernatant ja RNAlt soolade maha pesemiseks lisati tuubi üks ml jääkülma 75% etanooli ja fuugiti 7500 xg 4℃ juures viis minutit. Taaskord eemaldati supernatant, tuubi täielikuks kuivatamiseks pandi see lühiajaliselt lahtise kaanega 37℃ termostaadile. Seejärel suspendeeriti proove 50 µl destilleeritud vees (ddH_2O) ja mõõdeti RNA kontsentratsioon lahuses. Kontsentratsiooni mõõtmine sooritati NanoDrop ND-1000 spektrofotomeetriga (NanoDrop Technologies). Antud RNA proove säilitati -80℃ juures.

2.2.3. cDNA süntees

Töös kasutati pöördtranskriptsiooniks FIREScript® RT cDNA juhusliku järjestusega kuuenukleotiidiste praimerite segu (*random hexamer*) (Solis BioDyne) vastavalt tootja juhendile. Peale RNA eraldamist sünteesiti komplementaarne DNA ehk cDNA. Selleks võeti eelnevalt saadud lahused jääle sulama. Kõrge kontsentratsiooni korral pipeteeriti uude eppendorfi 10 µl RNA lahust ja madala kontsentratsiooni korral kuni 16,5 µl. Uude tuubi tõstetud lahusele lisati kaks µl juhusliku järjestusega kuuenukleotiidiste praimerite segu (*random hexamer*). Seejärel lisati vastavalt vajadusele nukleaasi vaba ddH_2O, kogumahuni 18,5 µl. Vee lisamise järel proov segati vorteksil ja tsentrifuugiti lauatsentrifuugis põhja. Kõige viimasena suspendeeriti pipetiga lahusesse 1,5 µl FIREScript® Enzyme Mix, et proovi kogumaht oleks 20 µl. Saadud segu inkubeeriti viis minutit toatemperatuuril. Pöördtranskriptsiooni toimumiseks asetas autor lahuse 30 minutiks kuni üheks tunniks 50°C kuumablokile. Pärast asetati proov viieks minutiks 85°C kuumablokile, et inaktiveerida ensüüme. Antud cDNA proove hoiustati -80°C juures.

2.2.4. Reaalaja-PCR

Enne PCR reaktsioone lahjendati cDNA proove 20 µl vees. Geeni arvu kvantitatiivseks mõõtmiseks sooritati geenikspressioon reaalaja-PCR (qRT-PCR) meetodiga. qRT-PCR-ga uuriti türosiini hüdroksülaasi (*Tyrosine hydroxulase, Th*), dopamiini retseptor 1 (*Dopamine*

receptor 1, Dr1), dopamiini retseptor 2 (*Dopamine receptor 2, Dr2*), dopamiini transporteri (*Dopamine transporter, Dat*), monoamiini oksüdaas A (*Maoa*), monoamiini oksüdaas B (*Maob*) ja katehhool-O-metüültransferaasi (*Catechol-O-methyltransferase, Comt*) ekspresseerumist VTA-s ning VST-s. Esmalt toimub DNA denaturatsioon, seejärel taas DNA kokkusulandumine. Kokkusulandumise käigus seonduvad praimerid DNA üksikahela komplementaarsetele aladele. Praimerile seonduv HOT FIREPol® DNA polümeraas sünteesib 3`-5`suunas DNA üksikahelale komplementaarse ahela. EvaGreen® värv seondub tekkinud DNA kaksikahelale. Värvi fluorestsentsi mõõdetakse pärast iga tsüklit, kokku mõõdeti 40 tsüklit.

Ekspressiooniks kasutati HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne) segu. Segu sisaldab HOT FIREPol® DNA polümeraasi, 5x EvaGreen® qPCR puhvrit (12,5 mM $MgCl_2$), dNTP-sid, EvaGreen® värvi, ROX värvi, GC-enhanserit ja sinist visualiseerimisvärvi. Eelnevale qPCR segule lisati 1,5 µl sünteesitud cDNA-d, ddH_2O. Kooslus segati vorteksil ning seejärel lisati vastavad praimerid (LISA 1): monoamiini oksüdaas A, monoamiini oksüdaas B, dopamiini transporter, katehool-O-metüültransferaas, dopamiini retseptor 1, dopamiini retseptor 2, türosiini hüdroksülaas (Varul et al., 2021). Kontrolliks tehti praimerite kõrvale koduhoidja beeta-aktiin (*ACT-B*). Reaktsiooni maht oli 10 µl ja igast proovist villiti neli kordust. Andmed analüüsiti ja teisendati kujule $2^{-\Delta CT}$.

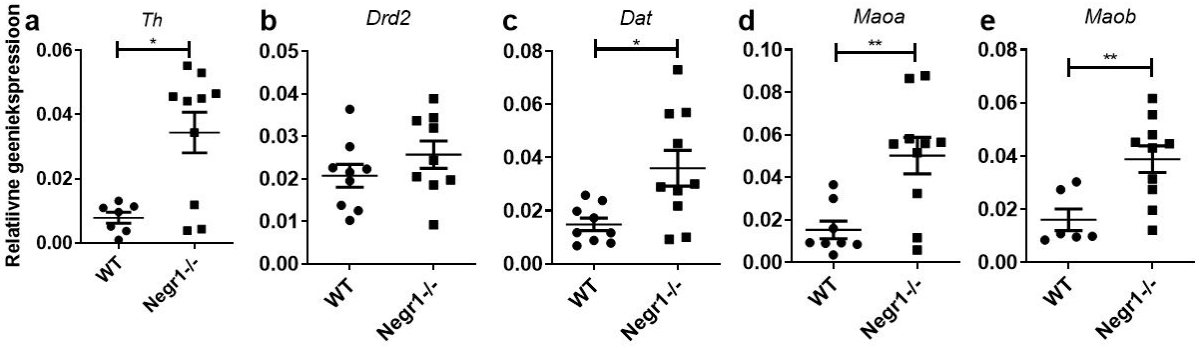
2.2.5. Statistiline analüüs

Käesolevas töös uuriti geeni ekspressiooni VTA-s (Joonis 3, Tabel 1) ja VST-s (Joonis 4, Tabel 2) metsiktüüpi (WT) ja *Negr1* puudulikel hiirtel. Vastavalt töö eesmärgile leida kõrvalekaldeid dopaminergilises mesolimbilises rajas võeti vaatluse alla seitse geeni: *Th*, *Dr1*, *Dr2*, *Dat*, *Maoa*, *Maob* ja *Comt*. Esmalt kontrolliti, kas andmed vastavad normaaljaotusele, kasutades Shapiro-Wilki testi. Seejärel viidi läbi reaalaja-PCRi andmete statistiline analüüs normaaljaotuse vastavusele, kas Studenti t-testi või Mann-Whitney U-testi abil. Kui andmed vastasid normaaljaotusele, siis kasutati Studenti t-testi, mitteparameetriliste andmete puhul kasutati analüüsiks Mann-Whitney U-testi. Tulemused väljendati keskmiste väärtustena koos keskväärtuse standardveaga (*standard error of the mean*, SEM). Kontroll- ja testgrupi vaheline seos loeti statistiliselt oluliseks kui p väärtus oli alla 0,05. Tulemused esitati $2^{-\Delta CT}$ kujul ja vastava arvtabelina. Andmete analüüsiks kasutati GraphPad Prism 8.

Kõik tulemused on kajastatud Kaare jt. 2022. aasta uuringus.

2.2.6. Tulemused ja arutelu

Antud töö eesmärgiks oli uurida kõrvalekaldeid *Negr1*-puudulikkusega hiirte dopaminergilises mesolimbilises juhtetees, keskendudes muutustele ventraalses tegmentaalses piirkonnas ja ventraalses striaatumis. Varasemalt on näidatud, et *DAT* vastutab dopamiini tagasihaare eest presünaptilistesse neuronitesse ja reguleerib dopamiinergilise ülekande lõpetamist (Hagerty et al., 2016). Tiihonen jt. (1995) näitasid, et alkohoolikutel on dopamiini retseptorite paiknemise tihedus oluliselt madalam. See viitab vähenenud dopamiini aktiivsusele ja muutused selles süsteemis avaldavad mõju tasusüsteemile (Hagerty et al., 2016). Selle töö käigus leiti mitu statistiliselt olulist tulemust, mis viitavad dopaminergilise mesolimbilise juhtetee muutustele *Negr1*$^{-/-}$ hiirte ajudes. Kõrvalekalded *Negr1*-puudulikkusega hiirte dopaminergilises mesolimbilises juhtetees, täpsemalt ventraalses tegmentaalses piirkonnas ja ventraalses striaatumis annavad alust oletada, et neuraalne adhesioonimolekul Negr1 mõjutab dopaminergilist mesolimbilist juhteteed, mis omakorda avaldab mõju tarbimiskäitumisele.



Joonis 3. Dopamiinisüsteemiga seotud relatiivse geenieksprессiooni tasemed VTA-s. Geenide **(a)** türosiini hüroksülaas (*Th)*, **(b)** dopamiini retseptor 2 (*Drd2)*, **(c)** dopamiini transporter (*Dat*), **(d)** monoamiini oksüdaas A (*Maoa*), **(e)** monoamiini oksüdaas B (*Maob*) mRNA ekspressioonitasemed metsiktüüpi (WT) ja *Negr1* puudulikel hiirtel ventraalses tegmentaalses piirkonnas. Joonisel on ühe tärniga (*) näidatud keskmised väärtused ± SEM, kus p<0,05 ja kahe tärniga (**) kui p<0,01. Tulemused on leitavad artiklis (LISA 2) "*Depression-associated Negr1 gene-deficiency induces alterations in the monoaminergic neurotransmission enhancing time-dependent sensitization to amphetamine in male mice*" (Kaare et al., 2022)

Tabel 1. **Dopamiinisüsteemiga seotud relatiivse geenieksprессiooni tasemed VTA-s.** Statistilise olulisuse määramiseks sooritati vastavalt normaaljaotusele kas Mann-Whitney U-test või Student t-test. Tulemused väljendati keskmiste väärtustena koos keskväärtuse standardveaga. Statistiliselt olulised väärtused on märgitult tumedamalt. *Negr1*$^{-/-}$ hiirtel oli *Dat* tase VTA-s märkimisväärselt kõrgem võrreldes WT hiirtega (p = 0,011). Statistiliselt olulisi erinevusi täheldati veel *Th* (p = 0,019), *Mao a* (p = 0,009) ja *Mao b* (p = 0,005) mRNA

ekspressiooni tasemetes, kus *Negr1*$^{-/-}$ hiirte VTA-s olid antud geenid oluliselt kõrgemalt ekspresseeritud. *Drd2* tulemus ei olnud statistiliselt oluline.

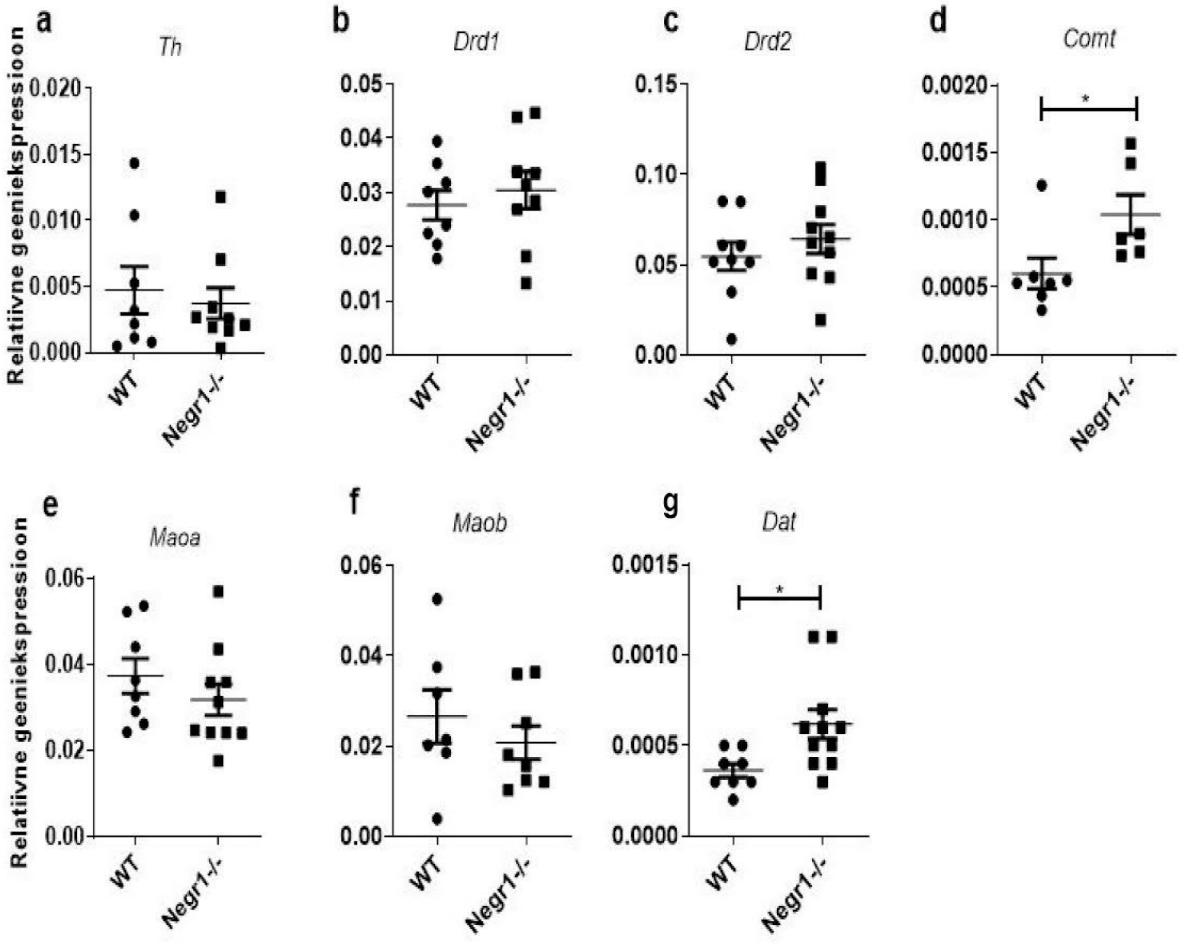
					p väärtus	
VTA	**WT** keskmine ± SEM	**n WT**	*Negr1*$^{-/-}$ keskmine ± SEM	**n *Negr1***	**Mann-Whitney U-test**	**t-test**
Th	0.008 ± 0.002	7	0.034 ± 0.006	10	**0.019**	-
Drd2	0.021 ± 0.003	9	0.026 ± 0.003	9	-	0.252
Dat	0.015 ± 0.002	9	0.050 ± 0.009	10	-	**0.011**
Maoa	0.015 ± 0.004	8	0.050 ± 0.009	10	**0.009**	-
Maob	0.016 ± 0.004	6	0.039 ± 0.005	10	**0.005**	-

