TARTU ÜLIKOOL
LOODUS- JA TÄPPISTEADUSTE VALDKOND
MOLEKULAAR- JA RAKUBIOLOOGIA INSTITUUT

Serotoniinisüsteem kui võimalik vahendaja *NEGR1* geeni ja autismispektri häire vahelisele seosele

Bakalaureusetöö
12 EAP
Helis Nõmm

Juhendajad: PhD Maria Kaare
PhD Mari-Anne Philips
PhD Tambet Tõnissoo

TARTU 2023

INFOLEHT

Serotoniinisüsteem kui võimalik vahendaja *NEGR1* geeni ja autismispektri häire vahelisele seosele

IgLON perekonda kuuluv neuronaalne kasvuregulaator NEGR1 on oluliseks kandidaadiks autismispektri häire (ASH) tekkes. NEGR1 mõjutab neurogeneesi, sünapsite moodustamist ja neuriitide väljakasvu. Neuronitega seotud areng on oluline serotoniinisüsteemile, kuna serotoniin innerveerib neuronite kaudu erinevaid ajuosasid, mõjutades ASH-le tunnuslikke funktsioone (nt emotsioone, kognitiivseid protsesse). Uurimustöö eesmärgiks oli *Negr1*$^{-/-}$ ja metsik-tüüpi (WT) hiirtes iseloomustada serotoniinisüsteemiga seotud geenide mRNA ekspressiooni raphes ning monoamiinide tasemeid raphes ja hippokampuses. Tulemuste analüüsimisel saadud olulisemad leiud: 1) *Negr1*$^{-/-}$ hiirte raphes on kõrgem serotoniini transporteri *Slc6a4* mRNA ekspressioonitase kui WT loomadel. 2) *Negr1*$^{-/-}$ hiirte hippokampuses oli tõusnud serotoniini metaboliidi (5-HIAA) tase. Tulemused viitavad sellele, et *NEGR1* geeni ja ASH-ga seotud tunnuste vahelised seosed võivad osaliselt tuleneda NEGR1 mõjust serotoniinisüsteemile.

Märksõnad: NEGR1, ASH, serotoniinisüsteem

CERCS: B640 Neuroloogia, neuropsühholoogia, neurofüsioloogia

The serotonin system as a possible mediator of the associations between the *NEGR1* gene and autism spectrum disorder

Neuronal growth regulator NEGR1 is an important candidate for autism spectrum disorder (ASD). NEGR1 affects neurogenesis, synapse formation and neurite outgrowth. Neuron-associated development is important for the serotonin system, as serotonin innervates through neurons to different brain regions, where serotonin influences functions also characterized in ASD (e.g., emotions, cognitive processes). The aim of this study was to characterize serotonin system related gene mRNA expression in raphe and the levels of monoamines in raphe and hippocampus in *Negr1*$^{-/-}$ and wild-type (WT) mice. Results showed increased mRNA expression of serotonin transporter gene (*Slc6a4*) in raphe from *Negr1*$^{-/-}$ mice and elevated levels of serotonin metabolite (5-HIAA) in the hippocampus of *Negr1*$^{-/-}$ mice. The results suggest that the association between *NEGR1* and ASD-related traits may partially arise from the influence of NEGR1 on the serotonin system.

Keywords: NEGR1, ASD, serotonin system

CERCS: B640 Neurology, neuropsychology, neurophysiology

SISUKORD

KASUTATUD LÜHENDID

5-HIAA – 5-hüdroksuindooläädikhape, 5-HT metaboliit (ingl. k. *5-hydroxyindoleacetic acid*)

5HIAA/5-HT – serotoniini käive ehk serotoniini metaboliidi ja serotoniini suhe (ingl. k. *ratio of serotonin metabolite to serotonin*)

5-HT – 5-hüdroksütrüptamiin ehk serotoniin (ingl. k. *5-hydroxytryptamine*)

5-HTP – 5-hüdroksütrüptofaan (ingl. k. *5-hydroxytryptophan*)

AAAD – aromaatne aminohappe dekarboksülaas (ingl. k. *aromatic amino acid decarboxylase*)

ASH – autismispektri häire

ATH - aktiivsus ja tähelepanuhäire (ingl. k. *attention deficit hyperactivity disorder*)

BBB – aju-vere barjäär (ingl. k. *blood-brain barrier*)

CAM - raku adhesioonimolekul (ingl. k. *cell adhesion molecul*)

GPI – glükosüül-fosfatidüül-inositool (ingl. k. *glycosylphosphatidylinositol*)

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

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

IgSF – immunoglobuliini superperekond (ingl. k. *immunoglobulin superfamily*)

KNS – kesknärvisüsteem

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

Mao-A – monoamiini oksüdaas A (ingl. k. *monoamine oxidase A*)

Mao-A – monoamiini oksüdaas A kodeeriv geen (ingl. k. *monoamine oxidase A encoding gene*)

Mao-B – monoamiini oksüdaas B (ingl. k. *monoamine oxidase B*)

Mao-B – monoamiini oksüdaas B kodeeriv geen (ingl. k. *monoamine oxidase B encoding gene*)

NEGR1/Kilon – neuronaalne kasvuregulaator (ingl. k. *neuronal growth regulator/kindred of IgLON*)

NEGR1 – neuronaalset kasvuregulaatorit (NEGR1) kodeeriv geen (ingl. k. *neuronal growth regulator encoding gene*)

Negr1$^{-/-}$– homosügootne *Negr1*-puudulik hiir (ingl. k. *homozygous Negr1-deficient mouse*)

NTM – neurotrimin

OPCML/OBCAM – opioide siduv raku adhesioonimolekul (ingl. k. *opioid-binding cell adhesion molecule*)

PNS – piirdenärvisüsteem

SERT1 – serotoniini transporter (ingl. k. *serotonin transporter*)

Slc6a4 – serotoniini transporterit (SERT1) kodeeriv geen (ingl. k. *serotonin transporter encoding gene*)

Tph – trüptofaani hüdroksülaas (ingl. k. *tryptophan hydroxylase*)

Tph2 – trüptofaani hüdroksülaasi kodeeriv geen (ingl. k. *tryptophan hydroxylase encoding gene*)

Trp – trüptofaan (ingl. k. *tryptophan*)

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

SISSEJUHATUS

Autismispektri häire on närvisüsteemi arengu käigus välja arenenud seisund, mida iseloomustab kahjustunud sotsiaalne käitumine ja kommunikatsioon ning ebatavalised korduvad käitumismustrid. Autismispektri häire täielik etioloogia on veel ebaselge, kuid on teada, et selle avaldumisel on oluline roll geneetilistel faktoritel. *NEGR1* geeni poolt kodeeritud adhesioonimolekulis NEGR1-s nähakse võimalikku alust autismispektri häire tekkele. NEGR1 valk on neuronaalne kasvuregulaator, mis kuulub immunogluboliinide LON valguperekonda ning ta funktsiooniks on kesknärvisüsteemi arengus neurogeneesi, sünapsite moodustamise ja neuriitide väljakasvu reguleerimine.

Serotoniin on neurotransmitter, mida toodetakse raphes aminohappest L-trüptofaanist. Raphest kantakse serotoniini mõju neuraalsete juhteteede kaudu laiali erinevatesse ajuosadesse. Sihtkohas, näiteks hippokampuses mõjutatakse seeläbi funktsioone, mille häirumine on tunnuslik autismispektri häirele. Serotoniinil on mõju näiteks kognitiivsetele protsessidele ja meeleolu reguleerimisele. Serotoniinisüsteemi regulatsioonile on oluline neuronite korrektne väljaareng ja regulatsioon, mida mõjutab NEGR1 tegevus. Varasemalt on leitud seoseid autismispektri häire ning serotoniinisüsteemi geenide ja monoamiinide töö vahel. Nende seoste põhjal võib serotoniinisüsteemi pakkuda üheks potentsiaalseks vahendajaks *NEGR1* geeni ja autismispektri häire vahelisele seosele.

Käesoleva töö eesmärk on uurida serotoniinisüsteemi geene ja monoamiine ning võrrelda nende regulatsiooni metsik-tüüpi kontrollhiirtes ja *Negr1* geeni puudulikkusega hiirtes. Lisaks hinnata, milline mõju võib *NEGR1* geenil olla autismispektri häirete avaldumisele. Töö eksperimentaalne osa teostati Tartu Ülikooli Arstiteaduskonna bio- ja siirdemeditsiini instituudi füsioloogia õppetoolis.

1. KIRJANDUSE ÜLEVAADE

1.1. IgLON perekond

Raku adhesioonimolekulid (CAM) on valgud, mis osalevad homo- ja heterofiilsetes rakkudevahelistes seondumistes ja kommunikatsioonis (Siegel et al., 2006). CAM-d jagunevad nelja perekonda. Integriinid, kadheriinid ja selektiinid on kaltsiumist-sõltuvad ning immunoglobuliinide (Ig) superperekond (IgSF) kuulub kaltsiumist sõltumatu perekonna alla (Siegel et al., 2006).

Immunoglobuliinide LON (IgLON) perekonna valgud on neuraalsed raku adhesioonimolekulid ning nad kuuluvad kaltsiumist-sõltumatute IgSF alla (Siegel et al., 2006). IgLON valgud on glükoproteiinid, mis sisaldavad kolme Ig-sarnast domeeni ning need kinnituvad plasma membraanile C-terminaalselt glükosüül-fosfatidüül-inositool (GPI) ankru abil (Tan et al., 2017). IgLON perekond moodustati pärast OPCML (*opioid-binding cell adhesion molecule*), LSAMP (*limbic system-associated membrane protein*) ja Neurotrimin (NTM) valkude kirjeldamist, kuna omasid tugevaid sarnasusi ning erinesid teistest perekondadest (Schofield et al., 1989; Pimenta et al., 1995; Struyk et al., 1995). 1999. aastal kirjeldati uut valku, mis tunnuste poolest klassifitseerus IgLON perekonda (Funatsu et al., 1999). Valk nimetati Kiloniks (*Kindred of IgLON*), mille sünonüümideks on Neurotractin ja NEGR1 (edaspidi: NEGR1) (Funatsu et al., 1999). Nüüdseks on lisandunud IgLON perekonda viies valk nimega IgLON5 (Sabater et al., 2014).

1.1.1. NEGR1

Käesolevas töös keskendutakse IgLON perekonna neuraalsele adhesioonimolekulile NEGR1 (ingl. k. *neuronal growth regulator*) valgule ja seda kodeerivale geenile *NEGR1*. *NEGR1* geeni asukoht inimesel on kromosoomis 1p31.1 (NCBI) ning sellelt transkribeeritakse 46 kDa suurune valk nimega NEGR1 (Funatsu et al., 1999). NEGR1 valgu struktuuris on IgLON perekonnale vastavalt kolm Ig-sarnast domeeni ja GPI-ankur, mille abil seondub valk C-terminaalselt rakumembraanile (Funatsu et al., 1999). Sim et al. (2022) on pakkunud välja, et NEGR1 valgu struktuuril olevad kuus N-glükosüülimiskohta (Funatsu et al., 1999) hoiavad valgu stabiilsust ja osalevad homofiilsetes seondumistes. *NEGR1* ekspresseerub erinevates aju piirkondades, peamiselt suurajukoores, ajutüves ja hippokampuses (Funatsu et al., 1999), kus ta on seotud erinevate neuronaalsete protsessidega. NEGR1-l on oluline roll neurogeneesi regulatsioonil (Noh et al., 2019). Lisaks soodustab NEGR1 neuriitide väljakasvu ja mõjutab

sünapsite moodustamist (Hashimoto et al., 2008; Schäfer et al., 2005). Nende protsesside reguleerimine on oluline juhteteede võrgustiku moodustumiseks.

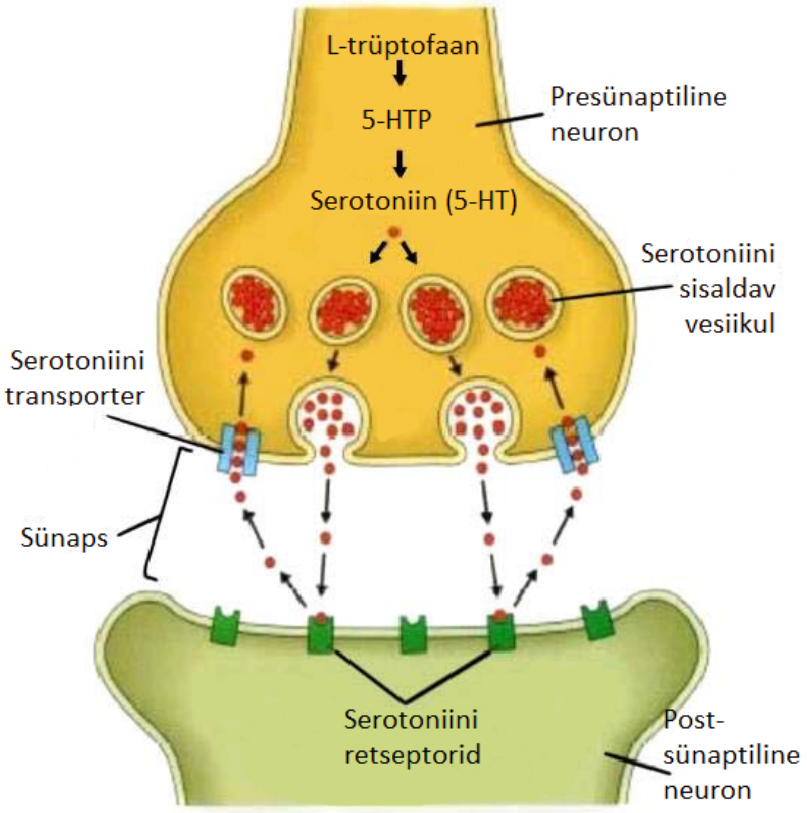
NEGR1-te seostatakse erinevate psühhiaatriliste häiretega, sealhulgas autismispektri häirega (ASH) (Grove et al., 2019). ASH üheks sümptomiks on häirunud käitumine sotsiaalsetes olukordades (World Health Organization, 1992), mida on leitud ka *Negr1*-puudulike (*Negr1*$^{-/-}$) hiirte puhul (Singh et al., 2018, 2019). Singh et al. (2019) näitab, et *Negr1*$^{-/-}$ hiirtel on vähenenud huvi sotsiaalse stiimuli vastu. 3-kambri testis ei esinenud *Negr1*$^{-/-}$ hiirtel tuttava ja tundmatu liigikaaslasega sotsialiseerumise vahel erinevusi. Metsik-tüüpi kontrollhiired (*wild type,* WT) hiired seevastu eelistasid liigikaaslase kohalolu. Singh et al. (2019) pakkus, et vastav käitumine *Negr1*$^{-/-}$ hiirtel võis tuleneda sotsiaalse mälu/äratundmise puudujäägist.

