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Rola stresu oksydacyjnego i nitracyjnego oraz szlaku katabolitów tryptofanu w patogenezie depresji

THE ROLE OF OXIDATIVE AND NITROSATIVE STRESS
AND THE TRYPTOPHAN CATABOLITES PATHWAY
IN THE PATHOGENESIS OF DEPRESSION

Praca doktorska
wykonana w Katedrze Genetyki
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Promotor:
prof. dr. hab. Tomasz Śliwiński

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Praca przeglądowa:

- **Wigner P**, Czarny P, Galecki P, Su KP, Sliwinski T. *The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression*. Psychiatry Res. 2018; 262: 566-574. doi: 10.1016/j.psychres.2017.09.045. **30 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 2,208**; **IF 5-letni = 2,670**

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- **Wigner P**, Czarny P, Synowiec E, Bijak M, Białek K, Talarowska M, Galecki P, Szemraj J, Sliwinski T. *Variation of genes involved in oxidative and nitrosative stresses in depression*. Eur Psychiatry. 2018; 48: 38-48. doi: 10.1016/j.eurpsy.2017.10.012. **35 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 3,941**; **IF 5-letni = 4,268**

- **Wigner P**, Czarny P, Synowiec E, Bijak M, Białek K, Talarowska M, Galecki P, Szemraj J, Sliwinski T. *Association between single nucleotide polymorphisms of TPH1 and TPH2 genes, and depressive disorders*. J Cell Mol Med. 2018; 22(3): 1778-1791. doi: 10.1111/jcmm.13459. Erratum in: J Cell Mol Med. 2018; 22(10): 5171. **35 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 4,658**; **IF 5-letni = 4,408**

- **Wigner P**, Czarny P, Synowiec E, Bijak M, Talarowska M, Galecki P, Szemraj J, Sliwinski T. *Variation of genes encoding KAT1, AADAT and IDO1 as a potential risk of depression development*. Eur Psychiatry. 2018; 52: 95-103. doi: 10.1016/j.eurpsy.2018.05.001. **35 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 3,941**; **IF 5-letni = 4,268**

- **Wigner P**, Synowiec E, Czarny P, Bijak M, Jóźwiak P, Szemraj J, Gruca P, Papp M, Śliwiński T. *Effects of venlafaxine on the expression level and methylation status of genes involved in oxidative stress in rats exposed to a chronic mild stress*. J Cell Mol Med. 2020. doi: 10.1111/jcmm.15231. **100 pkt MNiSW** (według punktacji MNiSW z dnia 31.07.2019 r.); **IF = 4,658**; **IF 5-letni = 4,408**

- **Wigner P**, Synowiec E, Jóźwiak P, Czarny P, Bijak M, Białek K, Szemraj J, Gruca P, Papp M, Śliwiński T. *The effect of chronic mild stress and venlafaxine on the expression and methylation levels of genes involved in the tryptophan catabolites pathway in the blood and brain structures of rats*. J Mol Neurosci. 2020. doi: 10.1007/s12031-020-01563-2. **70 pkt MNiSW** (według punktacji MNiSW z dnia 31.07.2019 r.); **IF = 2,577**; **IF 5-letni = 2,294**

Suma: 135 pkt MNiSW według punktacji MNiSW z dnia 09.12.2019 r. oraz 170 pkt MNiSW według punktacji MNiSW z dnia 31.07.2019 r.; łączna suma punktów MNiSW wynosi 305; IF = 21,983; IF 5-letni = 22,316

Współczynnik *Impact Factor* został podany zgodnie z rokiem opublikowania pracy, a dla prac z roku 2019 i 2020 został użyty wskaźnik dla wykazu czasopism naukowych z roku 2018. W przypadku punktów MNiSW użyto wykazu opublikowanego dnia 09.12.2016 r. dla prac publikowanych do 31.07.2019 r., a dla prac publikowanych po 31.07.2019 r. – wykazu z dnia 31.07.2019 r.

Pozostały dorobek naukowy:

Publikacje:

1. Sliwinska A, Kwiatkowski D, Czarny P, Toma M, **Wigner P**, Drzewoski J, Fabianowska-Majewska K, Szemraj J, Maes M, Galecki P, Sliwinski T. *The levels of 7,8-dihydrodeoxyguanosine (8-oxoG) and 8-oxoguanine DNA glycosylase 1 (OGG1) - A potential diagnostic biomarkers of Alzheimer's disease*. J Neurol Sci. 2016; 368: 155-9. doi: 10.1016/j.jns.2016.07.008. **25 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 2,295**; **5-letni IF = 2,447**

2. Krupa R, Czarny P, **Wigner P**, Wozny J, Jablkowski M, Kordek R, Szemraj J, Sliwinski T. *The Relationship Between Single-Nucleotide Polymorphisms, the Expression of DNA Damage Response Genes, and Hepatocellular Carcinoma in a Polish Population*. DNA Cell Biol. 2017, 36(8): 693-708. doi: 10.1089/dna.2017. **20 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 2,634**; **5-letni IF = 2,029**

3. Czarny P, **Wigner P**, Galecki P, Sliwinski T. *The interplay between inflammation, oxidative stress, DNA damage, DNA repair and mitochondrial dysfunction in depression*. Prog Neuropsychopharmacol Biol Psychiatry. 2018; 80(Pt C): 309-321. doi: 10.1016/j.pnpbp.2017.06.036. **35 pkt MNiSW** (według punktacji MNiSW z dnia 09.12.2016 r.); **IF = 4,315**; **5-letni IF = 4,159**

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11. Bialek K, Czarny P, Watala C, Synowiec E, **Wigner P**, Bijak M, Talarowska M, Galecki P, Szemraj J, Sliwinski T. *Preliminary Study of the Impact of Single-Nucleotide Polymorphisms of IL-1α, IL-1β and TNF-α Genes on the Occurrence, Severity and Treatment Effectiveness of the Major Depressive Disorder*. Cell Mol Neurobiol. 2020. doi: 10.1007/s10571-019-00782-5. **100 pkt MNiSW** (według punktacji MNiSW z dnia 31.07.2019 r.); **IF = 3,811**; **5-letni IF = 3,126**

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Sumaryczny dorobek naukowy: 365 pkt MNiSW według punktacji MNiSW z dnia 09.12.2016 r. oraz 610 pkt MNiSW według punktacji MNiSW z dnia 31.07.2019 r., łączna suma punktów MNiSW wynosi 975; IF = 59,972; IF 5-letni = 54,144.

Współczynnik Impact Factor został podany zgodnie z rokiem opublikowania pracy, a dla prac z roku 2019 i 2020 został użyty wskaźnik dla wykazu czasopism naukowych z roku 2018. W przypadku punktów MNiSW użyto wykazu opublikowanego dnia 09.12.2016 r. dla prac publikowanych do 31.07.2019 r., a dla prac publikowanych po 31.07.2019 r. – wykazu z 31.07.2019 r.

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10. **Wigner P**, *Molekularne aspekty depresji*, VI Konferencja Biologii Molekularnej, 06-08.04.2017, Łódź
11. **Wigner P**, Czarny P, Śliwiński T, *Polimorfizmy pojedynczego nukleotydu w genach szlaku naprawy DNA przez wycinanie zasad w depresji*, III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 11-12.05.2017, Łódź
12. **Wigner P**, Synowiec E, Śliwiński T, *Zaburzenia szlaku katabolitów tryptofanu jako podłoże rozwoju choroby Alzheimera*, VII Konferencja Biologii Molekularnej, 12-14.04.2018, Łódź **wyróżnienie**
13. Basak J, Dziuk Ż, **Wigner P**, *Rola stresu oksydacyjnego w zaburzeniach depresyjnych*, VII Konferencja Biologii Molekularnej, 12-14.04.2018, Łódź
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16. Dziedzic A, **Wigner P**, Bijak M, Saluk-Bijak J, *Zmiana poziomu ekspresji genów zaangażowanych w szlak katabolitów tryptofanu w stwardnieniu rozsianym*, IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 24-25.05.2018, Łódź
17. **Wigner P**, Dziedzic A, Bijak M, Śliwński T, *Rola stresu oksydacyjnego i nitracyjnego w rozwoju chorób cywilizacyjnych*, Ogólnopolska Konferencja Naukowa Doktorantów, 10.03.2018, Łódź
18. Dziedzic A, **Wigner P**, Bijak M, Saluk-Bijak J, *Molekularne podłoże rozwoju stwardnienia rozsianego i udaru niedokrwiennego mózgu*, Ogólnopolska Konferencja Naukowa Doktorantów, 10.03.2018, Łódź
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22. Szelenberger R, **Wigner P**, Kacprzak M, Bijak M, Saluk-Bijak J, *Analiza wpływu polimorfizmów genów SOD2, CAT i GPX4 na ryzyko wystąpienia ostrego zespołu wieńcowego*, V Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 30-31.05.2019, Łódź
23. **Wigner P**, Synowiec E, Śliwiński T, *Molecular aspects of oxidative stress in course of chronic mild stress and antidepressant therapy of agomelatine* e-Factory of Science, 01.06.2019
24. **Wigner P**, Synowiec E, Śliwiński T, *Effects of venlafaxine of the expression levels and methylation status of Gpx4, Nos1 and Nos2 genes in rats exposed to a chronic mild stress model of depression*, National Scientific Conference Science and Young Researchers III edition, 15.06.2019, Łódź, **nagroda**
25. **Wigner P**, Synowiec E, Śliwiński T, *The effects of chronic mild stress and treatment of agomelatine on the expression and methylation status of genes involved in tryptophan catabolites pathway*, National Scientific Conference Understand the Science III edition, 28.09.2019, Łódź

Streszczenie

Wstęp

Zaburzenia psychiczne występują znacznie częściej niż którakolwiek przewlekła choroba somatyczna. Wśród tych zaburzeń najliczniejszą grupę, dotykającą około 10-15% populacji, stanowią zaburzenia afektywne, do których zaliczana jest depresja (Anderson i wsp., 2011). Depresja jest niejednorodną jednostką chorobową, o zróżnicowanym obrazie klinicznym i nasileniu objawów, takich jak: obniżenie nastroju, anhedonia, uczucie zmęczenia i utrata energii, bezsenność lub nadmierna senność, spadek lub wzrost masy ciała niezwiązany z dietą, podniecenie lub spowolnienie ruchowe, bezpodstawne poczucie winy, braku wartości, a także obniżenie sprawności intelektualnej, koncentracji, niemożność podjęcia decyzji oraz nawracające myśli samobójcze (Kessler, 2003). Szacuje się, że na całym Świecie 350 milionów ludzi cierpi na depresję, co stanowi około 5% globalnej populacji (Demyttenaere i wsp., 2004). Co więcej, w krajach rozwiniętych problem ten może dotykać aż 10% mieszkańców (Vilagut i wsp., 2016). W Polsce szacuje się, że co jedenasta osoba cierpi z powodu depresji. Choroba ta dotyka zarówno kobiety, jak i mężczyzn we wszystkich grupach wiekowych, przy czym najczęściej obserwowana jest u osób w wieku 20-40 lat, a kobiety chorują dwukrotnie częściej niż mężczyźni. Ponadto, ryzyko zachorowania wrasta wraz z wiekiem. W grupie osób po 65. roku życia około 20% cierpi na depresję (Kessler, 2003; Demyttenaere i wsp. 2004; Wang i wsp., 2008; Vilagut i wsp., 2016; GBD, 2018). Co ciekawe, prognozy Światowej Organizacji Zdrowia (WHO, ang. *World Health Organization*) sugerują, że depresja w 2020 roku stanie się drugą najczęstszą przyczyną niepełnosprawności społeczeństwa, a do 2040 pierwszą (Reddy, 2012).

Pomimo, że depresja staje się jednym z najpoważniejszych zaburzeń zdrowotnych, ponad połowa chorych nie korzysta z pomocy lekarza, a około jedna trzecia pacjentów poddanych leczeniu nie odpowiada na tradycyjną farmakoterapię. W związku z tym, u około 15-20% pacjentów może rozwinąć się przewlekła postać depresji (Al-Harbi, 2012; Iwata i wsp., 2013). Konsekwencją długotrwałej, nasilającej się i nieleczonej depresji mogą być próby samobójcze. Na Świecie rocznie odnotowuje się około miliona samobójczych zgonów spowodowanych depresją (Marcus i wsp.,

2012). Konwencjonalna terapia antydepresyjna obejmuje głównie leki działające jako inhibitory zwrotnego wychwytu neuroprzekaźników, leki o receptorowych mechanizmach działania oraz inhibitory monoaminooksydazy. Najczęściej stosowane są leki pierwszej grupy, do której zaliczane są trójcykliczne leki przeciwdepresyjne (TCA, ang. *tricyclic antidepressants*), inhibitory zwrotnego wychwytu serotoniny i noradrenaliny (SNRIs, ang. *serotonin norepinephrine reuptake inhibitors*) oraz inhibitory zwrotnego wychwytu serotoniny (SSRIs, ang. *selective serotonin reuptake inhibitors*) (Wciórka i wsp., 2010).

Ponadto, depresja to również poważny problem ekonomiczny. W Stanach Zjednoczonych całkowity koszt leczenia depresji sięga około 83,1 miliardów dolarów rocznie, a w Europie jest to około 118 miliardów dolarów. Dla porównania leczenie niewydolności serca to koszt około 39,2 miliardów, a AIDS – około 50 miliardów dolarów rocznie (Dutta i wsp. 2015; Greenberg i wsp., 2015; Voigt i wsp., 2015; Osińska i wsp., 2017). Przewlekła depresja może również przyczynić się do rozwoju innych patologii, m. in.: chorób serca, udaru mózgu, osteoporozy czy cukrzycy (Clarke i Currie, 2009).

Pomimo intensywnych badań, molekularne podstawy rozwoju depresji nadal pozostają niejasne. Jednakże, dotychczasowe badania wskazują na wieloczynnikowy charakter choroby. Rozwój i przebieg depresji mogą zależeć od: indywidualnych cech biologicznych, czynników genetycznych oraz środowiskowych (Lopizzo i wsp., 2015). Pojawiające się doniesienia wskazują na rolę powiązanych ze sobą szlaków biochemicznych, tj.: stresu oksydacyjnego i nitracyjnego oraz nieprawidłowości szlaku katabolitów tryptofanu (szlak TRYCATs, ang. *tryptophan catabolites pathway*) w rozwoju zaburzeń depresyjnych (Maes et al., 2011a; b; c).

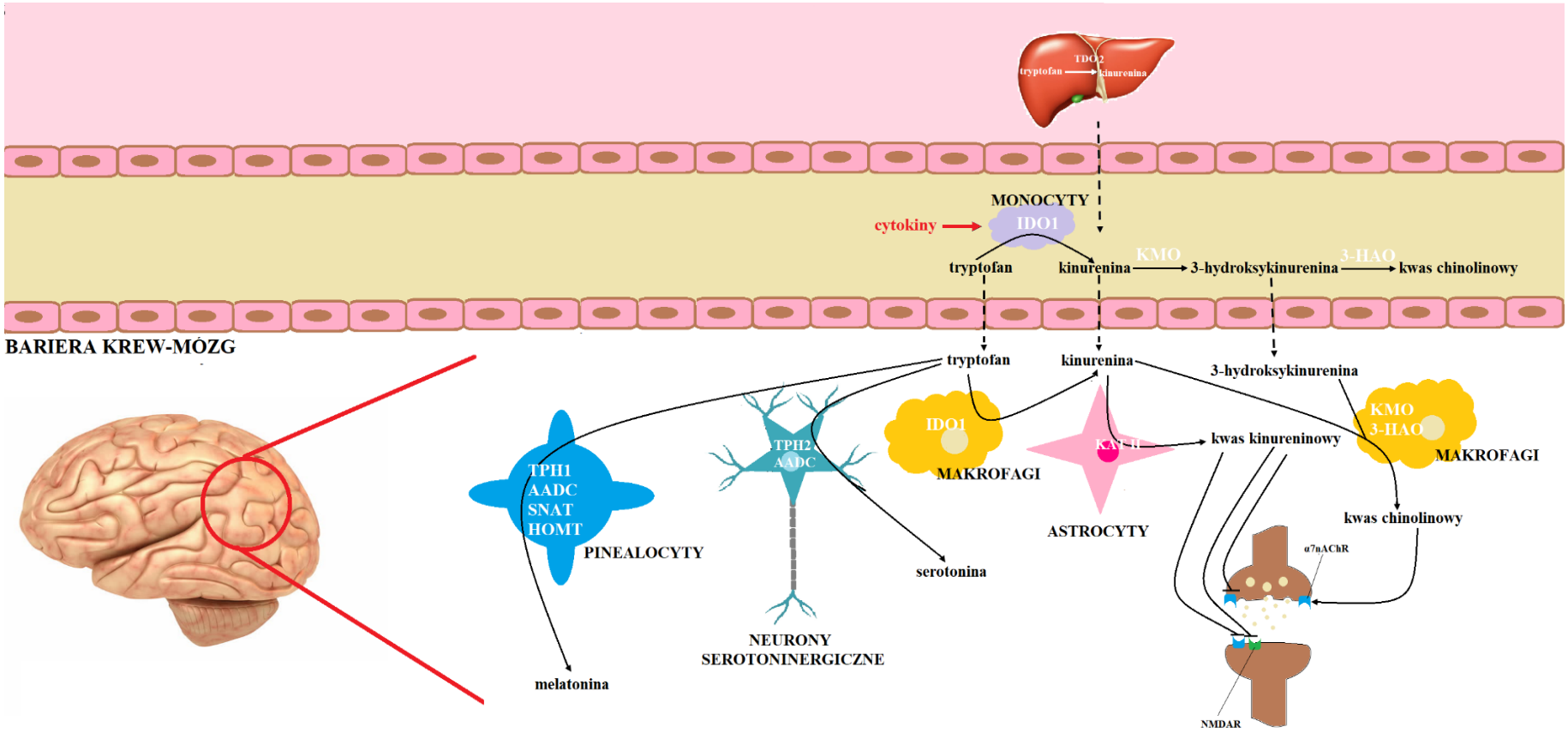
Przyczyną rozwoju depresji wydaje się być brak równowagi między produkcją a neutralizacją reaktywnych form tlenu (ROS, ang. *reactive oxygen species*) i azotu (RNS, ang. *reactive nitrogen species*). Pacjenci z depresją cechują się zmniejszoną aktywnością niskocząsteczkowych antyoksydantów, w tym obniżonym poziomem cynku, koenzymu Q10, witamin A, E, C oraz glutationu w osoczu, przy jednoczesnej intensyfikacji procesów oksydacyjnych (Pandya i wsp., 2013). W przebiegu depresji obserwuje się również zmiany aktywności enzymów zaangażowanych w stres oksydacyjny. Dotychczasowe badania wykazały, że depresja może być związana