Joonisel 3 ja tabelis 1 esitatud transkriptidest olid statistilised olulised *Th, Dat*, *Maoa* ja *Maob*. *Th* ekspressiooni keskmine tase metsik-tüüpi kontrollhiirtes oli 0,008 ja 0,034 *Negr1*$^{-/-}$ hiirtel. *Th* SEM oli WT hiirtel ±0,002 ja *Negr1*$^{-/-}$ ±0,006, kahe grupi vaheline p=0,019 (Joonis 3, a; Tabel 1, *Th*). *Drd2* ekspressiooni keskmine tase WT oli 0,021 ja 0,026 *Negr1*$^{-/-}$ hiirtel. *Drd2* SEM oli WT ja *Negr1*$^{-/-}$ mõlemal ±0,003, kahe grupi vaheline p=0,252 (Joonis 3, b; Tabel 1, *Drd2*). *Dat* ekspressiooni keskmine tase WT oli 0,015 ja 0,050 *Negr1*$^{-/-}$ hiirtel. WT *Dat* SEM oli ±0,002 ja *Negr1*$^{-/-}$ hiirtel ±0,009, kahe grupi vaheline p=0,011 (Joonis 3, c; Tabel 1, *Dat*). *Maoa* ekspressiooni keskmine tase WT hiirtel oli 0,015 ja *Negr1*$^{-/-}$ hiirtel 0,050. *Maoa* SEM oli WT hiirtel ±0,004 ja *Negr1*$^{-/-}$ ±0,009, kahe grupi vaheline p=0,009 (Joonis 3, d; Tabel 1, *Maoa*). *Maob* keskmine ekspressiooni tase WT hiirtel oli 0,016 ja *Negr1*$^{-/-}$ hiirtel 0,039. *Maob* SEM oli WT hiirtel ±0,004 ja *Negr1*$^{-/-}$ ±0,005, kahe grupi vaheline p=0,005 (Joonis 3, e; Tabel 1, *Maob*).

Pidev ligipääs toidule ehk normaalne toidu tarbimine madaldab DA vabanemist (Bassareo & Di Chiara, 1997), kuid Lindblom jt. on näidanud, et kui piirata pidevalt kättesaadavat toitu, siis vastuseks on türosiini hüdroksülaasi ja dopamiini transporteri mRNA ekspressiooni tõus VTA-s. Lisaks toimub sel juhul VTA-s DAT valgu üleregulatsioon. (Lindblom et al., 2006)

Sarnased tulemused esinevad ka antud töös *Negr1*$^{-/-}$ hiirte puhul, kus *Negr1* puudulikkusega hiirte VTA-s olid kõrgemalt ekspresseerunud eelkõige *Th* ja *Dat*. See võib viidata, et *Negr1*-puudulikkus tekitab ajus samasugust efekti nagu krooniline söögi piiramine.

Ühtlasi selgus antud töö tulemustest, et *Negr1*-puudulikkusega hiirtel on *Maoa, Maob* ja *Comt* kõrgemalt ekspresseeritud kui liigikaaslastest WT hiirtel. On täheldatud, et anoreksiaga rottide juttkehas on DA metaboliitide tase madalam (Giunti et al., 2023). Võib oletada, et rottidel, kes söövad rohkem, nagu antud olukorras *Negr1*$^{-/-}$ hiired seda teha võivad, on dopamiini metaboliseerivate geenide ekspressioon seega kõrgem ja dopamiini käive (*turnover*) toimub kiiremini.



Joonis 4. Dopamiinisüsteemiga seotud relatiivse geenieksprессiooni tasemed ventraalses striaatumis. Geenide **(a)** türosiini hüroksülaas (*Th)*, **(b)** dopamiini retseptor 1 (*Drd1)*, **(c)** dopamiini retseptor 2 (*Drd2),* **(d)** katehhool-O-metüültransferaas (*Comt*), **(e)** monoamiini oksüdaas A (*Maoa*), **(f)** monoamiini oksüdaas B (*Maob*) **(g)** dopamiini transporter (*Dat*) ekspressioonitasemed metsiktüüpi (WT) ja *Negr1*-puudulikel hiirtel ventraalses stria(a)tumis. Joonisel on ühe tärniga (*) näidatud keskmised väärtused ± SEM, kus $p<0,05$. Tulemused on avaldatud artiklis (LISA 2) "*Depression-associated Negr1 gene-deficiency induces alterations in the monoaminergic neurotransmission enhancing time-dependent sensitization to amphetamine in male mice*" (Kaare et al., 2022).

Tabel 2. Dopamiinisüsteemiga seotud geenieksрessiooni tasemed ventraalses striaatumis. Statistilise olulisuse määramiseks sooritati vastavalt normaaljaotusele kas Mann-Whitney U-test või Student t-test. Tulemused väljendati keskmiste väärtustena koos keskväärtuse standardveaga. Statistiliselt olulised väärtused on märgitult tumedamalt. VTS-s dopamiinisüsteemiga seotud geenid *Dat* (p = 0,011) ja Comt (p = 0,014) tasemed olid *Negr1*$^{-/-}$ hiirtel võrreldes WT-hiirtega märkimisväärselt kõrgemad. *Th, Drd1, Drd2, Maoa* ja *Maob* tulemused ei olnud statistiliselt olulised.

					p väärtus	
VTS	**WT** keskmine ± SEM	**n WT**	***Negr1*$^{-/-}$** keskmine ± SEM	**n *Negr1***	**Mann-Whitney U-test**	**t-test**
Th	0.005 ± 0.002	8	0.004 ± 0.001	9	0.962	-
Drd1	0.028 ± 0.003	8	0.03 ± 0.003	9	-	0.543
Drd2	0.055 ± 0.008	9	0.064 ± 0.008	10	-	0.401
Comt	0.0006 ± 0.0001	7	0.001 ± 0.0001	6	**0.014**	-
Maoa	0.037 ± 0.004	8	0.032 ± 0.004	10	-	0.332
Maob	0.027 ± 0.006	7	0.021 ± 0.004	8	0.332	-
Dat	0.0003 ±0 .00004	8	0.0006 ± 0.00008	10	**0.011**	-

Joonisel 4 ja tabelis 2 esitatud transkriptidest olid statistilised olulised *Dat* ja *Comt*. *Th* ekspressiooni keskmine tase WT oli 0,005 ja 0,004 *Negr1*$^{-/-}$ hiirtel. *Th* SEM oli WT hiirtel ±0,002 ja *Negr1*$^{-/-}$ ±0,001, kahe grupi vaheline p=0,962 (Joonis 4, a; Tabel 2, *Th*). *Drd1* ekspressiooni keskmine tase WT oli 0,028 ja 0,03 *Negr1*$^{-/-}$ hiirtel. *Drd1* SEM oli WT ja *Negr1*$^{-/-}$ mõlemal ±0,003, kahe grupi vaheline p=0,543 (Joonis 4, b; Tabel 2, *Drd1*). *Drd2* ekspressiooni keskmine tase WT oli 0,055 ja 0,056 *Negr1*$^{-/-}$ hiirtel. *Drd2* SEM oli WT ja *Negr1*$^{-/-}$ mõlemal ±0,008, kahe grupi vaheline p=0,401 (Joonis 4, c; Tabel 2, *Drd2*). *Comt* ekspressiooni keskmine tase WT oli 0,0006 ja 0,001 *Negr1*$^{-/-}$ hiirtel. WT *Comt* SEM oli WT ja

Negr1$^{-/-}$ mõlemal ±0,0001, kahe grupi vaheline p=0,014 (Joonis 4, d; Tabel 2, *Comt*). *Maoa* ekspressiooni keskmine tase WT hiirtel oli 0,037 ja *Negr1*$^{-/-}$ hiirtel 0,032. *Maoa* SEM oli WT ja *Negr1*$^{-/-}$ mõlemal ±0,004, kahe grupi vaheline p=0,332 (Joonis 4, e; Tabel 2, *Maoa*). *Maob* keskmine ekspressiooni tase WT hiirtel oli 0,027 ja *Negr1*$^{-/-}$ hiirtel 0,021. *Maob* SEM oli WT hiirtel ±0,006 ja *Negr1*$^{-/-}$ ±0,004, kahe grupi vaheline p=0,332 (Joonis 4, f; Tabel 2, *Maob*). *Dat* keskmine ekspressiooni tase oli WT hiirtel oli 0,0003 ja *Negr1*$^{-/-}$ hiirtel 0,0005. *Dat* SEM oli WT hiirtel ±0,00004 ja *Negr1*$^{-/-}$ ±0,00008, kahe grupi vaheline p=0,011 (Joonis 4, g; Tabel 2, *Dat*).

Dopamiin on oluline motivatsioonisüsteemi komponent, mis paneb inimesi ja loomi otsima tasu. See paneb haarama toidu järele, et rahuldada nälga, ja sunnib sõltlast otsima meelemürki, et rahuldada vajadust. Selle käigus on oht süüa kuni rasvumiseni ja juua kuniks tervis enam ei kannata, sest ei tunta enam seda sama tasu tunnet, mis ennist. Lootuses kogeda seda tunnet uuesti langetakse tarbimise nõiaringi (Volkow et al., 2016). Nagu käesoleva bakalaureusetöö tulemustest näha, siis mõjutab *Negr1*-puudulikkus ajus *DAT* ekspressiooni tõusu. Ekspressiooni tõusu tulemuseks on suurem DA tagasihaare ajus ehk sünaptilisse pilusse jääb väheb dopamiini, mis interakteeruks retseptoritega. 2015. aastal näitas üks uurimisrühm, et kõrge *DAT* ekspressioon võib endaga kaasa tuua oksüdatiivset stressi ja isegi dopamiini neuronite kaotust (Masoud et al., 2015). Kuna *Negr1* osaleb neuriitide väljakasvus ja rändel sihtpiirkonda ning ühtlasi ka neurogeneesis, siis geeni puudumine ja valgu mitte transkribeerimine loob ebasoodsamad võimalused neuriitide arenguks ja sünapsite moodustamiseks. Lisaks sellele, kõrge *DAT* ekspressiooni ja võimaliku neuronite kaotuse tulemuseks, võib olla dopaminergilise mesolimbilise süsteemi altereerumine. Kokku võivad sellised muudatused kaasa tuua tarbimiskäitumise muutused, sõltuvushäired ja meeleolu languse.

2.2.7. Peamised järeldused

- Statistiliselt olulised muutused *Negr1*$^{-/-}$ hiirte VTA-s ja VST-s toimusid kokku viie geenieksрessiooni tasemetes: türosiini hüdroksülaasi (*Th)*, katehhool-O-metüültransferaasi (*Comt*), monoamiini oksüdaas A (*Maoa*), monoamiini oksüdaas B (*Maob*) ja dopamiini transporteri (*Dat*). Mõlemas uuritavas ajuosas kerkis esile *Dat* kõrge ekspressioonitase, mis olla seotud muutustega Negr1 puudulikkusega hiirte tasu süsteemis. Need muutused võivad põhjendada inimese *NEGR1* geeni seotust alkoholismi või liigsöömisega.
- Dopamiini sünteesis osaleva türosiin hüdroksülaasi ning dopamiini lammutavaid valke kodeerivate *Maoa, Maob* ja *Comt* kõrgem tase võib viidata, et *Negr1*-puudulikkusega hiirte mesolimbilises juhtetees esineb kõrgem dopamiini käive (*turnover*), mis väljendub dopamiini suuremas sünteesis, aga ka kõrgemas lammutamise tasemes. Need muutused Negr1-puudulikkusega hiirte sõltuvusega seotud ajuosades võivad samuti selgitada *NEGR1* geeni seoseid sõltuvuskäigumistega.

KOKKUVÕTE

Dopaminergilise mesolimbilise juhtetee moodustavad keskajus asuv ventraalne tegmentaalne piirkond (VTA), mis suubub ventraalsesse striaatumi (VST). Antud rada peetakse seotuks motivatsiooni ja sõltuvuskäitumisega. Lisaks saab tarbimiskäitumise probleeme seostada ka geneetiliste teguritega. Muutused adhesioonimolekulide ekspressioonis võivad oma panuse anda dopamiini süsteemi altereerimisse. Uuringud on näidanud, et *NEGR1* polümorfismid mõjutavad tarbitava alkoholi ja toidu koguseid, pannes näiteks madala ekspressiooniga hiired vabalt kättesaadavat toitu pidurdamatult sööma. Seetõttu võib välja kujuneda liigsöömine ja rasvumine või alkoholisõltuvus.

Käesoleva töö eesmärgiks oli uurida *Negr1*-puudulikkuse tulemusena aset leidvaid ekspressiooni taseme muutusi ventraalses tegmentaalses piirkonnas ja ventraalses striaatumis. Uuriti nelja kuu vanuseid isased hiiri. Leiti, et võrreldes metsik-tüüpi hiirtega on *Negr1*$^{-/-}$ hiirtel türosiini hüroksülaasi (*Th)*, katehhool-O-metüültransferaasi (*Comt*), monoamiini oksüdaas A (*Maoa*), monoamiini oksüdaas B (*Maob*) ja dopamiini transporteri (*Dat*) geenid kõrgemalt ekspresseerunud. Eelkõige kerkis esile *Dat* kõrge ekspressioon, mis võib põhjustada närvirakkude kaotust. *Negr1*-puudulikkuse ja *Dat* kõrge ekspressiooni koostoimel võib avalduda negatiivne mõju dopamiini süsteemile ja anda tõuke sõltuvuskäitumise kujunemisele. Kõrgemad *Th, Maoa, Maob ja Comt* ekspressioonitasemed viitavad asjaolule, et Negr1 puudumine soodustab nii dopamiini suuremat tootmist, aga ka kiiremat lagunemist. Saadud tulemused viitavad üldiselt *Negr1* võimele altereerida mesolimbilise dopamiini juhtetee tööd, põhjustades muutusi toitumis- ja alkoholi tarbimiskäitumises.