1.2. Serotoniinisüsteem

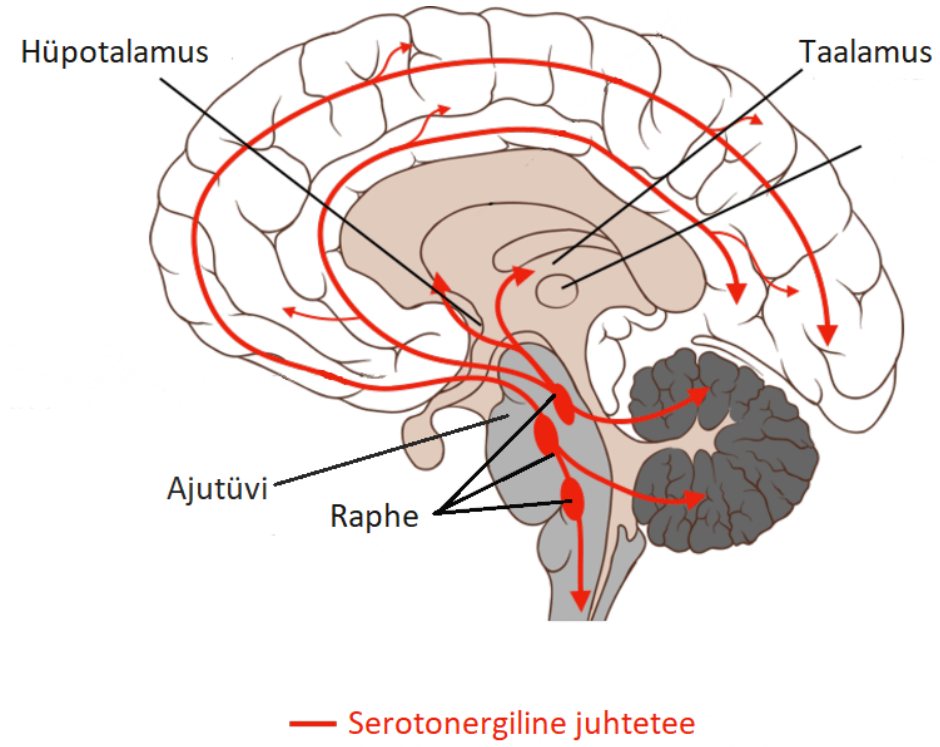
Serotoniin ehk 5-hüdroksütrüptamiin (5-HT) on neurotransmitter (Twarog, 1954), mida leidub lisaks kesknärvisüsteemile (KNS) ka piirdenärvisüsteemis (PNS) (Iversen et al., 2009). PNS-i ja KNS-i serotoniinisüsteemid ei ole omavahel seotud, kuna PNS-s toodetud serotoniin ei pääse aju-vere barjääri (BBB) tõttu KNS-i, mistõttu toodavad ajurakud ise serotoniini (Iversen et al., 2009). KNS-s on primaarseks 5-HT tootmise kohaks ajutüves asuv raphe (Iversen et al., 2009). Raphe presünaptilistes serotonergilistes neuronites sünteesitakse 5-HT asendamatust aminohappest trüptofaanist kahe ensümaatilise sammuga (Clark et al., 1954). Esmalt L-trüptofaani hüdroksüülitakse trüptofaani hüdroksülaasiga (Tph), mille tulemusel saadakse 5-hüdroksütrüptofaan (5-HTP) (Clark et al., 1954). Seejärel 5-HTP dekarboksüleeritakse aminohappe dekarboksülaasiga (AAAD) ning moodustub 5-hüdroksütrüptamiin (5-HT) ehk serotoniin (Clark et al., 1954; joonis 1).

Serotoniinisüsteem koosneb mitmetest neuraalsetest juhteteedest (joonis 2) ning nende kaudu transporditakse 5-HT aju erinevatesse piirkondadesse (nt limbilistesse struktuuridesse) (Clark et al., 2010). Juhteteede alguseks on raphe, kus presünaptilistes serotonergilistes neuronites toodetakse 5-HT-d (Iversen et al., 2009). Toodetud 5-HT hoiustatakse neuroni presünaptilise osa vesiikulites, kust 5-HT vabastatakse sünaptilisse pilusse (Schatzberg ja Nemeroff, 2004). Seejärel seondub 5-HT postsünaptilise neuroni membraanil asetsevatele 5-HT-spetsiifilistele retseptoritele, et neuronite kaudu anda edasi impulss, mis liigub lõpuks vastavatesse ajuosadesse (Schatzberg ja Nemeroff, 2004). 5-HT vastuvõtvaid retseptoreid on post- kui presünaptilistel neuronitel kokku vähemalt 14 erinevat alamtüüpi (Hannon & Hoyer, 2008). Sünaptilises pilus olev 5-HT eemaldatakse tagasihaarde protsessiga, millega lõpetatakse

postsünaptilisele neuronile mõju andmine (Schatzberg & Nemeroff, 2004). Selle protsessi eest vastutab presünaptilise neuroni membraanil asetsev valk serotoniini transporter (SERT1), mis on kodeeritud *Slc6a4* geeni poolt (Heils et al., 1995). 5-HT tagasihaarde käigus 5-HT transporditakse tagasi presünaptilisse neuronisse. Seal 5-HT kas metaboliseeritakse või sisestatakse uuesti vesiikulisse, kus ta on kaitstud metabolismi eest ja teda saab uuesti kasutada (Schatzberg & Nemeroff, 2004). 5-HT metabolismi viib läbi ensüümi monoamiini oksüdaasi kaks eri vormi, monoamiini oksüdaas A (Mao-A) ja monoamiini oksüdaas B (Mao-B) (Schatzberg & Nemeroff, 2004). Ensüümid lagundavad 5-HT inaktiivseks metaboliidiks 5-hüdroksuindoolääadikhappeks (5-HIAA), mis väljutatakse uriini kaudu (Schatzberg & Nemeroff, 2004). 5-HT tagasihaarde ja metaboliseerimisega reguleeritakse 5-HT taset ning hoitakse ära liigseid 5-HT signaale neuronite vahel. Häirumist serotoniinisüsteemi regulatsioonis seostatakse erinevate neuroloogiliste ja psühhiaatriliste häiretega, sealhulgas ASH-ga (Cook Jr et al., 1997).



Joonis 1. Serotoniini süntees ja transport.
Kirjeldab signaaliülekannet presünaptilisest serotoniini (punane) tootvast neuronist (kollane) postsünaptilisele neuronile (heleroheline), mida vahendab serotonergiline sünaptiline ülekanne ehk sünaps. Serotoniini sünteesitakse trüptofaanist, hoiustatakse vesiikulis ning seejärel serotoniin vabaneb vesikulaarse transpordi kaudu sünapsisse. Signaali postsünaptilisele neuronile vahendatakse retseptorite (tumeroheline) kaudu. Presünaptilisel neuronil asub serotoniini transporter (sinine), mis reguleerib serotoniini tagasihaaret sünaptilisest pilust tagasi presünaptilisse neuronisse. (Modifitseeritud Madsen et al., 2009 järgi)



Joonis 2. Serotonergilised juhteteed inimese peaajus.
Joonisel on punase joonega näidatud serotoniinisüsteemis serotoniini innervatsiooni võimalikke radasid erinevatesse ajuosadesse. (Modifitseeritud Nummenmaa et al., 2020 järgi)

1.3. Autismispektri häire

Autismispektri häire (ASH) uurimise alguseks loetakse 1940. aastaid kui kaks psühhiaatrit Leo Kanner ja Hans Asperger avaldasid eraldi artiklid autismi teemal (Folstein ja Rosen-Sheidley, 2001). 80 aastat hiljem on mõlema psühhiaatri esialgsed arusaamad ASH kohta küll muutunud, kuid need panid aluse autismi uurimisele. Rahvusvaheliste Haiguste Klassifikatsiooni 10. versioonis (RHK-10) on kasutusel termin "pervasiivsed arenguhäired", kuid vastavas töös kasutatakse edaspidi terminit "autismispektri häire (ASH)" (World Health Organization, 1992). "ASH" on kasutusel RHK 11. versioonis ning lisaks on Eesti Autismiliit täpsustanud, et "pervasiivsete arenguhäirete" asemel kasutatakse terminit "autismispektri häire" (World Health Organization, 2019; Eesti Autismiliit, i.a.).

Autismispektri häire (ASH) on psüühikat mõjutav aju arenguhäire, mida iseloomustab kahjustunud sotsiaalne areng ja korduvad käitumismustris (World Health Organization, 1992). ASH kujuneb välja närvisüsteemi arengu käigus ning seetõttu on see eluaegne seisund (World Health Organization, 1992). Ülemaailmselt on autismispektri häire keskmiseks levimuseks 100/10 000 ehk maailmas sünnib hinnanguliselt 1 laps 100-st ASH-ga (Zeidan et al., 2022). Eesti kohta andmed puuduvad, kuid Soomes on see suhtarv 10 000 elaniku kohta 76 ja

Euroopas 63,5 (Zeidan et al., 2022). Autismispektri häired kuuluvad RHK-10 V peatüki alla, mis käsitleb psüühika- ja käitumishäireid (World Health Organization, 1992). Eestis kasutatakse endiselt ASH diagnoosimiseks RHK 10. versiooni, kuigi Maailma Terviseorganisatsioon (WHO) andis 2019. aastal välja RHK-11 ingliskeelse versiooni. Praeguseks pole avastatud üksikuid kindlaid usaldusväärseid biomarkereid, mille alusel ASH-d diagnoosida ning seetõttu tuvastatakse seda hetkel käitumise põhjal (Lord et al., 2018). RHK-10 järgi on ASH-le omane triaad käitumuslikke kriteeriume: sotsiaalne areng, suhtlemine ning korduvad ja piiratud käitumismustrid (World Health Organization, 1992). Autismispektri häirete alla kuulub lapse autism, atüüpiline autism, Retti sündroom, lapse muu desintegratiivne häire, hüperaktiivsus motoorsete stereotüüpiate ja vaimse alaarenguga, Aspergeri sündroom, muud täpsustatud pervasiivsed arenguhäired ning täpsustamata pervasiivne arenguhäire (World Health Organization, 1992). Lapse autismi diagnoos pannakse siis, kui eelpool nimetatud kõigis kolmes kriteeriumis esineb häireid ning sümptomid väljenduvad enne kolmandat eluaastat (World Health Organization, 1992). Autismi levimus on sooliselt erinev. Poistel esineb autismi neli korda rohkem kui tüdrukutel (Maenner, 2020). Lisaks on ASH-ga inimestel suurem risk teistele psühhiaatrilistele probleemidele, nagu erinevad foobiad, une- ja söömishäired, ärevushäired, agressiivsushood, sage enesevigastamine, intellektipuue, aktiivsus- ja tähelepanuhäire (ATH) ning depressioon (World Health Organization, 1992).

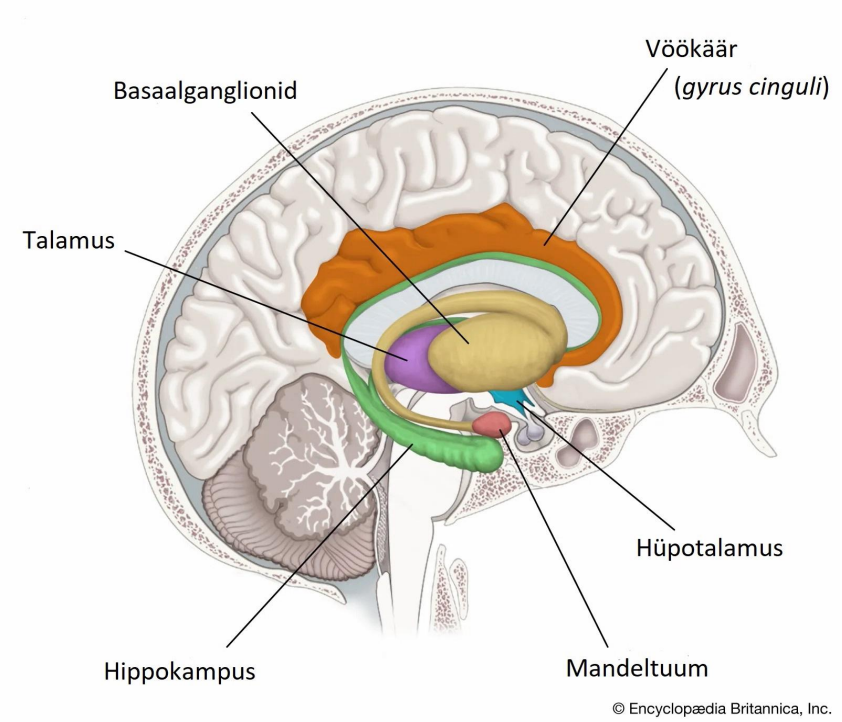
1.3.1. ASH-ga seotud ajupiirkonnad

ASH on paljudest teguritest mõjutatud häire, mis hõlmab muutusi erinevate ajupiirkondade struktuuris ja funktsioonis. ASH-i täpsemad põhjused on veel ebaselged, kuid laialdased uuringud on tuvastanud mitu ajupiirkonda (nt raphe, hippokampus), mis võivad olla ASH-ga seotud.

Ajutüve keskjoone läheduses asuvad serotonergiliste neuronite kehad, mille kogumikke nimetatakse rapheks (Clark et al., 2010; joonis 2). Raphe neuronite kehades toodetakse serotoniini, mille signaal viiakse neuronite aksonite kaudu teistesse ajupiirkondadesse (Siegel et al., 2006). Seeläbi mõjutab serotoniin inimese sotsiaalset käitumist (Luo et al., 2017). Seetõttu on pakutud, et muutused raphes on seotud ASH-i sotsiaalse puudujäägi tekkimisega (Luo et al., 2017).

Ajutüve kohal asub limbiline süsteem, mis koosneb paljudest ajustruktuuridest, sealhulgas hippokampusest (Clark et al., 2010; joonis 3). Limbilise süsteemi funktsiooniks on

emotsioonide töötlemine ja sotsiaalse käitumise kontrollimine (Clark et al., 2010). Hippokampuse funktsioon on seotud mälu kujunemise ja taastamisega ning ta seob omavahel pikaajalist mälu ja töömälu (Clark et al., 2010). Uuringud on näidanud, et häired limbilises süsteemis on seotud ASH inimeste sotsiaalsete ja emotsionaalsete raskustega (Stephens et al., 2021).



Joonis 3. Limbilise süsteemi peamised komponendid.
Aju külgvaade, mis näitab limbilise süsteemi peamisi komponente: mandeltuum, hüpotalamus, vöökäär (*gyrus cinguli*), basaalganglionid, talamus ja hippokampus. (Modifitseeritud Raikar, 2023 järgi)

1.3.2. ASH-i seos *NEGR1* geeniga.

ASH-i avaldumist mõjutavad paljud erinevad geneetilised ja keskkonnategurid (Rylaarsdam ja Guemez-Gamboa, 2019). ASH-i tuvastamise muudab keeruliseks häire heterogeenne olemus. Leitud on palju erinevaid võimalikke ASH-d põhjustavaid geene, kuid siiski pole kindlaid biomarkereid, mille abil diagnoosida ASH-i (Lenroot & Yeung, 2013). *NEGR1* geeni on pakutud üheks võimalikuks biomarkeriks erinevatele psüühikahäiretele, sealhulgas ASH-le (Grove et al., 2019). *NEGR1* ja ASH seost on näidanud ka GWAS uuringud (Lee et al., 2019). Lisaks sellele, et *NEGR1*-te seostatakse erinevate häiretega, mõjutab *NEGR1* ASH-i tõenäoliselt koos teiste geenidega. Szczurkowska et al., (2018) näitas, et *Negr1* koostöös *Fgfr2* geeniga muudab hiire käitumist. *Negr1*$^{-/-}$ hiirtel oli suurenenud enesehooldamine, mis võib-olla tunnuslik ASH-le (Szczurkowska et al., 2018).

1.3.3. ASH-i seos serotoniinisüsteemiga

ASH-i täpsed põhjused on veel ebaselged, siiski on leitud ASH-i ja serotoniinisüsteemi häirumise vahel seoseid. Serotoniin (5-HT) on neurotransmitter, mis mõjutab palju erinevaid psüühilisi funktsioone (Tan et al., 2004). 5-HT-l on oluline roll emotsioonide ning erinevate kognitiivsete protsesside, näiteks tähelepanu ja mälu, reguleerimisel (Tan et al., 2004). ASH-i diagnoosi puhul on need funktsioonid häiritud (Reyes et al., 2020). Lisaks neurotransmitteri funktsioonile on 5-HT-l oluline roll ka kasvufaktorina (Sodhi & Sanders-Bush, 2004). Inimese varajases arengujärgus on 5-HT tootmine suurem, mille läbi reguleerib 5-HT närvisüsteemi arengut (Muller et al., 2016). ASH-ga lastel on see arenguline protsess häiritud (Chugani et al., 1999).

Arvatakse, et 5-HT inaktiivse vormi 5-HIAA kogus tserebrospinaalvedelikus peegeldab serotonergiliste neuronite sünaptilist aktiivsust, mis omakorda võib olla ASH-i diagnoosimise biomarker (De Grandis et al., 2010). Uuringus leiti neuropsühhiaatriliste häiretega patsientidel tserebrospinaalvedelikus 5-HIAA puudulikkus (De Grandis et al., 2010). On leitud ka, et ASH-ga võib olla seotud kromosoomi piirkond 17q11.2. (Cook Jr et al., 1997; Lauritsen & Ewald, 2001). Vastavas piirkonnas asub geen *Slc6a4*, mis kodeerib serotoniini transporteri (SERT1) valku ning kontrollib serotoniini tagasihaaret sünapsist neuronisse (Heils et al., 1995).