ze zwiększoną aktywnością oksydazy ksantynowej (XO, ang. *xanthine oxidase*) w surowicy i mózgu (Herken i wsp., 2007; Michael i wsp., 2010; Morris i wsp., 2017). Niemniej jednak, w przypadku wielu enzymów antyoksydacyjnych otrzymane wyniki okazują się być sprzeczne. Gałecki i wsp. (2009a) odnotowali podwyższoną aktywność dysmutazy ponadtlenkowej (SOD, ang. *superoxide dismutase*), podczas gdy badania przeprowadzone przez Herken i wsp. (2007) wykazały obniżony poziom SOD w surowicy u chorych z depresją. Podobnie, zespół Bilici i wsp. (2001) dowiódł, że depresja może być związana z nadmierną aktywnością peroksydazy glutationowej (Gpx, ang. *glutathione peroxidase*), podczas gdy inne badania nie potwierdziły tej zależności (Kodykova i wsp., 2009; Kotan i wsp., 2011). Dodatkowo pacjenci z depresją cechują się obniżoną aktywnością paroksonazy 1 (PON1, ang.*paroxonase 1*), która chroni lipoproteiny o niskiej gęstości przed procesami utleniania (Bortolasci i wsp., 2014). Nadprodukcja ROS obserwowana w przebiegu depresji skutkuje uszkodzeniami kwasów nukleinowych, białek i lipidów, prowadzącymi do powstania specyficznych produktów, które mogą być uznawane za biomarkery depresji. Pacjenci z depresją cechują się podwyższonym poziomem 8-oksyguaniny (8-oxoG, ang. *8-oxoguanine*) w moczu, płynie mózgowo-rdzeniowym, osoczu i jednojądrzastych komórkach krwi obwodowej (PBMCs, ang. *peripheral blood mononuclear cells*), co świadczy o uszkodzeniach oksydacyjnych DNA (Irie i wsp., 2003; Forlenza i Miller, 2006; Kupper i wsp., 2009). W przebiegu depresji obserwowany jest również podwyższony poziom dialdehydu malonowego (MDA, ang. *malondialdehyde*), produktu ubocznego peroksydacji wielonienasyconych kwasów tłuszczowych (Gałecki i wsp., 2009b) oraz 8-izo-prostaglandyny F2 (8-iso-PGF2, ang. *8-iso-prostaglandin F2*), która powstaje w wyniku utleniania kwasu arachidonowego (Dimopoulos i wsp., 2008; Chung i wsp., 2013).

Depresja związana jest również z nadprodukcją RNS, określaną mianem stresu nitracyjnego. W przebiegu depresji obserwuje się podwyższony poziom tlenku azotu (II) (NO, ang. *nitric oxide*). Co więcej, pacjenci po próbach suicydalnych cechowali się wyższym poziomem NO w surowicy niż pacjenci, którzy nie dokonywali tych prób (Savass i wsp., 2002; Kim i wsp., 2006; Selek i wsp., 2008). Konsekwencją zbyt wysokiego stężenia NO może być nitrowanie i hipernitrozylacja aminokwasów i białek. Prowadzi to do produkcji reaktywnych związków, w tym NO-tyrozyny, NO-tryptofanu i NO-argininy, które w konsekwencji powodują wzrost poziomu przeciwciał IgM

przeciwko NO-tyrozynie, NO-tryptofanie i NO-argininie w surowicy. Z drugiej strony, ograniczenie syntezy NO może wykazywać działanie o charakterze antydepresyjnym (Maes i wsp., 2011a, 2013). Ponadto, badania na zwierzętach wykazały, że depresja może być związana ze zwiększoną aktywnością śródbłonkowej syntetazy tlenku azotu (eNOS, ang. *endothelial nitric oxide synthase*) oraz zwiększoną ekspresją na poziomie białka i mRNA neuronalnej syntetazy tlenku azotu (nNOS, ang. *neuronal nitric oxide synthase*) w hipokampie (Wegener i wsp., 2010). Jednakże, leczenie przeciwdepresyjne może zwiększać ekspresję mRNA nNOS w hipokampie, śródmózgowiu, móżdżku i opuszce węchowej oraz ekspresję mRNA indukowanej syntetazy tlenku azotu (iNOS, ang. *inducible nitric oxide synthase*) w korze czołowej i śródmózgowiu, a obniżać ekspresję mRNA eNOS w większości regionów mózgu (Yoshino i wsp., 2017).

Rozwój depresji może być również konsekwencją nieprawidłowego przebiegu szlaku katabolitów tryptofanu. Szlak TRYCATs jest związany z syntezą serotoniny z tryptofanu (Rycina 1).


TDO2
tryptofan
kinurenina
MONOCYTY
IDO1
cytokiny
tryptofan
kinurenina
KMO
3-hydroksykinurenina
3-HAO
kwas chinolinowy
BARIERA KREW-MÓZG
tryptofan
kinurenina
3-hydroksykinurenina
IDO1
MAKROFAGI
KMO
3-HAO
MAKROFAGI
TPH1
AADC
SNAT
HOMT
PINEALOCYTY
TPH2
AADC
NEURONY
SEROTONINERGICZNE
serotonina
melatonina
ASTROCYTY
kwas kinureninowy
kwas chinolinowy
α7nAChR
NMDAR

Rycina 1. Przemiany tryptofanu w organizmie. 2,3-dioksygenazy tryptofanu 2 (TDO2) oraz 2,3-dioksygenazy indolaminowej 1 (IDO1) powoduje rozszczepienie pierścienia indolowego tryptofanu (egzogenny aminokwas) z jednoczesnym przyłączeniem atomu tlenu w pozycji 2 i 3 prowadząc do powstania kinureniny. TDO2 wykazuje najwyższą ekspresję w wątrobie, a IDO1 ulega ekspresji w większości tkanek, w tym w komórkach OUN. W kolejnym etapie kinurenina jest metabolizowana do 3-hydroksykinureniny dzięki aktywności 3-monooksygenazy kinureninowej (KMO). 3-hydroksykinurenina jest przekształcana do silnie toksycznego kwasu chinolinowego. Tryptofan, kinurenina i 3-hydroksykinurenina mogą przenikać przez barierę krew-mózg. W mózgu, tryptofan ulega przemianom prowadzącym do powstania melatoniny (dzięki aktywności hydroksylazy tryptofanu 1 – TPH1; dekarboksylazy aromatycznych L-aminokwasów – AADC; N-acetylotransferaza serotoniny – SNAT; 5-hydroksyindolo-O-metylotransferaza – HOMT) , serotoniny (dzięki aktywności hydroksylazy tryptofanu 2 – TPH2; dekarboksylazy aromatycznych L-aminokwasów – AADC), kwasu kinureninowego (dzięki aktywności aminotransferazy kinureniny II – KATII) i kwasu chinolinowego (dzięki aktywności KMO, 3,4-dioksygenaza kwasu antranilowego – 3-HAO). Kwas kinureninowy wykazuje aktywność antagonisty receptora N-metylo-D-asparaginowy (NMDAR) i receptora α7 nikotynowych receptorów cholinergicznych (α7nAChR), podczas gdy kwas chinolinowy aktywuje receptor α7nAChR.

Hipotezę wpływu zaburzeń poziomu serotoniny na rozwój depresji przedstawiono już w latach 60. XX wieku. Późniejsze badania rozwinęły tę hipotezę, wskazując również na udział zaburzeń funkcjonowania i ilości receptorów serotoninergicznych w mózgu. Teoria ta stała się podstawą do opracowania terapii, która zakłada stosowanie SSRIs w leczeniu depresji (Albert i wsp., 2012). Niedobory serotoniny mogą być związane z niskim poziomem tryptofanu, który obserwuje się surowicy pacjentów z depresją. Obniżony poziom tryptofanu u tych pacjentów może być konsekwencją zwiększonej aktywność 2,3-dioksygenazy tryptofanu 2 (TDO2, ang. *tryptophan 2,3-dioxygenase 2*) i 2,3-dioksygenazy indoloaminowej 1 (IDO1, ang. *indoleamina 2,3-dioxygenase 1*), enzymów limitujących szybkość metabolizmu tryptofanu (Maes i wsp., 2011b; c). W wyniku aktywności TDO2 i IDO1 tryptofan jest przekształcany w kinureninę, która w kolejnych etapach jest metabolizowana do toksycznego kwasu chinolinowego. W związku z tym osoby z depresją cechują się również zwiększonym stosunkiem kinurenina/tryptofan, wskazującym na nadprodukcję toksycznych metabolitów szlaku TRYCATs (Maes i wsp., 2011b; c). Nadprodukcja innego toksycznego metabolitu tryptofanu – 3-hydroksykinureniny – może indukować produkcję opisanych wyżej ROS, prowadząc w konsekwencji do aktywacji apoptozy neuronów (Stone i wsp., 2001). Obok kwasu chinolinowego i 3-hydroksykinureniny, również kwas 3-hydroksyantranilowy wykazuje aktywność oksydacyjną, stymulując produkcję ROS, w tym nadtlenku wodoru, oraz powodując peroksydację lipidów (Dykens i wsp., 1987; Rios i Santamaria, 1991; Okuda i wsp., 1998; Guidetti i Schwarcz, 1999; Goldstein i wsp., 2000; Murakami i wsp., 2006; Santamaria i wsp., 2001; Smith i wsp., 2009). Z drugiej strony, inne badania sugerują, że 3-hydroksykinurenina, kwas 3-hydroksyantranilowy oraz kwas ksanturenowy mogą również wykazywać właściwości antyoksydacyjne. Metabolity te zmiatają wolne rodniki, zmniejszają peroksydację lipidów, zapobiegają spontanicznemu utlenianiu glutationu i uszkodzeniom 2-deoksyrybozy (Christen i wsp., 1990; Goda i wsp., 1999; Leipnitz i wsp., 2007).

Kolejnymi ważnymi enzymami zaangażowanymi w metabolizm tryptofanu, których zaburzenia mają wpływ na rozwój depresji, są hydroksylaza tryptophanu 1 i 2 (TPH1/2 ang. *tryptophan hydroxylase 1/2*), limitujące szybkość syntezy serotoniny. Badania na zwierzętach, potwierdziły, że stresowane zwierzęta wykazywały zmniejszony poziom ekspresji mRNA genów, kodujących TPH1 i TPH2 (Chen et al., 2017). Co więcej, w wyniku przemian szlaku TRYCATs mogą być generowane związki o działaniu neuroprotekcyjnym.

Aminotransferazy kinureniny I i II (KATI i KATII, ang. *kynurenine aminotransferase I and II*) przekształcają kinureninę do kwasu kinureninowego o właściwościach przeciwutleniających i neuroprotekcyjnych. W konsekwencji, rozwój depresji związany jest z obniżeniem aktywności KATI i II, a także z obniżeniem poziomu kwasu kinureninowego (Stone i wsp., 2001; Maes i wsp., 2011d). Nieprawidłowy przebieg szlaku TRYCATs ma również wpływ na zaburzenia syntezy melatoniny, zaangażowanej w regulację rytmów okołodobowych człowieka. 80% pacjentów ze zdiagnozowaną depresją charakteryzuje się zaburzeniami snu. Dodatkowo, nasilająca się bezsenność może przyczyniać się do ryzyka nawrotu i zwiększenia ciężkości przebiegu epizodu depresji. Ponadto, wykazano, że bezsenność jest charakterystyczna dla osób, które podejmowały próby samobójcze. Nieleczona bezsenność może być również przyczyną nawrotu pełnoobjawowej depresji (Kodykova i wsp., 2009).

Jak wspomniano wcześniej proces rozwoju depresji na charakter wieloczynnikowy. Co więcej, dotychczasowe badania wskazują, że w przebiegu depresji intensyfikacja procesów oksydacyjnych i zaburzenia szlaku katabolitów tryptofanu mogą wystąpić zarówno w mózgu jak i obwodowo – we krwi czy surowicy. W związku z tym, stopień nasilenia tych procesów może być uzależniony od rodzaju tkanki. Ponadto, sugeruje się, że proces rozwoju depresji może skutkować różnymi zmianami w różnych strukturach mózgu, co sugeruje, że niektóre części mózgu mogą być bardziej lub mniej narażone na negatywne działanie neurotoksycznych związków, w tym kwasu chinolinowego i 3-hydroksykinureniny oraz reaktywnych form tlenu (Di Chiara i wsp., 1999; Mayberg i wsp., 2000; Arango i wsp., 2001; Scheggi i wsp., 2002; Mayberg i wsp., 2005; Pariante i wsp., 2008; Matthews i wsp., 2012). To wszystko sprawia, że prawidłowa diagnostyka i odpowiednia, skuteczna terapia jest trudna. Biorąc pod uwagę powyższe, dokładne poznanie molekularnych mechanizmów rozwoju depresji może pozwolić w przyszłości na opracowanie skutecznych biomarkerów, które umożliwią wczesną i trafną diagnostykę. Regulacja określonych procesów, zaangażowanych w mechanizm rozwoju depresji, na poziomie molekularnym może pozwolić na opracowanie nowego, skutecznego i spersonalizowanego leczenia. Dlatego też w niniejszej pracy podjęto próbę określenia roli polimorfizmów pojedynczego nukleotydu, zlokalizowanych w pięciu genach, kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny oraz w pięciu genach, zaangażowanych w szlak katabolitów tryptofanu na częstość występowania depresji. Ponadto, dokonano oceny wpływu procedury chronicznego łagodnego stresu na ekspresję

na poziomie mRNA i białka oraz stopień metylacji regionów promotorowych genów zaangażowanych w wyżej wymienione szlaki. Charakterystyka enzymów, kodowanych przez badane w niniejszej pracy geny, została przedstawiona w Tabeli 1.

Tabela 1. Charakterystyka badanych enzymów na podstawie baz *The Human Protein Atlas* oraz *GeneCards*® oraz *The Human Gene Database*.

Stres oksydacyjny					
Nazwa enzymu (skrót)	**Funkcja**	**Lokalizacja genu (chromosom)**	**Ekspresja w tkankach**	**Związek z depresją**	**Choroby związane zaburzeniami funkcjonowania badanego białka**
Katalaza (CAT)	Enzym hemowy o aktywności katalozowej i peroksydazowej, obecny w peroksysomach każdej oddychającej komórki organizmu, przy dużym stężeniu nadtlenku wodoru jest on rozkładany do wody i tlenu (aktywność katalozowa), przy małym stężeniu substratami donorów wodoru są m. in.: etanol, metanol, fenol (aktywność peroksydazowa)	11p13	We wszystkich tkankach	U osób z depresją obserwowano podwyższony poziom CAT	Łupież pstry, akatalazja
Peroksydaza glutationowa 1 (GPx1)	Enzym (selenoproteina) katalizujący redukcję wodoronadtlenków organicznych i nadtlenku wodoru (H_2O_2) przez glutation, a tym samym chroniący komórki przed uszkodzeniem oksydacyjnym; chroni hemoglobinę w erytrocytach przed rozkładem oksydacyjnym	3p21.31	We wszystkich tkankach	Zmniejszona aktywność Gpx1 u osób z depresją	Niedobór peroksydazy glutationowej, choroba Keshana, stwardnienie zanikowe boczne (ALS), choroby nowotworowe
Peroksydaza glutationowa 4 (GPx4)	Enzym, zawierający selen, katalizuje redukcję wodoronadtlenku fosfolipidów, nawet jeśli są one wbudowane w błony i lipoproteiny, a także wodoronadtlenku kwasu tłuszczowego, wodoronadtlenku cholesterolu i wodoronadtlenku tyminy, a tym samym chroniący komórki przed peroksydacją lipidów błonowych i ich śmiercią	19p13.3	We wszystkich tkankach	Zmniejszona aktywność Gpx4 u osób z depresją	Dysplazja kręgosłupa pod postacią sedaghatian (SMDS), niepłodność męska
Dysmutaza ponadtlenkowa 1 (SOD1)	Cytoplazmatyczny enzym zawierający miedź i cynk, przekształca rodniki ponadtlenkowe w tlen i nadtlenek wodoru	21q22.11	We wszystkich tkankach	Niejednoznaczne obserwacje	Stwardnienie zanikowe boczne, tetraplegia spastyczna i osiowa hipotonia, neurodegeneracja, choroby nowotworowe
Dysmutaza ponadtlenkowa 2 (SOD2)	Mitochondrialny enzym, zawierający mangan, wiąże się z nadtlenkowymi produktami	6q25.3	We wszystkich tkankach, przy czym najwyższą ekspresję	Niejednoznaczne obserwacje	Idiomatyczna kardiomiopatią (IDC), przedwczesne starzeniem się, sporadyczna