Mõned uuringud on näidanud, et *Negr1* mõjutab alkoholi tarbimisharjumusi ja kuna alkohol mõjutab dopaminergilist mesolimbilist juhteteed ja on tihedalt seotud motivatsiooni, anhedoonia ning tarbimiskäitumisega, siis tasuks süvitsi uurida seoseid *Negr1*-puudulikkuse ja alkoholismi vahel. Lisaks peaks tulevikus tehtavad uuringud hõlmama emaseid liigikaaslasi.

Involvement of the Negr1 gene in the mesolimbic dopamine pathway associated with addictive disorders and anhedonia

Äli Leontjev

Summary

The dopaminergic mesolimbic pathway is formed by the ventral tegmental area (VTA) in the midbrain, which projects into the ventral striatum (VST). This pathway is thought to be associated with motivation and addictive behavior. In addition, problems in consumption behavior can also be linked to genetic factors. Alterations in the expression of adhesion molecules may contribute to alterations in the dopamine system. Studies have shown that polymorphisms in *NEGR1* affect the amount of alcohol and food consumed, for example by inducing low-expressing mice to eat freely available food without stopping. As a result, overeating and obesity or alcohol dependence can develop.

The aim of the present work was to investigate the changes in expression levels in the ventral tegmental area and ventral striatum that result from Negr1 deficiency. Four-month-old male mice were studied. It was found that compared to wild-type mice, *Negr1*$^{-/-}$ mice have higher expression of tyrosine hydroxylase (*Th)*, catechol-O-methyltransferase (*Comt*), monoamine oxidase A (*Maoa*), monoamine oxidase B (*Maob*) and dopamine transporter (*Dat*). In particular, the high expression of *Dat* was found in both areas of the brain. Studies have pointed out that high expression of *Dat* can cause nerve cell loss. The interaction between Negr1 deficiency and higher expression of Dat may have a negative effect on the dopamine system and may trigger the development of addictive behavior. Higher expression levels of *Th, Maoa, Maob* and *Comt* suggest that the lack of *Negr1* promotes both increased dopamine production but also faster dopamine degradation. Overall, the results obtained suggest the ability of *Negr1* to modulate the mesolimbic dopamine pathway by inducing changes in eating and alcohol consumption behavior.

Some studies have shown that *NEGR1* influences alcohol consumption habits, and since alcohol affects the dopaminergic mesolimbic pathway and is closely related to motivation, anhedonia and consumption behavior, it would be worthwhile to further investigate the links between *Negr1* deficiency and alcoholism. In addition, future studies should include female conspecifics.

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LISAD

LISA 1. Dopamiinisüsteemiga seotud geenide uurimiseks praimerite järjestused (Varul et al., 2021)

Türosiini hüdroksülaas	Th_mm_F	ACCGCACATTTGCCCAGTTC
	Th_mm_R	ACACAGCCCAAACTCCACAG
Dopamiini retseptor 1	Drd1_mm_F	GAGCAGGACATACGCCATTT
	Drd1_mm_R	CCTCTCCAAAGCTGAGATGC
Dopamiini retseptor 2	Drd2_mm_F	TCATTGCCAACCCTGCCTTC
	Drd2_mm_R	TTGGTGTTGACCCGCTTCC
Dopamiini transporter	DAT_mm_F	GCATCCTGTTCACATATTACAC
	DAT_mm_R	TTGTCTCCCAACCTGAATTC
Katehol-O-metüültransferaas	Comt_mm_F	GAGATGGACCGGAACTTTGA
	Comt_mm_R	AGTTGCCAGCACTGAGGTTT
Monoamiinide oksüdaas A	Maoa_mm_F	AGCCTACTTCCCTCCTGGTATC
	Maoa_mm_R	AGCTTCAACTGCACCTTCCATG
Monoamiinide oksüdaas B	Maob_mm_F	GAATCTTTGGATGTCCCTGCAC
	Maob_mm_R	TGTTGCTGACAAGATGGTGGTC
Beeta-aktiin	ActB_mm_F	ACCATGTACCCAGGCATTGC
	ActB_mm_R	AGCCACCGATCCACACAGAG

LISA 2. Depression-associated *Negr1* gene-deficiency induces alterations in the monoaminergic neurotransmission enhancing time-dependent sensitization to amphetamine in male mice **(**Kaare et.al, 2022)

brain sciences

MDPI

Article

Depression-associated *Negr1* gene-deficiency induces alterations in the monoaminergic neurotransmission enhancing time-dependent sensitization to amphetamine in male mice

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Abstract: In GWAS studies, the neural adhesion molecule encoding Neuronal growth regulator 1 (*NEGR1*) gene has been consistently linked with both depression and obesity. Although the linkage between *NEGR1* and depression is the strongest, evidence also suggests involvement of *NEGR1* in a wide spectrum of psychiatric conditions. Here we show expression of NEGR1 both in tyrosine- and tryptophan hydroxylase-positive cells. *Negr1*$^{-/-}$ mice show a time-dependent increase in behavioral sensitization to amphetamine associated with increased dopamine release in both dorsal and ventral striatum. Upregulation of transcripts encoding for dopamine and serotonin transporters and higher levels of several monoamines, and their metabolites was evident in distinct brain areas of *Negr1*$^{-/-}$ mice. Chronic (23 days) escitalopram-induced reduction of serotonin and dopamine turnover is enhanced in *Negr1*$^{-/-}$ mice and escitalopram rescued reduced weight of hippocampi in *Negr1*$^{-/-}$ mice. The current study is the first to show alterations in the brain monoaminergic systems in *Negr1*-deficient mice suggesting that monoaminergic neural circuits contribute to both depressive and obesity-related phenotypes linked to the human *NEGR1* gene.

Keywords: Negr1, depression, dopamine, serotonin, genetic models

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1. Introduction

The 1p31.1 locus in the human genome, encoding *Neuronal growth regulator 1* (*NEGR1)* gene has been recently identified as one of the most significant risk loci for both depression [1, 2, 3, 4, 5] and obesity [6, 7, 8]. Transcriptome and protein analysis suggest increased expression of NEGR1 in depression patients; an increased level of *NEGR1* has been reported in the brain, namely in the dorsolateral prefrontal cortex (DLPFC) and in the hypothalamic area [5] of patients with major depressive disorder (MDD) in comparison with healthy controls. Additional data from MDD patients suggest that the functional impact of NEGR1 might involve systemic regulation, as significant upregulation of NEGR1 has been shown in the cerebrospinal fluid [9] and peripheral blood of MDD patients [10, 11].

In the major studies that have recently linked NEGR1 with depression, the diagnosis has been specified as MDD (major depressive disorder) [1, 2, 5]; broad depression [3] and unipolar depression [4]. Although the linkage between NEGR1 and depression is the strongest, evidence also suggests involvement of NEGR1 in a wide spectrum of psychiatric conditions [12]. The levels of NEGR1 protein and transcripts are elevated in the post-mortem prefrontal cortex (PFC) [13] and DLPFC [14] of schizophrenic patients. NEGR1 has also been shown to be associated with intelligence [15], dyslexia [16] and autism spectrum disorders (ASD) [17, 18]. In addition, a microdeletion in the *NEGR1* gene was described in two siblings who presented cognitive disabilities, attention deficit hyperactivity disorder (ADHD), speech problems and one of them also features of autism [19].

NEGR1 is a member of the IgLON superfamily of cell adhesion molecules (CAMs), which also include limbic system associated membrane protein (Lsamp), neurotrimin (Ntm), opioid-binding protein/cell adhesion molecule like (Opcml) and IgLON5 [20]. IgLONs dimerize homophilically and heterophilically to shape synaptic connections and neural circuits by spanning cellular junctions (*in trans*) and/or at the same side of a junction (*in cis*) [20]. NEGR1 has been shown to interact with other IgLONs, such as NTM, through the first Ig domain [21]. Direct interaction between another IgLON, LSAMP, and NEGR1 has been shown in protein microarray experiments [22] and also confirmed in the mouse brain [23]. IgLONs act synergistically, each forming the context for the work of the other in the regulation of neural circuit formation which manifests both at the level of neuronal morphology and behavior [24, 20]. Therefore, NEGR1, together with other IgLONs, plays an important role in cell-to-cell adhesion and neurite outgrowth and synaptogenesis [25, 26, 27]. NEGR1 is reported to localize to the postsynaptic sites of dendritic and somatic synapses and is highly expressed in the cerebral cortex, hippocampus, and cerebellum during postnatal development [28].

Homozygous deletion of *Negr1* gene in mice (*Negr1*$^{-/-}$) induces no robust changes in sensory and motor development but causes impairment in social behavior and reversal learning deficits compared to WT littermates [27, 29]. *Negr1*$^{-/-}$ mice also displayed neuroanatomical alterations, such as enlargement of ventricles and a decrease in the volume of the

whole brain, including corpus callosum, hippocampus and globus-pallidus. In addition, a decreased number of parvalbumin-positive inhibitory interneurons was evident in *Negr1*$^{-/-}$ hippocampi [27]. In independently created *Negr1*$^{-/-}$ mice, it has been shown that *Negr1* deficiency results in alterations in adult neurogenesis and hippocampal dentate gyrus (DG) synaptic transmission and leads to anxiety- and depression-like behaviors [30]. Szczurkowska et al showed that downregulation of *Negr1* in mice impairs neuronal migration and proper development of the somatosensory cortex resulting in behavioral phenotypes related to ASD [31]. We have also shown that *Negr1*$^{-/-}$ mice eat smaller amounts of food both in case of standard and high fat diets which may be related with alterations in their metabolic profiles [32] but it is not clear if these mice have alterations in the motivational/reward system which may also explain reduced food intake. Again, the data from mice supports the strong associations of *NEGR1* gene with both psychiatric and obesity related phenotypes in human studies.

The monoamine hypothesis of depression states that dysfunction in the monoamine neurotransmitter system is the cause of the symptoms of depression. Although accumulating evidence also suggests involvement of other pathways, monoamines still play a crucial role in the mood disorders and are the main targets of anti-depressant drugs that are currently available [33]. The expression of *Negr1* has been shown in both dopaminergic and serotonergic nuclei and pathways. *Negr1* is expressed in the whole fasciculus retroflexus, which serves as a molecular scaffold for dopaminergic axons that grow from the midbrain toward the habenula. Furthermore, the interaction partner of NEGR1, LSAMP, has been shown to mediate guidance of the dopaminergic axons to the habenula [34]. High *Negr1* expression has been detected specifically in the islands of Calleja [27] which receive dense dopaminergic projections from the ventral tegmental area (VTA) and the substantia nigra [35]. The modest signal of *Negr1* and *Lsamp* was detected in the serotonergic neurons in the dorsal raphe of macaques. In another study, *Negr1* was identified as a differentially expressed gene across molecularly defined serotonergic neuron subtypes, the expression of *Negr1* was highest in the medial raphe, especially ventral areas of medial raphe (clusters R2 and R3) and lowest in the dorsal raphe [36].

The expression of *Negr1* has been shown to be altered after administration of several antidepressants that target monoaminergic neurotransmission. Chronic treatment with one of the most commonly used antidepressants, venlafaxine, a serotonin-norepinephrine reuptake inhibitor, has been shown to increase *Negr1* expression in the cerebral cortex in rats [37]. Carboni et al. however, showed a decrease of *Negr1* transcripts after administration of selective serotonin reuptake inhibitors escitalopram in the hypothalamus and fluoxetine in the hippocampus in rodent models [38]. Moreover, tricyclic antidepressant nortriptyline downregulates *Negr1* in hippocampal primary neurons. Another study showed increased NEGR1 levels in human cell lines which are treated with clozapine, which binds to both dopaminergic and serotonergic receptors, suggesting NEGR1 as a target of antipsychotic drugs [39].

Alterations in serotonergic neurotransmission, namely increased serotonin turnover, has been repeatedly described in *Lsamp*-deficient mice [40, 41] which might be the explanation for the decreased anxiety and social deviations in these mice [42, 43]. Decreased sensitivity to the stimulating locomotor effect of amphetamine has been described in mice deficient for *Lsamp* [39] and also in mice deficient for *Ntm* [44], further supporting the hypothesis of altered reactivity in the monoaminergic, especially dopaminergic neurotransmission, in mice deficient for IgLONs. Monoamines are also important regulators of reward and motivational processes. As mentioned above, feeding behavior is altered in *Negr1*-deficient mice. Characterization of monoaminergic neurotransmission in *Negr1*-deficient mice could clarify the mechanisms behind this observation.

The aim of this study was to assess the effects of deletion of *Negr1* on the monoaminergic circuitry as one of the mechanisms through which NEGR1 could be involved in the pathogenesis of depression and possibly in the pathogenesis of obesity.

2. Materials and Methods

2.1. Animals

Male wild-type (WT) mice and their homozygous *Negr1*-deficient littermates ($Negr1^{-/-}$), generated and described previously in Lee et al (2012) were used in the F2 background [(129S5/SvEvBrd × C57BL/6N) × (129S5/SvEvBrd × C57BL/6N)] in the present study [45]. Mice were group-housed in standard laboratory cages measuring 42.5 (L) × 26.6 (W) × 15.5 (H) cm, 10 animals per cage in the animal colony at 22 ± 1 °C, under a 12:12 h light/dark cycle (lights off at 19:00 h). A 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei) were used in each cage and changed every week. Water and food pellets (R70, Lactamin AB, Sweden) were available *ad libitum*. Breeding and the maintenance of the mice were performed at the animal facility of the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. All behavioral experiments were conducted between 8am and 5pm. Each cohort of mice were prior to experiment in group housing conditions. During the experiment cohort I mice went in single cages 6 days before the experiment and stayed there for the rest of the experiment. Mice were single caged before the experiment since chronic administration of amphetamine increases fighting in mice [46]. The rest of the cohorts were in group housing conditions throughout all the experiments. The use of mice was conducted in accordance with the regulations and guidelines approved by the Laboratory Animal Center at the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. All animal procedures were conducted in accordance with the European Communities Directive (2010/63/EU) with permit (No 150, September 27, 2019) from the Estonian National Board of Animal Experiments.