2. EKSPERIMENTAALOSA

2.1. Töö eesmärgid

Antud bakalaureusetöös püstitati hüpotees, et neuraalne kasvuregulaator NEGR1 mõjutab serotoniinisüsteemi kaudu autismispektri häire avaldumist. Sellest lähtuvalt oli töö eesmärgiks välja selgitada, kas *Negr1* geeni väljalülitamine hiires põhjustab muutusi serotoniinisüsteemiga seotud geenide (*Slc6a4, Tph2, MaoA, MaoB*) mRNA suhtelises ekspressioonis raphes ja serotoniinisüsteemiga seotud monoamiinide tasemetes katseloomade (*Negr1*$^{-/-}$ ja WT isased hiired) raphes ja hippokampuses ning seeläbi teha järeldusi, kas NEGR1 võib mõjutada autismispektri häire avaldumist serotoniinisüsteemi kaudu.

2.2. Materjal ja metoodika

2.2.1. Katseloomad ja koematerjal

Bakalaureusetöös kasutatud katseloomade paljundamine ja hooldus viidi läbi Tartu Ülikooli bio- ja siirdemeditsiini katseloomakeskuses. Katseloomadeks olid isased homosügootsed *Negr1*- puudulikud hiired (*Negr1*$^{-/-}$) ja nende pesakonnakaaslastest metsik-tüüpi kontrollhiired (WT, *Negr1*$^{+/+}$), kes olid 129S5/SvEvBrd ja C57BL/6N segatüüpi geneetilise taustaga. Hiired paigutati rühmadesse standardsetesse laboratoorsetesse puuridesse mõõduga 42,5 (P) × 26,6 (L) × 15,5 (K) cm, 22 ± 1°C, 12:12 valguse/pimeduse tsükliga. Igas puuris oli kasutusel haavapuust allapanu (Tapvei, Eesti) ja 0,5 l haavapuust pesamaterjali (Tapvei, Eesti), mida vahetati iga nädal. Toidugraanulid ja vesi olid kättesaadavad *ad libitum*. Serotoniinisüsteemiga seotud geenide ekspressioonitasemete uurimiseks (raphes) kasutatavad katseloomad olid ohverdamise hetkel 4 kuu vanused (*Negr1*$^{-/-}$ n=11 ja *Negr1*$^{+/+}$ n=11) ja monoamiinide tasemete määramiseks (hippokapmuses ja raphes) kasutatavad katseloomad olid dekapiteerimise hetkel 3 kuu vanused (*Negr1*$^{-/-}$ n= 13-15 ja *Negr1*$^{+/+}$ n=13-15). Dekapiteeritud katseloomadelt eraldati dissekteerimise käigus vastavad ajupiikonnad (hippokampus ja raphe), mis külmutati vedelas lämmastikus. Katseloomade käsitlemise ja dissekteerimise viis läbi vastavat FELASA sertifikaati omav spetsialist.

2.2.2. RNA eraldamine

RNA eraldamiseks ajukudedest kasutati Trizoli® reagenti (Invitrogen) vastavalt tootja protokollile. Koeproovid tõsteti sulamiseks jääle ning seejärel alustati koeproovide homogeniseerimisega. Väiksematele koetükkidele lisati 500 µl Trizoli® ja suuremate koetükkide puhul lisati Trizoli® 1000 µl. Esialgu homogeniseeriti uhmriga koed väikese

koguse Trizoli® reagendiga ning edasi homogeniseeriti ülejäänud Trizoliga® vähemalt 1 mm paksuseks. Seejärel inkubeeriti homogenisaate 5 minutit toatemperatuuril. 1,5 ml tuubi (Axygen®) lisati 100 µl kloroformi, raputati 15 sekundit käes ning pandi 2 minutiks toatemperatuurile inkubeerima. Segu tsentrifuugiti (15 minutit, 12 000 x g) 4°C juures. Tekkis kolm kihti, kust pealmine RNA-d sisaldav vesifaas tõsteti uude 1,5 ml tuubi (Eppendorf). Vesifaasile lisati 250 µl isopropanooli. Tuub segati hoolega läbi tuubi pöörates ning inkubeeriti 10 minutit toatemperatuuril. RNA sadestati tsentrifuugimisel (10 minutit, 12 000 x g) 4°C juures põhja ja supernatant eemaldati. RNA sademele lisati soolade maha pesemiseks 1 ml jääkülma 75% etanooli. Tsentrifuugiti 5 minutit 7500 x g 4°C juures ning eemaldati supernatant. Seekord tehti tuub supernatandist täiesti kuivaks vaakumi abiga. Tuubidesse lisati 50 µl destilleeritud vett (ddH_2O) ning RNA suspendeeriti lahusesse. RNA kontsentratsioonid lahuses mõõdeti NanoDrop ND-1000 spektrofotomeetriga (NanoDrop Technologies). RNA kontsentratsioon viidi ligikaudu 500 ühikud. Eraldatud RNA proovid hoiustati -80°C juures.

2.2.3. cDNA süntees

Töös kasutati pöördtranskriptsiooniks FIREScript® RT cDNA juhusliku järjestusega kuuenukleotiidiste praimerite segu (*random hexamer*) (Solis BioDyne) vastavalt tootja protokollile. Komplementaarse DNA (cDNA) sünteesiks võeti RNA eraldamisest saadud lahused jääle sulama. Uude 1,5 ml tuubi pipeteeriti 10 µl RNA lahust. Seejärel lisati lahusele 2 µl kuuenukleotiidiste praimerite segu. Tuubi lisati veel 6,5 µl nukleaasivaba ddH_2O ning tekkinud segu segati läbi. Viimasena lisati lahusesse 1,5 µl FIREScript® ensüümisegu, mis suspendeeriti õrnalt pipetiga lahusesse. Kokku tekkis 20 µl lahust, mida inkubeeriti praimerite seondumiseks 5 minutit toatemperatuuril. Järgnevalt pöördtranskriptsiooni alustamiseks inkubeeriti lahust 30 minutit 50°C juures. Ensüümide inaktivatsiooniks hoiti proove 5 minutit 85°C juures. Saadud cDNA proovid hoiustati -80°C juures.

2.2.4. qPCR

Uuritavate geenide: monoamiini oksüdaas A (*MaoA*), monoamiini oksüdaas B (*MaoB*), serotoniini transporter (*Slc6a4*) ja trüptofaani hüdroksülaas (*Tph2*) uurimiseks kasutati kvantitatiivset polümeraasi ahelreaktsiooni (qPCR). Uuritavate mRNA-de suhtelise ekspressiooni määramiseks kasutati kontrollina koduhoidja-geeni β-aktiin (*ActB*). Geenidekspressiooniks 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 segule lisati 1,5 µl sünteesitud cDNA-d, ddH_2O ja uuritavad praimerid (Lisa 1). Igast proovist villiti neli kordust ning ühe reaktsiooni maht oli 10 µl. Geenieksprессiooni tuvastamiseks qPCR meetodiga kasutati ABI Prism 7900HT Sequence Detection System (Applied Biosystems) ja ABI Prism 7900 SDS 2.4.2. tarkvara.

qPCR-s esmalt toimub denaturatsioon, kus DNA kaksikahelad seonduvad lahti. Seejärel toimub DNA kokkusulandumine, mille käigus praimerid seonduvad DNA üksikahela komplementaarsetele aladele. HOT FIREPol® DNA polümeraas seondub praimerile ning sünteesib 3`-5`suunas DNA üksikahelale komplementaarse ahela. EvaGreen® värv seondub tekkinud DNA kaksikahelale ning värvi fluorestsentsi mõõdetakse pärast iga tsüklit. Kokku mõõdeti 40 tsüklit. Andmed analüüsiti ja teisendati kujule $2^{-\Delta CT.}$

2.2.5. Monoamiinide mõõtmine

Monoamiinide tasemete mõõtmine telliti bio- ja siirdemeditsiini instituudi biokeemia õppetoolilt. Raphe ja hippokampusega seotud monoamiinide kvantifitseerimine sooritati vedelikkromatograafia-massispektromeetriga (LC/MS). Uuritavateks serotoniinisüsteemiga seotud monoamiinideks oli trüptofaan (Trp), serotoniin (5-HT), serotoniini metaboliit (5-HIAA) ning serotoniini käive ehk serotoniini metaboliidi ja serotoniini suhe (5HIAA/5-HT). Monoamiinide tasemed esitati ühikuga pmol/mg. Põhjalikum protokoll kasutatud metoodikast on leitav Kaare et al. (2022) artiklist (lisa 2). Tulemuste analüüs sooritati käesoleva töö raames.

2.2.6. Statistiline analüüs

Andmete analüüsiks kasutati GraphPad Prism 8. versiooni tarkvara. qPCR-st saadud geenide mRNA ekspressioone ja monoamiinide tasemeid hinnati Studenti t-testiga. Tulemused esitati keskmiste väärtustena koos standardveaga (SEM). Kontroll- ja testgrupi vaheline seos loeti statistiliselt oluliseks kui p-väärtus oli alla 0,05 ($p<0,05$).

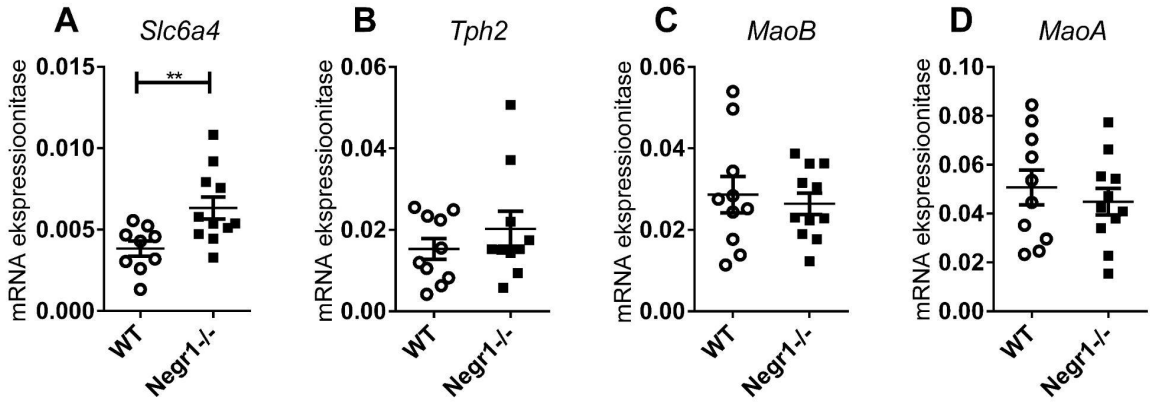
2.3. Tulemused

Alljärgnevalt kirjeldatud bakalaureusetöö eksperimentide tulemused ja analüüs on teostatud käesoleva töö autori poolt, mis on avaldatud Kaare et al. (2022) (lisa 2).

2.3.1. Serotoniinisüsteemiga seotud geenide mRNA suhteline ekspressioon raphes

Antud bakalaureusetöö eesmärgiks oli välja selgitada, kas *Negr1* geeni väljalülitamine põhjustab muutusi katseloomade serotoniinisüsteemis ning hinnata, kas NEGR1 mõjutab autismispektrihäire (ASH) avaldumist. Kuna varasemalt on seostatud ASH kujunemist muutustega teatud ajupiirkondades, nagu raphe (Luo et al., 2017), siis otsustati uurida võimalikke serotoniinisüsteemiga seotud geenide ekspressiooni muutusi *Negr1*$^{-/-}$ hiirte vastavas ajupiirkonnas võrrelduna WT pesakonnakaaslastega. Selleks valiti välja serotoniinisüsteemis olulised geenid: serotoniini transporter (*Slc6a4*), trüptofaani hüdroksülaas (*Tph2*), monoamiini oksüdaas A (*MaoA*) ja monoamiini oksüdaas B (*MaoB*), mille suhtelisi ekspressiooni tasemeid hinnati qPCR-ga.

Analüüsi tulemusena selgus, et serotoniini transporterit kodeeriva geeni *Slc6a4* mRNA ekspressiooni tase raphes oli statistiliselt oluliselt (p=0,01) kõrgem *Negr1*$^{-/-}$ hiirtel kui WT kontroll-isastel (joonis 4A, tabel 1). Trüptofaani hüdroksülaasi (*Tph2*) mRNA ekspressiooni tasemetes uuritud katseloomade (*Negr1*$^{-/-}$) raphetes märkimisväärseid erinevusi ei täheldatud (p=0,338; joonis 4B; tabel 1). Järgnevalt hinnati monoamiini oksüdaaside *MaoA* (joonis 4C) ja *MaoB* (joonis 4D, tabel 1) suhtelist mRNA ekspressiooni taset ja leiti, et *Negr1*$^{-/-}$ ja WT kontrollide monoamiinide oksüdaaside ekspressioon raphes oli sarnane (*MaoA*, p=0,521; *MaoB*, p=0,663).



Joonis 4. Serotoniinisüsteemiga seotud geenide mRNA suhteline ekspressioon raphes. (**A**) serotoniini transporteri (*Slc6a4*), (**B**) trüptofaani hüdroksülaasi (*Tph2*), (**C**) monoamiini oksüdaas B (*MaoB*) ja (**D**) monoamiini oksüdaas A (*MaoA*) suhtelised mRNA ekspressioonitasemed metsik-tüüpi (WT) ja *Negr1*-puudulikel (*Negr1*$^{-/-}$) hiirtel raphes. Ekspressiooni taseme määramiseks on võrdlusena kasutatud koduhoidja-geeni β-aktiini suhtelist ekspressiooni ja $2^{-\Delta CT}$ metoodikat. Tulemused on esitatud keskmiste väärtustena (neli kordust) koos standardveaga ± SEM. ** p=0,01

Tabel 1. Raphes mõõdetud serotoniinisüsteemiga seotud geenide suhtelise mRNA ekspressiooni tase (WT) ja *Negr1*-puudulikel (*Negr1*$^{-/-}$) hiirtel.