	ubocznymi fosforylacji oksydacyjnej i przekształca je w nadtlenek wodoru i tlen		zaobserwowano w fibroblastach		choroba neuronu ruchowego i choroby nowotworowe
Stres nitracyjny					
Nazwa enzymu (skrót)	**Funkcja**	**Lokalizacja genu (chromosom)**	**Ekspresja w tkankach**	**Związek z depresją**	**Choroby związane zaburzeniami funkcjonowania badanego białka**
Syntetaza tlenku azotu 1 (NOS1)	Enzym, który syntetyzuje tlenek azotu z L-argininy. Tlenek azotu jest reaktywnym wolnym rodnikiem, który działa jako biologiczny mediator w różnych procesach, w tym neurotransmisji, mechanizmach przeciwdrobnoustrojowych i przeciwnowotworowych. W mózgu i obwodowym układzie nerwowym tlenek azotu działa jako neuroprzekaźnik	12q24.22	Mózg, mięśnie szkieletowe, skóra, męski układu moczowy i rozrodczy, a w mniejszych ilościach również w płucach i żeńskim układzie rozrodczym	Pacjenci z depresją cechują się podwyższonym poziomem NO, wskazującym tym samym na wzrost aktywności NOS	Achalazja, rodzinne zwężenie przełyku i odźwiernika, stwardnienie zanikowe boczne (ALS)
Syntetaza tlenku azotu 2 (NOS2)	Enzym, który syntetyzuje tlenek azotu, będący silnym mediatorem w licznych procesach	17q11.2	Tkanka limfatyczna, jelita, płuca	Pacjenci z depresją cechują się podwyższonym poziomem NO, wskazującym tym samym na wzrost aktywności NOS	Oponiak wywołany przez promieniowanie
Szlak katabolitów tryptofanu					
Nazwa enzymu (skrót)	**Funkcja**	**Lokalizacja genu (chromosom)**	**Ekspresja w tkankach**	**Związek z depresją**	**Choroby związane zaburzeniami funkcjonowania badanego białka**
2,3-dioksygenaza indoloaminowa (IDO1)	Enzym hemowy, który katalizuje pierwszy i limitujący szybkość etap katabolizmu tryptofanu w szlaku kinureninowym, przekształca tryptofan do N-formylo-kinureniny, enzym ten prawdopodobnie bierze również udział w różnych procesach patofizjologicznych,	8p11.21	Krew, łożysko, w mniejszych ilościach również w męskim i żeńskim układzie rozrodczym, w mózgu	Zwiększona aktywność IDO1 u pacjentów z depresją	Zakażenia drobnoustrojowe, choroby nowotworowe

	takich jak obrona przeciwdrobnoustrojowa i przeciwnowotworowa				
2,3-dioksygenza tryptofanu (TDO2)	Enzym hemowy, który katalizuje pierwszy i limitujący szybkość etap katabolizmu tryptofanu w szlaku kinureninowym, przekształca tryptofan do N-formylo-kinureniny	4q32.1	Wątroba, w mniejszych ilościach również w tkance limfatycznej i mózgu	Zwiększona aktywność TDO2 u pacjentów z depresją	Autyzm, choroby nowotworowe
Hydroksylaza tryptofanu 1 (TPH1)	Enzym katalizujący pierwszy i limitujący etap biosyntezy serotoniny	11p15.1	Mózg, jelito, przysadka mózgowa, żołądek	Zmniejszenie ekspresji *TPH1* na poziomie mRNA	Schizofrenia, lęk somatyczny, zaburzenia dwubiegunowe, zachowania samobójcze, uzależnienia
Hydroksylaza tryptofanu 2 (TPH2)	Enzym katalizujący pierwszy i limitujący szybkość etap biosyntezy serotoniny	12q21.1	Mózg, w mniejszych ilościach również w trzustce i pęcherzu moczowym	Zmniejszenie ekspresji *TPH2* na poziomie mRNA	Choroba afektywna dwubiegunowa, depresja
Aminotranseraza kinureninowa I (KAT1)	Enzym katalizujący nieodwracalną transaminację metabolitu L-tryptofanu, L-kinureninę do kwasu kinureninowego, który wykazuje właściwości neuroprotekcyjne	9q34.11	We wszystkich tkankach	Pacjenci z depresją cechują się obniżonym poziomem kwasu kinureninowego i obniżoną aktywnością KATI	Schizofrenia
Aminotranseraza kinureninowa II (AADAT; KATII)	Transaminaza o szerokiej specyficzności substratowej, wykazuje aktywność aminotransferazy wobec aminoadypinianu, kinureniny, metioniny i glutaminianu, tryptofanu, asparaginianu i hydroksykynureniny. Katalizuje transaminację kinureniny, prowadząc do wytworzenia kwasu kinureninowego, który ma właściwości neuroprotekcyjne	4q33	We wszystkich tkankach, przy czym największa ilość w wątrobie	Pacjenci z depresją cechują się obniżonym poziomem kwasu kinureninowego i obniżoną aktywnością KATII	Choroba Huntingtona, dyssynergia zwieracza pęcherza
Monooksygenaza kinureninowa (KMO)	Enzym, katalizujący hydroksylację L-kinureniny, prowadząc do powstania 3-hydroksy-L-kinureniny, który jest wymagany syntezy kwasu chinolinowego, neurotoksycznego antagonisty receptora NMDA	1q43	Krew, wątroba, łożysko, nerki, płuca	Pacjenci z depresją cechują się podwyższonym poziomem kwasu chinolonowego	Choroba Huntingtona, anomalie zębów, przejściowe niedokrwienie mózgu
Kinureninaza (KYNU)	Katalizuje rozszczepienie L-kinureniny i L-3-hydroksykynureniny odpowiednio do kwasu antranilowego i kwasu 3-hydroksyantranilowego. Wykazuje również aktywność beta-liazy sprzężonej z cysteiną	2q22.2	W większości tkanek, najwyższa ekspresja w krwi, wątrobie, łożysku	Pacjenci z depresją cechują się podwyższonym poziomem kwasu antranilowego	Zespół wad kręgosłupa, serca, nerek i kończyn

Cel pracy

Depresja to złożone zaburzenie psychiczne o wieloczynnikowym mechanizmie rozwoju. Pomimo, że stanowi wiodącą przyczynę niepełnosprawności społeczeństwa, etiologia choroby nadal pozostaje niejasna. Brak skutecznych metod diagnostycznych opartych na mierzalnych biomarkerach przyczynia się do błędnego rozpoznania schorzenia, a także doboru nieskutecznej terapii przeciwdepresyjnej. Dotychczasowe badania wskazują na udział czynników genetycznych w rozwoju depresji. W związku z tym, celem niniejszej pracy było wyjaśnienie roli stresu oksydacyjnego i nitracyjnego oraz zaburzeń przebiegu szlaku katabolitów tryptofanu w molekularnym podłożu depresji. Postawiony w ten sposób cel ogólny został zrealizowany poprzez następujące cele szczegółowe:

- określenie związku genotypów i alleli 16 polimorfizmów pojedynczego nukleotydu (SNPs, ang. *single nucleotide polymorphisms*) zlokalizowanych w genach zaangażowanych w stres oksydacyjny (*SOD2*, *Gpx4*, *CAT*) i nitracyjny (*NOS1*, *NOS2*) oraz szlak katabolitów tryptofanu (*TPH1*, *TPH2*, *IDO1*, *KATI*, *KATII*), ich kombinacji i haplotypów z ryzykiem wystąpienia depresji z uwzględnieniem płci,
- określenie wpływu procedury chronicznego łagodnego stresu i terapii wenlafaksyną na poziom ekspresji i stopień metylacji regionów promotorowych genów zaangażowanych w stres oksydacyjny (*SOD1*, *SOD2*, *Gpx1*, *Gpx4*, *CAT*) i nitracyjny (*NOS1*, *NOS2*) oraz szlak katabolitów tryptofanu (*Tph1*, *Tph2*, *Ido1*, *KatI*, *KatII*, *Kynu*, *Kmo*).

Materiały i metody

Charakterystyka pacjentów

Materiał do badań obejmował 510 próbek krwi obwodowej pobranej od 229 osób należących do grupy kontrolnej oraz od 281 pacjentów ze zdiagnozowaną depresją hospitalizowanych w Klinice Psychiatrii Dorosłych Uniwersytetu Medycznego w Łodzi. Próbki te otrzymano dzięki współpracy z zespołem Pana Profesora Piotra Gałeckiego. Pacjenci i grupa kontrolna zostali dobrani pod względem wieku i płci. Kwalifikacja pacjentów do badania była oparta na kryteriach Międzynarodowej Statystycznej Klasyfikacji Chorób i Problemów Zdrowotnych klasyfikacji ICD-10 (ang. *International Statistical Classification of Diseases and Related Health Problems*) (F32.0-7.32.32, F33.0-33.8). Kryteria wykluczenia obejmowały: stany zapalne, choroby autoimmunologiczne, choroby nowotworowe, uszkodzenia ośrodkowego układu nerwowego, chroniczne lub ostre choroby somatyczne oraz obecność innych zaburzeń osi I i II. Nasilenie objawów depresji oceniono i klasyfikowano za pomocą 21-stopniowej Skali Hamiltona (HDRS, ang. *Hamilton Depression Rating Scale*) przez tego samego lekarza psychiatrę zarówno przed i po terapii lekami z grupy SSRIs. Każdy uczestnik badania został zapoznany z przebiegiem, celem badania i sposobem przetwarzania danych osobowych oraz złożył pisemną zgodę na uczestnictwo w badaniu. Na wykonanie badań została udzielona zgoda Komisji Bioetycznej Uniwersytetu Medycznego w Łodzi nr RNN/70/14/KE.

Oznaczanie genotypów polimorfizmów pojedynczego nukleotydu w genach kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny oraz szlak katabolitów tryptofanu

Rozkład genotypów i alleli badanych SNPs przeprowadzono na genomowym DNA wyizolowanym z krwi obwodowej komercyjnie dostępnym zestawem Blood Mini Kit (A&A Biotechnology, Gdynia, Polska). Analizie poddano łącznie 16 SNPs, które przedstawiono w Tabeli 2. Oznaczenie genotypów polimorfizmów wykonano za pomocą sond TaqMan™ (TaqMan SNP Genotyping Assay; Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) oraz komercyjnie dostępnego zestawu TaqMan Universal Master Mix, no UNG II (Thermo Fisher Scientific, Waltham, Massachusetts, USA) w łańcuchowej reakcji polimerazy z detekcją produktu specyficznego dla jednego allelu i/bądź drugiego allelu w czasie rzeczywistym (*real-time* PCR ang. *real-time polymerase chain reaction*)

z wykorzystaniem termocyklera CFX96 Touch™ Real-Time PCR Detection System BIO-RAD (BIO-RAD Laboratories Inc., Hercules, CA, USA), a wyniki analizowano przy użyciu oprogramowania CFX Manager™ Software wersja 3.1 (BIO-RAD Laboratories Inc., Hercules, CA, USA).

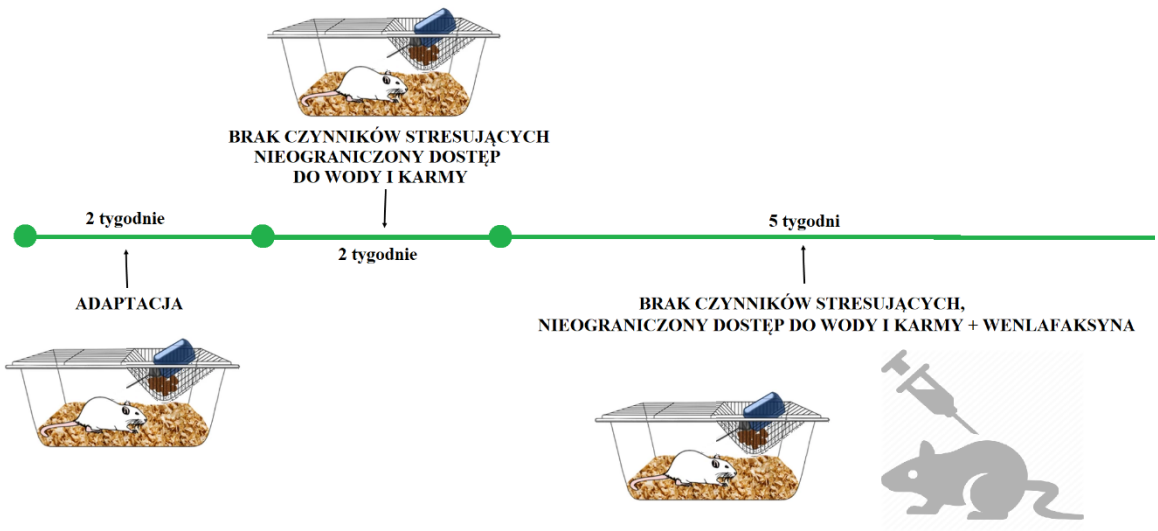
Tabela 2. Charakterystyka analizowanych SNPs.

Stres oksydacyjny i nitracyjny				
Gen	**Nr rs badanego polimorfizmu**	**Polimorfizm**	**Lokalizacja**	**Zmiana sekwencji aminokwasowej**
SOD2	rs4880	c.47T>C	Ekson	Val16Ala
CAT	rs7943316	c.-89A>T	5'UTR	-
GPx4	rs713041	c.660T>C	3'UTR	-
NOS1	rs1879417	c.-420-34221G>A	Intron	-
NOS2	rs2297518	c.1823C>T	Exon	Ser608Leu
	rs10459953	c.-227G>C	5'UTR	-
Szlak katabolitów tryptofanu				
Gen	**Nr rs badanego polimorfizmu**	**Polimorfizm**	**Lokalizacja**	**Zmiana sekwencji aminokwasowej**
TPH1	rs1799913	c.804-7C>A	5'UTR	-
	rs623580	c.-1668T>A	Intron	-
	rs1800532	c.803+221C>A	Intron	-
	rs10488682	c.-173A>T	Intron	-
TPH2	rs7963803	c.-1449C>A	5'UTR	-
	rs4570625	c.-844G>T	5'UTR	-
KATI	rs10988134	c.*456G > A	3'UTR	-
ADDAT	rs1480544	c.975-7T > C	Intron	-
IDO1	rs3824259	c.-1849C > A	Intron	-
	rs10089084	c.-1493G > C	Intron	-

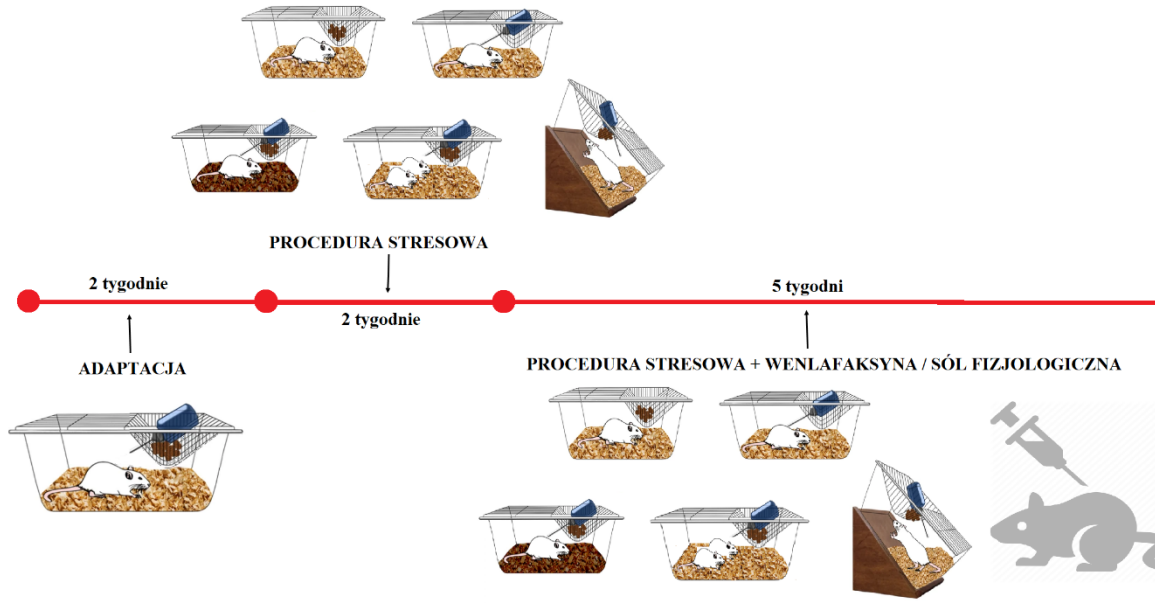
Charakterystyka zwierząt

Badanie przeprowadzono na samcach szczurów rasy Wistar Han (Charles River, Sulzfeld, Germany), które losowo podzielono na pięć grup (zwierzęta kontrolne, stresowane, kontrolne po terapii wenlafaksyną, stresowane po terapii solą fizjologiczną, stresowane po terapii wenlafaksyną) po sześć osobników każda. Schemat przebiegu procedury chronicznego łagodnego stresu (CMS, ang. *chronic mild stress*) i terapii przeciwdepresyjnej z zastosowaniem wenlafaksyny (lek przeciwdepresyjny z grupy SNRIs) lub terapii solą fizjologiczną przedstawiono na Rycinie 2. Przed rozpoczęciem ekspozycji na bodźce CMS zwierzęta poddawano dwutygodniowej adaptacji do warunków laboratoryjnych. Procedura CMS polegała na długotrwałej ekspozycji zwierząt na łagodne bodźce stresowe, obejmujące pozbawienie zwierząt pokarmu lub wody, przechylanie klatki pod kątem 45°, zmiana dobowych rytmów oświetlenia pomieszczeń (włączanie i wyłączanie światła co dwie godziny), zabrudzanie klatki (250 ml wody w trocinowej ściółce) czy światło stroboskopowe (150 błysków/minutę). Bodźce stresowe stosowano indywidualnie i nieprzerwanie w dzień i w nocy. Po dwóch tygodniach u tak stresowanych zwierząt pojawia się szereg zmian behawioralnych, fizjologicznych i biochemicznych, wykazujących podobieństwo do objawów depresji. Podstawowym następstwem ekspozycji na bodźce stresowe jest zmniejszenie wrażliwości stresowanych szczurów na działanie bodźców nagradzających, które jest uznawane jako przejaw głównego objawu depresji, czyli anhedonii. Miarą tego efektu jest obniżenie spożycia 1% roztworu sacharozy. Zwierzęta kontrolne nie miały kontaktu z grupami stresowanymi. Po dwóch tygodniach ekspozycji na bodźce stresowe zwierzęta poddano terapii, która obejmowała iniekcje roztworu wenlafaksyny o stężeniu 10 mg/kg masy ciała lub soli fizjologicznej w ilości 1 ml/kg masy ciała podawane przez pięć tygodni (Papp, 2012). Po zakończeniu procedury stresowej bądź terapii zwierzęta dekapitowano, a do dalszych analiz pobierano próbki krwi obwodowej i tkanki mózgowej z odpowiednio oddzielonymi strukturami mózgu (hipokamp, ciała migdałowate, śródmózgowie, podwzgórze, kora mózgowa, jądra zwojów podstawy). Procedura CMS została przeprowadzona w Instytucie Farmakologii Polskiej Akademii Nauk w Krakowie dzięki współpracy z Panem Profesorem Mariuszem Pappem. Próbki krwi obwodowej wykorzystano do izolacji komórek PBMCs z zastosowaniem Gradisolu L (Aqua-Med, Łódź, Polska) metodą wirowania różnicowego. Wszystkie badania zostały przeprowadzone na podstawie zgody Komisji Bioetycznej Instytutu Farmakologii Polskiej Akademii Nauk w Krakowie nr 1272/2015.