Amphetamine and escitalopram were used as pharmacological agents to challenge monoaminergic neurotransmission in *Negr1*-deficient mice. Behavioral profiles were assessed together with monoamine and gene expression levels of related enzymes.

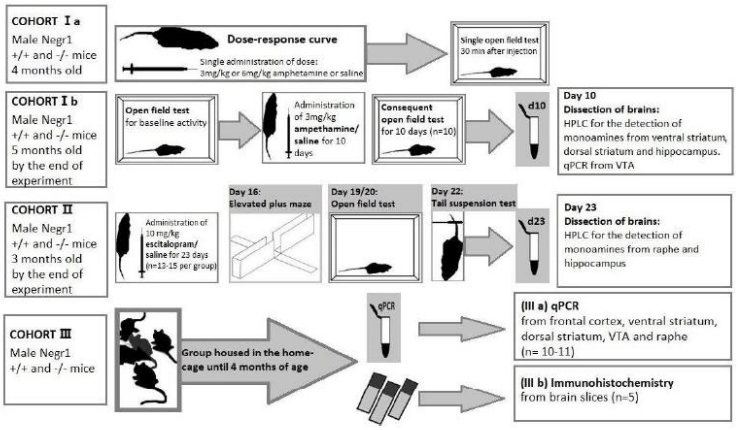

Figure 1. Schematic overview of the cohorts of mice and tests/measurements performed in the current study. Small arrows indicate the timing of behavioral tests in the schematic timeline. For the estimation of the treatment of acute and chronic amphetamine (cohort I), two subgroups of mice were used: cohort Ia for the estimation of dose curve (data shown in Supplementary figure S1) and cohort Ib for the chronic amphetamine administration. Open field test for baseline activity of cohort Ib mice was performed 7 days before administration of amphetamine. cohort II was used for the estimation of the treatment of chronic escitalopram and cohort III was used for baseline measurement of gene expression and IHC stainings.

2.2. Acute and chronic amphetamine treatment

For the estimation of the treatment of acute and chronic amphetamine (cohort I), two subgroups of 5 months old mice were used: cohort Ia for the estimation of dose-response curve and cohort Ib for the chronic amphetamine administration. Schematic overview of the cohorts of mice and tests/measurements performed can be found in the Figure 1. All *Negr1*-deficient mice and their age-matched WT littermates were randomly assigned to groups (n=10 per group). In the dose-response (acute administration) group (Ia), mice received a single dose of d-amphetamine in two different dosages: 3 mg/kg and 6 mg/kg or saline (Supplementary figure S1). In the chronic amphetamine experiment (cohort Ib), all mice were tested in the open field test (I testing) two weeks prior to the amphetamine injections in order to detect their baseline motor/exploratory activity. Thereafter, cohort Ib mice received an i.p. injection of saline or 3 mg/kg amphetamine for 10 days followed by a daily open field test 30 min after injection. The 3 mg/kg dose of amphetamine for repeated injection induced behavioral sensitization has been shown to be sufficient in our earlier studies [47, 48], still, the results were analyzed daily to make sure that there is no need to make corrections (decrease or increase) in the dose. Amphetamine (Sigma-Aldrich, St. Louis, MO,

USA) was freshly prepared in a sterile, pyrogen free, 0.9% solution of sodium chloride (B. Braun, Melsungen, Germany). In this experiment we used the D-isomer of amphetamine (d-amphetamine) because l-amphetamine is a weaker agonist of the dopamine system. All drugs were injected intraperitoneally (i.p.) at a volume of 10 ml/kg 30 min before testing.

2.3. Escitalopram treatment

All *Negr1*$^{-/-}$mice were age-matched with littermates and were tested at 2 – 3 months of age. *Negr1*$^{-/-}$ and WT mice were randomly divided into groups that received an i.p. injection of either saline (B. Braun) or 10 mg/kg of selective serotonin reuptake inhibitor escitalopram (Sigma-Aldrich) at a volume of 10 ml/kg for 23 consecutive days. The dosage of escitalopram was chosen based on Bregin et al. [41]. Mice were injected every day at 9 am and allocated as follows: 13 mice in *Negr1*$^{-/-}$ and 15 mice in WT escitalopram groups; in saline groups there were 13 mice in both WT and *Negr1*$^{-/-}$ groups. Escitalopram (Sigma-Aldrich) was freshly prepared in a sterile pyrogen free 0.9% solution of sodium chloride (B. Braun). Body weight was measured weekly for 8 weeks before administration of escitalopram and on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 during the period of injections. Behavioral changes were evaluated in the elevated plus maze (day 16), open field test (half of the mice on day 19 and other half of the mice on day 20) and tail suspension (day 22) tests (Figure 1).

2.4. Elevated plus maze

The elevated plus maze apparatus consisted of two opposite open (17.5 × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. The apparatus was elevated to a height of 30 cm and placed in a room with the light intensity of 100 lx in open arms. Testing began by placing the animal to the central platform of the maze, facing an open arm. After each mouse, the floor of the testing apparatus was cleaned with 70% ethanol and dried thoroughly. Standard 5 min test duration was employed, and all the sessions were video recorded. An arm entry was counted only when all four limbs were within a given arm.

2.5. Open field test

Locomotor activity of individual mice was measured with the illumination level of 450 lx for 30 min in soundproof photoelectric motility boxes (44.8 × 44.8 × 45 cm) connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). The floor of the testing apparatus was cleaned with 70% ethanol and dried thoroughly after each mouse. The system automatically registered the movement of the animal: the distance travelled, the number of rearings, corner visits, time spent and distance covered in the central part of the box.

2.6. Tail Suspension Test

Mice were suspended for 6 min from the edge of a shelf 60 cm above a tabletop by adhesive tape, placed approximately 1 cm from the tip of the tail. The duration of immobility, the number of immobility episodes (an episode defined as hanging passively and being motionless for at least 3 s), and the number of short immobility episodes lasting 1–2 s were scored during the last 4 min from the recorded videos by an observer blind to the genotype.

2.7. Measurement of monoamines

Immediately after the last behavioral test, all mice were decapitated. Brains were dissected into five parts and frozen in liquid nitrogen. Dorsal striatum (*caudate putamen*), ventral striatum (including *nucleus accumbens* and *olfactory tubercle*), hippocampus and frontal cortex were dissected from the brains of all mice; from the brains of mice receiving chronic amphetamine (cohort Ib), ventral tegmental area, was dissected and from the mice receiving chronic escitalopram (cohort II), the raphe nuclei (including both dorsal and median groups of the raphe nuclei) were dissected. The brain dissection was performed according to the coordinates presented in the mouse brain atlas [49]. Monoamine measurements from striatum were done differently compared to raphe nuclei and hippocampi. Monoamines – serotonin (5-HT), noradrenaline (NA) and dopamine (DA) – and their metabolites – 5-hydroxyindoleacetic acid (5-HIAA), normetanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) – were assayed by high performance liquid chromatography (HPLC) with electrochemical detection. Monoamines and their metabolites were measured from ventral and dorsal striatum (VSTR and DSTR) tissues of the mice from the chronic amphetamine experiment.

Monoamine quantification in raphe nuclei and hippocampi were done by liquid chromatography mass spectrometry. Briefly, the samples were weighed and transferred into 50 µl PBS. Fifty µl internal standard [$^{2}H_{3}$]leucine, [$^{13}C_{6}$]tyrosine, [$^{2}H_{5}$]phenylalanine (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and 0.9-2.0 mm stainless steel beads (Next Advance, Troy, NY, USA) were added. Homogenization was achieved within 2 min in bullet blender (Next Advance). Thereafter 400 µl ice-cold methanol (resulting the final concentration of 80% methanol) was added and the samples were let stand at -20 °C for 20 min. After centrifugation 10 min at 21,000 ×g two 200 µl aliquots were taken from supernatant and dried under a stream of nitrogen. First aliquot was treated with 50 µl phenylisothiocyanate in water and pyridine (v/v/v 50/320/635) for 40 min at 40 °C. The second aliquot was treated with 100 µl 200 mM 2-nitrophenylhydrazine and 20 µl 120 mM 1(3-dimethylaminopropyl)-N-ethylcarbodiimide for 1 h at room temperature. After subsequent drying under nitrogen the samples were resolved in 100 µl 5 mM ammonium acetate in methanol. Ten µl was injected into Acquity Premier 1.7 µm CSH Phenyl-Hexyl 2.1 × 100 mm column in Waters Acquity UPLC H-class – Xevo TQ-XS mass spectrometer (Waters, Milford MA, USA). Gradient was composed of solvent A: $H_{2}O$ with 0.2% formic acid and solvent B: acetonitrile with 0.2% formic acid. Starting from 85% solvent A for 0.5 min the gradient rose to 50% B in 1 min and to 90% B in next 0.5 min. Total run time was 4.5 min with flow

rate 0.5 ml/min. The phenylisothiocyanate derivatives were analyzed in positive ionization multiple reaction monitoring mode with the following quantification ion pairs: 5-HT 312/160, NA 287/135, DA 289/137, NMN 301/166, 5-HIAA 192/146 and 3-MT 303/94. Nitrophenylhydrazine derivatives were quantified in negative ionization mode with the following ion pair signals: DOPAC 302/137, HVA 316/146.

2.8. Immunohistochemistry

To specify the location of NEGR1 in the monoaminergic pathways, anti-NEGR1 immunostaining was performed along with co-immunostainings specific for tyrosine hydroxylase and tryptophan hydroxylase. Fluorescent immunohistochemistry was performed on floating 30 μm thick coronal sections collected after every 300 μm into phosphate buffered saline (PBS). Incubations were performed with gentle rocking and at room temperature unless mentioned otherwise. After washing with PBS for 10 min, sections were permeabilized with 0.25% Triton X-100 (Naxo, Tartu, Estonia)/PBS solution for 45 min. Sections were subsequently blocked in solution containing 0.3 M glycine/5% donkey serum//1% bovine serum albumin (BSA, Sigma-Aldrich)/PBS for 2 h at room temperature and incubated with rat anti-dopamine transporter/DAT (1:100, Santa Cruz Biotechnology, Heidelberg, Germany; sc-32258), sheep anti-TH (tyrosine hydroxylase) antibody (1:1000, Abcam, Cambridge, United Kingdom, Cat# ab113, RRID:AB_297905) in combinations with rabbit anti-TPH2 (tryptophan hydroxylase isoform 2) (1:500, Abcam Cat# ab26092, RRID:AB_2207690) and mouse anti-Negr1 antibody (1:50, Santa Cruz Biotechnology, H-12: sc-393293) dilutions in 0.1% Tween-20/1% BSA/PBS 72 hours at 4 °C. Subsequently sections were then washed with 5 times 0.1% Tween-20//PBS for 10 min and incubated with the appropriate secondary antibody Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H+L) (1:1000, Jackson ImmunoResearch Labs, West Grove, PA, USA; 712-607-003), FITC AffiniPure donkey anti-rabbit (1:1000, Jackson ImmunoResearch Lab., 711–095–152, RRID:AB_2315776), donkey anti-sheep IgG (H+L) Alexa Fluor 594 (1:1000, Thermo Fisher Scientific, Waltham, MA, USA, Cat# A11016, RRID:AB_10562537), donkey anti-mouse IgG (H+L) Alexa Fluor 647 (1:1000, Thermo Fisher Scientific, Cat# A-31571, RRID:AB_162542) at room temperature for 2 h. After subsequent washes with PBS 3 times 10 min nuclei were stained with 5 μg/ml Bisbenzimide H 33258 (Hoechst 33258, Sigma Aldrich) in PBS for 2 min. Subsequently sections were rinsed with PBS for 5 min, mounted in Fluoromount mounting medium (Sigma Aldrich), and covered with a 0.17 mm coverslip (Deltalab). Specificity of the immunohistochemistry was determined by incubations without the primary antibodies. Fluorescent images were obtained with the Olympus FV1200MPE (Olympus, Hamburg, Germany) laser scanning confocal microscope and Leica Aperio VERSA Brightfield, Fluorescence & FISH Digital Scanner.

Fluorescent intensity of dopamine transporter (DAT) immunostaining in the striatum was quantified using the Positive pixel count 2004-08-11 algorithm of Aperio Image Scope [v12.4.3.5008]. The image of striatal surface was divided into DSTR containing caudate-putamen and VSTR consisting of nucleus accumbens and olfactory tubercle, according to the Scalable Brain Atlas [50]. Isosurface was created separately from both the parts of the

striatum and was used to make quantitative measurements on area and surface fluorescent intensity.

2.9. RT-qPCR analysis in mouse brain areas

Gene expression was determined by two-step RT-qPCR (qPCR). Total RNA was extracted from each tissue sample by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized by using FIREScript RT cDNA Synthesis MIX with Oligo (dT) and Random primers (Solis BioDyne, Tartu, Estonia) according to the manufacturer's protocol.

In qPCR, 8 dopamine related genes were studied, tyrosine hydroxylase (*Th*), dopamine receptor 1 (*Drd1*), dopamine receptor 2 (*Drd2*), dopamine receptor 5 (*Drd5*), dopamine transporter (*Dat*), catechol-O-methyltransferase (*Comt*), monoamine oxidase A (*MaoA*) and monoamine oxidase B (*MaoB*). Dopamine system-related primers used in the experiment have been previously described in Varul et al. [51]. Additionally, two serotonin system related genes were studied: serotonin transporter (*Slc6a4*) and tryptophan hydroxylase (*Tph2*). As a housekeeper gene, beta-actin (ActB_mm_F ACCATGTACCCAGGCATTGC, ActB_mm_R AGCCACCGATCCACACAGAG) was used. Every reaction was made in four parallel replicates to minimize possible errors. All reactions were performed in a final volume of 10 µL, using 5 ng of cDNA. Real-time qPCR was performed using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne). ABI Prism 7900HT Sequence Detection System with ABI Prism 7900 SDS 2.4.2 software (Applied Biosystems) was used for qPCR detection. qRT-PCR data in figures is presented on a linear scale, calculated as $2^{-\Delta\Delta CT}$, where ΔCT is the difference in cycle threshold (CT) between the target genes and the housekeeper gene.