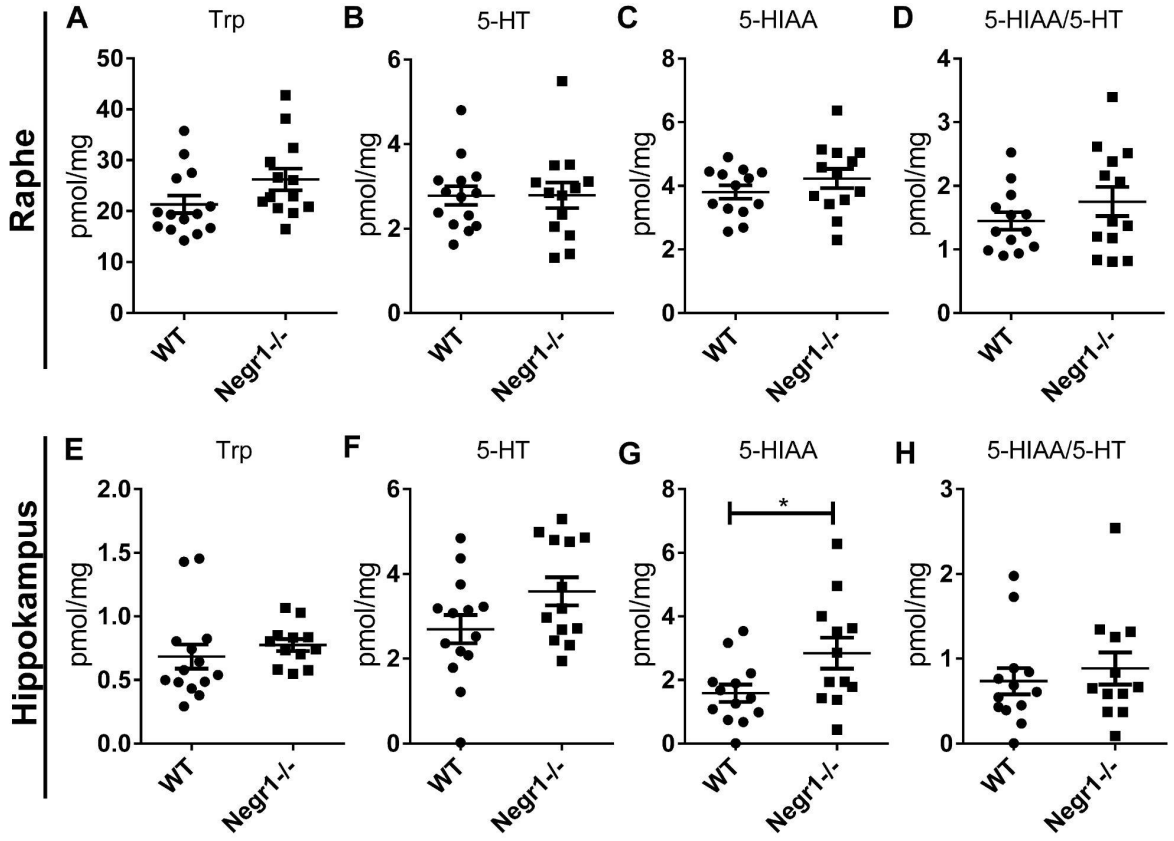
Raphe	**n (WT)**	**n (*Negr1-/-)***	**WT** keskmine ± SEM	***Negr1-/-*** keskmine ± SEM	**t-test (p-väärtus)**
Slc6a4	9	11	0,004 ± 0,0005	0,006 ± 0,0007	0,010**
Tph2	10	10	0,015 ± 0,003	0,026 ± 0,004	0,338
MaoA	10	11	0,051 ± 0,007	0,045 ±0,005	0,521
MaoB	10	11	0,029 ± 0,004	0,026 ± 0,003	0,663

**statistiliselt väga oluline tulemus (p<0,01)

2.3.2. Serotoniinisüsteemiga seotud monoamiinide tasemed raphes ja hippokampuses

Varasemalt oli teada, et häired limbilises süsteemis on seotud ASH kujunemisega (Stephens et al., 2021) ja samuti muutused raphes (Luo et al., 2017) põhjustavad ASH-i. Seetõttu vaadeldi järgnevalt serotoniinisüsteemiga seotud monoamiinide tasemeid raphes kui ka hippokampuses. Analüüsiti olulisi serotoniinisüsteemi monoamiinide: trüptofaani (Trp), serotoniini (5-HT) ja serotoniini metaboliiti (5-HIAA). Lisaks hinnati ka serotoniini käivet (5-HIAA/5-HT).

Analüüsi tulemusena oli märgatav tendents, et trüptofaani tase on tõusnud *Negr1*$^{-/-}$ hiirte raphes, kuid see tulemus ei leidnud statistiliselt kinnitust (p=0,084; joonis 5A; tabel 2). Hippokampuses ei leitud Trp puhul olulist (p=0,427) muutust *Negr1*$^{-/-}$ hiirtel (joonis 5E, tabel 3). Järgnevalt uuriti serotoniini (5-HT) tasemeid *Negr1*$^{-/-}$ hiirtel võrrelduna WT-kontrollidega raphes (p=0,987; joonis 5B; tabel 2) ja hippokampuses (p=0,07; joonis 5F, tabel 3) ning olulisi muutusi ei täheldatud. Märkimisväärseks leiuks oli serotoniini metaboliidi (5-HIAA) kõrgem tase *Negr1*$^{-/-}$ hiirte hippokampuses võrreldes WT kontrollhiirtega (p=0,031; joonis 5G, tabel 3). Raphes seevastu 5-HIAA taseme puhul katseloomadel olulist tõusu ei esinenud (joonis 5C, tabel 2). Lõpuks analüüsiti ka serotoniini käivet (5-HIAA/5-HT) *Negr1*$^{-/-}$ ja WT loomade raphes ja hippokampuses, mille tulemusena leiti, et serotoniini käibe osas katseloomadel olulisi muutusi ei ole (raphe, p=0,267; joonis 5D; tabel 2; hippokampus, p=0,542; joonis 5H, tabel 3).



Joonis 5. Serotoniinisüsteemiga seotud monoamiinide tasemed *Negr1*$^{-/-}$ ja WT hiirte raphes ja hippokampuses. (**A**) trüptofaani (Trp), (**B**) serotoniini (5-HT), (**C**) serotoniini metaboliidi (5-HIAA) ning (**D**) serotoniini käibe (5-HIAA/5-HT) tasemed raphes. (**E**) trüptofaani (Trp), (**F**) serotoniini (5-HT), (**G**) serotoniini metaboliidi (5-HIAA) ning (**H**) serotoniini käibe (5-HIAA/5-HT) tasemed hippokampuses. * p=0,031

Tabel 2. Serotoniinisüsteemiga seotud monoamiinide keskmised tasemed metsik-tüüpi (WT) ja *Negr1*-puudulike (*Negr1*$^{-/-}$) hiirte raphetes.

Raphe	**n (WT)**	**n (*Negr1-/-*)**	**WT** keskmine ± SEM	***Negr1-/-*** keskmine ± SEM	**t-test (p-väärtus)**
Trp	14	13	21,33 ± 1,720	26,22 ± 2,132	0,084
5-HT	14	13	2,783 ± 0,222	2,788 ± 0,303	0,987
5-HIAA	13	13	3,806 ± 0,208	4,233 ± 0,303	0,257
5HIAA/5-HT	13	13	1,450 ± 0,137	1,743 ± 0,229	0,267

Tabel 3. Serotoniinisüsteemiga seotud monoamiinide keskmised tasemed metsik-tüüpi (WT) ja *Negr1*-puudulike (*Negr*$^{-/-}$) hiirte hippokampustes.

Hippo-kampus	**n (WT)**	**n (*Negr1-/-)***	**WT** keskmine ± SEM	***Negr1-/-*** keskmine ± SEM	**t-test (p-väärtus)**
Trp	14	12	0,685 ± 0,095	0,775 ± 0,047	0,427
5-HT	14	13	2,699 ± 0,334	3,59 ± 0,331	0,07
5-HIAA	13	12	1,584 ± 0,274	2,845 ± 0,488	0,031*
5HIAA/5-HT	13	12	0,735 ± 0,154	0,885 ± 0,189	0,542

*statistiliselt oluline tulemus ($p<0,05$)

2.4. Arutelu

Autismispektri häire (ASH) kujuneb välja närvisüsteemi arengu käigus, mille häirumine põhjustab puudujääke inimese käitumuslikes protsessides. ASH-i avaldumisega seostatakse tuhandeid erinevaid geene, kuid pole leitud ühtegi kindlat biomarkerit (Xiong et al., 2019). Üheks potentsiaalseks markergeeniks ASH-le peetakse *NEGR1*-te (Szczurkowska et al., 2018). NEGR1 on neuronaalne kasvuregulaator, mis tähendab, et NEGR1 osaleb närvisüsteemi arengus neuronite väljakujunemise reguleerimisel. ASH avaldub varajases eas närvisüsteemi häirumise tõttu ning seetõttu võib arvata, et muutused NEGR1 ekspressioonis või regulatsioonis on aluseks ASH avaldumisele. Szczurkowska et al. (2018) on näidanud, et *Negr1* geeni mahasurumine põhjustab *Negr1*-puudulikkusega (*Negr1*$^{-/-}$) hiirtes ASH-le omast häiritud käitumist. Täpne NEGR1-e regulatsiooni mehhanismi mõju neuronitele ja sünapsitele on veel ebaselge. Kaare et al. (2022) on aga välja pakkunud, et NEGR1-l on oluline roll valguvõrgustiku organiseerimisel monoaminergilises sünaptilises pilus ja/või sünaptiliste vesiikulite liikuvuse ja kokkupaneku reguleerimisel. Seetõttu võib arvata, et NEGR1-e ja ASH-i vahendajaks võib olla serotoniinisüsteem. Neuronite ja sünapsite düsregulatsiooni korral võib otseselt häiritud olla serotoniinisüsteemi töö. Seda seetõttu, et serotoniini sünteesimine ja transport toimub neuronites ja neuronitevahelistes sünapsites. Neuronite väljaarengu ja regulatsiooni potentsiaalne häirumine võib tuua muutusi serotoniinisüsteemis ning seeläbi võib ajuosadesse jõuda ebatavaline serotoniini impulss, mis võib esile tuua ASH-i sümptomeid.

Käesoleva töö eesmärgiks oli iseloomustada serotoniinisüsteemiga seotud geenide ekspressiooni raphes ning monoamiinide taset raphes ja hippokampuses metsik-tüüpi

kontrollhiirtes (WT) ja *Negr1*-puudulikkusega (*Negr1*$^{-/-}$) hiirtes ning seeläbi hinnata, kas *NEGR1* mõjutab serotoniinisüsteemi kaudu autismispektri häire avaldumist. Uuriti serotoniinisüsteemiga seotud geenide mRNA ekspressiooni ja monoamiinide tasemeid. Antud uuring sai läbi viidud, et uurida varasemalt näidatud ASH-i ja serotoniinisüsteemi seoselisust. Eelnevalt on Cook Jr et al. (1997) näidanud, et muutused serotoniini transporterit kodeerivas (*Slc6a4*) alas on korrelatsioonis ASH-ga.

Käesolevas töös leiti kaks statistiliselt olulist tulemust, mis viitavad serotoniinisüsteemi muutustele *Negr1*-puudulikkusega hiirte ajudes. Esimene statistiliselt oluline väärtus leiti serotoniini transporteri geenile (*Slc6a4*). Raphe piirkonnas tuvastati *Negr1*$^{-/-}$ hiirtel kõrgem *Slc6a4* mRNA suhteline ekspressioon. Selline leid viitab, et *Negr1*-puudulikkusega isastel hiirtel on raphes ka valgu tasandil rohkem serotoniini transporterit. Seeläbi on võimalik, et serotoniini haaratakse raphe piirkonnas sünaptilisest pilust intensiivsemalt tagasi kui see toimub kontrollhiirtes. Suurenenud serotoniini transporteri valgu seondumist raphes on varasemalt täheldatud ka emakakaela düstoonia uurimise käigus (Smit et al., 2018).

Serotoniinisüsteemi normaalseks toimimiseks on oluline, et serotoniini tekke ja selle lagundamine metaboliitideks oleks tasakaalus. Seda väljendab serotoniini käive, mis näitab serotoniini metaboliidi taseme suhet serotoniini tasemesse (5-HIAA/5-HT). Seetõttu peaks töös leitud suurenenud *Slc6a4* geeni mRNA ekspressioon mõjutama ka serotoniini käibe suurenemist. Monoamiinide mõõtmisel aga ei tuvastatud, et raphe piirkonnas oleks oluline erinevus serotoniini käibes. Siiski käesolevas uurimuses mõõdeti monoamiinid ja nende metaboliidid tervest dissekteeritud ajukoe tükist ning seetõttu ei saa eristada nende molekulide tasemeid sünaptilises pilus ja rakusiseselt. Sellest tulenevalt ei saa välistada suurenenud serotoniini käivet *Negr1*-puudulike hiirte raphe piirkonnas. Teiste uuritud geenide puhul raphe piirkonnas (*Tph2, Maoa, Maob*) statistiliselt olulisi muutusi geeniekspressioonis genotüüpide vahel ei tuvastatud.

Teiseks statistiliselt oluliseks leiuks käesolevas töös oli serotoniini metaboliidi 5-HIAA tõus isaste *Negr1*-puudulikkusega hiirtel hippokampuse piirkonnas. Seetõttu võib see tulemus viidata suurenenud serotoniini käibele selles ajukoes. Serotoniini transporterit hippokampuses mõõta ei saa, sest serotonergiliste rakkude kehad, kus serotoniini transporter sünteesitakse, paiknevad üksnes raphe piirkonnas. Varasemalt on pakutud, et 5-HIAA kogus tserebrospinaalvedelikus peegeldab serotonergiliste neuronite sünaptilist aktiivsust ning seega võiks olla potentsiaalseks biomarkeriks psühhiaatriliste häirete tuvastamisel (De Grandis et

al., 2010). De Grandis et al. (2010) uuringus leiti neuropsühhiaatriliste häiretega patsientidel tserebrospinaalvedelikus 5-HIAA puudulikkus.

Serotoniinisüsteemiga seotud muutused *Negr1*-puudulikkusega isaste hiirte ajuosades, raphes ja hippokampuses, viitavad sellele, et inimese *NEGR1* geeni ja ASH-ga seotud fenotüüpide tugevad seosed võivad vähemalt osaliselt tuleneda sellest, et NEGR1 mõjutab neuraalse adhesioonimolekulina serotoniinisüsteemi.

2.5. Peamised järeldused

- Isastes hiirtes *Negr1* geeni väljalülitamise tagajärjel suureneb serotoniini transporteri (*Slc6a4*) geeni mRNA ekspressioon raphe piirkonnas. mRNA taseme tõus on suure tõenäosusega seotud ka kõrgema serotoniini transportervalgu tasemega sünaptilises pilus, mis võib olla seotud suurema serotoniini käibega. Raphes mõõdetud monoamiinide tasemetes see ei väljendunud. Käesoleva uurimuse raames aga ei saa välistada serotoniini käive muutust, kuna monoamiinide tasemeid ei mõõdetud eraldi sünaptilises pilus ja rakusiseselt.
- Serotoniinisüsteemi metaboliitidest esines isastel hiirtel *Negr1* geeni väljalülitamise tagajärjel suurenenud serotoniini metaboliidi 5-HIAA tase hippokampuse piirkonnas.
- Käesoleva töö tulemused annavad varasemale kirjandusele toetudes aluse hüpoteesile, et *NEGR1* geen võib mõjutada autismispektri häire avaldumist serotoniinisüsteemi regulatsiooni kaudu.

KOKKUVÕTE

Käesolevas töös püstitati hüpotees, et NEGR1 (neuraalne kasvuregulaator) valk mõjutab serotoniinisüsteemi kaudu autismispektri häire avaldumist. Hüpoteesi uurimiseks leiti qPCR meetodiga serotoniinisüsteemiga seotud geenide: serotoniini transporteri (*Slc6a4*), trüptofaani hüdroksülaasi (*Tph*), monoamiini oksüdaasi A (*MaoA*) ja monoamiini oksüdaasi B (*MaoB*) mRNA ekspressiooni tasemeid raphes metsik-tüüpi kontrollhiirtes (WT) ja *Negr1*-puudulikes hiirtes (*Negr1*$^{-/-}$). Lisaks uuriti vedelikkromatograafia-massispektromeetriga saadud serotoniinisüsteemiga seotud monoamiinide: trüptofaani (Trp), serotoniini (5-HT), serotoniini metaboliidi (5-HIAA) ning serotoniini käive ehk serotoniini metaboliidi ja serotoniini suhte (5-HIAA/5-HT) tasemeid WT ja *Negr1*$^{-/-}$ hiirte raphes ja hippokampuses.

Varasemalt on leitud, et muutused serotoniinisüsteemiga seotud ajupiirkondades, näiteks raphes ja hippokampuses, mõjutavad autismispektri häire (ASH) avaldumist. Käesolevas töös uuriti hiirtes *Negr1* geeni väljalülitamise mõju serotoniinisüsteemi geenide mRNA ekspressioonidele raphes ning monoamiinide tasemetele raphes ja hippokampuses. Negr1-puudulikkusega hiirtel leiti serotoniinisüsteemis muutusi. Märkimisväärset tulemust täheldati isaste *Negr1*$^{-/-}$ hiirte *Slc6a4* geeni mRNA suhtelises ekspressioonis. Raphe piirkonnas ekspresseerus *Slc6a4* kõrgemalt *Negr1*$^{-/-}$ hiirtes kui WT hiirtes. Monoamiinide mõõtmistest esines oluliselt suurenenud tase 5-HIAA isaste *Negr1*$^{-/-}$ hiirte hippokampuses. *Negr1*-puudulikkusega hiirte kõrgenenud serotoniinisüsteemiga seotud *Slc6a4* geeni mRNA ekspressiooni ja 5-HIAA monoamiini tasemed kontrollgrupiga võrreldes annavad hüpoteesile tõestust, et NEGR1 adhesioonimolekulina mõjutab serotoniinisüsteemi ning seeläbi on vahendajaks NEGR1-e ja ASH-i vahelistel seostel.