A



B



Rycina 2. Schemat przeprowadzonego eksperymentu. (A) Przebieg eksperymentu w grupach kontrolnych. Zwierzęta każdej grupy kontrolnej po zakupieniu były poddawane dwutygodniowemu okresowi adaptacji do warunków laboratoryjnych. W tym czasie następowało również przyuczenie do wykonywanego cotygodniowo testu spożycia 1% roztworu sacharozy, który traktowano jako pomiar reakcji na nagrodę. Zwierzęta grupy kontrolnej były poddawane dwutygodniowemu okresowi adaptacji, po którym następował dwutygodniowy okres, w którym miały nieograniczony

dostęp do wody i karmy oraz nie były poddane działaniu czynników stresowych. Zwierzęta grupy kontrolnej, które poddano terapii otrzymywały wenlafaksynę po czterech tygodniach od przybycia do laboratorium przez okres pięciu tygodni. (B) Przebieg procedury chronicznego łagodnego stresu. Zwierzęta każdej grupy stresowanej po zakupieniu były poddawane dwutygodniowemu okresowi adaptacji do warunków laboratoryjnych. W tym czasie następowało również przyuczenie do wykonywanego cotygodniowo testu spożycia 1% roztworu sacharozy, który traktowano jako pomiar reakcji na nagrodę. Zwierzęta grupy stresowanej były poddawane dwutygodniowemu okresowi adaptacji, po którym następował dwutygodniowy okres, w którym były poddawane działaniu różnych czynników stresowych, w tym przechylanie klatki o kąt 45°, zabrudzanie ściółki wodą, brak dostępu do wody lub pokarmu, czy ekspozycja na światło stroboskopowe. Po tym czasie, zwierzęta, które podawano terapii otrzymywały iniekcje wenlafaksyny lub soli fizjologicznej przez okres pięciu tygodni (Na podstawie Sequeira-Cordero et al., 2019; zmodyfikowano).

Oznaczenie ekspresji genów kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny oraz szlak katabolitów tryptofanu na poziomie mRNA

Analizę ekspresji genów kodujących enzymy zaangażowane w stres oksydacyjny (*SOD1, SOD2, GPx1, GPx4, CAT*) i nitracyjny (*NOS1, NOS2*) oraz szlak TRYCATs (*Tph1, Tph2, Ido1, KatI, KatII, Kynu, Kmo*) przeprowadzono, wykorzystując cDNA otrzymane w reakcji odwrotnej transkrypcji, której poddano mRNA wyizolowane z osadu PBMCs i tkanki mózgowej komercyjnie dostępnymi zestawami do izolacji opartymi na zdolności wiązania RNA do złóż krzemionkowych. W przypadku próbek krwi izolacji RNA dokonano za pomocą komercyjnie dostępnego zestawu GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) oraz za pomocą zestawu ISOLATE II RNA/DNA/Protein Kit (Bioline, Gdańsk, Polska) dla próbek tkanki mózgowej. Reakcję odwrotnej transkrypcji przeprowadzono zgodnie z protokołem komercyjnie dostępnego zestawu High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Ocenę poziomu ekspresji wykonano za pomocą sond TaqMan™ Gene Expression Assay (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) oraz komercyjnego zestawu TaqMan Universal Master Mix, no UNG II (Thermo Fisher Scientific, Waltham, Massachusetts, USA) w łańcuchowej reakcji polimerazy z detekcją w czasie rzeczywistym z wykorzystaniem termocyklera CFX96 Touch ™ Real-Time PCR Detection System (BIO-RAD Laboratories Inc., Hercules, CA, USA). Poziom ekspresji badanych genów określono według wzoru $2^{-\Delta ct}$, gdzie $\Delta Ct = C_{t\ genu\ badanego} - C_{t\ genu\ referencyjnego}$, a jako gen referencyjny wykorzystano *18S*, zaliczany do grupy genów metabolizmu

podstawowego (Schmittgen i Livak, 2008). Wartości progowe cyklu (C_t) zostały wyznaczane automatycznie przy użyciu oprogramowania CFX Manager™ Software wersja 3.1. (BIO-RAD Laboratories Inc., Hercules, CA, USA).

Oznaczanie stopnia metylacji regionów promotorowych genów kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny oraz szlak katabolitów tryptofanu

Jedną z przyczyn zmian ekspresji genów na poziomie mRNA może być metylacja cytozyny w obrębie wysp CpG. Niski stopień metylacji występuje w regionach genomu, kodujących geny aktywne transkrypcyjnie (Piletič i Kunej, 2016). Analizę stopnia metylacji w obrębie regionów promotorowych genów, kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny oraz szlak katabolitów tryptofanu przeprowadzono, wykorzystując DNA wyizolowane z osadu jednojądrzastych komórek krwi obwodowej i tkanki mózgowej komercyjnie dostępnymi zestawami do izolacji opartymi na zdolności wiązania DNA do złóż krzemionkowych (odpowiednio QIAamp DNA Mini Kit, Qiagen, Hilden, Niemcy – dla próbek krwi oraz ISOLATE II RNA/DNA/Protein Kit, Bioline, Gdańsk, Polska – dla próbek tkanki mózgowej). Następnie otrzymane DNA poddano reakcji bisulfidacji przeprowadzonej zgodnie z protokołem komercyjnego zestawu CiTi Converter DNA Methylation Kit (A&A Biotechnology, Gdynia, Polska). W wyniku reakcji bisulfidacji następuje deaminacja niemetylowanej cytozyny, która jest przekształcana do uracylu, podczas gdy metylowana cytozyna nie podlega tym przekształceniom. DNA jest następnie amplifikowane w łańcuchowej reakcji polimerazy, w której uracyl jest przekształcany w tyminę (Hayatsu, 2008). W celu oceny stopnia metylacji regionów promotorowych badanych genów zastosowano metodę analizy topnienia DNA o wysokiej rozdzielczości (MS-HRM, ang. *methylation sensitive – high resolution melting*) z wykorzystaniem starterów zaprojektowanych w Methyl Primer Express™ Software v 1.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) dostępnym na stronie http://www.urogene.org/methprimer2/ oraz komercyjnego zestawu 5 x HOT FIREPol® EvaGreen® HRM Mix (no ROX) (Solis BioDyne Tartu, Estonia) (Wojdacz i wsp., 2007; 2008; 2009). Startery, które zaprojektowano dla badanych regionów promotorowych, zawierających wyspy CpG, przedstawiono w Tabeli 3. Ocenę stopnia metylacji przeprowadzono, wykorzystując wzorce DNA o różnej zawartości zmetylowanego DNA (100%, 75%, 50%, 25% i 10%), które otrzymano poprzez zmieszanie komercyjnie dostępnych standardów w pełni zmetylowanego i niezmetylowanego DNA (CpGenome™

Rat Methylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA oraz CpGenome™ Rat Unmethylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA). Wszystkie reakcje przeprowadzono w termocyklerze CFX96™ Real-TIME PCR Detection System (BIO-RAD Laboratories Inc., Hercules, CA, USA) i analizowano je przy wykorzystaniu oprogramowania Precision Melt Analysis ™ Software wersja 3.1. (BIO-RAD Laboratories Inc., Hercules, CA, USA). W celu określenia poziomu metylacji, profil HRM prób badanych porównano z profilem otrzymanym dla wzorców.

Tabela 3. Charakterystyka starterów wykorzystanych do oznaczenia poziomu metylacji regionów promotorowych badanych genów.

Stres oksydacyjny i nitracyjny			
Gen	**Sekwencja starterów**	**Wielkość produktu [pz]**	**Tm* [ºC]**
CAT	F:TTTGAGATTATTGTGTTTGAAA R:TACCTACACCCAAAAAAAAATA	148	59
GPx1	F:GTTGTTTTAGGTTTTGTTGTTG R:AAAACTAAAATCCTCCAACTCT	102	65
GPx4 (promotor 2)	F:AGGTTGGAGGTTTAGAGGTTTA R:TCCCCTAAATACAAAAATCTCT	118	59
GPx4 (promotor 3)	F:AGGTTGGAGGTTTAGAGGTTTA R:AAAACATAACAAAATCATCTCCC	147	65
SOD1	F: AAGGAGGTGTGTTTAATTGGTA R: AACCCCTCTCACAAATTTCTAA	144	65
SOD2	F: GGGGAAGGTTATTTAGGGTATA R: CCTTTTCCATTCCTAATTCTAAA	133	59
NOS1 (promotor 3)	F: GGGTTTTTAATTTTTTTATTGTG R: CAACCCTCATTAAAAAAACC	124	59
NOS1 (promotor 7)	F: GTTTGAGATTGGAATTTTTTGG R: CCAAAACATCCAAAAATACACA	124	59
Szlak katabolitów tryptofanu			
Gen	**Sekwencja starterów**	**Wielkość produktu [pz]**	**Tm* [ºC]**
Tph1 (promotor 2)	F:GGGAGTTTTGTTTTGGTTTTTA R:TCCTCAACCACAAAAAATCTAA	132	55
Ido1 (promotor 2)	F:TTTGAGTTTTAGTGATTTTGGG R:TTAATATCTAATCCCAATCTCTAAAAC	100	59
Tdo2 (promotor 1)	F:GATGATTTAGGTGGTTTGAGGT R:CAAAAAAAACAAAATTCATCCA	123	59
TDO2 (promotor 2)	F:ATGATTTAGGTGGTTTGAGGTT R:ACCCAATCTACCTAACTAACAAC	187	61.4
Kmo (promotor 7)	F: TTGGTTTAGGGAAGGAAAT R: ATAAAAAACTAAACCCAAAACAC	150	55.7

*Temperaturę przyłączania starterów wyznaczono eksperymentalnie

Oznaczanie ilości białek zaangażowanych w stres oksydacyjny i nitracyjny oraz szlak katabolitów tryptofanu

Ekspresja na poziomie białka została oznaczona za pomocą techniki Western Blot. W tym celu wyizolowano próbki białka z sześciu badanych struktur mózgowych z zastosowaniem sonikacji w buforze RIPA (ang. *radioimmunoprecipitation assay buffer*), zawierającym dodatkowo 1mM fluorek fenylometylosulfonylu (PMSF, ang. *phenylmethylsulfonyl fluoride*) jako inhibitor proteaz. Stężenie białka w otrzymanych próbkach oznaczono metodą Lowry'ego, a jako standard do przygotowania krzywej wzorcowej wykorzystano roztwór surowicy bydlęcej (BSA, Sigma-Aldrich, Saint Louis, Missouri, US) (Lowry i wsp., 1951). Próbki białka rozdzielono elektroforetycznie w 10% żelu poliakrylamidowym w warunkach denaturujących, a następnie przeprowadzono całonocny transfer białek na membranę nitrocelulozową Immobilon-P (Millipore, Bedford, Massachusetts, USA) w temperaturze 4°C. Po zakończeniu transferu przeprowadzono jednogodzinne blokowanie membrany w 5% roztworze odtłuszczonego mleka w temperaturze pokojowej, którą po przepłukaniu 0,1% buforem TBST (roztwór soli fizjologicznej buforowany TRIS-em, zawierającym 0,1% detergent Tween 20) inkubowano początkowo z przeciwciałami pierwszorzędowymi, a po odpłukaniu ich nadmiaru 0,1% buforem TBST, z przeciwciałami drugorzędowymi znakowanymi peroksydazą chrzanową. Warunki inkubacji dla danego przeciwciała były zgodne z zaleceniami producenta i zostały przedstawione w Tabeli 4. Po odpłukaniu nadmiaru przeciwciał drugorzędowych 0,1% buforem TBST, membrany inkubowano w obecności komercyjnego roztworu substratu peroksydazy chrzanowej SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Końcowo produkty białkowe były wizualizowane na kliszy rentgenowskiej. Zmiany w ekspresji białka oceniono na podstawie densytometrycznego pomiaru intensywności uzyskanych prążków białek, którego dokonano za pomocą oprogramowania Gel-Pro® Analyzer Software (Media Cybernetics Inc., USA). Poziom białek był normalizowany względem poziomu ekspresji białka referencyjnego, β-aktyny zgodnie ze wzorem $IOD_{\text{badany gen}} / IOD_{\beta\text{-aktyny}}$.

Tabela 4. Warunki inkubacji przeciwciał pierwszo- i drugorzędowych podczas oznaczania poziomu białek metodą Western Blot.

	Przeciwciało pierwszorzędowe	**Przeciwciało drugorzędowe**
β-aktyna (białko referencyjne)	mysie, rozcieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), inkubacja 1 h w temperaturze pokojowej	anty-mysie, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Katalaza	mysie, rozcieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), całonocna inkubacja w 4°C	anty-mysie, rozcieńczenie1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Dysmutaza ponadtlenkowa 2	mysie, rozieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), inkubacja 2 h w temperaturze pokojowej	anty-mysie, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Peroksydaza glutationowa 4	królicze, rozcieńczenie 1:6000, (Abcam), całonocna inkubacja w4°C	anty-królicze, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Hydroksylaza tryptofanu 1	królicze, rozcieńczenie 1:1000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), całonocna inkubacja w 4°C	anty-królicze, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Hydroksylaza tryptofanu 2	królicze, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), całonocna inkubacja w 4°C	anty-królicze, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
2,3-dioksygenaza indoloaminowa	mysie, rozcieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), całonocna inkubacja w 4°C	anty-mysie, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej
Aminotransferaza kinureninowa	mysie, rozcieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), całonocna inkubacja w 4°C	anty-mysie, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej

Kinureninaza	mycie, rozcieńczenie 1:1000, (Santa Cruz Biotechnology Inc.), całonocna inkubacja w 4°C	anty-mysie, rozcieńczenie 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), inkubacja 1 h w temperaturze pokojowej

Analiza statystyczna

Analizy statystyczne wszystkich prezentowanych wyników wykonano przy użyciu programów Statistica 12 (Statsoft, Tulsa, OK, USA), SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) i GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Do analizy statystycznej rozkładu genotypów w grupach badanych użyto regresji logistycznej, za pomocą, której wyznaczono iloraz szans (OR, ang. *odds ratio*) nieskorygowany i skorygowany dla płci przy przedziale ufności 95%. Dla wyników otrzymanych z eksperymentów z udziałem zwierząt normalność rozkładu otrzymanych wyników oceniono testem Shapiro-Wilka. Jeżeli rozkład danych miał cechy rozkładu normalnego stosowano jednoczynnikową analizę wariancji ANOVA oraz test *post hoc* Tukey'a. Kiedy rozkład danych odbiegał od rozkładu normalnego wówczas stosowano test Kruskala-Wallisa oraz test *post hoc* Student-Newman-Keuls. Dla wszystkich przeprowadzonych analiz poziom $p < 0,05$ przyjęto jako istotny statystycznie.

Wyniki

Związek genotypów polimorfizmów genów zaangażowanych w odpowiedź na stres oksydacyjny: SOD2, CAT, GPx4, NOS1 i NOS2 z występowaniem depresji

Analiza rozkładu genotypów oraz częstości występowania alleli wykazała, że genotyp T/T polimorfizmu c.47T>C (rs4880) genu *SOD2* był związany z większą częstością występowania depresji. W przypadku polimorfizmu c.-89A>T (rs7943316) genu *CAT*, wykazano, że genotyp A/A jest pozytywnie skorelowany z występowaniem depresji, podczas gdy genotyp A/T znacznie częściej występował w grupie kontrolnej. Co więcej, genotyp T/T i allel T polimorfizmu c.660T>C (rs713041) genu *GPx4* był związany ze zwiększoną częstością występowania depresji, podczas gdy heterozygota T/C oraz allel C wykazywały odwrotną zależność. W przypadku pozostałych badanych polimorfizmów 420-34221G>A (rs1879417) genu *NOS1*, c.1823C> T (rs2297518) i c.-227G>C (rs10459953) genu *NOS2* nie stwierdzono korelacji między rozkładem ich genotypów/alleli, a zmianą częstości występowania depresji.