2.10. Statistical analysis

Results are expressed as mean values ± SEM. Normal distribution of data was evaluated with the Shapiro-Wilk test. Results from qPCR and other data comparing two groups were assessed using Student's t-test or Mann–Whitney test for nonparametric data. Detailed information about the analysis (normality estimates, statistical test used and p-values) of RT-qPCR data, comparing only two groups (WT and *Negr1*$^{-/-}$), can be found in Supplementary tables S2-S6. Comparison of RT-qPCR data from chronic amphetamine experiment in the VTA, of the behavioral results from the escitalopram experiment, levels of monoamines and their metabolites, and body weight differences was performed using two-way ANOVA followed by a Bonferroni *post hoc* test (Supplementary table S7). The body weight dynamics from day -10 to day -3 were analyzed using repeated measures two-way ANOVA (time x genotype) followed by Bonferroni *post hoc* test. Behavioral data from the chronic amphetamine experiment was analyzed by repeated measures three-way ANOVA followed by a Tukey´s *post hoc* test. All differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 8 software.

3. Results

3.1. Negr1$^{-/-}$ mice display higher sensitivity to amphetamine compared to WT mice

In the dose-response curve experiment, 6 mg/kg amphetamine induced highly increased motor activity in both genotypes (Supplementary figure S1A) and therefore the 6 mg/kg dose was considered to be too high for chronic experiment, as the behavioral sensitization effect of amphetamine is well known [52]. Therefore, chronic amphetamine experiment was performed by using 3mg/kg dose, which, if first time injected, induced only tendency towards increased activity in batch 1a mice (Supplementary figure S1A) but also statistically significant increase in the distance travelled in wt mice in bach 1b ($p < 0.05$, two-way ANOVA (Bonferroni *post hoc* test) (for more details Supplementary figure S1D and figure legend). In order to assess the impact of amphetamine on behavior, *Negr1*$^{-/-}$ and WT mice received 10 days of amphetamine in the dose of 3 mg/kg. The administration of amphetamine for 10 days induced significantly higher motor activity in *Negr1*$^{-/-}$ mice compared to WT mice. In particular, amphetamine-treated *Negr1*$^{-/-}$ mice had travelled, visited corners, and rotated more compared to WT mice, whereas the control (saline) groups for both genotypes did not show any differences in these activities (Figure 2A-F). Clockwise and anticlockwise rotations were summed up in these experiments as there was no difference in the direction of rotation. Three-*Way Repeated-Measures ANOVA* showed that distance travelled was affected by the time ($F_{3.4,62.6} = 5.65$; $p = 0.001$), treatment ($F_{1,18} = 67.24$; $p < 0.0001$) and genotype x treatment interaction ($F_{1,18} = 22.84$; $p < 0.001$) (Figure 2A, more details can be found in Supplementary table S8). The number of corner visits showed significant time ($F_{3.2,57.9} = 5.06$; $p = 0.002$), treatment ($F_{1,18} = 63.32$; $p < 0.0001$) and genotype x treatment effects ($F_{1,18} = 17.36$; $p < 0.001$) (Figure 2B). Rotations were also affected by the time ($F_{3.6,64.8} = 5.48$; $p = 0.001$), treatment ($F_{1,18} = 43.95$; $p < 0.0001$) and genotype x treatment interaction ($F_{1,18} = 21.03$; $p < 0.001$) (Figure 2C) (Supplementary table S8).

Analyzing the AUC showed that amphetamine increased the travelled distance for both WT and *Negr1*$^{-/-}$ mice ($p < 0.0001$; Figure 2D). Nevertheless, the travelled distance was longer in *Negr1*$^{-/-}$ mice that received amphetamine compared to their WT littermates ($p = 0.016$; Figure 2D). A similar situation was also seen in case of corner visits (Figure 2B, E) and rotations (Figure 2C, F): while both genotypes visited more corners as well as made more rotations ($p < 0.001$ for WTs and $p < 0.0001$ for *Negr1*$^{-/-}$ mice), (Figure 2E) upon chronic amphetamine administration. Besides that, in summary, the amphetamine induced more corner visits ($p = 0.012$) and rotations ($p = 0.032$) in *Negr1*$^{-/-}$ mice compared to their WT littermates.

The body weight of the mice was measured 10 days before amphetamine or saline injections (marked as day -10). Body weight dynamics prior to the amphetamine injection (day -10 until -3) showed significant genotype ($F_{1,38} = 9.17$; $p = 0.004$) and genotype x time interaction effects ($F_{2,76} = 5.97$; $p = 0.004$). WT mice showed slight decrease in body weight, whereas *Negr1*$^{-/-}$mice gained weight during the first week of measurements (Figure 2G),

at the time when handling due to daily weighing was the only interfering activity. According to Bonferroni's *post hoc* test on day -10 the difference between the body weight of the WT mice and *Negr1*$^{-/-}$ mice was statistically significant ($p = 0.001$). On day -7, the baseline open field test was performed and on day -6 all the mice were single housed. The difference in body weight was still statistically significant on day -6 ($p = 0.018$) but was not anymore seen on day -3. The change of body weight during the first week (days -10 vs day -3) was also calculated (Figure 2H). The average weight loss for WT mice was 0.27 g and the average weight gain of *Negr1*$^{-/-}$ mice was 0.69 g. Mann-Whitney test showed that weight change differences between WT and *Negr1*$^{-/-}$ mice were statistically significant ($p = 0.008$). The weight from the beginning of the experiment to the end of the experiment (day -10 vs day 10) was also calculated (Figure 2I), but there were no statistically significant changes in the body weight change caused by amphetamine. There was, however, a significant genotype effect ($F_{1,36} = 11.62$; $p = 0.002$) showing that *Negr1*$^{-/-}$ mice lost less body weight than WT both upon saline and amphetamine injections.

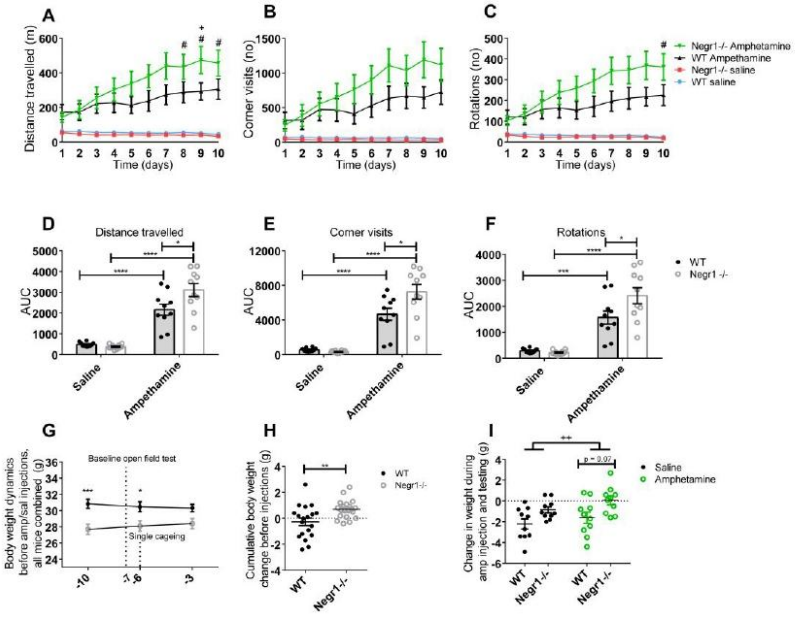

Figure 2. ***Negr1*$^{-/-}$ mice are more sensitive to chronic amphetamine administration.** Effect of chronic amphetamine on (A, D) distance travelled, (B, E) corner visits and (C, F) rotations in open field test. In G and H saline/amphetamine groups have not been separated yet, the body weight change is a reaction for non-pharmacological environmental manipulations. (G) Body weight dynamics measured during 1 week of period before amphetamine injection (from day -10 until day -3) (H) Cumulative body weight change before saline/amphetamine injections (from day -10 until day -3). (I) Body weight change caused by chronic saline or amphetamine injections and behavioral testing (from day 1 until day 10). Data represents mean ± SEM, + – $p < 0.01$ – difference in treatment in

WT mice, # – p< 0.05 – difference in treatment in *Negr1*$^{-/-}$ mice (Tukey post hoc test, A-C); ++ – p< 0.01 – genotype effect, *p < 0.05, ***p < 0.001, ****p < 0.0001 (Mann-Whitney, D-F), *p < 0.05, **p < 0.01, ***p < 0.001 (Bonferroni *post hoc* test, G-I). AUC, area under curve.

If the data from saline groups was analyzed separately (Supplementary figure S9), genotype differences were evident in the distance travelled in the center and rotations, as well as in the number of rearings in the open field test. The *post hoc* test showed no significant changes between *Negr1*$^{-/-}$ and WT mice for individual days. For each mouse, the AUC was calculated, and the results analyzed using the Mann-Whitney U test showed that *Negr1*$^{-/-}$ mice performed significantly less rearings compared to WT mice (p = 0.012). The AUC of distance travelled in the center of the box was significantly lower in *Negr1*$^{-/-}$ mice (p = 0.035).

3.2. Chronic administration of amphetamine increases the level of tyrosine hydroxylase (TH) in VTA

To assess the level of dopamine system related genes in VTA, qPCR was performed using both cohort I (Figure 3A-D) and cohort III mice (Figure 3-I). There was a genotype x treatment interaction ($F_{1,34}$ = 8.3; p = 0.007) effect on the level of *Th* in the VTA. *Post hoc* tests showed that chronic amphetamine treatment significantly increased the level of *Th* only in WT mice (p = 0.027). The level of *Th* was significantly higher in the WT amphetamine group compared to the *Negr1*$^{-/-}$ amphetamine group (p = 0.045) (Figure 3B). Post hoc analysis confirmed no other significant changes in the level of dopamine system related genes *(Dat*, *Drd2* and *Comt*) between the groups in the VTA of cohort I mice (Figure 3A, C, D). The detailed results of ANOVA have been shown in the Supplementary table S7.

Mann-Whitney U Test or t- test (according to normality distribution) was used to compare WT and *Negr1*$^{-/-}$ groups, statistical details can be found in Supplementary table S2. In the VTA, the level of *Dat* was significantly higher in *Negr1*$^{-/-}$ mice (p = 0.011) (Figure 3E). The levels of *Th* (p = 0.019) (Figure 3F), *MaoA* (p = 0.009) (Figure 3H) and *MaoB* (p = 0.005) (Figure 3I) were also significantly higher in the VTA of *Negr1*$^{-/-}$ mice compared to WT mice. There were no significant changes in the level of *Drd2* in the VTA of cohort II mice (Figure 3G).

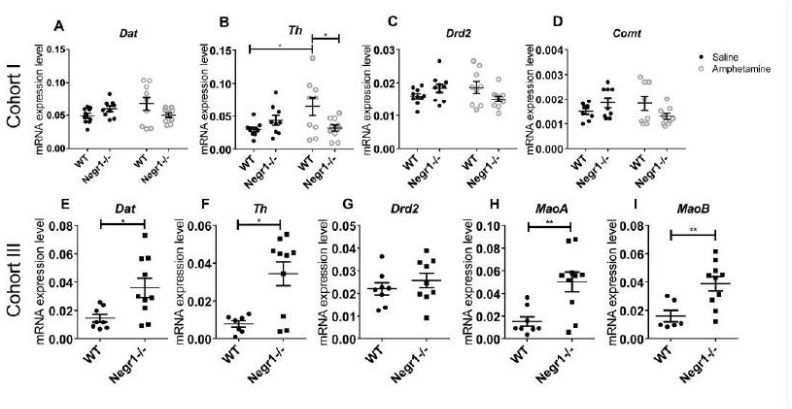

Figure 3. The level of dopamine system-related genes in the VTA of mice. Relative mRNA expression levels of (A) dopamine transporter (*Dat*), (B) tyrosine hydroxylase (*Th*), (C) dopamine receptor D2 (*Drd2*), (D) catechol-*O*-methyltransferase (*Comt*) in *Negr1*$^{-/-}$ mice and their WT littermates after 10 days of chronic saline or amphetamine i.p. injection (cohort I). The levels of (E) dopamine transporter (*Dat*), (F) tyrosine hydroxylase (*Th*), (G) dopamine receptor D2 (*Drd2*), (H) monoamine oxidase A (*MaoA*) and (I) monoamine oxidase B (*MaoB*) in home-cage *Negr1*$^{-/-}$ mice and their WT littermates (cohort III). Data represents mean ± SEM, *p < 0.05, **p < 0.01, ordinary two-way ANOVA (Bonferroni *post hoc* test) (A-D), Mann-Whitney U test (E-I).

3.3. Amphetamine increases the level of dopamine in dorsal striatum (DSTR)

To identify the differences caused by chronic amphetamine administration between WT and *Negr1*$^{-/-}$ two-way ANOVA [treatment (amphetamine or saline) × genotype (WT or *Negr1*$^{-/-}$)] and Bonferroni *post hoc* test was used. The level of dopamine and its metabolites were measured in DSTR (Supplementary table S3; S10; figure S12) and VSTR (Table S11). In the DSTR the level of DA was affected by the treatment (treatment: $F_{1,36} = 9.19$; $p = 0.005$; Bonferroni's *post hoc* tests showed that chronic treatment with amphetamine significantly increased the level of DA in *Negr1*$^{-/-}$ ($p = 0.026$) but not in control mice (Figure 4A). Dopamine turnover (3-MT/DA) in the DSTR was affected by the genotype ($F_{1,36} = 5.30$; $p = 0.027$) (Figure 4B), *post hoc* test showed no statistically significant changes between the groups. Immunohistochemical DAT staining of DSTR and VSTR did not show any significant differences between WT and *Negr1-/-* mice (Figure 4C).

In the VSTR, the level of DA displayed no significant changes between the groups (Figure 4D, Table S11). Whereas dopamine turnover to 3-MT (3-MT/DA) was again affected by the genotype ($F_{1,34} = 12.51$; $p = 0.001$) (Figure 4E). The level of DA metabolite 3-MT itself was affected by the treatment ($F_{1,34} = 9.77$; $p = 0.004$) (Figure 4F) and *post hoc* test revealed that the level of 3-MT was significantly increased in the *Negr1*$^{-/-}$ group receiving amphetamine ($p = 0.047$) in comparison to the saline-injected *Negr1*$^{-/-}$. The level of serotonin metabolite 5-HIAA was significantly affected by both treatment ($F_{1,34} = 10.67$; $p = 0.003$) and genotype ($F_{1,34} = 10.06$; $p = 0.003$) (Figure 4G, Table S11). In the VSTR, the levels of DA system related

genes *Dat* (p = 0.011) (Figure 4H) and *Comt* (p = 0.014) (Figure 4I) were significantly higher in *Negr1*$^{-/-}$ mice compared to WT mice (Supplementary table S4).