Edasised uuringud peaksid keskenduma *Negr1*-puudulikkusega emaste hiirte serotoniinisüsteemiga seotud geenide mRNA ekspressiooni ja monoamiinide tasemete mõõtmisele. Lisaks tuleks monoamiinide mõõtmistel eristada sünaptilise pilu tasemeid rakusisestest tasemetest, mille läbi saaks teada täpsemad molekulide tasemed, seeläbi ka serotoniini käibe ehk serotoniini metaboliidi ja serotoniini suhte.

SUMMARY

The serotonin system as a possible mediator of the associations between the *NEGR1* gene and autism spectrum disorder

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by impaired social behavior and communication and abnormal repetitive patterns. Full etiology of ASD is still unknown, but genetic factors play an important role. Possible biomarker for ASD is neuronal growth regulator NEGR1, that is encoded by *NEGR1* gene. NEGR1, a member of the IgLON family, is a cell adhesion molecule that takes part in regulating neurogenesis, synapse formation and neurite outgrowth in the development of central nervous system. Neuron-associated development is important for the serotonin system. Serotonin is synthesised from tryptophan in raphe nuclei`s serotonergic neurons. Serotonin is released into synaptic cleft where it binds on postsynaptic neurons. Then, through neurons serotonin innervates different brain regions thereby influencing functions also characterized in ASD, such as emotions and cognitive processes.

In this study, the hypothesis was proposed that NEGR1 influences the manifestation of ASD through the serotonin system. To investigate the hypothesis, the expression levels of genes associated with serotonin system were examined using qPCR method. These genes included serotonin transporter (*Slc6a4*), tryptophan hydroxylase (*Tph*), monoamine oxidase A (*MaoA*) and monoamine oxidase B (*MaoB*) in the raphe nuclei of wild-type control mice (WT) and *Negr1*-deficient mice (*Negr1*$^{-/-}$). In addition, the levels of monoamines associated with serotonin system were investigated using liquid chromatography-mass spectrometry. These monoamines included tryptophan (Trp), serotonin (5-HT), serotonin metabolite (5-HIAA) and serotoniini turnover represented by the ratio of serotonin metabolite to serotonin (5-HIAA/5-HT) in the raphe nuclei and hippocampus of WT and *Negr1*$^{-/-}$ mice.

Previously, it has been shown that alterations in brain regions (raphe nuclei and hippocampus) associated with the serotonin system impact the manifestation of ASD. Present study examined the impact of *Negr1* gene knockout (*Negr1*$^{-/-}$) in mice on the mRNA expression of serotonin system-related genes (raphe nuclei) and on the levels of monoamines (raphe nuclei and hippocampus). Changes in the serotonin system were found in *Negr1*-deficient mice. A significant effect was found in the *Slc6a4* gene of male *Negr1*$^{-/-}$ mice. In the raphe region, the *Slc6a4* gene was more highly expressed in *Negr1*$^{-/-}$ mice than in WT mice. Monoamine measurements showed significantly increased levels of 5-HIAA in the hippocampus of male *Negr1*$^{-/-}$ mice. Elevated serotonin system-related *Slc6a4* gene mRNA expression and 5-HIAA

monoamine levels in *Negr1*-deficient mice compared to controls provide evidence for the hypotheses that Negr1 as an adhesion molecule affects the serotonin system and thereby serves as a mediator in the associations between NEGR1 and ASD.

Future studies should focus on measuring *Negr1*-deficient female mice on serotonin system-related gene mRNA expression and monoamine levels. In addition measurement of monoamine should be distinguished between synaptic cleft and intracellular levels, which would provide more precise information about molecular levels, including serotonin turnover, represented by the ratio of serotonin metabolite to serotonin.

TÄNUSÕNAD

Soovin tänada oma juhendajaid Maria Kaaret ja Mari-Anne Philipsit huvitava bakalaureusetöö teema ja juhendamise eest ning Tambet Tõnissood igakülgse abistamise eest. Suur tänu meie töögrupi koostööpartnerile bio- ja siirdemeditsiiniinstituudi biokeemia õppetoolile, kus viidi läbi vedelikkromatograafia-massispektromeetria.

KASUTATUD KIRJANDUS

Kasutatud artiklid

Chugani, D. C., Muzik, O., Behen, M., Rothermel, R., Janisse, J. J., Lee, J., & Chugani, H. T. (1999). Developmental changes in brain serotonin synthesis capacity in autistic and nonautistic children. *Annals of Neurology*, *45*(3), 287–295. https://doi.org/10.1002/1531-8249(199903)45:3<287::aid-ana3>3.0.co;2-9

Clark, C. T., Weissbach, H., & Udenfriend, S. (1954). 5-hydroxytryptophan decarboxylase: Preaparation and properties. *Journal of Biological Chemistry*, *210*(1), 139–148. https://doi.org/10.1016/S0021-9258(18)65440-7

Cook Jr, E., Courchesne, R., Lord, C., Cox, N. J., Yan, S., Lincoln, A., Haas, R., Courchesne, E., & Leventhal, B. L. (1997). Evidence of linkage between the serotonin transporter and autistic disorder. *Molecular Psychiatry*, *2*(3), Article 3. https://doi.org/10.1038/sj.mp.4000266

De Grandis, E., Serrano, M., Pérez-Dueñas, B., Ormazábal, A., Montero, R., Veneselli, E., Pineda, M., González, V., Sanmartí, F., Fons, C., & others. (2010). Cerebrospinal fluid alterations of the serotonin product, 5-hydroxyindolacetic acid, in neurological disorders. *Journal of Inherited Metabolic Disease*, *33*(6), 803–809. https://doi.org/10.1007/s10545-010-9200-9

Folstein, S. E., & Rosen-Sheidley, B. (2001). Genetics of autism: Complex aetiology for a heterogeneous disorder. *Nature Reviews Genetics*, *2*(12), Article 12. https://doi.org/10.1038/35103559

Funatsu, N., Miyata, S., Kumanogoh, H., Shigeta, M., Hamada, K., Endo, Y., Sokawa, Y., & Maekawa, S. (1999). Characterization of a novel rat brain glycosylphosphatidylinositol-anchored protein (Kilon), a member of the IgLON cell adhesion molecule family. *The Journal of Biological Chemistry*, *274*(12), 8224–8230.

https://doi.org/10.1074/jbc.274.12.8224

Grove, J., Ripke, S., Als, T. D., Mattheisen, M., Walters, R. K., Won, H., Pallesen, J., Agerbo, E., Andreassen, O. A., Anney, R., & others. (2019). Identification of common genetic risk variants for autism spectrum disorder. *Nature Genetics*, *51*(3), Article 3. https://doi.org/10.1038/s41588-019-0344-8

Hannon, J., & Hoyer, D. (2008). Molecular biology of 5-HT receptors. *Behavioural Brain Research*, *195*(1), 198–213. https://doi.org/10.1016/j.bbr.2008.03.020

Hashimoto, T., Yamada, M., Maekawa, S., Nakashima, T., & Miyata, S. (2008). IgLON cell adhesion molecule Kilon is a crucial modulator for synapse number in hippocampal neurons. *Brain Research*, *1224*, 1–11. https://doi.org/10.1016/j.brainres.2008.05.069

Heils, A., Teufel, A., Petri, S., Seemann, M., Bengel, D., Balling, U., Riederer, P., & Lesch, K.-P. (1995). Functional promoter and polyadenylation site mapping of the human serotonin (5-HT) transporter gene. *Journal of Neural Transmission / General Section JNT*, *102*(3), 247–254. https://doi.org/10.1007/BF01281159

Kaare, M., Jayaram, M., Jagomäe, T., Singh, K., Kilk, K., Mikheim, K., Leevik, M., Leidmaa, E., Varul, J., Nõmm, H., & others. (2022). Depression-Associated Negr1 Gene-Deficiency Induces Alterations in the Monoaminergic Neurotransmission Enhancing Time-Dependent Sensitization to Amphetamine in Male Mice. *Brain Sciences*, *12*(12), Article 12. https://doi.org/10.3390/brainsci12121696

Lauritsen, M., & Ewald, H. (2001). The genetics of autism. *Acta Psychiatrica Scandinavica*, *103*(6), 411–427. https://doi.org/10.1034/j.1600-0447.2001.00086.x

Lee, P. H., Anttila, V., Won, H., Feng, Y.-C. A., Rosenthal, J., Zhu, Z., Tucker-Drob, E. M., Nivard, M. G., Grotzinger, A. D., Posthuma, D., & Wang, M. M.-J. (2019). Genomic relationships, novel loci, and pleiotropic mechanisms across eight psychiatric disorders. *Cell*, *179*(7), 1469-1482.e11. https://doi.org/10.1016/j.cell.2019.11.020

Lenroot, R. K., & Yeung, P. K. (2013). Heterogeneity within Autism Spectrum Disorders: What have We Learned from Neuroimaging Studies? *Frontiers in Human Neuroscience*, *7*, 733. https://doi.org/10.3389/fnhum.2013.00733

Lord, C., Elsabbagh, M., Baird, G., & Veenstra-Vanderweele, J. (2018). Autism spectrum disorder. *The Lancet*, *392*(10146), 508–520. https://doi.org/10.1016/S0140-6736(18)31129-2

Luo, J., Feng, Q., Wei, L., & Luo, M. (2017). Optogenetic activation of dorsal raphe neurons rescues the autistic-like social deficits in Shank3 knockout mice. *Cell Research*, *27*(7), Article 7. https://doi.org/10.1038/cr.2017.52

Madsen, K., Steen, G., Hasselbalch, Dmsc, Knudsen, G., Kessing, L., Chairperson, G., Waldemar, Varrone, A., & Herholz, K. (2009). *Review Committee*.

Maenner, M. J. (2020). Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years—Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2016. *MMWR. Surveillance Summaries*, *69*. https://doi.org/10.15585/mmwr.ss6904a1

Muller, C. L., Anacker, A. M. J., & Veenstra-VanderWeele, J. (2016). The serotonin system in autism spectrum disorder: From biomarker to animal models. *Neuroscience*, *321*, 24–41. https://doi.org/10.1016/j.neuroscience.2015.11.010

Noh, K., Lee, H., Choi, T.-Y., Joo, Y., Kim, S.-J., Kim, H., Kim, J. Y., Jahng, J. W., Lee, S., Choi, S.-Y., & Lee, S. J. (2019). Negr1 controls adult hippocampal neurogenesis and affective behaviors. *Molecular Psychiatry*, *24*(8), Article 8. https://doi.org/10.1038/s41380-018-0347-3

Nummenmaa, L., Seppälä, K., & Vesa, P. (2020). *Molecular imaging of the human emotion circuit*. https://doi.org/10.31234/osf.io/5w63q

Pimenta, A. F., Zhukareva, V., Barbe, M. F., Reinoso, B. S., Grimley, C., Henzel, W., Fischer,

I., & Levitt, P. (1995). The limbic system-associated membrane protein is an Ig superfamily member that mediates selective neuronal growth and axon targeting. *Neuron*, *15*(2), 287–297. https://doi.org/10.1016/0896-6273(95)90034-9

Raikar, S. P. (2023, aprill 11). *Limbic system*. Encyclopedia Britannica. https://www.britannica.com/science/limbic-system

Reyes, N. M., Factor, R., & Scarpa, A. (2020). Emotion regulation, emotionality, and expression of emotions: A link between social skills, behavior, and emotion problems in children with ASD and their peers. *Research in Developmental Disabilities*, *106*, 103770. https://doi.org/10.1016/j.ridd.2020.103770

Rylaarsdam, L., & Guemez-Gamboa, A. (2019). Genetic Causes and Modifiers of Autism Spectrum Disorder. *Frontiers in Cellular Neuroscience*, *13*, 385. https://doi.org/10.3389/fncel.2019.00385

Sabater, L., Gaig, C., Gelpi, E., Bataller, L., Lewerenz, J., Torres-Vega, E., Contreras, A., Giometto, B., Compta, Y., Embid, C., & others. (2014). A novel NREM and REM parasomnia with sleep breathing disorder associated with antibodies against IgLON5: A case series, pathological features, and characterization of the antigen. *Lancet neurology*, *13*(6), 575–586. https://doi.org/10.1016/S1474-4422(14)70051-1

Schofield, P. R., McFarland, K. C., Hayflick, J. S., Wilcox, J. N., Cho, T. M., Roy, S., Lee, N. M., Loh, H. H., & Seeburg, P. H. (1989). Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact. *The EMBO Journal*, *8*(2), 489–495.

Schäfer, M., Bräuer, A. U., Savaskan, N. E., Rathjen, F. G., & Brümmendorf, T. (2005). Neurotractin/kilon promotes neurite outgrowth and is expressed on reactive astrocytes after entorhinal cortex lesion. *Molecular and Cellular Neuroscience*, *29*(4), 580–590. https://doi.org/10.1016/j.mcn.2005.04.010

Siegel, G. J., Albers, R. W., Brady, S. T., & Price, D. L. (2006). Cell adhesion molecules. *Basic Neurochemistry: Molecular, cellular and medical aspects* (lk 111–121).

Sim, G., Jeong, M., Seo, H., Kim, J., & Lee, S. (2022). The Role of N-Glycosylation in the Intracellular Trafficking and Functionality of Neuronal Growth Regulator 1. *Cells*, *11*(7), Article 7. https://doi.org/10.3390/cells11071242

Singh, K., Jayaram, M., Kaare, M., Leidmaa, E., Jagomäe, T., Heinla, I., Hickey, M. A., Kaasik, A., Schäfer, M. K., Innos, J., & others. (2019). Neural cell adhesion molecule Negr1 deficiency in mouse results in structural brain endophenotypes and behavioral deviations related to psychiatric disorders. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-41991-8

Singh, K., Loreth, D., Pöttker, B., Hefti, K., Innos, J., Schwald, K., Hengstler, H., Menzel, L., Sommer, C. J., Radyushkin, K., & others. (2018). Neuronal Growth and Behavioral Alterations in Mice Deficient for the Psychiatric Disease-Associated Negr1 Gene. *Frontiers in Molecular Neuroscience*, *11*, 30. https://doi.org/10.3389/fnmol.2018.00030

Smit, M., Vállez García, D., de Jong, B. M., Zoons, E., Booij, J., Dierckx, R. A., Willemsen, A. T., de Vries, E. F., Bartels, A. L., & Tijssen, M. A. (2018). Relationships between Serotonin Transporter Binding in the Raphe Nuclei, Basal Ganglia, and Hippocampus with Clinical Symptoms in Cervical Dystonia: A [11C]DASB Positron Emission Tomography Study. *Frontiers in Neurology*, *9*. https://www.frontiersin.org/articles/10.3389/fneur.2018.00088

Sodhi, M. S. K., & Sanders-Bush, E. (2004). Serotonin and brain development. *International Review of Neurobiology* (Kd 59, lk 111–174). Academic Press. https://doi.org/10.1016/S0074-7742(04)59006-2

Szczurkowska, J., Pischedda, F., Pinto, B., Managò, F., Haas, C. A., Summa, M., Bertorelli,

R., Papaleo, F., Schäfer, M. K., Piccoli, G., & Cancedda, L. (2018). NEGR1 and FGFR2 cooperatively regulate cortical development and core behaviours related to autism disorders in mice. *Brain*, *141*(9), 2772–2794. https://doi.org/10.1093/brain/awy190

Stephens, K., Silk, T. J., Anderson, V., Hazell, P., Enticott, P. G., & Sciberras, E. (2021). Associations Between Limbic System White Matter Structure and Socio-Emotional Functioning in Children with ADHD + ASD. *Journal of Autism and Developmental Disorders*, *51*(8), 2663–2672. https://doi.org/10.1007/s10803-020-04738-3

Struyk, A. F., Canoll, P. D., Wolfgang, M. J., Rosen, C. L., D'Eustachio, P., & Salzer, J. L. (1995). Cloning of neurotrimin defines a new subfamily of differentially expressed neural cell adhesion molecules. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *15*(3 Pt 2), 2141–2156. https://doi.org/10.1523/JNEUROSCI.15-03-02141.1995

Zeidan, J., Fombonne, E., Scorah, J., Ibrahim, A., Durkin, M. S., Saxena, S., Yusuf, A., Shih, A., & Elsabbagh, M. (2022). Global prevalence of autism: A systematic review update. *Autism Research*, *15*(5), 778–790. https://doi.org/10.1002/aur.2696

Tan, H., Zhong, P., & Yan, Z. (2004). Corticotropin-Releasing Factor and Acute Stress Prolongs Serotonergic Regulation of GABA Transmission in Prefrontal Cortical Pyramidal Neurons. *The Journal of Neuroscience*, *24*(21), 5000–5008. https://doi.org/10.1523/JNEUROSCI.0143-04.2004

Tan, R. P. A., Leshchyns'ka, I., & Sytnyk, V. (2017). Glycosylphosphatidylinositol-Anchored Immunoglobulin Superfamily Cell Adhesion Molecules and Their Role in Neuronal Development and Synapse Regulation. *Frontiers in Molecular Neuroscience*, *10*, 378. https://doi.org/10.3389/fnmol.2017.00378

Twarog, B. M. (1954). Responses of a molluscan smooth muscle to acetylcholine and

5-hydroxytryptamine. *Journal of Cellular and Comparative Physiology*, *44*(1), 141–163. https://doi.org/10.1002/jcp.1030440112

Xiong, J., Chen, S., Pang, N., Deng, X., Yang, L., He, F., Wu, L., Chen, C., Yin, F., & Peng, J. (2019). Neurological Diseases With Autism Spectrum Disorder: Role of ASD Risk Genes. *Frontiers in Neuroscience*, *13*. https://www.frontiersin.org/articles/10.3389/fnins.2019.00349

Kasutatud raamatud

Clark, D. L., Boutros, N. N., & Mendez, M. F. (2010). *The Brain and Behavior. An Introduction to Behavioral Neuroanatomy*.