Dotychczasowe badania wykazały, że kobiety cechują się dwukrotnie wyższym ryzykiem rozwoju depresji niż mężczyźni (Kessler, 2003). W związku z tym dokonano analizy rozkładu genotypów badanych polimorfizmów z uwzględnieniem płci, która potwierdziła różnice między populacją kobiet i mężczyzn. W przypadku polimorfizmu c.47T>C (p.Val16Ala) genu *SOD2* (rs4880), genotyp T/T występował znacznie częściej u mężczyzn z depresją, podczas gdy w populacji kobiet nie obserwowano wpływu badanego polimorfizmu na częstość występowania depresji. Genotyp A/A polimorfizmu c.89A>T genu *CAT* (rs7943316) u mężczyzn, a genotypy A/T i T/T u kobiet były związane ze zmniejszeniem częstości występowania depresji. W przypadku polimorfizmu c.660T>C (rs713041) genu *GPx4* genotyp T/T był pozytywnie skorelowany z występowaniem depresji zarówno w populacji kobiet jak i mężczyzn, jednakże heterozygota T/C tego SNP była związana ze wzrostem częstości depresji tylko w grupie mężczyzn. W grupie kobiet genotyp G/C polimorfizmu c.227G>C genu *NOS2* (rs10459953) był związany ze zmniejszeniem częstości występowania depresji, podczas gdy genotyp G/G tego SNP wykazywał odwrotny związek. W populacji mężczyzn nie zaobserwowano wpływu badanego polimorfizmu na częstość występowania depresji. Co więcej, przeprowadzone analizy nie wykazały wpływu wystąpienia określonego polimorfizmu na skuteczność terapii lekami z grupy SSRIs.

Związek genotypów polimorfizmów genów zaangażowanych w szlak katabolitów tryptofanu: TPH1, TPH2, IDO1, KATI, AADAT (KATII) z występowaniem depresji

Wśród badanych polimorfizmów, genotyp C/C oraz allel C polimorfizmu c. 804–7C>A (rs1799913) zlokalizowanego w genie *TPH1*, homozygota A/A polimorfizmu c. 803+221C>A (rs1800532) genu *TPH1*, T/G oraz genotyp T/T i allel T polimorfizmu c.-173A>T (rs10488682) genu *TPH1* były związane ze zwiększoną częstością występowania depresji. Z kolei genotyp T/T polimorfizmu c.-1668T>A (rs623580) genu *TPH1* był ujemnie skorelowany z występowaniem depresji, podczas gdy genotyp A/A i allel A tego SNP były związany ze wzrostem częstości rozwoju depresji. Ponadto, genotyp G/G oraz allel G polimorfizmu c.-844G>T (rs4570625) genu *TPH2* były pozytywnie skorelowane z wystąpieniem depresji, podczas gdy heterozygota G/T i allel T tego SNP wykazywały odwrotną korelację. Genotyp C/C oraz allel C polimorfizmu c.-1449C>A (rs7963803) genu *TPH2* zmniejszał częstość wystąpienia depresji, natomiast heterozygota C/A i allel A tego polimorfizmu wykazywała odwrotną zależność. W przypadku polimorfizmu c.*456G>A (rs10988134) genu *KATI* genotyp A/A zwiększał częstość wystąpienia depresji. W przypadku pozostałych badanych polimorfizmów c.975-7T>C genu *AADAT* (rs1480544), c.-1849C>A (rs3824259) i c.-1493G >C (rs10089084) genu *IDO1* nie wykazano ich wpływu na częstość wystąpienia depresji. Co ciekawe, analiza wieku pierwszego epizodu depresji wykazała, że heterozygoty polimorfizmu c.-844G>T (rs4570625) zlokalizowanego w genie *TPH2* (rs4570625) doświadczały pierwszego epizodu depresji w młodszym wieku niż homozygoty G/G.

Analiza wpływu płci na częstość wystąpienia depresji wykazała, że genotyp A/A polimorfizmu c. *456G>A (rs10988134) genu *KAT1* był pozytywnie skorelowany z rozwojem depresji, podczas gdy genotyp G/G był związany ze zmniejszeniem częstości przypadków depresji w populacji mężczyzn. Natomiast w populacji kobiet nie zaobserwowano wpływu tego polimorfizmu na częstość rozwoju depresji. Przeprowadzone badania wykazały wpływ wystąpienia genotypu T/T polimorfizmu c.975-7T>C (rs1480544) genu *AADAT (KATII)* na obniżenie skuteczności konwencjonalnej terapii SSRIs.

Oznaczenie poziomu spożycia roztworu sacharozy jako miary zdolności reagowania na bodźce nagradzające

Po pierwszych dwóch tygodniach ekspozycji na bodźce stresowe spożycie 1% roztworu sacharozy zmniejszyło się o 40%. Jednakże, pięciotygodniowa terapia wenlafaksyną skutkowała normalizacją ilości 1% roztworu sacharozy, spożywanego przez stresowane zwierzęta.

Wpływ chronicznego łagodnego stresu i terapii wenlafaksyną na zmiany ekspresji na poziomie mRNA i białka oraz stopień metylacji regionów promotorowych genów SOD1, SOD2, GPx1, GPx4, CAT, NOS1 i *NOS2*

Terapia wenlafaksyną powodowała wzrost poziomu ekspresji mRNA *SOD1, SOD2* i *NOS2* w komórkach PBMCs. Zmiany ekspresji badanych genów obserwowane w mózgu zależały od analizowanej struktury. Procedura chronicznego łagodnego stresu obniżała poziom ekspresji *CAT* w hipokampie, podczas gdy terapia wenlafaksyną powodowała dalsze obniżenie ekspresji *CAT*, a także *GPx1*, *SOD1* i *SOD2* w hipokampie stresowanych szczurów. Ekspresja na poziomie mRNA genów *GPx1*, *GPx4* i *NOS1* wzrastała w śródmózgowiu, a *GPX1* i *NOS1* w hipokampie na skutek ekspozycji na bodźce stresowe, podczas gdy terapia wenlafaksyną normalizowała poziom ekspresji tych genów. Podobne zmiany zaobserwowano również dla ekspresji *NOS1* w jądrach zwojów podstawy. Ponadto, ekspozycja na bodźce stresowe skutkowała wzrostem stopnia metylacji regionu promotorowego *GPx1* w komórkach PBMCs. Co więcej, wzrost stopnia metylacji drugiego regionu promotorowego *GPx4* w śródmózgowiu i jądrach zwojów podstawy oraz regionów promotorowych *SOD1* i *SOD2* w hipokampie był obserwowany u szczurów poddanych procedurze CMS. Z drugiej strony, procedura CMS powodowała zmniejszenie ilości białka GPx4 w korze mózgowej. W wyniku terapii wenlafaksyną obserwowano dalsze obniżenie poziomu tego białka w badanej strukturze mózgu. Jednakże, na skutek procedury CMS ilość białka GPx4 wzrastała, a terapia wenlafaksyną prowadziła do dalszego wzrostu ilości tego białka w hipokampie. Wzrost ilości białka CAT na skutek terapii wenlafaksyną obserwowano w śródmózgowiu i korze mózgowej stresowanych szczurów.

Wpływ chronicznego łagodnego stresu i terapii wenlafaksyną na zmiany ekspresji na poziomie mRNA i białka oraz stopień metylacji regionów promotorowych genów Tph1, Tph2, KatI, KatII, Ido1, Kmo, Kynu

Wykazano, że ekspresja badanych genów na poziomie mRNA zależy nie tylko od rodzaju badanej tkanki (PBMCs izolowane z krwi obwodowej oraz tkanka mózgowa), ale również od rodzaju analizowanej struktury mózgu. Procedura CMS powodowała wzrost ekspresji *KatI* w śródmózgowiu, podczas gdy terapia wenlafaksyną obniżała ją w podwzgórzu i korze mózgowej stresowanych zwierząt. W ciałach migdałowatych i śródmózgowiu stresowanych zwierząt obserwowano zwiększony poziom ekspresji *KatII*. Podwyższony poziom ekspresji *Tph2* w śródmózgowiu stresowanych zwierząt ulegał normalizacji po podaniu wenlafaksyny. Co ciekawe, ani procedura CMS, ani terapia wenlafaksyną nie wpływały istotnie na zmiany poziomu ekspresji badanych genów w komórkach PBMCs. Z drugiej strony, stopień metylacji regionów promotorowych genów *Tph1* i *Kmo* wzrastał w komórkach PBMCs stresowanych szczurów po podaniu wenlafaksyny. Co więcej, stresowane szczury cechowały się zwiększonym stopniem metylacji regionu promotorowego *Ido1* w śródmózgowiu. Analizy ekspresji na poziomie białka wykazały, że terapia wenlafaksyną powodowała zmniejszenie ilości białka Tph1 i Ido1 w jądrach zwojów podstawy stresowanych szczurów w porównaniu do stresowanych zwierząt otrzymujących sól fizjologiczną.

Podsumowanie

Depresja jest poważnym schorzeniem psychicznym, które w ostatnich latach dotyka coraz większą populację osób. Obecne metody diagnostyczne obejmujące subiektywną ocenę lekarzy opartą jedynie na wywiadzie lekarskim, często prowadzą do błędnej diagnozy. Niewłaściwa diagnostyka uniemożliwia dobór spersonalizowanej i skutecznej terapii. Mimo to, etiologia rozwoju depresji wciąż pozostaje niejasna. Jednakże, dotychczasowe badania podkreślają wieloczynnikowy charakter depresji, której rozwój może zależeć zarówno od czynników środowiskowych jak i genetycznych. Przypuszcza się, że mechanizm rozwoju depresji może być związany z zaburzeniami równowagi oksydacyjno-anyoksydacyjnej oraz nieprawidłowościami przemian w szlaku katabolitów tryptofanu na poziomie molekularnym. W związku z tym, w ramach realizacji rozprawy doktorskiej zbadano 16 polimorfizmów pojedynczego nukleotydu zlokalizowanych w pięciu genach kodujących enzymy zaangażowane w stres oksydacyjny i nitracyjny (sześć SNPs) oraz w pięciu genach kodujących enzymy zaangażowane w szlak katabolitów tryptofanu (dziesięć SNPs). Stwierdzono, że polimorfizmy genów *CAT, GPx4, SOD2, NOS2, TPH1, TPH2, KAT1, AADAT (KATII)* mogą wpływać na częstość występowania depresji.

W celu potwierdzenia wpływu genów, kodujących enzymy, zaangażowane w badane procesy, wykonano również analizy ekspresji na poziomie mRNA i białka oraz stopnia metylacji regionów promotorowych na skutek wpływu bodźców procedury chronicznego łagodnego stresu i terapii wenlafaksyną. Uzyskane wyniki wskazują na udział stresu oksydacyjnego i nitracyjnego oraz szlaku katabolitów tryptofanu w rozwoju depresji. Jednakże, pełne potwierdzenie tej tezy wymaga dalszych analiz poszerzonych o geny kodujące inne enzymy. Istotne dla dalszych badań jest również uwzględnienie interakcji międzygenowych oraz wpływu czynników środowiskowych na mechanizm rozwoju depresji. Niemniej jednak, wykonane w ramach prezentowanej rozprawy doktorskiej badania mogą przyczynić się do opracowania skutecznej i wczesnej diagnostyki tego schorzenia. Przeprowadzenie badań zarówno na jednojądrzastych komórkach krwi obwodowej, jak i tkance sześciu struktur mózgowych pozwala lepiej zrozumieć mechanizm rozwoju depresji. Co więcej, wyjaśnienie patogenezy depresji może pozwolić w przyszłości na stosowanie spersonalizowanych i skutecznych terapii.

Wnioski

Na podstawie badań przeprowadzonych w ramach tej pracy można sformułować następujące wnioski:

1. Stres oksydacyjny i nitracyjny, a także zaburzenia w przebiegu szlaku katabolitów tryptofanu biorą udział w molekularnym mechanizmie rozwoju depresji.

2. Polimorficzne warianty genów zaangażowanych w stres oksydacyjny (*SOD2*, *GPx4*, *CAT*) i nitrowanie (*NOS1*, *NOS2*) oraz szlak katabolitów tryptofanu (*TPH1*, *TPH2*, *IDO1*, *KATI*, *KATII*) wpływają na ryzyko wystąpienia depresji.

3. Depresja i terapia wenlafaksyną mogą modulować poziom ekspresji i stopień metylacji regionów promotorowych genów zaangażowanych w stres oksydacyjny (*SOD1*, *SOD2*, *GPx1*, *GPx4*, *CAT*) i nitrowanie (*NOS1*, *NOS2*) oraz szlak katabolitów tryptofanu (*Tph1*, *Tph2*, *Ido1*, *KatI*, *KatII*, *Kynu*, *Kmo*), a zmiany te zależą od rodzaju tkanki, a także od badanych struktur mózgu.

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Summary

Introduction

Mental disorders occur much more than any chronic somatic diseases. Among them, the most numerous groups, affecting about 10-15% of the population, are affective disorders, including depression (Anderson et al., 2011). Depression is a nonhomologous disease about the differential clinical picture and worsening of symptoms, such as depressed mood, anhedonia, fatigue and loss of energy, insomnia or excessive drowsiness, weight loss or loss not related to diet, excitement or slowing of movement, unfounded guilt, lack of self-esteem, as well as mental decline, concentration, inability to make decisions and recurrence suicidal thoughts (Kessler, 2003). It is estimated that 350 million people suffer from depression in the world, which is about 5% of the global population (Demyttenaere et al., 2004). Moreover, in the developed countries depression may be affected up to 10% of inhabitants (Vilagut et al., 2016). Moreover, it is estimated that every eleventh person suffers from depression in Poland. This disease affects all age groups of men and women, however, it is most often observed in people of between 20 and 40 years of age, and women are diagnosed about two times as often as men. Moreover, the risk of depression development also increases with age (Kessler, 2003; Demyttenaere et al. 2004; Wang et al., 2008; Vilagut et al., 2016, GBD, 2018). Interestingly, the prognosis of the World Health Organization suggest that depression will be second the most common disability cause of society in 2020 and in 2014 it will be first (Reddy, 2012).

In spite of that depression is becoming one of the most serious health disorders, more than half of the patients do not benefit from medical help, and about a third of treated patients do not respond to conventional pharmacotherapy. Therefore, about 15-20% of people may suffer from severe chronic depression (Al-Harbi, 2012; Iwata et al., 2013). The consequence of prolonged, worsening, and untreated depression may be suicidal attempts. Around one million suicidal deaths due to depression are reported annually in the world (Marcus et al., 2012). The conventional antidepressant therapy is mainly consisting of drugs that act as neurotransmitter reuptake inhibitors, drugs with receptor mechanisms of action, and monoamine oxidase inhibitors. The conventional antidepressant therapy mainly consists of drugs that act as neurotransmitter reuptake inhibitors, drugs with receptor mechanisms of action, and monoamine oxidase inhibitors. The most common of used drugs are the first

group drugs, which includes tricyclic antidepressants (TCAs), serotonin and noradrenaline reuptake inhibitors (SNRIs) and serotonin reuptake inhibitors (SSRIs).

Moreover, depression is also a serious economic problem. The total cost of depression treatment is about 83.1 milliard dollars a year in the US, whereas in Europe this cost is about 118 milliard dollars. By comparison, the total cost of heart failure therapy or AIDS treatment is about 39.2 milliard or 50 milliard dollars a year, respectively (Dutta et al., 2015; Greenberg et al., 2015; Voigt et al., 2015; Osińska et al., 2017). Chronic depression may also contribute to the development of other pathologies, including heart disease, stroke, osteoporosis or diabetes (Clarke and Currie, 2009). Despite intensive research, the molecular mechanism of depression development still remains unclear. The development and course of depression may depend on individual biological characters, genetic and environmental factors (Lopizzo et al., 2015). Emerging reports point to the role of related biochemical pathways, including oxidative and nitrative stress, and abnormalities of the tryptophan catabolites (TRYCATs) pathway, in the development of depressive disorders.

The cause of depression development appears to be lack of balance between generation and the neutralisation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Patients with depression were characterised by reduced activity of low molecular weight antioxidants, including decreased level of zinc coenzyme Q10, vitamins A, E, C and glutathione in plasma. On the other hand, it observed an intensification of oxidative processes. Patients with depression were characterised by reduced activity of low molecular weight antioxidants, including zinc, coenzyme Q10, vitamins A, E, C and glutathione in plasma. Moreover, depression may be associated with an intensification of oxidative processes (Pandya et al., 2013). Activity changes of enzymes involved in oxidative stress are also observed in the course of depression. Previous studies showed that depression may be associated with increased activity of xanthine oxidase (XO) in serum and brain (Herken et al., 2007; Michael et al., 2010; Morris et al., 2017). Nevertheless, in the case of many enzymes, received results are inconsistent. Gałecki et al. (2009a) found increased activity of superoxide dismutase, whereas studies by Herken et al. (2007) showed a reduced SOD level in serum of patients with depression. Similarly, Bilici et al. (2001) proved that depression may be associated with glutathione peroxidase (Gpx) overactivity while other studies did not confirm this dependence (Kodykova et al., 2009; Kotan et al., 2011). Additionally, depressed patients were characterised by a decreased activity

of paroxonase 1 (PON1), which protects low density lipoproteins against oxidation processes (Bortolasci et al., 2014). ROS overproduction observed in course of depression causes the damage of nucleic acids, proteins and lipids. As a result, these damages lead to a generation of specific products, which may be declared biomarkers of depression. Depressed patients were characterised by an elevated level of 8-oxoguanine in urine, cerebrospinal fluid, plasma and peripheral blood mononuclear cells (PBMCs), which indicates oxidative DNA damage (Irie et al., 2003; Forlenza and Miller, 2006; Kupper et al., 2009). Depression also manifests an increased level of malondialdehyde (MDA), which is the by-product of polyunsaturated fatty acids peroxidation (Gałecki et al., 2009b) and 8-iso-prostaglandin F2, which is the product of arachidonic acid oxidation (Dimopoulos et al., 2008; Chung et al., 2013).