Amphetamine reduced the turnover of DA to DOPAC and DA to HVA (Supplementary figure S12). In the dorsal striatum, the levels of DOPAC ($F_{1,35}$ = 38.61; p < 0.0001, DOPAC/DA, ($F_{1,35}$ = 45.23; p < 0.0001, HVA ($F_{1,35}$ = 90.40; p < 0.0001 were affected by treatment. The ratio HVA/DA was significant if genotype ($F_{1,34}$ = 6.870; p = 0.013) and treatment ($F_{1,34}$ = 100.6; p < 0.0001) were considered. In the ventral striatum, the level of DOPAC, ($F_{1,36}$ = 9.19; p = 0.0045), DOPAC/DA, ($F_{1,36}$ = 136.2; p < 0.0001), HVA ($F_{1,36}$ = 75.26; p < 0.0001) and HVA/DA were affected by treatment ($F_{1,36}$ = 92.09; p < 0.0001).

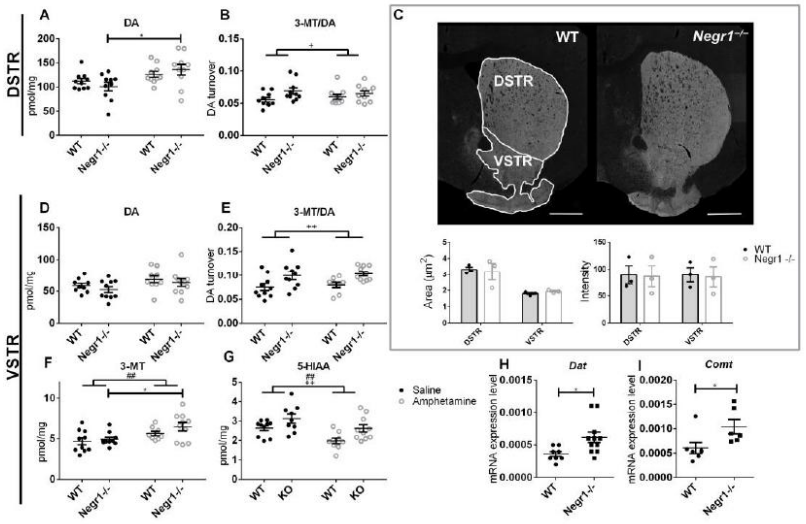

Figure 4. Effect of chronic amphetamine in the dorsal striatum (DSTR) and ventral striatum (VSTR). The level of (A) dopamine (DA) and (B) dopamine turnover (3-MT/DA) in the DSTR. (C) Immunohistochemical DAT stainings of DSTR and VSTR. The level of (D) dopamine (DA), (E) dopamine turnover (3-MT/DA), (F) 3MeOTyramine (3-MT), (G) 5-Hydroxyindoleacetic acid (5-HIAA), (H) dopamine transporter (*Dat*) and (I) catechol-*O*-methyltransferase (*Comt*) in the VSTR. Data represents mean ± SEM, ##p < 0.01 - treatment effect, +p < 0.05, ++p < 0.01 - genotype effect, *p < 0.05 - *post hoc* test, ordinary two-way ANOVA (Bonferroni *post hoc* test), Mann-Whitney U test (H, I).

In the striatum of WT mice, NEGR1 antibody gave signal in the vicinity of dopaminergic projection area, which was marked with anti-tyrosine hydroxylase (TH) - the rate limiting enzyme of dopamine production (Figure 5A, B, C, A1, B1, C1).

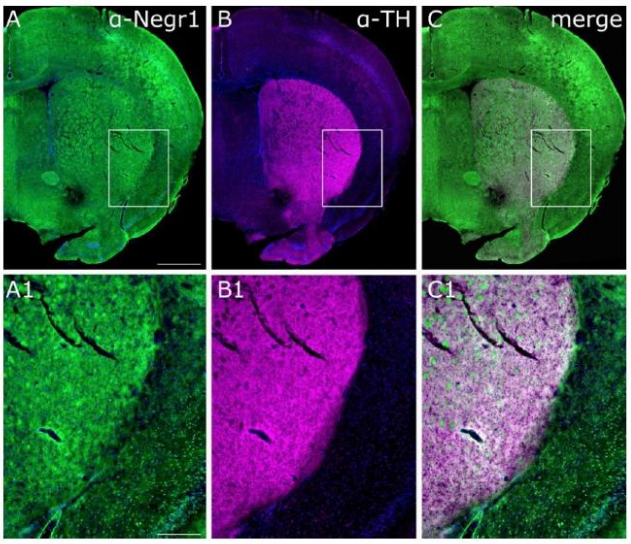

Figure 5. Expression of NEGR1 and TH in the striatum. Representative confocal images of striatum show co-immunohistochemical stainings of anti-NEGR1 in green (A, A1), with anti-tyrosine hydroxylase in magenta (TH) (B, B1) and NEGR1 staining in the vicinity of dopaminergic projection area can be seen with white color merged images (C, C1). Boxed areas show the localization of the close-ups in the images (A1, B1, C1). Scale bars: (A-C) 1mm, (A1-C1) 300 μm. LV - lateral ventricles, Ctx - central cortex, Sept - septum, CPu - caudate putamen, NAc - nucleus accumbens, OT - olfactory tubercule, Pir - piriform cortex.

3.4. Chronic amphetamine administration alters the level of monoamines in hippocampus and chronic escitalopram treatment causes weight difference of the hippocampus

Hippocampi of the *Negr1*$^{-/-}$ mice weigh less compared to WT hippocampi (Figure 6C-D). Amphetamine had no effect on the hippocampal weights of either *Negr1*$^{-/-}$ or WT mice (Figure 6A-B). In the escitalopram treatment experiment, the weight of hippocampi was significantly affected by the genotype ($F_{1,51}$ = 7.38; p = 0.009) and there was a significant genotype x treatment interaction ($F_{1,51}$ = 5.06; p = 0.03). The *post hoc* test showed that hippocampi of the *Negr1*$^{-/-}$ mice that received saline weigh less compared to WT saline group mice hippocampi (p = 0.006) and *Negr1*$^{-/-}$ escitalopram group hippocampi weigh significantly more compared to *Negr1*$^{-/-}$ saline group hippocampi (p = 0.03) (Figure 6C). If the weight of hippocampi was divided by the body weight of mice it was seen that the hippocampi/body weight relationship was affected by genotype x treatment interaction ($F_{1,51}$ = 8.01; p = 0.007). The *post hoc* test showed that this relationship was significantly higher in the *Negr1*$^{-/-}$ escitalopram group compared to *Negr1*$^{-/-}$ saline group (p = 0.03) (Figure 6D). On the other hand, chronic administration of either of amphetamine or escitalopram doesn't have effect on body weight of mice (Supplementary figure S13, S14).

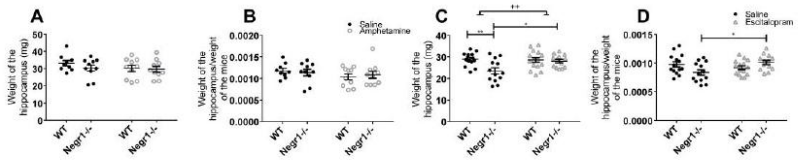

Figure 6. Hippocampi of *Negr1*$^{-/-}$ mice weigh less compared to WT mice and escitalopram restores the weight of hippocampi of *Negr1*$^{-/-}$ mice. (A) The weight of the hippocampi of the mice after receiving 10 days of saline or amphetamine. (B) Weight of hippocampi divided by the weight of mice (after receiving 10 days of saline or amphetamine). (C) Weight of hippocampi of the mice receiving 23 days of saline or escitalopram. (D) Weight of hippocampi divided by the weight of mice (after receiving 23 days of saline or escitalopram). Data represents mean ± SEM, ++p < 0.01 - genotype effect, *p < 0.05, **p < 0.01 - *post hoc* test, ordinary two-way ANOVA (Bonferroni *post hoc* test).

The level of monoamines and their metabolites were measured in the hippocampi of mice receiving either chronic treatment of amphetamine (Supplementary table S15) (Figure 7A-H) or escitalopram (Supplementary table S16) (Figure 7I-P). In case of chronic amphetamine administration, the level of tyrosine (Tyr) was affected by the treatment ($F_{1,34}$ = 7.78; p = 0.009). Bonferroni's *post hoc* test revealed that the level of tyrosine was significantly decreased by amphetamine in the *Negr1*$^{-/-}$ group (p = 0.039) (Figure 7A). The level of 3-MT was affected by the genotype ($F_{1,35}$ = 6.00; p = 0.019) (Figure 7C) and the level of tyramine was affected by the genotype x treatment interaction ($F_{1,35}$ = 5.82; p = 0.021) (Figure 7D), but *post hoc* test showed no significant changes in case of neither of them. The level of 5-HT was affected by the treatment ($F_{1,35}$ = 17.51; p < 0.001), the *post hoc* test showed that amphetamine increased the level of 5-HT in the WT mice group (p = 0.005) (Figure 7E). There was a treatment effect ($F_{1,35}$ = 4.18; p = 0.049) on level of 5-HIAA (Figure 7F), post hoc comparison did not indicate significant differences in *Negr1*$^{-/-}$ or WT mice separately. The level of NA was affected by the genotype ($F_{1,35}$ = 4.36; p = 0.044) (Figure 7G) and its metabolite NMN was affected by the treatment ($F_{1,33}$ = 19.44, p < 0.001), *post hoc* test showed that amphetamine increased the level of NMN of the WT mice (p = 0.002) (Figure 7H).

In the chronic escitalopram experiment, there was a genotype effect ($F_{1,51}$ = 4.25; p = 0.045) on the level of Tyr (Figure 7I, Table S16), Bonferroni's *post hoc* test showed no significant changes. The level of 3-MT was affected by the treatment ($F_{1,51}$ = 10.36; p = 0.002) (Figure 7K). The change of levels of tyramine ($F_{1,51}$ = 5.93; p = 0.018) (Figure 7L), 5-HT ($F_{1,51}$ = 5.40; p = 0.024) (Figure 7M), 5-HIAA ($F_{1,49}$ = 8.64; p = 0.005) (Figure 7N) and NA ($F_{1,50}$ = 4.14; p = 0.047) (Figure 7O) were affected by the genotype. The level of NMN was affected by the both treatment ($F_{1,50}$ = 4.57; p = 0.038) and genotype ($F_{1,50}$ = 10.55; p = 0.002), the *post hoc* test showed that the level of NMN was higher in the *Negr1*$^{-/-}$ saline group compared to WT saline group (p = 0.023) (Figure 7P).

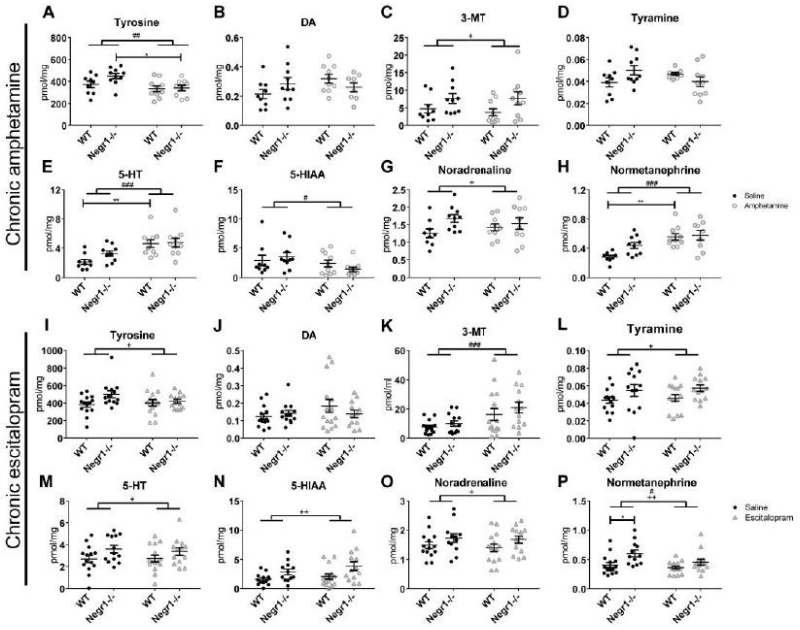

Figure 7. Effect of chronic administration of amphetamine or escitalopram on the level of monoamines and their metabolites in the hippocampus. The levels of (A) tyrosine, (B) dopamine (DA), (C) 3MeOTyramine (3-MT), (D) tyramine, (E) serotonin (5-HT), (F) 5-hydroxyindoleacetic acid (5-HIAA), (G) noradrenaline and (H) normetanephrine in the hippocampus of *Negr1*$^{-/-}$ mice and their WT littermates after 10 days of chronic amphetamine ip injections. The levels of (I) tyrosine, (J) dopamine (DA), (K) 3MeOTyramine (3-MT), (L) tyramine, (M) serotonin (5-HT), (N) 5-Hydroxyindoleacetic acid (5-HIAA), (O) noradrenaline and (P) normetanephrine in the hippocampus of *Negr1*$^{-/-}$ mice and their WT littermates after 23 days of chronic escitalopram ip injections. Data represents mean ± SEM, #$p < 0.05$, ###$p < 0.001$- treatment effect, +$p < 0.05$, ++$p < 0.01$ - genotype effect, *$p < 0.05$, **$p < 0.01$ - *post hoc* test, ordinary two-way ANOVA (Bonferroni *post hoc* test).

3.5. Chronic administration of escitalopram alters the level of monoamines and their metabolites in raphe

Levels of different monoamines and their metabolites were measured in the raphe nuclei (Figure 8A-J) (Supplementary table S17). In the raphe, the levels of 5-HT ($F_{1,48} = 6.36$; $p = 0.015$) (Figure 8B) and its metabolite 5-HIAA ($F_{1,47} = 6.23$; $p = 0.016$) (Figure 8C) were affected by the treatment, but Bonferroni's *post hoc* test did not show any significant changes. In the raphe, escitalopram significantly decreased the 5-HT turnover (5-HIAA/5-HT) in the *Negr1*$^{-/-}$ group ($p = 0.002$) (Figure 8D), 5-HT turnover was affected by the treatment ($F_{1,46} = 19.11$; $p < 0.0001$). The level of tyrosine showed no statistically significant differences between the groups (Figure 8E).