Iversen, L. L., Iversen, S. D., Bloom, F. E., & Roth, R. H. (2009). Serotonin. *Introduction to neuropsychopharmacology* (lk 214–237).

Schatzberg, A. F., & Nemeroff, C. B. (2004). Serotonergic System. *The American Psychiatric Publishing textbook ofpsychopharmacolog* (lk 9–13).

World Health Organization. (1992). Pervasiivsed arenguhäired. *RHK-10 Psüühika- ja käitumishäirete klassifikatsioon: Kliinilised kirjeldused ja diagnostilised juhised* (3. tr, lk 245–253).

KASUTATUD VEEBIAADRESSID

Eesti Autismiliit. (i.a.). Salvestatud 28.05.2023, https://www.autismiliit.ee/autismist/autismihairete-spekter/

NCBI. (s.a.). *NEGR1 neuronal growth regulator 1 [Homo sapiens (human)]—Gene—NCBI*. Salvestatud 30. märts 2023, https://www.ncbi.nlm.nih.gov/gene/257194

Raikar, S. P. (2023, aprill 11). *Limbic system*. Encyclopedia Britannica. Salvestatud 27.05.2023, https://www.britannica.com/science/limbic-system

World Health Organization. (2019). 6A02 Autism spectrum disorder. In *International statistical classification of diseases and related health problems* (11th ed.). Salvestatud 28.05.2023, https://icd.who.int/browse11/l-m/en#/http%3a%2f%2fid.who.int%2ficd%2fentity%2f437815624

LISAD

LISA 1. Serotoniinisüsteemiga seotud geenide uurimiseks kasutatud praimerite järjestused

Serotoniini transporter	Slc6a4_F	AAGCCAAGCTGATGATGTAA
	Slc6a4_R	TCCTCACATATCCCAGTCAG
Trüptofaani hüdroksülaas	Tph2_F	CAGGGTCGAGTACACAGAAG
	Tph2_R	CTTTCAGAAACATGGAGACG
Monoamiini oksüdaas A	MaoA_F	AGCCTACTTCCCTCCTGGTATC
	MaoA_R	AGCCTACTTCCCTCCTGGTATC
Monoamiini oksüdaas B	MaoB_F	GAATCTTTGGATGTCCCTGCAC
	MaoB_R	TGTTGCTGACAAGATGGTGGTC
Beeta-aktiin	ActB_F	ACCATGTACCCAGGCATTGC
	Act_R	AGCCACCGATCCACACAGAG

(Varul et al., 2021; Vogelgesang et al., 2017)

LISA 2. Depression-associated *Negr1* gene-deficiency induces alterations in the monoaminergic neurotransmission enhancing time-dependent sensitization to amphetamine in male mice.

brain sciences

MDPI

Article

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

Maria Kaare[1,2], Mohan Jayaram[1,2], Toomas Jagomäe[1,2,4], Katyayani Singh[1,2], Kalle Kilk[1,3], Kaie Mikheim[1,2], Marko Leevik[1,2], Este Leidmaa[5] ,Jane Varul[1,2], Helis Nõmm[1,2], Kristi Rähn[3], Tanel Visnapuu[1,2], Mario Plaas[1,2,4], Kersti Lilleväli[1,2] , Michael K.E. Schäfer[6], Mari-Anne Philips[1,2*], Eero Vasar[1,2]

[1] Institute of Biomedicine and Translational Medicine, Department of Physiology, University of Tartu, 19 Ravila Street, Tartu 50411, Estonia

[2] Centre of Excellence in Genomics and Translational Medicine, University of Tartu, 50411 Tartu, Estonia

[3] Institute of Biomedicine and Translational Medicine, Department of Biochemistry, University of Tartu, 19 Ravila Street, Tartu 50411, Estonia

[4] Institute of Biomedicine and Translational Medicine, Laboratory Animal Centre, University of Tartu, 14B Ravila Street, Tartu 50411, Estonia

[5] Institute of Molecular Psychiatry, Medical Faculty, University of Bonn, Bonn, Germany

[6] Department of Anesthesiology, Focus Program Translational Neurosciences, Research Center for Immunotherapy, University Medical Center of the Johannes Gutenberg-University Mainz, Germany.

***Correspondence: marian@ut.ee**

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

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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.

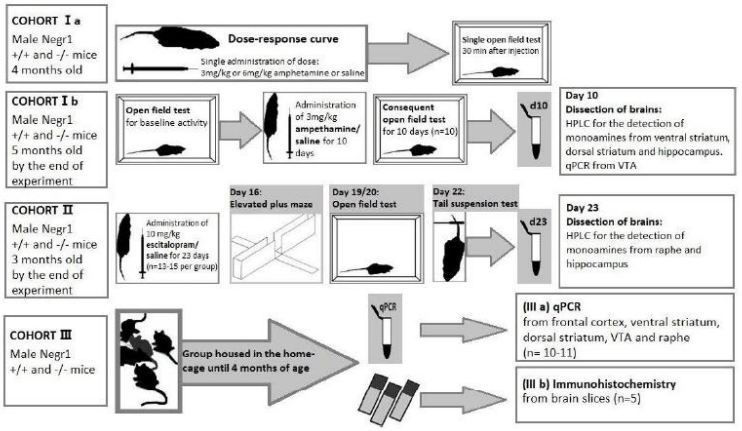


Figure 1. Schematic overview of the cohorts of mice and tests/measurements performed in the current study. Small arrows indicate the timing of behavioral tests in the schematic timeline. For the estimation of the treatment of acute and chronic amphetamine (cohort I), two subgroups of mice were used: 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 ($F_{1,36} = 9.17$; $p = 0.004$) and genotype x time interaction effects ($F_{2,72} = 5.97$; $p = 0.004$). 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.

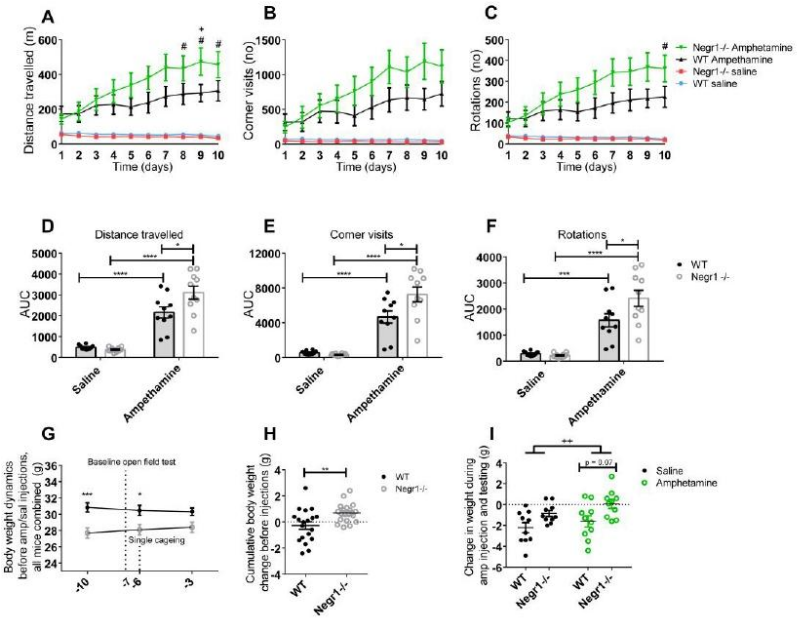


Figure 2. ***Negr1*$^{-/-}$ mice are more sensitive to chronic amphetamine administration.** Effect of chronic amphetamine on (A, D) distance travelled, (B, E) corner visits and (C, F) rotations in open field test. In G and H saline/amphetamine groups have not been separated yet, the body weight change 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).

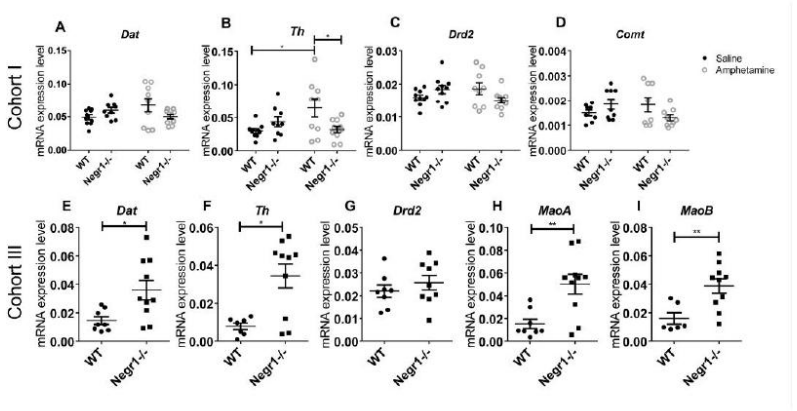


Figure 3. The level of dopamine system-related genes in the VTA of mice. Relative mRNA expression levels of (A) dopamine transporter (*Dat*), (B) tyrosine hydroxylase (*Th*), (C) dopamine receptor D2 (*Drd2*), (D) catechol-*O*-methyltransferase (*Comt*) in *Negr1*$^{-/-}$ mice and their WT littermates 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).

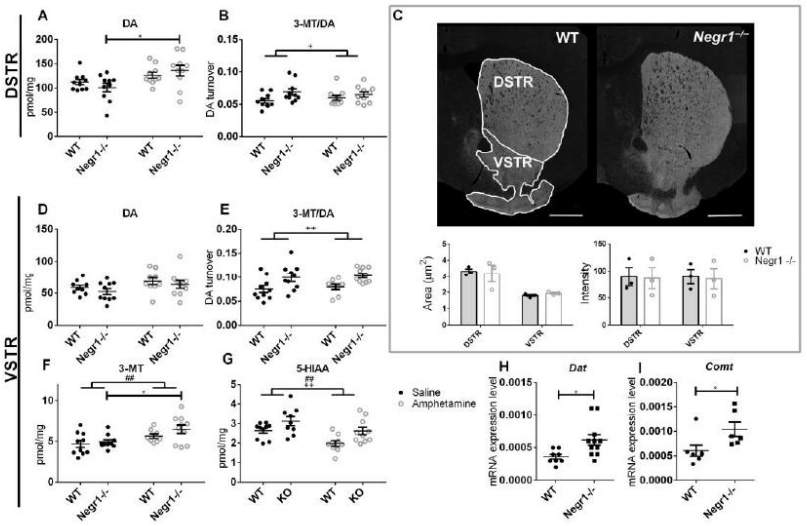


Figure 4. Effect of chronic amphetamine in the dorsal striatum (DSTR) and ventral striatum (VSTR). The level of (A) dopamine (DA) and (B) dopamine turnover (3-MT/DA) in the DSTR. (C) Immunohistochemical DAT stainings of DSTR and VSTR. The level of (D) dopamine (DA), (E) dopamine turnover (3-MT/DA), (F) 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).

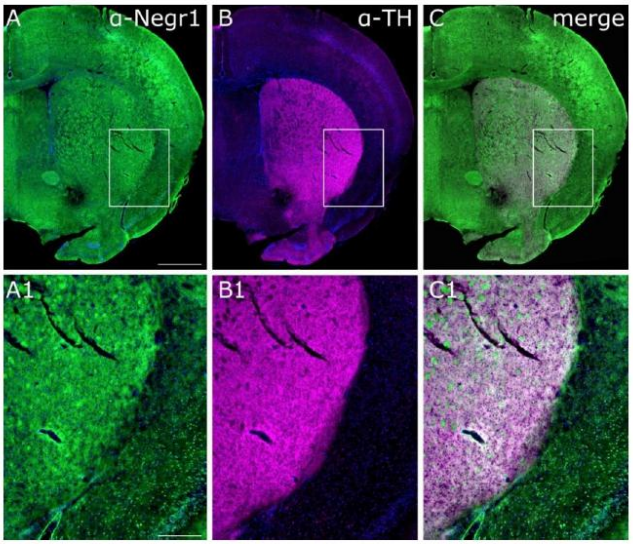


Figure 5. Expression of NEGR1 and TH in the striatum. Representative confocal images of striatum show co-immunohistochemical stainings of anti-NEGR1 in green (A, A1), with anti-tyrosine hydroxylase in magenta (TH) (B, B1) and NEGR1 staining in the vicinity of dopaminergic projection area can be seen 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).

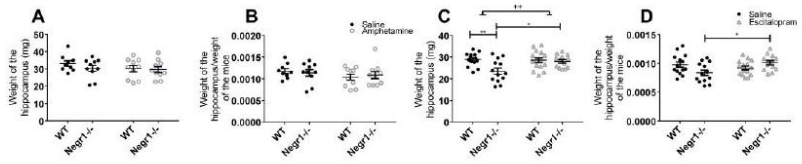


Figure 6. Hippocampi of *Negr1*$^{-/-}$ mice weigh less compared to WT mice and escitalopram restores the weight of hippocampi of *Negr1*$^{-/-}$ mice. (A) The weight of the hippocampi of the mice after receiving 10 days of saline or amphetamine. (B) Weight of hippocampi divided by the weight of mice (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).

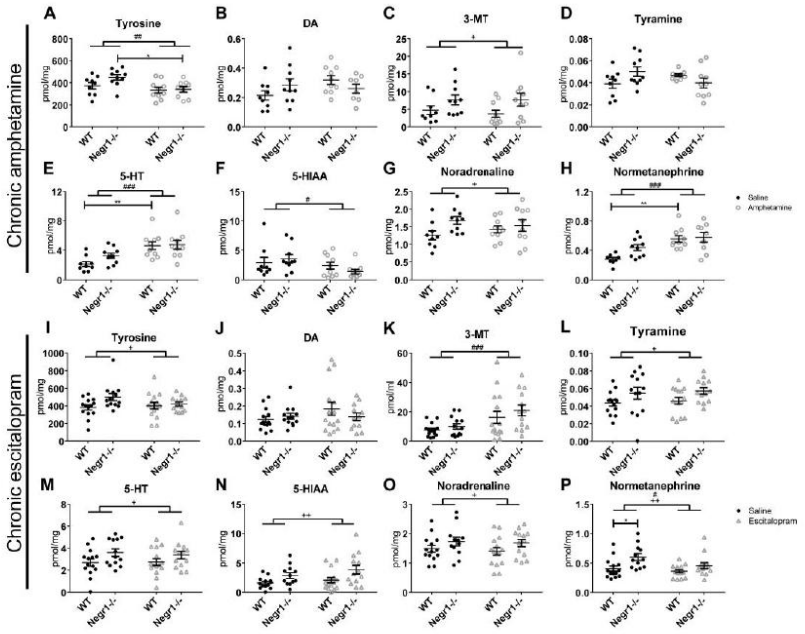


Figure 7. Effect of chronic administration of amphetamine or escitalopram on the level of monoamines and their metabolites in the hippocampus. The levels of (A) tyrosine, (B) dopamine (DA), (C) 3MeOTyramine (3-MT), (D) tyramine, (E) serotonin (5-HT), (F) 5-hydroxyindoleacetic acid (5-HIAA), (G) noradrenaline 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.