Depression is also associated with RNS overproduction, which is declared nitrosative stress. The elevated level of nitric oxide (NO) is observed in the course of depression. Moreover, depressed patients after suicide attempts were characterised by a higher NO level in plasma than patients without these attempts (Savass et al., 2002; Kim et al., 2006; Selek et al., 2008). The consequence of too high NO concentration may be nitration and hypernitrosylation of amino acids and proteins. The processes lead to reactive compound, including NO-tyrosine, NO-tryptophan and NO-arginine, which cause an increase of IgM antibody anty-NO-tyrosine, anty-NO-tryptophan and anty-NO-arginine level in serum. On the other hand, the limitation of NO synthesis may show an antioxidant action (Maes et al., 2011a, 2013). Moreover, an animal study showed that depression may be associated with increased activity of endothelial nitric oxide synthase (eNOS), elevated protein and mRNA expression of neuronal nitric oxide synthase (nNOS) in the hippocampus. However, antidepressant therapy may increase mRNA expression of nNOS in the hippocampus, midbrain, cerebellum and olfactory bulb, and mRNA expression of inducible nitric oxide synthase (iNOS) in the frontal cortex and midbrain, and may reduce mRNA expression of eNOS in most regions of the brain (Yoshino et al., 2017).

The depression development may also be a consequence of an abnormal course of the tryptophan catabolites pathway. TRYCATs pathway is associated with the synthesis of serotonin from tryptophan (Figure 1).

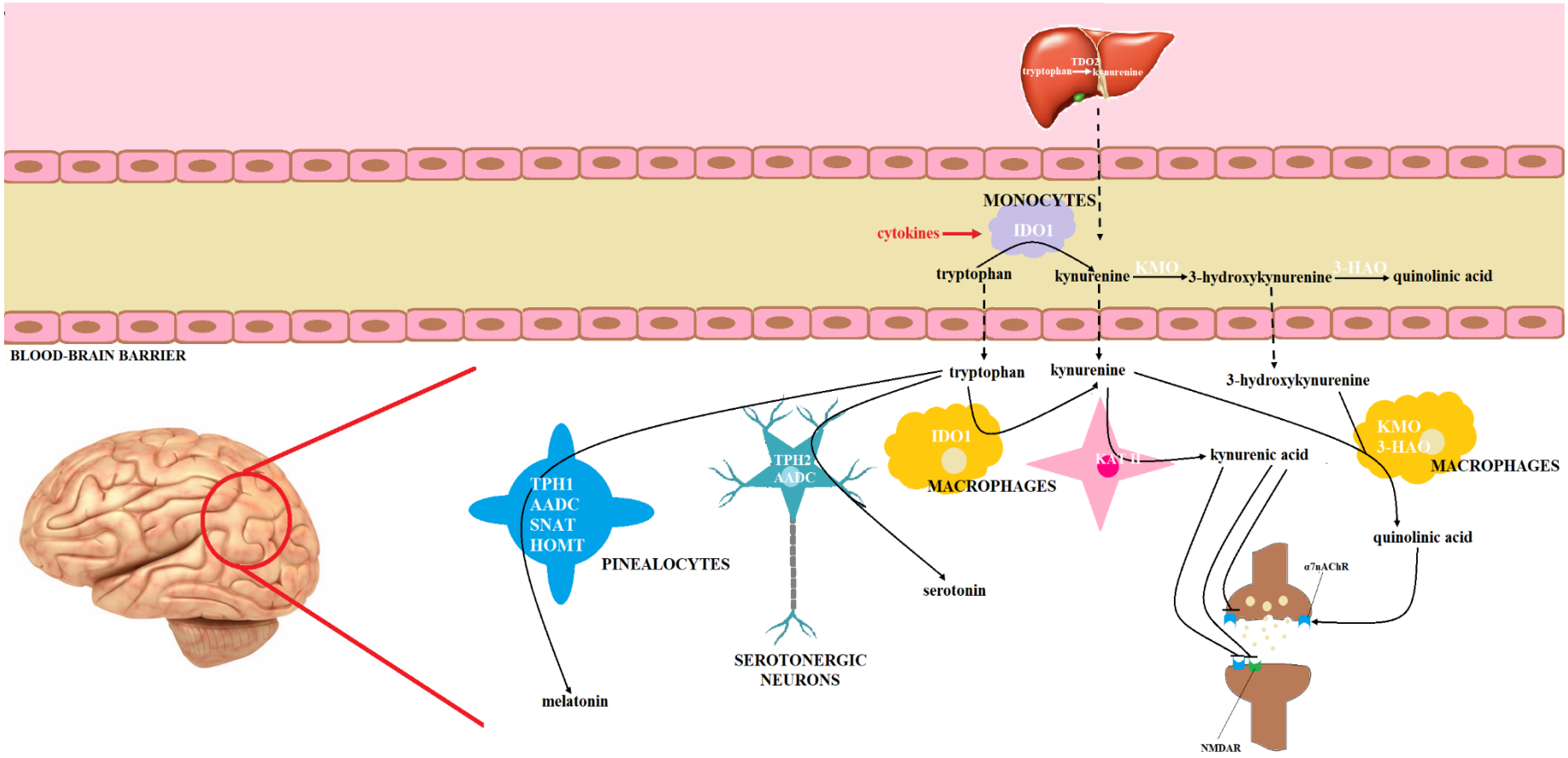
TDO2
tryptophan
kynurenine
MONOCYTES
cytokines
IDO1
tryptophan
kynurenine
KMO
3-hydroxykynurenine
3-HAO
quinolinic acid
BLOOD-BRAIN BARRIER
tryptophan
kynurenine
3-hydroxykynurenine
IDO1
MACROPHAGES
KAT II
kynurenic acid
KMO
3-HAO
MACROPHAGES
quinolinic acid
TPH1
AADC
SNAT
HOMT
PINEALOCYTES
TPH2
AADC
serotonin
melatonin
SEROTONERGIC
NEURONS
α7nAChR
NMDAR

Figure 1. The transformations of tryptophan in the body. Tryptophan 2,3-dioxygenase 2 (TDO2) and indoleamine 2,3-dioxygenase 1 (IDO1) cause cleavage of the tryptophan indole ring (exogenous amino acid) with the simultaneous attachment of oxygen at positions 2 and 3 leading to the formation of kynurenine. TDO2 shows the highest expression in the liver, and IDO1 is expressed in most tissues, including CNS cells. In the next stage, kynurenine is metabolized to 3-hydroxykynurenine by kynurenine 3-monooxygenase (KMO). 3-hydroxykynurenine is converted to highly toxic quinoline acid. Tryptophan, kynurenine and 3-hydroxykynurenine can cross the blood-brain barrier. In the brain, tryptophan undergoes changes leading to the formation of melatonin (by the tryptophan hydroxylase 1 activity - TPH1; aromatic L-amino acid decarboxylase activity - AADC; serotonin N-acetyltransferase activity - SNAT; 5-hydroxyindole-O-methyltransferase activity - HOMT), serotonin (by the hydroxylase tryptophan 2 activity - TPH2; aromatic L-amino acid decarboxylase activity - AADC), kynurenine acid (by the kynurenine transaminase II activity - KATII) and quinoline acid (by the KMO activity, anthranilic acid 3,4-dioxygenase activity - 3-HAO). Kynurenic acid exhibits N-methyl-D-aspartic receptor (NMDAR) antagonist and α7 nicotinic cholinergic receptor (α7nAChR) antagonist activity, while quinolinic acid is activating the α7nAChR receptor.

The hypothesis of the impact of serotonin level disorders on depression development was presented as early as in the 1960s. The next studies have developed this hypothesis also pointing to the involvement of functional and number disorders of serotonin receptors in the brain. This theory has become the basis for developing a therapy that involves the use of SSRIs in the treatment of depression (Albert et al., 2012). Serotonin insufficiencies may be associated with low tryptophan level, which is observed in serum of depressed patients. The reduced level of tryptophan may be a consequence of the increased activity of tryptophan 2,3-dioxygenase 2 (TDO2) and indoleamine 2,3-dioxygenase 1 (IDO1), whichare rate-limiting enzymes of tryptophan metabolism (Maes et al., 2011b; c). As a result of TDO and IDO activity, tryptophan is converted into kynurenine, which is metabolised into toxic quinolinic acid in the next step. Thus, patients with depression are also characterised by an elevated kynurenine/tryptophan ratio, indicating the overproduction of toxic TRYCATs (Maes et al., 2011b, c). The overproduction of another toxic tryptophan metabolite – 3-hydroxykynurenine – may induce ROS production, leading to activation of neurons apoptosis (Stone, 2001). In addition to quinolinic acid and 3-hydroxykynurenine, 3-hydroxyanthranilic acid has oxidative activity, generating ROS, including hydrogen peroxide, and causing lipid peroxidation (Dykens et al., 1987; Rios and Santamaria, 1991; Okuda et al., 1998; Guidetti and Schwarcz, 1999; Goldstein et al., 2000; Murakami et al., 2006; Santamaria et al., 2001; Smith et al., 2009). On the other hand, other studies suggest that 3-hydroxykynurenine, 3-hydroxyanthranilic acid and xanthurenic acid may also have antioxidative properties. These metabolites sweep free radicals, reduce lipid peroxidation, prevent spontaneous oxidation of glutathione and damage to 2-deoxyribose (Christen et al., 1990; Goda et al., 1999; Leipnitz et al., 2007). The next important enzymes involved in tryptophan metabolism are tryptophan hydroxylases 1 and 2 (TPH1/2), which limit the synthesis of serotonin. Animal study confirmed that the stressed animals were characterised by a decreased mRNA expression of *TPH1/TPH2* (Chen et al., 2017). Moreover, as a result of TRYCATs pathway transformations, neuroprotective compounds may be generated. Kynurenine aminotransferases I/II (KAT I/II) convert kynurenine into kynurenic acid, which have antioxidant and neuroprotective properties. Therefore, the depression development is associated with reduced activity of KAT I, II and also kynurenic acid (Stone 2001, Maes et al., 2011d). The abnormal course of TRYCATs pathway also impacts on disorders of melatonin synthesis. Melatonin is involved in the regulation of circadian rhythms. 80% of patients with diagnosed depression suffer from sleep disorders. Additionally, worsening of insomnia may contribute to the risk of depression recurrence and severity

of the depressive episode. Moreover, insomnia primarily affects depressed patients aftersuicide attempts. An untreated insomnia may also cause recurrent of full-blown depression (Kodykova et al., 2009).

As mentioned above, the process of depression development has multifactorial character. Moreover, previous studies show that intensification of the oxidative process and abnormalities of TRYCATs pathway may occur in the brain, blood and serum in the course of depression. Thus, the severity degree of these process may be tissue-specific. Moreover, it is suggested that the mechanism of depression development may involve different changes in different parts of the brain. Therefore, some parts of the brain may be more or less exposed to the action of neurotoxic compounds, including quinolinic acid and 3-hydroxyquinurenine, and reactive oxygen species (Di Chiara et al., 1999; Mayberg et al., 2000; Arango et al., 2001; Scheggi et al., 2002; et al., 2005; Pariante et al., 2008; Matthews et al., 2012). All that makes the correct diagnosis and effective therapy are difficult. Therefore, the detailed knowledge of the molecular mechanism of depression development may allow the development of effective diagnosis biomarkers. The regulation of the specific processes, involved in the mechanism of depression development, may allow the development of a new, effective and personalised antidepressant therapy. Therefore, in this paper, the effect of the occurrence of single nucleotide polymorphisms (SNPs) of five genes encoding enzymes involved in oxidative and nitrative stress and five genes involved in the tryptophan catabolites pathway on the risk of developing depression was examined. Moreover, the impact of the chronic mild stress procedure on expression at the mRNA and protein level as well as the status of methylation of the promoter regions of the genes involved in the abovementioned pathways were assessed. The characteristics of enzymes encoded by the genes studied in this work are presented in Table 1.

Table 1. Characteristics of the studied enzymes based on *The Human Protein Atlas oraz GeneCards*® and *The Human Gene Database.*

Oxidative stress					
Name of the enzyme (abbreviation)	**Function**	**Gene location (chromosome region)**	**Tissue expression**	**The association with the depression**	**Diseases associated with abnormal function of the studied genes**
catalase (CAT)	Heme enzyme with catalase and peroxidase activity, present in the peroxisomes of every breathing body cell, at high concentration of hydrogen peroxide, it breaks down into water and oxygen (catalase activity), at low concentration, hydrogen donor substrates are, among others: ethanol, methanol, phenol (peroxidase activity)	11p13	In all tissues	The elevated levels of CAT were observed in patients with depression	Variegated dandruff, akatalasia
Glutathione peroxidase 1 (Gpx1)	The enzyme (selenoprotein), catalysing the reduction of organic hydroperoxides and hydrogen peroxide (H_2O_2) by glutathione, and thus protects cells from oxidative damage, protects haemoglobin in erythrocytes against oxidative degradation	3p21.31	In all tissues	The reduced Gpx1 activity in people with depression	Glutathione peroxidase deficiency, Keshan's disease, amyotrophic lateral sclerosis (ALS), cancer
Glutathione peroxidase 4 (Gpx4)	The selenium-containing enzyme catalyses the reduction of phospholipid hydroperoxide, even if they are embedded in membranes and lipoproteins, as well as fatty acid hydroperoxide, cholesterol hydroperoxide and thymine hydroperoxide, and thus protects cells from membrane lipid peroxidation and their death	19p13.3	In all tissues	The reduced Gpx4 activity in people with depression	Spinal dysplasia in the form of sedaghatian (SMDS), male infertility
Superoxide dismutase 1 (SOD1)	The cytoplasmic enzyme containing copper and zinc converts superoxide radicals into oxygen and hydrogen peroxide	21q22.11	In all tissues	Ambiguous observations	Amyotrophic lateral sclerosis, spastic tetraplegia and axial hypotension, neurodegeneration, neoplastic diseases
Superoxide dismutase 2 (SOD2)	Mitochondrial containing manganese enzyme binds to peroxide by-products of oxidative	6q25.3	In all tissues, with the highest expression observed in fibroblasts	Ambiguous observations	Idiomatic cardiomyopathy (IDC), premature aging,

	phosphorylation and converts them into hydrogen peroxide and oxygen				sporadic motor neuron disease and cancer
Nitrosative stress					
Name of the enzyme (abbreviation)	**Function**	**Gene location (chromosome region)**	**Tissue expression**	**The association with the depression**	**Diseases associated with abnormal function of the studied genes**
Nitric oxide synthetase 1 (NOS1)	The enzyme synthesizes nitric oxide from L-arginine. NO is a reactive free radical that acts as a biological mediator in various processes, including neurotransmission, antimicrobial and anti-cancer mechanisms. In the brain and peripheral nervous system, nitric oxide exhibits act as a neurotransmitter	12q24.22	Brain, skeletal muscles, skin, male urinary and reproductive system, and in smaller amounts also in the lungs and female reproductive system	Depressed patients are characterized by an increased NO levels, thus indicating an increase in NOS activity	Achalasia, familial esophageal and pyloric stenosis, amyotrophic lateral sclerosis (ALS)
Nitric oxide synthetase 2 (NOS2)	The enzyme synthesizes nitric oxide, which is a strong mediator in numerous processes	17q11.2	Lymphatic tissue, intestines, lungs	Depressed patients are characterized by an increased NO levels, thus indicating an increase in NOS activity	Meningioma caused by radiation
Tryptophan catabolites pathway					
Name of the enzyme (abbreviation)	**Function**	**Gene location (chromosome region)**	**Tissue expression**	**The association with the depression**	**Diseases associated with abnormal function of the studied genes**
Indoleamine-2,3-dioxygenase (IDO1)	The heme enzyme, which catalyses the first and rate-limiting step of tryptophan catabolism in the kynurenine pathway, converts tryptophan to N-formyl-kynurenine, this enzyme probably also participates in various pathophysiological processes such as antimicrobial and antitumor defence	8p11.21	Blood, placenta, in smaller amounts also in the male and female reproductive system and the brain	The increased IDO1 activity in depressed patients	Microbial infections, cancer
Tryptophan-2,3-dioxygenase (TDO2)	The heme enzyme, which catalyses the first and rate-limiting stage of tryptophan catabolism in the kynurenine pathway, converts tryptophan to N-formyl-kynurenine	4q32.1	Liver, in smaller amounts, also in lymphatic tissue and the brain	The increased TDO2 activity in depressed patients	Autism, cancer

Tryptophan hydroxylase 1 (TPH1)	The enzyme catalysing the first and limiting step of the serotonin biosynthesis	11p15.1	Brain, intestine, pituitary gland, stomach	The decreased *TPH1* mRNA expression	Schizophrenia, somatic anxiety, bipolar disorder, suicidal behavior, addiction
Tryptophan hydroxylase 2 (TPH2)	The enzyme catalysing the first and limiting step of the serotonin biosynthesis	12q21.1	Brain, in smaller amounts, also in the pancreas and bladder	The decreased *TPH2* mRNA expression	Bipolar disorder, depression
Kynurenine aminotransferase I (KAT1)	The enzyme catalysing the irreversible transamination of the L-tryptophan metabolite, L-kynurenine to kynurenic acid, which has neuroprotective properties	9q34.11	In all tissues	Depressed patients have reduced levels of kynurenine acid and reduced KATI activity	Schizophrenia
Kynurenine aminotransferase II (KATII)	The transaminase with broad substrate specificity exhibits aminotransferase activity against aminoadipate, kynurenine, methionine and glutamate, tryptophan, aspartate and hydroxykynurenine. It catalyses kynurenine transamination, leading to the production of kynurenine acid, which has neuroprotective properties	4q33	In all tissues, with the highest amount in the liver	Depressed patients have reduced levels of kynurenine acid and reduced KATII activity	Huntington's disease, bladder sphincter dyssynergy
Kynurenine 3-monooxyhenase (KMO)	The enzyme catalyses the hydroxylation of L-kynurenine, resulting in the formation of 3-hydroxy-L-kynurenine, which is required for the synthesis of quinolinic acid, a neurotoxic NMDA receptor antagonist	1q43	Blood, liver, placenta, kidneys, lungs	Depressed patients are characterized by elevated levels of quinolone acid	Huntington's disease, tooth anomalies, transient cerebral ischemia
Kynureninase (KYNU)	he enzyme catalyses the cleavage of L-kynurenine and L-3-hydroxykynurenine to anthranilic acid and 3-hydroxyanthranilic acid, respectively. Also exhibits cysteine-conjugated beta-lyase activity	2q22.2	In most tissues, the highest expression in the blood, liver, placenta	Depressed patients have elevated level of anthranilic acid	Syndrome of the spine, heart, kidneys and limbs

Aim of the study

Depression is a complex mental disorder with a multifactorial mechanism of development. In spite of that depression is the root cause of social disability, its aetiology remains unclear. Consequently, the lack of effective diagnostic methods, based on measurable biomarkers, contributes to the misdiagnosis of the disease as well as the selection of ineffective antidepressant therapy. Previous studies show that genetic factors are also involved in depression development. Therefore, the presented study aimed to explore the role of oxidative and nitrative stress and the disorders of the tryptophan catabolite pathway in the molecular basis of depression.