The level of DA was affected by treatment x genotype interaction ($F_{1,47} = 4.11$; $p = 0.048$). Escitalopram significantly increased the level of DA in the WT group ($p = 0.048$) but not in *Negr1*$^{-/-}$ mice (Figure 8F). The level of DA metabolite DOPAC was also affected by the treatment x genotype interaction ($F_{1,51} = 5.80$; $p = 0.02$), the level of DOPAC was statistically significantly higher in the *Negr1*$^{-/-}$ group receiving saline, compared to the *Negr1*$^{-/-}$ escitalopram group ($p = 0.025$) (Figure 8G). There was a treatment ($F_{1,49} = 17.53$; $p < 0.001$) and treatment x genotype interaction ($F_{1,49} = 6.03$; $p = 0.018$) effect on DA turnover (DOPAC/DA). DA turnover was significantly higher in the *Negr1*$^{-/-}$ saline group compared to the WT saline group ($p = 0.035$) and escitalopram significantly decreased the DA turnover in the *Negr1*$^{-/-}$ group ($p < 0.0001$) (Figure 8H). There was a significant genotype effect on the level of tyramine ($F_{1,49} = 5.38$; $p = 0.025$). The level of tyramine was statistically significantly higher in the *Negr1*$^{-/-}$ saline group compared to the WT saline group ($p = 0.043$) (Figure 8I). There were no significant changes in the level of 3-MT (Figure 8J).

The serotonin system-related genes were measured in the raphe using qPCR. Mann-Whitney U Test or t- test (according to normality distribution) was used to compare WT and *Negr1*$^{-/-}$ groups, statistical details can be found in supplementary table S5. (Figure 8K-N). In the raphe, the level of *Slc6a4* was significantly higher in *Negr1*$^{-/-}$ mice ($p = 0.009$) (Figure 8K). There were no significant differences between *Negr1*$^{-/-}$ and WT mice in the level of *Tph2* (Figure 8L), *MaoA* (Figure 8M) and *MaoB* (Figure 8N). The levels of dopamine system related genes were found to be unaltered in the frontal cortex (Supplementary table S6). In the DSTR, the level of *Dat* showed significant difference between *Negr1*$^{-/-}$ mice compared to WT mice, p=0.029, the details of statistical analysis and p-values can be found in Supplementary table S3.

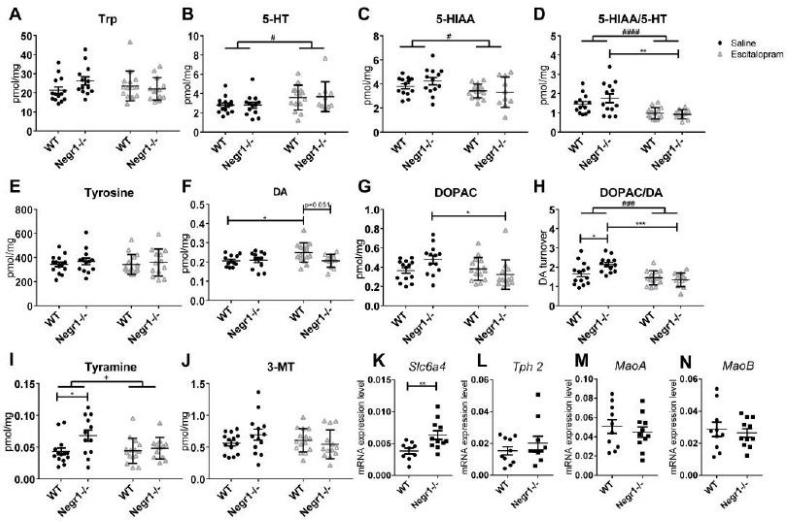

Figure 8. Effect of chronic escitalopram on the level of monoamines and their metabolites in the raphe nuclei. Levels of (A) tryptophan (Trp) (B) serotonin (5-HT), (C) 5-Hydroxyindoleacetic acid (5-HIAA), (D) serotonin turnover (5-HIAA/5-HT), (E) tyrosine, (F) dopamine (DA), (G) 3,4-Dihydroxyphenylacetic acid (DOPAC), (H) dopamine turnover (DOPAC/DA), (I) tyramine and (J) 3MeOTyramine in raphe. The mRNA expression level of (K) serotonin transporter (SLC6A4), (L) tryptophan hydroxylase 2 (*Tph2*), (M) monoamine oxidase A (*MaoA*) and (N) monoamine oxidase B (*MaoB*). Data represents mean ± SEM, #$p < 0.05$, ###$p < 0.001$, ####$p < 0.0001$ - treatment effect, +$p < 0.05$ - genotype effect, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ - *post hoc* test, ordinary two-way ANOVA (Bonferroni *post hoc* test) (A-J), Mann-Whitney U test (K-N).

Triple immunohistochemical stainings of WT mice midbrain to pons area, revealed expression of tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH), the rate limiting enzyme of serotonin production, throughout the dopaminergic and serotonergic neurons (Figure 9A-E). Localization of NEGR1 is also observed in cells expressing TH in substantia nigra pars reticulata and TPH in dorsal, median raphe, indicating the involvement of NEGR1 in the both dopaminergic as well as serotonergic neurotransmission (Figure 9F-Q). The Supplementary image panel S presents specificity of Alexa 647 secondary antibody binding.

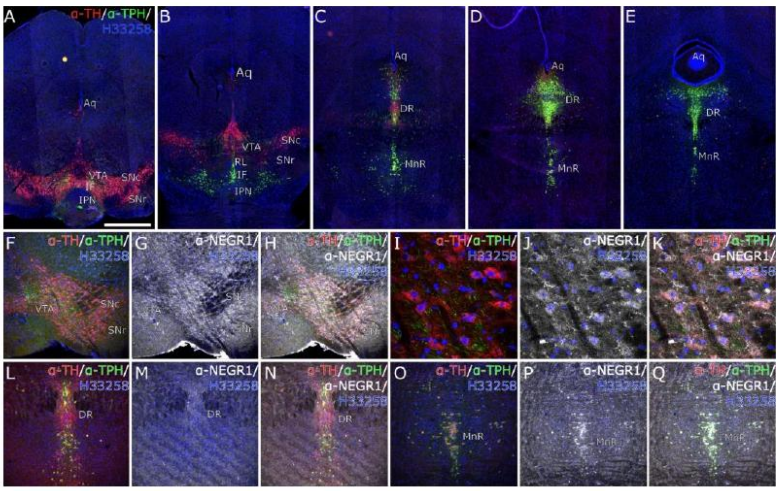

Figure 9. Immunohistochemical staining of WT mouse brain coronal sections displaying expression of Tyrosine hydroxylase (TH), Tryptophan hydroxylase 2 (TPH) with NEGR1. (A-E) epifluorescent images display localization of TH and TPH throughout dopaminergic and raphe nuclei. (F-K) laser scanning confocal microscope images displaying localization of NEGR1 in TH positive cells in substantia nigra pars reticulata (SNr). (L-N) laser-scanning confocal microscope images show diffuse localization of NEGR1 in dorsal raphe (DR) whereas (O-Q) the localization in medial raphe (MnR) is observable in cells expressing TH and TPH. (A-Q) Nuclei were stained using H33258 stain (blue). Scale bars: (A-E) 1mm, (F-H, L-Q) 0.5 mm, (I-K) 100 μm. Aq - aqueduct, VTA - ventral tegmental area, IF - interfacicular nucleus, IPN - interpeduncular nucleus, SNc - substantia nigra pars compacta, SNr - substantia nigra pars reticulate, RL - rostral linear nucleus, DR - dorsal raphe nucleus, MnR - median raphe nucleus.

3.6. Chronic administration of escitalopram causes no alterations in the behavior of Negr1-/- mice.

Negr1$^{-/-}$ and WT received 23 days of escitalopram and the behavior of the mice was assessed in the elevated plus maze (day 16), open field test (days 19 and 20), and tail suspension test (day 22). The test results showed that in the tail suspension test latency to freeze was affected by the treatment ($F_{1,50} = 4.189$; $p = 0.046$). In the elevated plus maze test frequency to enter closed arms was affected by the genotype ($F_{1,50} = 4.713$; $p = 0.035$). In the open field test corner visits made within first 5 minutes of the experiment was affected by the genotype ($F_{1,50} = 4.356$; $p = 0.042$). There were no other significant behavioral changes in the chronic escitalopram treatment experiment (Supplementary figure S19; S20).

4. Discussion

4.1. NEGR1 expression in monoaminergic brain circuits

In human GWAS studies, the neural adhesion molecule encoding *NEGR1* gene has been linked to both depression and obesity [6, 7, 1, 3]. Altered monoaminergic neurotransmission has also been linked to both obesity [53, 54] and depression [33] and these conditions have been associated with neural pathways that are guided and maintained in the presence of NEGR1 protein. The expression of *Negr1* has been shown in both dopaminergic nuclei and projection areas such as substantia nigra pars compacta, VTA, islands of Calleja in the VSTR [27] and in the fasciculus retroflexus, which serves as a molecular scaffold for dopaminergic axons that grow from the midbrain towards the habenula [34]. Additionally, *Negr1* has been identified as a differentially expressed gene across 5-HT neuron subtypes, whereas the expression of *Negr1* was highest in the median raphe [36].

The current study is the first to explore the brain monoaminergic system in NEGR1-deficient mice to gain novel insights whether these neural circuits could be responsible for the link between NEGR1 polymorphisms and phenotypes of depression and obesity. We challenged the monoaminergic neurotransmission of mice lacking *Negr1* and their WT littermates with chronic injection of either amphetamine or escitalopram. The behavior of these mice was tested, and brain monoamines and gene expression were measured from the brain consequently. In our earlier studies, we have shown by using in situ hybridization that compared to other brain areas, the mRNA expression of *Negr1* is sparse in the striatal areas, especially in the DSTR of adult mice [55]. To estimate NEGR1 protein expression and impact in the dopaminergic signaling in the striatal area, we performed NEGR1 and tyrosine hydroxylase co-staining in the striatal area (Figure 5). We found that *Negr1* in the striatum is highly expressed in the fibers and moderately on cell bodies where *Negr1* also shows co-localization with tyrosine hydroxylase, indicating that *Negr1* is expressed in the same cells where dopamine is synthesized. The staining indicates that at least some amount of the NEGR1 protein in the striatum is not synthesized on the cell bodies in the striatum but is expressed on the axon bundles projecting through it. The more specific identification of these bundles remains to be clarified in future studies.

Next, we also studied potential expression of NEGR1 in the region from midbrain to pons, by using triple immunostainings for tryptophan hydroxylase, tyrosine hydroxylase and NEGR1 (Figure 10). Additionally, to striatum, we found co-expression of tyrosine hydroxylase and NEGR1 also in the VTA/substantia nigra region. Our findings of more prevalent NEGR1 expression in the median raphe compared to dorsal raphe are in line with the findings from Okaty et al. [36]. In the median raphe, NEGR1 is present in both the tyrosine hydroxylase and tryptophan hydroxylase-positive cells, whereas in the dorsal raphe, the expression of NEGR1 is minor. In conclusion, we show that NEGR1 protein is present in both dopaminergic and serotonergic pathways; in the tyrosine hydroxylase-positive cells in both striatum and midbrain and in the raphe where NEGR1 is mostly present in the median raphe. Considering our current knowledge about the function of NEGR1 in the

nervous system [56], we propose that NEGR1 plays role in the organization of protein networks at the monoaminergic synaptic cleft and/or in the regulation of motility and assembly of synaptic vesicles.

4.2. Increased behavioral sensitization to amphetamine and upregulation of Dat transcript in Negr1$^{-/-}$ mice

In the current study, 10-day administration of amphetamine induced significantly higher motor and stereotypic activity in *Negr1*$^{-/-}$ mice compared to WT mice, indicating a higher sensitivity to amphetamine. It has been shown earlier that time-dependent changes in behavioral sensitization to amphetamine are associated with time-dependent changes in amphetamine-stimulated DA release in the striatum [57]. In our current study, significantly increased DA levels after 10-day administration of amphetamine were evident in the dorsal striatum in *Negr1*$^{-/-}$ mice. However, the DA metabolite 3-MT was elevated in the ventral striatum in *Negr1*$^{-/-}$ mice, indicating that DA release was increased there as well. Increased levels of *Comt* transcript in the ventral striatum is further supporting higher dopamine release along with higher DA turnover in the ventral striatum in *Negr1*$^{-/-}$ mice. The difference that we see between dorsal and ventral striatum, could indicate differential effect of amphetamine on the dopamine release and uptake in dorsal and ventral striatum [58, 59].

Transcripts encoding proteins regulating dopaminergic neurotransmission were mostly significantly increased in the striatum and VTA area of *Negr1*$^{-/-}$ mice. Namely, dopamine transporter (*Dat*) transcripts were significantly upregulated both in the VTA and ventral striatum in *Negr1*$^{-/-}$ mice. Transcripts encoding tyrosine hydroxylase, *MaoA* and *MaoB* were upregulated in the VTA, whereas *Comt* was upregulated in the ventral striatum (Figure 3 and 4). DAT plays a central role in the regulation of dopaminergic signaling; DAT overexpressing transgenic mice demonstrate markedly increased locomotor responses to amphetamine compared with WT animals [60]. Likewise, reduced DAT expression has been shown to diminish amphetamine's locomotor stimulatory effects [61]. Furthermore, similarly to our current finding in the ventral striatum in *Negr1*$^{-/-}$ mice, an increase in the amount of DA released by amphetamine has been shown in the DAT overexpressing mice [60].

From our previous studies we have shown that deletion of other IgLONs, both *Lsamp* and *Ntm*, which are closely related NEGR1 homologs, are causing reduced sensitivity for acute amphetamine administration in mice [40, 24]. Moreover, this phenotype is basically the only overlapping phenotype in mice deficient for either *Lsamp* or *Ntm* and the insensitivity for amphetamine is magnified in *Lsamp*$^{-/-}$*Ntm*$^{-/-}$ double mutant mice. Indeed, in the current study we could not see clear genotype difference in amphetamine treatment groups, however our data indicates tendency toward reduced sensitivity in case of acute administration of amphetamine. Therefore, members of the IgLON family of neural adhesion

molecules could be collectively responsible for the fine tuning of neural circuits involved in both acute and chronic responses to amphetamine.