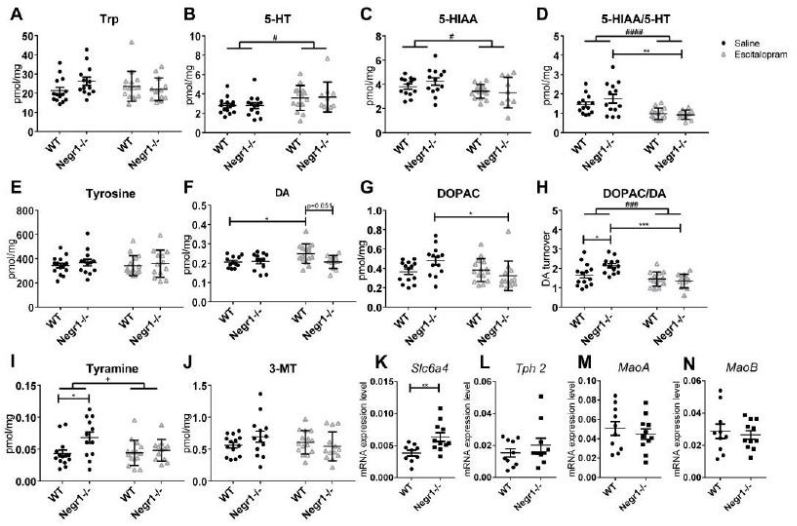
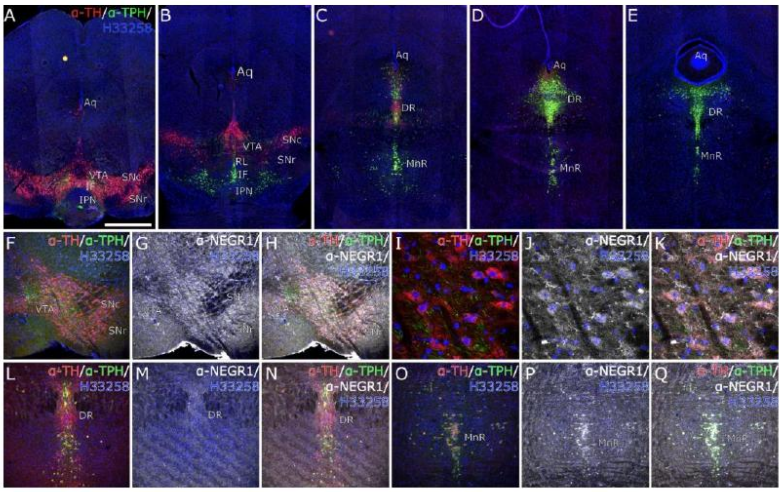


Figure 8. Effect of chronic escitalopram on the level of monoamines and their metabolites in the raphe nuclei. Levels of (A) tryptophan (Trp) (B) serotonin (5-HT), (C) 5-Hydroxyindoleacetic acid (5-HIAA), (D) serotonin turnover (5-HIAA/5-HT), (E) tyrosine, (F) dopamine (DA), (G) 3,4-Dihydroxyphenylacetic 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.



593

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. 594 595 596 597 598 599 600 601 602 603 604

3.6. Chronic administration of escitalopram causes no alterations in the behavior of Negr1-/- mice. 605 606

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). 607 608 609 610 611 612 613 614

615

616

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 Negr$1^{-/-}$ 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 Negr1$^{-/-}$ 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.

Author Contributions: Conceptualization: M.K., M.-A.P., E.V.; Methodology: M.K., M.J., M.-A.P., Software: M.K., T.J., K.S.; Validation: T.J., K.K.; Formal Analysis: M.K., M.J., T.J., K.S., K.K., K.M; Investigation: M.K., M.J., T.J., K.S, J.V., H.N., K.R, M.L., T.V.; Data Curation: M.K., M.J., M.-A.P.; Resources: E.V., M.K.E.S., M.P.; Writing – Original Draft: M.K., M.-A.P., K.S., E.L., K.L.; Writing – Review & Editing: M.K., M.J., T.J., K.S., K.M., K.K., M.L., J.V., E.L., T.V., H.N., K.R., M.P., K.L., M.K.E.S, M.-A.P., E.V.; Visualization: M.K., K.S., T.J., K.M; Supervision: M.-A.P., K.L., E.V.; Project Administration: M.-A.P., E.V.; Funding Acquisition: E.V.

Funding: This research was supported by the European Union through the European Regional Development Fund (Project No. 2014-2020.4.01.15-0012), and by the team grant from the Estonian Research Foundation (PRG 685). HPLC analysis was supported by the grant PRG1213 from the Estonian Research Council.

Institutional Review Board Statement: The animal study protocol was approved by Estonian National Board of Animal Experiments (permission No 150, September 27, 2019).

Data Availability Statement: The data will be available upon request from the corresponding author.

Acknowledgments: We would like to thank dr. Karita Laugus for the help with our initial HPLC measurements. We are thankful for Ruth Pooga for genotyping mice and Maia Järva and Marian Mitt for the help with breeding and gentle care of the animals during all of our studies.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hyde, C.L., Nagle, M. W., Tian, C., Chen, X., Paciga, S.A., Wendland, J.R., Tung, J.Y., Hinds, D.A., Perlis, R.H. and Winslow, A.R. Identification of 15 genetic loci associated with risk of major depression in individuals of European descent. *Nat Genet.* **2016,** *48(9)*, 1031-1036. 10.1038/ng.3623.
2. Wray, N.R., Ripke, S., Mattheisen, M., Trzaskowski, M., Byrne, E.M., Abdel, A., Adams, M.J., Agerbo, E., Air, T.M., Andlauer, T.M.F. et al. Genome-wide association analyses identify 44 risk variants and refine the genetic architecture of major depression. *Nat Genet.* **2018,** *50(5)*, 668-681. 10.1038/s41588-018-0090-3.
3. Howard, D.M., Adams, M.J., Shirali, M., Clarke, T.K., Marioni, R.E., Davies, G., Coleman, J.R.I., Alloza, C., Shen, X., Barbu, M.C. et al. Genome-wide association study of depression phenotypes in UK Biobank identifies variants in excitatory synaptic pathways. *Nat Commun.* **2018,** *9*, 1470. 10.1038/s41467-018-03819-3.
4. Howard, D.M., Adams, M.J., Clarke, T.K., Hafferty, J.D., Gibson, J., Shirali M., Coleman, J.R.I., Hagenaars, S.P., Ward, J, Eigmore, E.M. et al. Genome-wide meta-analysis of depression identifies 102 independent variants and highlights the importance of the prefrontal brain regions. *Nat Neurosci.* **2019**, *22*, 343-352. 10.1038/s41593-018-0326-7.
5. Levey, D.F., Stein, M.B., Wendt, F.R., Pathak, G.A., Zhou, H., Aslan, M., Quaden, R., Harrington, K.M., Nuñez, Y.Z., Overstreet, C. et al. Bi-ancestral depression GWAS in the Million Veteran Program and meta-analysis in >1.2 million individuals highlight new therapeutic directions. *Nat Neurosci*. **2021**, *24*, 954-963. 10.1038/s41593-021-00860-2.
6. Thorleifsson, G., Walters, G., Gudbjartsson, D., Steinthorsdottir, V., Sulem, P., Helgadottir, A., Strykarsdottir, U., Gretarsdottir, S., Thorlacius, S., Jonsdottir, I. et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat Genet*. **2009**, *41*, 18–24. 10.1038/ng.274.
7. Speliotes, E., Willer, C., Berndt, S., Monda, K.L., Thorleifsson, G., Jackson, A.U., Allen, H.L., Lindgren C.M., Luan, J., Mägi, R. et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet*. **2010**, *42*, 937–948. 10.1038/ng.686.
8. Wheeler E., Huang, N., Bochukova, E.G., Keogh, J.M., Lindsay, S, Grag, S., Henning, E., Blackburn, H., Loos, R.J.F. et al. Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity. *Nat Genet*. 2013, *45*, 513-7. 10.1038/ng.2607.
9. Maccarrone, G., Ditzen, C., Yassouridis, A., Rewerts, C., Uhr, M., Uhlen, M., Holsboer, F. and Turck, C.W. Psychiatric patient stratification using biosignatures based on cerebrospinal fluid protein expression clusters. *Journal of Psychiatric Research* **2013**, *47 (11)*, 1572-1580. 10.1016/j.jpsychires.2013.07.021.

10. Dall'Aglio, L., Lewis, C.M., Pain, O. Delineating the Genetic Component of Gene Expression in Major Depression. *Biol Psychiatry* **2021**, *89*, 627–636. 10.1016/j.biopsych.2020.09.010.
11. Deng Y.-T., Ou, Y.-N., Wu, B.-S., Yang, Y.X., Jiang, Y., Huang, Y.-Y., Liu, Y., Tan, L., Dong, Q., Suckling, J. et al. Identifying causal genes for depression via integration of the proteome and transcriptome from brain and blood. *Mol Psychiatry* **2022**, 27, 2849–2857. 10.1038/s41380-022-01507-9
12. Cross-Disorder Group of the Psychiatric Genomics Consortium. Genomic Relationships, Novel Loci, and Pleiotropic Mechanisms across Eight Psychiatric Disorders. *Cell* **2019**, *179(7)*, 1469–1482.e11. 10.1016/j.cell.2019.11.020.
13. Cox, D.A., Gottschalk, M.G., Wesseling, H., Ernst, A., Cooper, J.D and Bahn, S. Proteomic system evaluation of the molecular validity of preclinical psychosis models compared to schizophrenia brain pathology. *Schizophrenia Research* **2016**, *177 (1-3)*, 98-107. 10.1016/j.schres.2016.06.012.
14. Karis, K., Eskla, K.-L., Kaare, M., Täht, K., Tuusov, J., Visnapuu, T., Innos, J., Jayaram, M., Timmusk, T., Weickert, C.S. et al. Altered Expression Profile of IgLON Family of Neural Cell Adhesion Molecules in the Dorsolateral Prefrontal Cortex of Schizophrenia Patients. *Front. Mol. Neurosci.* **2018**. 10.3389/fnmol.2018.00008.
15. Sniekers, S., Stringer, S., Watanabe, K., Jansen, P.R., Coleman, J.R.I., Krapohl, E., Taskesen, E., Hammerschlag, A.R., Okbay, A., Zabaneh, D. et al. Genome-wide association meta-analysis of 78,308 individuals identifies new loci and genes influencing human intelligence. *Nat Genet.* **2017**, *49(7)*, 1107-1112. 10.1038/ng.3869.
16. Veerappa, A. M., Saldanha, M., Padakannaya, P. and Ramachandra, N. B. Family-based genome-wide copy number scan identifies five new genes of dyslexia involved in dendritic spinal plasticity. *J Hum Genet.* **2013**, *58(8)*, 539-47. 10.1038/jhg.2013.47.
17. Marshall, C.R., Noor, A., Vincent, J.B., Lionel, A.C., Feuk, L., Skaug, J., Shago, M., Moessner, R., Pinto, D., Ren, Y. et al. Structural variation of chromosomes in autism spectrum disorder. *Am J Hum Genet.* **2008**, *82(2)*, 477-88. 10.1016/j.ajhg.2007.12.009.
18. Michaelson, J. J., Shi, Y., Gujral, M., Zheng, H., Malhotra, D., Jin, X., Jian, M., Liu, G., Greer, D., Bhandari, A. et al. Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. *Cell* **2012**, *151(7)*, 1431-42. 10.1016/j.cell.2012.11.019.
19. Genovese, A., Cox, D. M. and Butler, M. G. Partial Deletion of Chromosome 1p31.1 Including only the Neuronal Growth Regulator 1 Gene in Two Siblings. *J Pediatr Genet.* **2015** 23-8. 10.1055/s-0035-1554977.
20. Vanaveski, T., Singh, K., Narvik, J., Eskla, K.-L., Visnapuu, T., Heinla, I., Jayaram, M., Innos, J., Lilleväli, K., Philips, M.-A. et al. Promoter-Specific Expression and Genomic Structure of IgLON Family Genes in Mouse**.** *Front Neurosci.* **2017**, 11, 38. 10.3389/fnins.2017.00038.
21. Ranaivoson, F.M., Turk, L.S., Ozgul, S., Kakehi, S., Daake, D., Lopez, N., Trobiani, L., Jaco, A.D., Denissova, N., Demeler, B. et al. A Proteomic Screen of Neuronal Cell-Surface Molecules Reveals IgLONs as Structurally Conserved Interaction *Modules at the Synapse. Structure* **2019**, *27(6)*, 893–906.e9. 10.1016/j.str.2019.03.004.
22. Ramani, S.R., Tom, I., Lewin-Koh, N., Wranik, B., De Palastis, L., Zhang, J., Eaton, D., Gonzalez, L.C. A secreted protein microarray platform for extracellular protein interaction discovery. *Anal. Biochem.* **2012**, *420 (2)*, 127–138. 10.1016/j.ab.2011.09.017.
23. Pourhaghighi, R., Ash, P.E.A., Phanse, S., Goebels, F., Hu, L.Z.M., Chen, S., Zhang, Y., Wierbowski, S.D., Boudeau, S., Moutaoufjk, M.T. et al. BraInMap Elucidates the Macromolecular Connectivity Landscape of Mammalian Brain. *Cell Syst.* **2020**, *10(4)*, 333–350.10.1016/j.cels.2020.03.003.
24. Singh, K., Lilleväli, K., Gilbert, S.F., Bregin, A., Narvik, J., Jayaram, M., Innos, J., Kaasik, A., Vasar, E. et al. The combined impact of IgLON family proteins Lsamp and Neurotrimin on developing neurons and behavioral profiles in mouse. *Brain. Res. Bull.* **2018a**, *140*, 5–18. 10.1016/j.brainresbull.2018.03.013.