This aim was accomplished by:

- determination of association between the genotypes and alleles of 16 single nucleotide polymorphisms and the risk of depression. The studied SNPs are localised in genes which are involved in oxidative (*SOD2, Gpx4, CAT*) and nitrosative stress (*NOS1*, *NOS2*) as well as tryptophan catabolites pathway (*TPH1*, *TPH2*, *IDO1*, *KATI*, *KATII*).
- determination of chronic mild stress and venlafaxine therapy impact on the level of expression and methylation genes involved in oxidative (*SOD2*, *Gpx4*, *CAT*) and nitrosative stress (*NOS1*, *NOS2*) as well as tryptophan catabolites pathway (*Tph1*, *Tph2*, *Ido1*, *KatI*, *KatII*).

Materials and methods

Characteristic of patients

The study material included 510 peripheral blood samples collected from 229 controls and 281 patients with diagnosed depression, who are admitted to hospitals at the Department of Adult Psychiatry of the Medical University of Lodz. These samples were obtained thanks to cooperation with the team of Professor Piotr Gałecki. Patients and controls were selected for age and gender. The International Statistical Classification of Diseases and Related Health Problems ICD-10 (F32.0-7.32.32, F33.0-33.8) were used to qualification for the study. Exclusion criteria comprised the autoimmune diseases, inflammation, cancer, damage of the central nervous system, chronic or acute somatic disease, and the presence of other axes I and II disorders, other than DD. The evaluation and classify depression severity were based on those outlined in the 21-item Hamilton Depression Rating Scale (HDRS-21) and were done by the same psychiatrist doctor before and after therapy with SSRIs. All study participants were fully informed regarding the course, the purpose of the study and the method of processing personal data, and submitted written consent to participate in the study. The research was approved by the Bioethics Committee of the Medical University of Lodz No. RNN/70/14/EC.

Determination of single nucleotide polymorphisms genotypes localised in genes encoding enzymes involved in oxidative and nitrative stress and the tryptophan catabolites pathway

The distribution of genotypes and alleles of the studied SNPs was performed on genomic DNA. Genomic DNA was isolated from peripheral blood according to the protocol of commercially available Blood Mini Kit (A&A Biotechnology, Gdynia, Poland). A total of 16 polymorphisms have been analysed (Table 2.). The studied SNPs were genotyped using the TaqMan ™ probes (TaqMan SNP Genotyping Assay; Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) and the commercially available TaqMan Universal Master Mix II, no UNG II (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Real-time polymerase chain reaction (real-time PCR) were carried out in the Bio-Rad CFX96 Real-Time PCR Detection System and analysed in the CFX Manager Software version 3.1. (BIO-RAD Laboratories Inc., Hercules, CA, USA).

Table 2. Characteristic of analysed SNPs.

Oxidative and nitrosative stress				
Gene	**SNP ID (rs number)**	**Polymorphism**	**Location (region)**	**Change of the amino acid sequence**
SOD2	rs4880	c.47T>C	Exon	Val16Ala
CAT	rs7943316	c.-89A>T	5'UTR	-
GPx4	rs713041	c.660T>C	3'UTR	-
NOS1	rs1879417	c.-420-34221G>A	Intron	-
NOS2	rs2297518	c.1823C>T	Exon	Ser608Leu
	rs10459953	c.-227G>C	5'UTR	-
Tryptophan catabolites pathway				
Gene	**No. of studied polymorphism**	**Polymorphism**	**Localisation**	**Change of the amino acid sequence**
TPH1	rs1799913	c.804-7C>A	5'UTR	-
	rs623580	c.-1668T>A	Intron	-
	rs1800532	c.803+221C>A	Intron	-
	rs10488682	c.-173A>T	Intron	-
TPH2	rs7963803	c.-1449C>A	5'UTR	-
	rs4570625	c.-844G>T	5'UTR	-
KATI	rs10988134	c.*456G > A	3'UTR	-
ADDAT	rs1480544	c.975-7T > C	Intron	-
IDO1	rs3824259	c.-1849C > A	Intron	-
	rs10089084	c.-1493G > C	Intron	-

Characteristics of animals

Studied male Wistar Han rats (Charles River, Germany) were randomly divided into five groups controls, stressed, controls after venlafaxine therapy, controls after venlafaxine therapy and stress after saline therapy. Each studied group included six animals. The course of chronic mild stress (CMS) procedure and antidepressant therapy with venlafaxine (SNRIs group of antidepressants) or therapy with saline were shown in Figure 2. Animals were subjected to the biweekly adaptation to laboratory conditions prior to commencing exposure to CMS stimuli .The stressed procedure included two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (light on and off every 2h), two periods of soiled cage (250 mL water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress for a week. Stress stimuli were used individually and continuously during the day and at night. After two weeks, the stressed animals showed a number of behavioural, physiological and biochemical changes showing similarity to depression symptoms. The main consequence of exposure to stress stimuli is a decrease in the sensitivity of stressed rats to the effect of rewarding stimuli, which is recognized as a manifestation of the main symptom of depression, i.e. anhedonia. The measure of this effect is the reduction in the consumption 1% sucrose solution. Non-stressed rats had no contact with the stressed animals in separated rooms. After two weeks of exposure to stress stimuli, animals treated with antidepressant therapy, which consisted of the injection of venlafaxine (daily 10 mL / kg, IP) or saline (daily 10 mg / kg, IP) administered for five weeks (Papp, 2012). Finally, after the stressed procedure, the studied animals were decapitated and samples of blood and brain structures were collected. The samples of brain structures included hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia. Finally, after the end of the stressed procedure, the studied animals were decapitated and samples of blood and brain structures were collected. The samples of brain structures included hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia. The CMS procedure was carried out at the Institute of Pharmacology of the Polish Academy of Sciences in Cracow thanks to cooperation with Professor Papp. The samples of Peripheral blood were used to PBMC isolation using Gradisol L (Aqua-Med, Lodz, Poland) and the differential centrifugation. All tests in the study were approved by the Bioethical Committee at the Institute of Pharmacology of the Polish Academy of Sciences in Krakow (Poland) (No. nr 1272/2015) and were conducted in compliance with the rules and principles of the 86/609/EEC Directive.

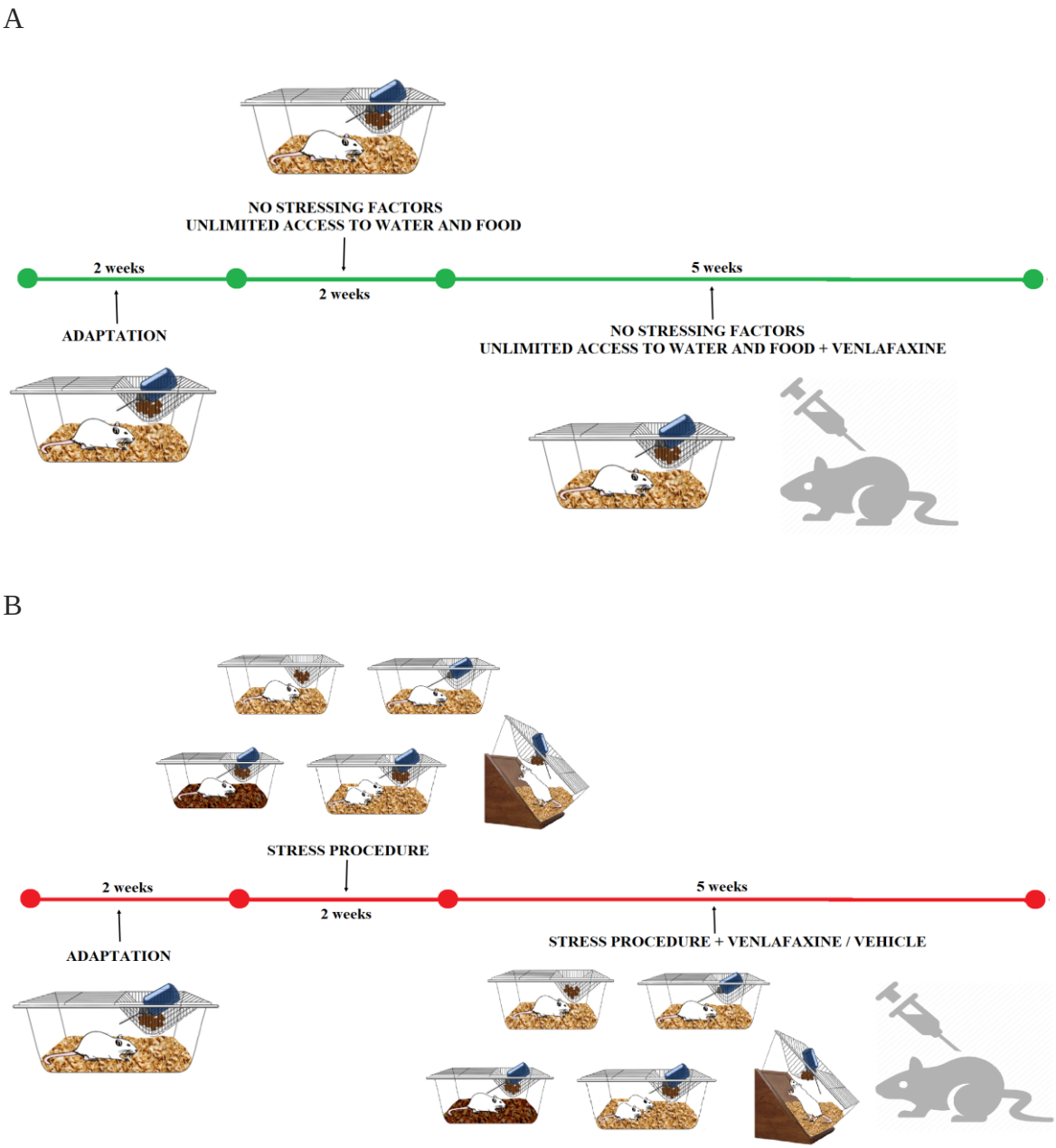

Figure 2. The scheme of the experiment. (A) The course of the experiment in control groups. Animals of each control group after purchase were subjected to a two-week period of adaptation to laboratory conditions. During this time, there was also apprenticeship to the weekly test of ingestion of a 1% sucrose solution as a measurement of the response to a reward. Control group animals were subjected to a two-week adaptation period, followed by a two-week period during which they had unlimited access to water and feed, and were not exposed to stress factors. Control-treated animals receiving venlafaxine four weeks after arrival at the laboratory for a period of five weeks. (B) Chronic mild stress procedure. Animals of each stressed group after purchase were subjected to a two-week adaptation period to the laboratory conditions. During this time, there was also apprenticeship to the weekly test of ingestion of a 1% sucrose solution as a measurement

of the response to a reward. Stressed group animals were subjected to a two-week adaptation period, followed by a two-week period during which they were exposed to various stress factors, including tilting the cage by 45°, litter contamination with water, lack of access to water or food, or exposure to a strobe light. After this time, the treated animals received venlafaxine or saline injections for a period of five weeks (The based on Sequeira-Cordero et al., 2019; modified)

Determination of mRNA expression of gene encoding enzymes involved in oxidative and nitrative stress and the tryptophan catabolites pathway

The analyses of mRNA expression included *SOD1, SOD2, GPx1, GPx4, CAT, NOS1, NOS2, Tph1, Tph2, Ido1, KatI, KatII, Kynu, Kmo* genes. mRNA expression levels were performed using total RNA, which was isolated from PBMCs and brain structures using the commercial spin column methods (GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich, ISOLATE II RNA/DNA/Protein Kit, Bioline, respectively). The RNA samples and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to the reaction of reversion transcription in a C1000^{TM} programmed Thermal Cycler (Bio Rad Laboratories Inc., Hercules, CA, USA). The expression levels were detected and identified by real-time PCR using a commercially available TaqMan Universal Master Mix, no UNG II (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) and species-specific TaqMan Gene Expression Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Real-time PCR were performed using a CFX96TM Real-Time PCR Detection System Thermal Cycler (BIO-RAD Laboratories Inc., Hercules, CA, USA) and analysed in the CFX Manager Software version 3.1. (BIO-RAD Laboratories Inc., Hercules, CA, USA). The expression levels were determined according to the $2^{-\Delta ct}$ methods, where $\Delta Ct = C_{t\ studied\ gene} - C_{t\ reference\ gene}$. The *18S* gene, belonging to the group of basic metabolism genes, was used as the reference gene (Schmittgen and Livak, 2008).

Determination of the methylation status of promoter regions of genes encoding enzymes involved in oxidative and nitrosative stress and the tryptophan metabolite pathway

One reason for changes in mRNA expression may be cytosine methylation within CpG islands. Low methylation occurs in regions of the genome encoding transcription active genes (Piletič and Kunej, 2016). The analysis of the methylation status of the promoter region was done by using a total DNA. Total DNA was extracted from PBMCs and brain structures by using commercial spin column methods (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany; ISOLATE II RNA/DNA/Protein Kit, Bioline, respectively). Then, the obtained DNA was subjected to a to sodium bisulfite modification using a CiTi Converter DNA Methylation Kit (A&A Biotechnology, Gdynia, Poland). The bisulfidation causes the deamination of unmethylated cytosine to uracil, while methylated cytosine is not subject to these transformations. Then, the DNA is amplified in a polymerization chain reaction in which uracil is converted into thymine (Hayatsu, 2008). The methylation status of the promoter regions of the studied genes was detected and identified using a methylation sensitive – high resolution melting method, primers which were designed according to the recommendations of Wojdacz in MethPrimer (http://www.urogene.org/methprimer2/) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 5 x HOT FIREPol® EvaGreen® HRM Mix (no ROX) (Solis BioDyne Tartu, Estonia) (Wojdacz et al., 2007; 2008; 2009). The primers that were designed for the promoter regions investigated containing CpG islands are shown in Table 3. The status of methylation was assessed using DNA standards with different content of methylated DNA (100%, 75%, 50%, 25% and 10%), which were obtained by mixing commercially available standards of fully methylated and unmethylated DNA (CpGenome^{TM} Rat Methylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA and CpGenome^{TM} Rat Unmethylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA) All reactions were carried out in a CFX96TM Real-TIME PCR Detection System thermocycler (BIO-RAD Laboratories Inc., Hercules, CA, USA) and were analysed using Precision Melt Analysis TM Software version 3.1 (BIO-RAD Laboratories Inc., Hercules, CA, USA). The assessment of the methylation level was made by comparing the profile of the studied sample curves compared with the profile obtained for the standards.

Table 3. Characteristic of primer used to determination of the methylation status of the studied genes.