4.3. Altered molecular reactivity to the amphetamine in the brains of Negr1$^{-/-}$ mice

The robust effect of amphetamine was similar in the striatum in both *Negr1*$^{-/-}$ mice and their WT controls. Amphetamine reduced the turnover of dopamine to DOPAC and dopamine to HVA in a similar rate. Higher amphetamine-induced dopamine levels in the dorsal striatum and higher 3-MT levels in the ventral striatum were induced by amphetamine only in the *Negr1*$^{-/-}$ mice. Increased DAT and tyrosine hydroxylase in the midbrain after amphetamine have been described earlier in wild type animals [62, 63], likewise, in the current study, amphetamine induced an increase in tyrosine hydroxylase and a trend towards increased levels of *Dat*, *Comt* and *Drd2* in WT mice in the VTA. In *Negr1*$^{-/-}$ mice, on the contrary, these transcripts showed a trend towards amphetamine-induced reduction, indicating altered molecular reactivity to amphetamine in the brains of *Negr1*$^{-/-}$ mice. Similarly, in the hippocampi of WT mice, amphetamine significantly increased the levels of serotonin and normetanephrine and induced a tendency for an increase of several monoamines including dopamine. In *Negr1*$^{-/-}$ mice, the amphetamine induced changes in monoamine profile were quite different in the hippocampus, while the only significant effect of amphetamine was reduced levels of tyrosine (Figure 7). We also explored the effect of acute and chronic amphetamine on the NEGR1 transcript in various brain areas, however, the downregulation of *Negr1* transcript induced by chronic amphetamine was present only in the frontal cortex and only in 129Sv mice (Supplementary figure S21).

The baseline levels of DA itself were not found to be altered in the brain areas of *Negr1*$^{-/-}$ mice, however, several significant alterations in several brain areas of the *Negr1*$^{-/-}$ mice suggest increased turnover of dopamine but also of serotonin. Increased turnover of DA to 3-MT was evident in both dorsal and ventral striatum. Interestingly, serotonin metabolite 5-HIAA was increased only in the ventral striatum of *Negr1*$^{-/-}$ mice. Although amphetamine suppressed 5-HIAA in both genotypes, the 5-HIAA still remained higher in *Negr1*$^{-/-}$ mice. The upregulation or the tendency for upregulation of monoamines and their metabolites in saline-injected *Negr1*$^{-/-}$ mice was most evident in the hippocampal area. The results from mice receiving saline chronically from amphetamine study and escitalopram study could be regarded as replicates (Figure 6) and they indicate genotype differences between knockout and control groups in two distinct age groups; the age of mice at the end of escitalopram study was 3 months and the age of mice in the end of amphetamine study was 5 months. Significantly higher levels in *Negr1*$^{-/-}$ mice compared to WT could be detected for tyrosine, 3-MT, tyramine, 5-HT, 5-HIAA, noradrenaline and normetanephrine.

The stronger behavioral effect of amphetamine in *Negr1*$^{-/-}$ mice could have also been modulated by the trace amine associated receptor 1 (TAAR1) that has been shown to serve as a direct intracellular target for amphetamines in dopaminergic neurons [64]. TAAR1 is

stimulated by amphetamine, but also by a variety of trace amines and monoamines, which are upregulated in the brains of Negr1$^{-/-}$ mice, such as tyramine and 3-MT. The increased levels of endogenous agonists could have an impact on the sensitivity of TAAR1, which could in turn, influence the effects of amphetamine.

Taken together, while the robust effect of amphetamine in the reduction of dopamine turnover in the striatum was similar in both genotypes, there were significant alterations in the response to amphetamine in the brains of *Negr1*$^{-/-}$ mice which indicated higher tone of dopaminergic neurotransmission in the dorsal striatum but blunted response of dopamine system related gene expression in the midbrain.

4.4. Negr1$^{-/-}$ *mice display reduced sensitivity to experimental manipulations and show less activity during chronic injections/testing*

In the chronic amphetamine study, the genotype differences appeared already prior to injections, as an open field test for baseline activity (7 days before injections started) and a consequent housing in single cages induced the expected decrease in the body weight of WT mice, whereas the body weight of *Negr1*$^{-/-}$ mice stayed stable or even increased slightly during the same time period (Figure 2) resulting in the disappearance of previously significant body weight difference between genotypes (Supplementary figure S13). This indicates that *Negr1*$^{-/-}$ mice could be less sensitive to the single-housing stress similarly to the phenotype we have previously described in *Lsamp*$^{-/-}$ mice [43]. In the baseline open field test, *Negr1*$^{-/-}$ mice spent significantly more time in the center of the field, indicating higher exploratory activity and reduced anxiety (the results of this experiment have been published in [27]. During the course of daily chronic injections, housing in single cages and testing, however, saline-receiving *Negr1*$^{-/-}$ mice became less active in most of the behavioral parameters that were measured, including total distance traveled and distance in the center (Figure 2). Interestingly, *Negr1*$^{-/-}$ mice performed significantly less rearings both during baseline testing [27] and during the course of chronic testing/saline injections in the current study. Previous studies have found that damage to the hippocampus impairs rearing due to failures in spatial memory, where novelty detection is impaired [65]. The hypothesis that reduced rearing in *Negr1*$^{-/-}$ mice could be the expression of impaired hippocampal morphology is supported by accumulating data of reduced size of hippocampus in *Negr1*$^{-/-}$ mice and numerous molecular and cellular alterations in the hippocampi of *Negr1*$^{-/-}$ mice [24, 27, 30].

4.5. Escitalopram-induced reduction of 5-HT and DA turnover is enhanced in Negr1$^{-/-}$ mice

Despite upregulation of the serotonin transporter (*Slc6a4*) which is the main target of escitalopram in the raphe of *Negr1*$^{-/-}$ mice, deletion of *Negr1* did not induce alterations in the behavior of mice after chronic administration of escitalopram (Supplementary figure S18). However, we found that escitalopram could rescue the significantly smaller volumes of hippocampi that *Negr1*$^{-/-}$ mice have compared to WT, the phenotype that we have also demonstrated earlier [27]. This result is intriguing, especially as it has been shown earlier that depression-related changes in the hippocampal volume could be prevented by antidepressant treatment [66] Still, several other studies indicate that hippocampal atrophy is persisting despite treatment of depression and long-term remission [67]. Interestingly, NEGR1 has been shown to be involved in neurogenesis [21, 25, 30]. However, it has to be noted that the hippocampal tissue weight, which could be comparable to MRI-based volumetric measures, cannot reflect subtle changes within different layers of the hippocampus (or dorsal vs. ventral region). Therefore the change in the weight/volume of the hippocampus is more likely to reflect dendritic arborization and not neurogenesis, that only takes place in sub-layers of the dentate gyrus [68].

It is questionable if *Negr1*$^{-/-}$ mice represent an appropriate model for depression despite the strong link of the human *NEGR1* gene with depression phenotypes in accumulating studies, because higher levels of NEGR1 have been described in the tissues and body fluids of depressed patients. However, it is likely that NEGR1 is regulating pathways that are linked with depression and therefore its role in the reactivity to escitalopram deserves further studies.

Nevertheless, we found clear genotype-specific alterations in biochemical reactions to escitalopram which were evident in the raphe nuclei but not in the hippocampus. Escitalopram induced a decrease in serotonin turnover in both genotypes, but this effect was highly significant only in the raphe of *Negr1*$^{-/-}$ mice. Furthermore, escitalopram induced a robust decrease in dopamine turnover only in *Negr1*$^{-/-}$ mice; this effect was amplified by the increased baseline turnover of dopamine (DOPAC/DA) in the *Negr1*$^{-/-}$ raphe compared to WT. In fact, the significant treatment effect suggests that escitalopram induced the elevation of serotonin in the raphe of both genotypes, but the significant elevation of dopamine was induced only in the raphe of wild-type mice, suggesting that alterations in the dopamine system could affect the serotonergic neurotransmission in *Negr1*$^{-/-}$ raphe. Interestingly, we have previously found that similarly to *Negr1*$^{-/-}$ mice, escitalopram-induced reduction of 5-HT turnover is enhanced in the raphe of *Lsamp*$^{-/-}$ mice [41] indicating overlapping functions of these homologous proteins. In *Negr1*$^{-/-}$ mice, however, we could not see increased 5-HT turnover, which was evident in various brain areas in *Lsamp*$^{-/-}$ mice. At the same time, we detected dopamine-related changes in the raphe of *Negr1*$^{-/-}$ mice that were not seen previously in *Lsamp*$^{-/-}$ mice suggesting distinct functions of these proteins in specific monoaminergic pathways. Accumulating evidence suggests that serotonergic system has an impact on the activity of dopaminergic neurons in the striatum [69], thus, the altered interplay of dopamine and serotonin might also be responsible for the

altered sensitivity to amphetamine in *Negr1*$^{-/-}$ mice, possibly indicated by the increased serotonin metabolite 5-HIAA in the ventral striatum of *Negr1*$^{-/-}$ mice.

4.6. Negr1 deficiency-induced alterations in the monoaminergic neurotransmission could explain links of NEGR1 with both depression and obesity phenotypes

Imbalance of monoaminergic neurotransmission in the brain areas, such as mesolimbic pathways [33], raphe [70] and hippocampus have been shown to underlie depressive conditions. Here we show that *Negr1*$^{-/-}$ mice display a time-dependent increase in behavioral sensitization to amphetamine associated with changes in amphetamine-stimulated DA release in the ventral and dorsal striatum, indicating altered reactivity of mesolimbic pathways lacking NEGR1 protein. Mesolimbic pathways underlying reward processing were our special interest as dysfunctional reward processing that has been described both in depressive patients [71, 72] and obese subjects [73] could be a shared mechanism underlying both obesity and depression. Anhedonia, one of the core symptoms of depression, has been linked to dysfunctions in the reward system, and in particular the dopamine system [74]. We have previously shown that *Negr1*$^{-/-}$ mice eat less palatable high fat food, both if the food consumption was measured for a longer period of time but also during the first 24 h of novel high fat food exposure [32]. Reduced intake of palatable food in a longer time period is more likely linked with altered metabolic homoeostasis in Negr*1*$^{-/-}$ mice; reduced intake of high fat food during the first 24 hours could be a sign of altered reward processing, which, however, needs further research. Serotonergic signaling contributes to the regulation of both homeostatic and hedonic feeding. In the hedonic circuitry it reduces reward related food consumption [75]. Increased serotonin levels found in the hippocampus of *Negr1*$^{-/-}$ mice may contribute to the reduced standard food intake we have previously observed in *Negr1*$^{-/-}$ mice [32] which in turn may explain the lower baseline body weight observed also in the current study. We also showed that *Negr1*$^{-/-}$ mice are less sensitive to stress/injection induced weight loss, which could reflect the impact of *Negr1* to the body mass index in both mice and humans.

5. Conclusions

We show that NEGR1 is expressed in both tyrosine- and tryptophan hydroxylase-positive cells and that *Negr1*$^{-/-}$ mice show altered reactivity to the substances that are targeting monoaminergic neurotransmission. The current study is the first to show alterations in the brain monoaminergic system in mice deficient of *Negr1*. We suggest that these neural circuits could underlay both depressive and obesity-related phenotypes that have been strongly linked with the *NEGR1* gene in human studies.

Limitations of the study

Due to technical reasons the monoamines from dorsal and ventral striatal areas have been measured by using different apparatus and protocol compared to the monoamines measured from hippocampi and raphe. Another limitation of the study was the usage of only

male mice. As we have shown in our previous paper [32] that male and female *Negr1*$^{-/-}$ mice show somewhat different metabolic profiles, female mice need to be included in the future studies. Heterozygous mice should be also included to specify the gene dose effects in altered phenotypes. Some of the neurodevelopmental and -chemical alterations in constitutive knockout mice may not be reversible or may need higher doses/duration of drug treatment. Additionally, important behavioral tests for depressive behavior, such as forced swim test and glucose preference tests have not been used in the current study and must be included in the future studies focusing on the depressive phenotypes in rodents. Studying the monoamine-related biochemistry and pharmacological reactions of mice lacking NEGR1 is only one option for understanding the role of NEGR1 in the depression. Accumulating evidence suggests that elevated levels on NEGR1 could be linked with pathological conditions such as obesity and depression, therefore, in the future studies, mice with NEGR1 overexpression would serve as a better mouse model for understanding the role of NEGR1 in the pathogenesis of depression and obesity.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1. Amphetamine dose curve and chronic amphetamine experiment; Table S2. The expression level of dopamine system related genes in the VTA; Table S3. The level of dopamine system related genes in the dorsal striatum; Table S4. The expression level of dopamine system related genes in the ventral striatum; Table S5. The level of serotonin system related genes in the raphe; Table S6. The level of dopamine system related genes in the frontal cortex; Table S7. The level of monoamines and their metabolites in VTA of amphetamine experiment mice; Table S8. Values (Mean ± SEM) and statistical parameters for the behavioral analysis of chronic amphetamine experiment; Figure S9. Effects of daily saline injections on activity of *Negr1*$^{-/-}$ and WT mice; Table S10. The level of monoamines and their metabolites in the dorsal striatum of amphetamine experiment mice; Table S11. The level of monoamines and their metabolites in the ventral striatum of amphetamine experiment mice; Figure S12. Effects of chronic administration of amphetamine to the levels of dopamine metabolites DOPAC and HVA in the striatal area; Figure S13. Effects of chronic administration of amphetamine (3 mg/kg) on the body weight of mice; Figure S14. Effects of chronic administration of escitalopram (10 mg/kg) on the body weight of mice; Table S15. The level of monoamines and their metabolites in the hippocampus of amphetamine experiment mice; Table S16. The level of monoamines and their metabolites in the hippocampus of escitalopram experiment mice; Table S17. The level of monoamines and their metabolites in the raphe of escitalopram experiment mice; Figure S18. Epifluorescent images from immunohistochemical staining of WT mouse hippocampal coronal sections; Figure S19. Escitalopram had no effect on the behavior of the mice; Table S20. Values (Mean ± SEM) and statistical parameters for chronic escitalopram experiment behavioral analysis; Figure S21. Expression of Negr1 in the ventral striatum was lower in 129Sv mice compared to Bl6 mice.

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