25. Hashimoto, T., Yamada, M., Maekawa, S., Nakashima, T. and Miyata, S. IgLON cell adhesion molecule Kilon is a crucial modulator for synapse number in hippocampal neurons. *Brain Research* **2008**, *1224*, 1-11. 10.1016/j.brainres.2008.05.069.
26. Pischedda, F., Szczurowska, J., Cirnaru, M.D., Gisert, F., Vezzoli, E., Ueffing, M., Sala, C., Francolini, M., Hauck, S.M., Cancedda, L. et al. (2024). Cell Surface Biotinylation Assay to Reveal Membrane-associated Neuronal Cues: Negr1 Regulates Dendritic Arborization. Mol *Cell Proteomics* **2024**, *13(3)*, 733-748. 10.1074/mcp.M113.03171.
27. Singh, K., Jayaram, M., Kaare, M., Leidmaa, E., Jagomäe, T., Heinla, I., Hickey, M.A., Kaasik, A., Schäfer, M.K., Innos, J. et al. Neural cell adhesion molecule *Negr1* deficiency in mouse results in structural brain endophenotypes and behavioral deviations related to psychiatric disorders. *Sci Rep*. **2019**, *9*, 5457. 10.1038/s41598-019-41991-8.
28. Schäfer, M., Bräuer, A.U., Savaskan, N.E., Rathjen, F.G. and Brümmendorf, T. Neurotractin/kilon promotes neurite outgrowth and is expressed on reactive astrocytes after entorhinal cortex lesion. *Mol Cell Neurosci.* **2005**, *29(4)*, 580-90. 10.1016/j.mcn.2005.04.010.
29. Singh, K., Loreth, D., Pöttker, B., Hefti, K., Innos, J., Schwald, K., Hengstler, H., Menzel, L., Sommer, C.J., Radyushkin. et al. (2018b). Neuronal Growth and Behavioral Alterations in Mice Deficient for the Psychiatric Disease-Associated *Negr1* Gene. *Front Mol Neurosci.* 2018b, *11*, 30. 10.3389/fnmol.2018.00030.
30. Noh, K., Lee, H., Choi, T.-Y., Joo, Y., Kim, S.-J., Kim, H., Kim, J.Y., Jahng, J.W., Lee, S., Choi, S.-Y. et al. Negr1 controls adult hippocampal neurogenesis and affective behaviors. *Mol Psychiatry* **2019**, 24, 1189–1205. 10.1038/s41380-018-0347-3.
31. Szczurkowska, J., Pischedda, F., Pinto, B., Managò, F., Haas, C.A., Summa, M., Bertorelli, R., Papaleo, F., Schäfer, M.K., Piccoli, G. et al. NEGR1 and FGFR2 cooperatively regulate cortical development and core behaviours related to autism disorders in mice. *Brain* **2018**, *141(9)*, 2772–2794. 10.1093/brain/awy190.
32. Kaare, M., Mikheim, K., Lilleväli, K., Kilk, K., Jagomäe, T., Leidmaa, E., Piirsalu, M., Porosk, R., Singh, K., Reimets, R. et al. High-Fat Diet Induces Pre-Diabetes and Distinct Sex-Specific Metabolic Alterations in Negr1-Deficient Mice. *Biomedicine* **2021**, *9(9)*, 1148. 10.3390/biomedicines9091148.
33. Belujon, P and Grace, A.A. (2017). Dopamine System Dysregulation in Major Depressive Disorder. *Int J Neuropsychophrmacolo*. **2017,** *20*, 1036-1046. 10.1093/ijnp/pyx056
34. Schmidt, E.E., Brignani, S., Adolfs, Y., Lemstra, S., Demmers, J., Vidaki, M., Donahoo, A.-L.S., Lilleväli, K., Vasar, E., Richards, L.J. et al. Subdomain-Mediated Axon-Axon Signaling and Chemoattraction Cooperate to Regulate Afferent Innervation of the Lateral Habenula. *Neuron* **2014**, *83(2)*, 372-387. 10.1016/j.neuron.2014.05.036.
35. Inta, D., Meyer-Lindenberg, A. and Gass, P. Alterations in postnatal neurogenesis and dopamine dysregulation in schizophrenia: a hypothesis. *Schizophr Bull.* **2010**, *37(4)*, 674-680. 10.1093/schbul/sbq134.
36. Okaty, B. W., Freret, M.E., Rood, B.D., Brust, R.D., Hennessy, M.L., Bairos, D., Kim, J.K., Cook, M.N., Dymecki, S.M. et al. Multi-Scale Molecular Deconstruction of the Serotonin Neuron System. *Neuron* **2015**, *88(4)*, 774-91. 10.1016/j.neuron.2015.10.007.
37. Tamási, V., Petschner, P., Adori, C., Kirilly, E., Ando, R. D., Tothfalusi, L., Juhasz, G. and Bagdy, G. Transcriptional Evidence for the Role of Chronic Venlafaxine Treatment in Neurotrophic Signaling and Neuroplasticity Including also Glutatmatergic- and Insulin-Mediated Neuronal Processes. *PLoS ONE* **2014**, *9(11)*, e113662. 10.1371/journal.pone.0113662.
38. Carboni, L., Pischedda, F., Piccoli, G., Lauria, M., Musazzi, L., Popoli, M., Mathé, A.A. and Domenici, E. Depression-Associated Gene *Negr1-Fgfr2* Pathway Is Altered by Antidepressant Treatment. *Cell* **2020**, *9(8)*, 1818. 10.3390/cells9081818.
39. Mustard, C., Whitfield, P., Megson, I. and Wei, J. P-1104 - The Effect of Clozapine on the Expression of Obesity Genes. *European Psychiatry* **2012**, *27(S1)*, 1-1. 10.1016/S0924-9338(12)75271-9.
40. Innos, J., Leidmaa, E., Philips, M.-A., Sütt, S., Alttoa, A., Harro, J., Kõks, S., Vasar, E. Lsamp−/− mice display lower sensitivity to amphetamine and have elevated 5-HT turnover. Biochem. Biophys. *Res. Commun.* **2013**, *430(1)*, 413–418. 10.1016/j.bbrc.2012.11.077.

41. Bregin, A., Kaare, M., Jagomäe, T., Karis, K., Singh, K., Laugus, K., Innos, J., Leidmaa, E., Heinla, I., Visnapuu, T. et al. Expression and impact of Lsamp neural adhesion molecule in the serotonergic neurotransmission system. *Pharmacol. Biochem. Behav.* **2020**, *198*, 173017. 10.1016/j.pbb.2020.173017.
42. Innos, J., Philips, M.A., Leidmaa, E., Heinla, I., Raud, S., Reemann, P., Plaas, M., Nurk, K., Kurrikoff, K., Matto, V. et al. (2011). Lower anxiety and a decrease in agonistic behaviour in Lsamp-deficient mice. *Behav. Brain Res.* **2011**, *217(1)*, 21–31. 10.1016/j.bbr.2010.09.019.
43. Innos, J., Philips, M.-A., Raud, S., Lilleväli, K., Kõks, S. and Vasar, E. Deletion of the Lsamp gene lowers sensitivity to stressful environmental manipulations in mice. *Behav. Brain Res.* **2012**, 228(7), 74–81. 10.1016/j.bbr.2011.11.033.
44. Mazitov, T., Brefgin, A., Philips, M.A., Innos, J. and Vasar, E. (2017). Deficit in emotional learning in neurotrimin knockout mice. Behav. *Brain Res.* **2017**, 28, 311–318. 10.1016/j.bbr.2016.09.064.
45. Lee, A.W.S.; Hengstler, H.; Schwald, K.; Diaz, M.B.; Loreth, D.; Kirsch, M.; Kretz, O.; Haas, C.A.; de Angelis, M.H.; Herzig, S.; et al. Functional Inactivation of the Genome-Wide Association Study Obesity Gene Neuronal Growth Regulator 1 in Mice Causes a Body Mass Phenotype. *PLoS ONE* **2012**, *7*, e41537.
46. Sokolov, B. P., Schindler, C. W. and Cadet, J. L. Chronic methamphetamine increases fighting in mice. Pharmacology Biochemistry and Behavior **2004**, *77*, 319.326. https://doi.org/10.1016/j.pbb.2003.11.006.
47. Visnapuu, T., Plaas, M., Reimets, R., Raud, S., Terasmaa, A., Kõks, S., Sütt, S., Luuk, H., Hundahl, C.A., Eskla, K.-L., Altpere, A., Alttoa, A., Harro, J. and Vasar, E. Evidence of impaired function of dopaminergic system in Wfs1-deficient mice. *Behavioural Brain Research* **2013**, *244*, 90-99. https://doi.org/10.1016/j.bbr.2013.01.046
48. Vanaveski, T., Narvik, J., Innos, J., Philips, M.-A., Ottas, A., Plaas, M., Haring, L., Zilmer, M. and Vasar, E. Repeated Administration of D-Amphetamine Induces Distinct Alterations in Behavior and Metabolite Level in 129Sv and Bl6 Mouse Strains. *Front. Neurosci.* **2018,** *12*. https://doi.org/10.3389/fnins.2018.00399.
49. Franklin, K.B.J. and Paxinos, G. The Mouse Brain in Stereotaxic Coordinates (Academic Press, San Diego, CA) **1997**.
50. Bakker, R., Tiesinga, P. and Kötter, R. The Scalable Brain Atlas: Instant Web-Based Access to Public Brain Atlases and Related Content. *Neuroinform* **2015**, *13*, 353–366. 10.1007/s12021-014-9258-x.
51. Varul, J., Eskla, K.-L., Piirsalu, M., Innos, J., Philips, M.-A., Visnapuu, T., Plaas, M. and Vasar, E. Dopamine System, NMDA Receptor and EGF Family Expressions in Brain Structures of Bl6 and 129Sv Strains Displaying Different Behavioral Adaptation. *Brain Sci.* **2021**, *11*, 725. 10.3390/brainsci11060725.
52. Scholl, J.L., Feng, N., Watt, M.J., Renner, K.J., Forster, G.L. Individual differences in amphetamine sensitization, behavior and central monoamines. *Physiol Behav.* **2009,** *96(3)*, 493-504. doi: 10.1016/j.physbeh.2008.12.001.
53. Geiger, B.M., Haburcak, M., Avena, N.M., Moyer, M.C., Hoebel, B.G. and Pothos, E.N. Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity. *Neuroscience* **2009**, *159(4)*, 1193-1199. 10.1016/j.neuroscience.2009.02.007.
54. Sjödin, A., Gasteyger, C., Nielsen, A.L., Raben, A., Mikkelsen, J.D., Jensen, J. K.S., Meier, D. and Astrup, A. The effect of the triple monoamine reuptake inhibitor tesofensine on energy metabolism and appetite in overweight and moderately obese men. *Int J Obes.* **2010**, *34*, 1634–1643. 10.1038/ijo.2010.87.
55. Jagomäe, T., Singh, K., Philips, M. A., Jayaram, M., Seppa, K., Tekko, T., Gilbert, S. F., Vasar, E., & Lilleväli, K. (2021). Alternative Promoter Use Governs the Expression of IgLON Cell Adhesion Molecules in Histogenetic Fields of the Embryonic Mouse Brain. *International journal of molecular sciences* **2021**, *22(13)*, 6955. 10.3390/ijms22136955.
56. Venkannagari, H., Kasper, J.M., Misra, A., Rush, S.A., Fan, S., Lee, H., Sun, H., Seshadrinathan, S., Machius, M., Hommel, J.D. et al. (2020). Highly Conserved Molecular Features in IgLONs Contrast Their Distinct Structural and Biological Outcomes. *J Mol Biol.* **2020**, *432*, 5287–5303. 10.1016/j.jmb.2020.07.014.

57. Paulson, P. E., and Robinson, T. E. Amphetamine-induced time-dependent sensitization of dopamine neurotransmission in the dorsal and ventral striatum: a microdialysis study in behaving rats. *Synapse (New York, N.Y.)* **1995**, *19(1)*, 56–65. 10.1002/syn.890190108.
58. Avelar, A.J., Juliano, S.A and Garris, P.A. Amphetamine augments vesicular dopamine release in the dorsal and ventral striatum through different mechanisms. *J Neurochem*. **2013**, *125(3)*, 373-385. 10.1111/jnc.12197.
59. Siciliano, C.A., Calipari, E.S. and Jones, S.R. Amphetamine potency varies with dopamine uptake rate across striatal subregions. *J Neurochem.* **2014**, *131(3)*, 348-355. 10.1111/jnc.12808.
60. Salahpour, A., Ramsey, A.J., Medvedev, I.O., Kile, B., Sotnikova, T.D., Holmstrand, E., Ghisi, V., Nicholls, P.J., Wong, L., Murphy, K. et al. Increased amphetamine-induced hyperactivity and reward in mice overexpressing the dopamine transporter. *Proc Natl Acad Sci U S A* **2008,** *105(11)*, 4405-4410. 10.1073/pnas.0707646105.
61. Cagniard,, B., Sotnikova, T.D., Gainetdinov, R.R. and Zhuang, X. The Dopamine Transporter Expression Level Differentially Affects Responses to Cocaine and Amphetamine. *Journal of Neurogenetics* **2014**, *28(1-2)*, 112-121. 10.3109/01677063.2014.908191.
62. Shilling, P.D., Kelsoe, J.R. and Segal, D.S. Dopamine transporter mRNA is up-regulated in the substantia nigra and the ventral tegmental area of amphetamine-sensitized rats. *Neuroscience Letters* **1997**, *236(3)*, 131-134. 10.1016/S0304-3940(97)00768-4.
63. Dietz, D.M., Tapocik, J., Gaval-Cruz, M. and Kabbaj, M. Dopamine transporter, but not tyrosine hydroxylase, may be implicated in determining individual differences in behavioral sensitization to amphetamine. *Physiol Behav.* **2005**, *86(3)*, 347-355. 10.1016/j.physbeh.2005.08.005.
64. Underhill, S.M., Hullihen, P.D., Chen, J., Fenollar-Ferre, C., Rizzo, M. A., Ingram, S.L. and Amara, S.G. Amphetamines signal through intracellular TAAR1 receptors coupled to $G\alpha_{13}$ and $G\alpha_s$ in discrete subcellular domains. *Mol Psychiatry* **2021**, *26*, 1208–1223. 10.1038/s41380-019-0469-2.
65. Barth, A.M., Domonkos, A., Fernandez-Ruiz, A., Freund, T.F. and Varga, V. Hippocampal Network Dynamics during Rearing Episodes. *Cell Rep.* **2018**, *23(6)*, 1706-1715. 10.1016/j.celrep.2018.04.021.
66. Czéh, B., Michaelis, T., Watanabe, T., Frahm, J., de Biurrun, G., van Kampen, M., Bartolomucci, A., & Fuchs, E. Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98(22)*, 12796–12801. 10.1073/pnas.211427898.
67. Sapolsky R. M. Depression, antidepressants, and the shrinking hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98(22)*, 12320–12322. 10.1073/pnas.231475998.
68. Kempermann G, Song H, Gage FH. Neurogenesis in the Adult Hippocampus. Cold Spring Harb Perspect Biol. 2015 Sep 1;7(9):a018812. doi: 10.1101/cshperspect.a018812.
69. Navailles, S. and De Deurwaerdère, P. Presynaptic control of serotonin on striatal dopamine function. *Psychopharmacology (Berl).* **2011**, *213(2-3)*, 213-242. 10.1007/s00213-010-2029-y.
70. Fazekas, C.L., Bellardie, M., Török, B., Sipos, E., Tóth, B., Baranyi, M., Sperlágh, B., Dobos-Kovács, M., Chaillou, E. and Zelena, D. Pharmacogenetic excitation of the median raphe region affects social and depressive-like behavior and core body temperature in male mice. *Life Sci.* **2021**, *286*, 120037. 10.1016/j.lfs.2021.120037.
71. Admon, R. and Pizzagalli, D.A. Dysfunctional Reward Processing in Depression. *Current opinion in psychology* **2015**, *4*, 114–118. 10.1016/j.copsyc.2014.12.011.
72. Ng, T.H., Alloy, L.B. and Smith, D.V. Meta-analysis of reward processing in major depressive disorder reveals distinct abnormalities within the reward circuit. *Transl Psychiatry* **2019**, *9*, 293. 10.1038/s41398-019-0644-x.

73. Kenny P. J. Reward mechanisms in obesity: new insights and future directions. *Neuron* **2011**, *69(4)*, 664–679. 10.1016/j.neuron.2011.02.016.
74. Yadid, G. and Friedman, A. Dynamics of the dopaminergic system as a key component to the understanding of depression. *Prog Brain Res*. **2008**, *172*, 265-286. 10.1016/S0079-6123(08)00913-8.
75. Van Galen, K. A., ter Horst, K. W. and Serlie, M. J. Serotonin, food intake, obesity. Etiology and Pathophysiology, **2021**, *22*, e13210. https://doi.org/10.1111/obr.13210.

LIHTLITSENTS

Lihtlitsents lõputöö reprodutseerimiseks ja üldsusele kättesaadavaks tegemiseks

Mina, Helis Nõmm,

1. annan Tartu Ülikoolile tasuta loa (lihtlitsentsi) minu loodud teose **Serotoniinisüsteem kui võimalik vahendaja *NEGR1* geeni ja autismispektri häire vahelisele seosele,** mille juhendajad on PhD Maria Kaare, PhD Mari-Anne Philips ja PhD Tambet Tõnissoo, reprodutseerimiseks eesmärgiga seda säilitada, sealhulgas lisada digitaalarhiivi DSpace kuni autoriõiguse kehtivuse lõppemiseni.
2. Annan Tartu Ülikoolile loa teha punktis 1 nimetatud teos üldsusele kättesaadavaks Tartu Ülikooli veebikeskkonna, sealhulgas digitaalarhiivi DSpace kaudu Creative Commonsi litsentsiga CC BY NC ND 4.0, mis lubab autorile viidates teost reprodutseerida, levitada ja üldsusele suunata ning keelab luua tuletatud teost ja kasutada teost ärieesmärgil, kuni autoriõiguse kehtivuse lõppemiseni.
3. Olen teadlik, et punktides 1 ja 2 nimetatud õigused jäävad alles ka autorile.
4. Kinnitan, et lihtlitsentsi andmisega ei riku ma teiste isikute intellektuaalomandi ega isikuandmete kaitse õigusaktidest tulenevaid õigusi.

Helis Nõmm

29.05.2023