Oxidative and nitrosative stress			
Gene	**Primers sequence**	**Product size [bp]**	**Tm [ºC]**
CAT	F:TTTGAGATTATTGTGTTTGAAA R:TACCTACACCCAAAAAAAAATA	148	59
GPx1	F:GTTGTTTTAGGTTTTGTTGTTG R:AAAACTAAAATCCTCCAACTCT	102	65
GPx4 (promotor 2)	F:AGGTTGGAGGTTTAGAGGTTTA R:TCCCCTAAATACAAAAATCTCT	118	59
GPx4 (promotor 3)	F:AGGTTGGAGGTTTAGAGGTTTA R:AAAACATAACAAAATCATCTCCC	147	65
SOD1	F: AAGGAGGTGTGTTTAATTGGTA R: AACCCCTCTCACAAATTTCTAA	144	65
SOD2	F: GGGGAAGGTTATTTAGGGTATA R: CCTTTTCCATTCCTAATTCTAAA	133	59
NOS1 (promotor 3)	F: GGGTTTTTAATTTTTTTATTGTG R: CAACCCTCATTAAAAAAACC	124	59
NOS1 (promotor 7)	F: GTTTGAGATTGGAATTTTTTGG R: CCAAAACATCCAAAAATACACA	124	59
Tryptophan catabolites pathway			
Gene	**Primers sequence**	**Product size [bp]**	**Tm [ºC]**
Tph1 (promotor 2)	F:GGGAGTTTTGTTTTGGTTTTTA R:TCCTCAACCACAAAAAATCTAA	132	55
Ido1 (promotor 2)	F:TTTGAGTTTTAGTGATTTTGGG R:TTAATATCTAATCCCAATCTCTAAAAC	100	59
Tdo2 (promotor 1)	F:GATGATTTAGGTGGTTTGAGGT R:CAAAAAAAACAAAATTCATCCA	123	59
Tdo2 (promotor 2)	F:ATGATTTAGGTGGTTTGAGGTT R:ACCCAATCTACCTAACTAACAAC	187	61.4
Kmo (promotor 7)	F: TTGGTTTAGGGAAGGAAAT R: ATAAAAAACTAAACCCAAAACAC	150	55.7

Determination of the amount of proteins involved in oxidative and nitrative stress as well as tryptophan catabolites pathway

Proteins expression levels were determined using Western Blotting analyses. The protein samples were isolated using sonication in RIPA buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF, serine protease inhibitor). Total protein content was estimated by the modified Lowry procedure (Lowry et al., 1951), using bovine serum albumin as calibration standard BSA, Sigma-Aldrich, Saint Louis, Missouri, US) (Lowry et al., 1951). Protein samples were run under reducing conditions on 10% SDS

polyacrylamide gels and then electroblotted overnight onto Immobilon-P (Millipore, Bedford, Massachusetts, USA) in 4°C temperature. After transfer, the blots were blocked with 5% non-fat dry milk for 1 hour at room temperature and then incubated with the primary antibodies. Then the membranes were washed with 0,1% TBST buffer (1X Tris-Buffered Saline, 0.1% Tween 20 Detergent) and incubated with the opportune secondary antibodies conjugated with horseradish peroxidase. The condition of primary and secondary antibody incubation was presented in Table 4. After flushing out excess antibody the blots were again washed and incubated peroxidase substrate solution SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Finally, the proteins were visualised on X-ray film. The integrated optical density (IOD) of the immunoreactivity bands was measured using Gel-Pro® Analyzer Software (Media Cybernetics Inc., USA). Results were normalised using the reference protein (β-actin) according to $IOD_{studied\ gene}$ / $IOD_{\beta\text{-actin}}$ formula.

Table 4. The condition of primary and secondary antibody incubation in Western Blotting analyses.

	Primary antibody	**Secondary antibody**
β-actin (reference protein)	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), incubation 1 h at room temperature	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
catalase	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), overnight incubation at 4°C	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
Superoxide dismutase 2	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), incubation 2 h at room temperature	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
Glutathione peroxidase 4	rabbit, dilution 1:6000, (Abcam), overnight incubation at 4°C	anti-rabbit, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
Tryptophan hydroxylase 1	rabbit, dilution 1:1000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), overnight incubation at 4°C	anti-rabbit, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation1 h at room temperature

Tryptophan hydroxylase 2	rabbit, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), overnight incubation at 4°C	Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation1 h at room temperature
Indoleamine 2,3-dioxygenase	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), overnight incubation at 4°C	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
Kynurenine aminotransferaze	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), overnight incubation at 4°C	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature
Kynureninase	mice, dilution 1:1000, (Santa Cruz Biotechnolgy Inc.), overnight incubation at 4°C	anti-mice, dilution 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), incubation 1 h at room temperature

Statistical analysis

All statistical analyses of presented data were performed using Statistica 12 (Statsoft, Tulsa, OK, USA), SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). An unconditional multiple logistic regression model was used to calculate the association between case/control and each polymorphism and the odds ratios (ORs) with 95% confidence interval (95% CI) were determined. Moreover, the OR was adjusted for gender. The Shapiro-Wilk test was used to estimate the distribution of the normality of the animal study results. When the data were normally distributed the data of gene expression and methylation were analysed using one-way analysis of variance (one-way ANOVA) and followed by post hoc Tukey's test. Otherwise, when the data were not normality distributed, the results were analysed using a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by post hoc Student-Newman-Keuls test. p values < 0.05 were considered as statistically significant.

Results

Correlation between genotypes of gene polymorphisms involved in response to oxidative stress – SOD2, CAT, GPx4, NOS1 and NOS2 – with the occurrence of depression

The distribution of genotypes and alleles showed that T/T genotype of the c.47T>C (rs4880) – *SOD2* polymorphism was associated with an increased frequency of depression occurrence. In the case of c.-89A>T (rs7943316) – CAT polymorphism, A/A genotype was positively correlated with depression occurrence, while A/T genotype was more frequent in the control group. Moreover, T/T genotype and T allele c.660T>C (rs713041) – *GPx4* polymorphism were associated with an increased frequency of depression occurrence, while T/C heterozygote and C allele showed opposite relation. No correlation was found between genotypes/alleles of the 420-34221G>A (rs1879417) – *NOS1*, c.1823C>T (rs2297518), and c.-227G> C (rs10459953) – *NOS2* polymorphisms, and depression occurrence.

The previous study showed that women are twice as likely to develop depression as men (Kessler, 2003). Thus, the distribution of studied polymorphism genotypes/alleles may be depended on gender. In the case of the c.47T>C (p.Val16Ala) (rs4880) – *SOD2* polymorphism, T/T genotype was more frequently in men with depression, while no correlation between this SNP and risk of the depression development in women wasdetected. Moreover, A/A genotype of the c. 89A>T – *CAT* (rs7943316) polymorphism in the male population and A/T and T/T genotypes in the female population were associated with reduced risk of depression occurrence. In the case of the c.660T>C (rs713041) – *GPx4*, T/T genotype was positively correlated with the incidence of depression in men and women however T/C genotype of the same SNP was associated with an increased frequency of depression in only men group. In the female population, G/C genotype of the c.227G>C (rs10459953) – *NOS2* polymorphism was associated with the decreased frequency of depression occurrence, while G/G genotype of the same polymorphism showed opposite correlation. However, no correlation was found between studied polymorphism and the frequency of depression in the male population. Moreover, no correlation was also found between studied polymorphism and effectiveness of SSRIs therapy.

Correlation between genotypes of gene polymorphisms involved in the tryptophan catabolites pathways – TPH1, TPH2, IDO1, KATI, AADAT (KATII) – and depression

Among the studied polymorphisms of *TPH1* gene, C/C genotype and C allele of the c. 804–7C>A (rs1799913), A/A homozygote c. 803+221C>A (rs1800532) and T/G, T/T genotypes and T allele of c.-173A>T (rs10488682) polymorphisms were associated with the increased risk of depression occurrence. On the other hand, T/T genotype of the c.-1668T>A (rs623580) – *TPH1* polymorphism was negatively correlated with the frequency of the depression development while A/A genotype and A allele of the same SNP were associated with the increased risk of the disease. Moreover, G/G genotype and G allele of the c.-844G>T (rs4570625) – *TPH2* polymorphism were positively correlated with the depression occurrence, whereas G/T heterozygote and T allele of the same SNP showed the opposite correlation. The C/C genotype of the c.-1449C>A (rs7963803) – *TPH2* polymorphism decreased the risk of depression while C/A heterozygote and A allele of the same SNP increased the risk. In the case of the c.*456 G>A (rs10988134) – *KATI* polymorphism, A/A genotype increased a frequency of depression occurrence. No correlation was found between other studied polymorphisms, including c.975-7T>C (rs1480544) – *AADAT*, c.-1849C>A (rs3824259) and c.-1493G >C (rs10089084) – *IDO1*, and the risk of depression development.

Interestingly, the analysis of the age distribution of the first depressive episodes showed that the G/T heterozygote of the c.-844G>T (rs4570625) – *TPH2* polymorphism experienced the first episode of depression at a younger age than G/G homozygotes. Analysis of the studied genotypes polymorphisms distribution in the male and female population showed that the A/A genotype of the c.*456G>A (rs10988134) – *KAT1* polymorphism was positively correlated with the development of depression, while the G/G genotype was associated with a reduction in the incidence of depression only in the male population. In contrast, no correlation between this polymorphism and the incidence of depression was observed in the female population. Interestingly, the T/T genotype of the c.975-7T>C (rs1480544) – *ADDAT* (*KATII*) polymorphism reduced the effectiveness of conventional SSRI therapy.

Determination of the level of consumption of sucrose solution as a measure of the ability to respond to rewarding stimuli

After the first two weeks of exposure to stress stimuli, the consumption of 1% sucrose solution decreased by 40%. However, five-week venlafaxine therapy normalised the consumption of 1% sucrose solution by stressed animals.

The effect of chronic mild stress and venlafaxine therapy on expression changes at mRNA and protein levels and the degree of methylation of the promoter regions of the SOD1, SOD2, GPx1, GPx4, CAT, NOS1 and NOS2 genes

The therapy of increased the mRNA expression *SOD1*, *SOD2* and *NOS2* in PBMCs. In the brain, mRNA expression changes of the studied genes depended on the structure studied. CMS procedure decreased the level of *CAT* expression in the hippocampus, while venlafaxine therapy caused the further reduction of the *CAT*, *GPx1*, *SOD1* and *SOD2* expression in the hippocampus of stressed rats. Moreover, *GPx1*, *GPx4*, *NOS1* mRNA expression increased in the midbrain and *GPx1*, *NOS1* in the hippocampus of stressed rat and restored by venlafaxine therapy. Similarly, *NOS1* expression was increased in basal ganglia of stressed animals and restored by treatment with venlafaxine. Moreover, CMS procedure caused an increase of methylation status of the second promoter of *GPx1* gene in PBMCs. Stressed rats were characterised by the increased methylation status of the second promoter region of GPx4 in the midbrain and basal ganglia as well as *SOD1* and *SOD2* promoter regions in the hippocampus. On the other hand, CMS procedure caused decreased of GPx4 protein expression in the cerebral cortex. The treatment with venlafaxine caused a further decreased of the protein expression in the cerebral cortex. However, the CMS procedure increased Gpx4protein expression, and venlafaxine therapy led to a further increase of this protein expression in the hippocampus. An increased Cat protein expression was observed in the midbrain and cerebral cortex of stressed rats.

The effect of chronic mild stress and venlafaxine therapy on expression changes at mRNA and protein levels and the degree of methylation of the promoter regions of the Tph1, Tph2, KatI, KatII, Ido1, Kmo, Kynu genes

The mRNA expression of the studied genes depends on the type of tissue examined (PBMCs isolated from peripheral blood and brain tissue) and also on the type of brain structure. The CMS procedure caused an increase of *KatI* mRNA expression in the midbrain, while the treatment with venlafaxine reduced this expression in the hypothalamus and cerebral cortex of stressed animals. The increased level of the *KatII* mRNA expression was observed in the amygdala and midbrain of stressed rats. The *Tph2* mRNA expression was increased midbrain of stressed animals and restored by treatment with venlafaxine. Interestingly, no correlation between the mRNA expression of studied genes and CMS procedure and venlafaxine therapy was found in PBMCs. On the other hand, the methylation status of *Tph1* and *Kmo* promoter regions increased in stressed animals after chronic venlafaxine administration. Moreover, stressed animals were characterised by the increased methylation status of *Ido1* promoter region in the midbrain. The analysis of protein expression showed that stressed animals after venlafaxine therapy were characterised the reduction of Tph1 and Ido1 in the basal ganglia as compared to stressed animals after saline therapy.

Resume

Depression is serious mental disorders, which are affecting more and more population in recent years. Moreover, diagnostic methods, including subjective assessment of doctors based on medical history, are often incorrect. The inadequate diagnosis prevents the selection of personalized and effective therapy. Nevertheless, an aetiology of the depression development still unclear. However, previous studies emphasise the multifactorial character of depression, which the development may depend environmental and genetic factors. It is presumed that the molecular mechanism of the depression development may be associated with the oxidative and antioxidative imbalance and abnormalities of tryptophan catabolites pathway. Therefore, as part of the doctoral dissertation, 16 single nucleotide polymorphisms were examined located in 5 genes encoding enzymes involved in oxidative and nitrative stress (6 SNPs) and in 5 genes encoding enzymes involved in the tryptophan catabolite pathway (10 SNPs).

The presented studies showed that polymorphisms of *CAT*, *GPx4*, *SOD2*, *NOS2*, *TPH1*, *TPH2*, *KAT1*, *KATII* genes may modulate the frequency of the depression occurrence. In order to confirm the effect of genes encoding enzymes involved in the studied processes, expression analysis at the level of mRNA and protein as well as the status of methylation of the promoter regions were performed as a result of the procedure of chronic mild stress and venlafaxine therapy. The obtained results indicate the role of oxidative and nitrative stress and the tryptophan catabolites pathway in the development of depression. However, full confirmation of this hypothesis requires further analysis extended by genes encoding other enzymes. It is also important for further research to consider intergenic interactions and the impact of environmental factors on the mechanism of depression development. Nevertheless, the studies of the presented doctoral dissertation may contribute to the development of the effective and early diagnosis of this disease. The studies, involving mononuclear blood cells as well as tissue of six brain structures, allows a better understanding of the mechanism of depression development. Moreover, the explanation of depression pathogenesis may allow for the use of personalized and effective therapies in the future.

Conclusion

Based on the research carried out as part of this work, the following conclusions can be made:

1. Oxidative and nitrative stress, as well as disorders in the course of tryptophan catabolites pathways, are involved in the molecular mechanism of depression development.
2. Polymorphic variants of genes involved in oxidative stress (*SOD2*, *GPx4*, *CAT*) and nitration (*NOS1*, *NOS2*) and the tryptophan catabolites pathway (*TPH1*, *TPH2*, *IDO1*, *KATI, KATII*) affect the risk of depression.
3. Depression and venlafaxine therapy can modulate the level of expression and methylation of the promoter regions of genes involved in oxidative stress (*SOD1*, *SOD2*, *GPx1*, *GPx4*, *CAT*) and nitration (*NOS1*, *NOS2*) and the tryptophan catabolites pathway (*Tph1*, *Tph2*, *Ido1*, *KatI*, *KatII*, *Kynu*, *Kmo*), and these changes depend on the type of tissue as well as the brain structures studied.

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The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression

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ABSTRACT

Depression is the most common mental disorder in the world. It is estimated that 350 million people suffer from depression worldwide. Depressive disorders will have become the second most frequent health problem globally by the year 2020, just behind ischemic heart disease. The causes of depressive disorders are not fully known. Previous studies showed that impaired tryptophan catabolites pathway, oxidative and nitrosative stress may play an important role in the pathogenesis of depression. Patients with depression have lower plasma levels of superoxide dismutase and glutathione peroxidise in comparison to controls. Moreover, depressed patients are characterized by decreased plasma levels of zinc, coenzyme Q10, albumin, uric acid, vitamin E and glutathione. Abnormal nitric oxidative production and nitric oxide synthase activity are also associated with depression. A dysfunction of the tryptophan catabolites pathway, indicated by increased levels of tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase, is also involved in the development of depression. Furthermore, increased levels of kynurenine and quinolinic acid might cause depression. Moreover, studies to date indicate that 8-oxyguanine, malondialdehyde, and 8-iso-prostaglandin F2α may serve as possible biomarkers. Additionally, regulation of defective mechanisms may provide a promising direction for the development of new and effective therapies.

1. Introduction

Mental disorders, including depressive disorders (DD), have a devastating impact on patients, their family and close relatives (Wittchen and Jacobi, 2005; Alonso et al., 2004), and are highly associated with the development of cardiovascular diseases, osteoporosis, diabetes and cerebral ischemia (Zardawi, 2013; Banaś et al., 2005; Jabłoński et al., 2008; Łabuz-Roszak et al., 2013). DD is a major global health issue, affecting one-tenth of the global population, and a leading cause of human disability (Demyttenaere et al., 2004). An early diagnosis of DD is very difficult. This is due to the lack of specific biomarkers. Moreover, treatment of depression is often ineffective; about one-third of patients do not respond to traditional antidepressant therapy (such as the application of selective serotonin reuptake inhibitor or tricyclic antidepressant) (Iwata et al., 2013; Thase and Schwartz, 2015). The causes of depressive disorders are not fully known (Marcus et al., 2012). There are several hypotheses to elucidate this. Disorders of related biochemical pathways, including oxidative and nitrosative stress as well as tryptophan catabolites (TRYCATs) pathway, may be considered an explanation for the development mechanism of DD (Fig. 1). The aim of this review is to underline the key role of oxidative and nitrosative stress, and TRYCATs pathway, in the development of depression and in the mechanism of action of antidepressant agents, based on the available literature.

Abbreviation: BDNF, brain-derived neurotrophic factor; CAT, catalase; CIRS, compensatory (anti)inflammatory reflex system; CoQ_{10}, coenzyme Q_{10}; DD, depressive disorders; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, glutathione; HDL-c, high-density lipoprotein cholesterol; IDO, indoleamine 2,3-dioxygenase; IRS, inflammatory responses system; LDL-c, low-density lipoprotein cholesterol; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthase; ω3PUFAs, omega-3 polyunsaturated fatty acids; PBMC, peripheral blood mononuclear cells; PICs, pro-inflammatory cytokines; PON1, paraoxonase 1; RBC, red blood cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SNPs, single-nucleotide polymorphisms; SSRIs, selective serotonin reuptake inhibitors; TDO, tryptophan 2,3-dioxygenase; TPH-2, tryptophan hydroxylase 2; TRYCATs, tryptophan catabolites pathway; Zn^{2+}, zinc; 8-oxo, 8-oxyguanine

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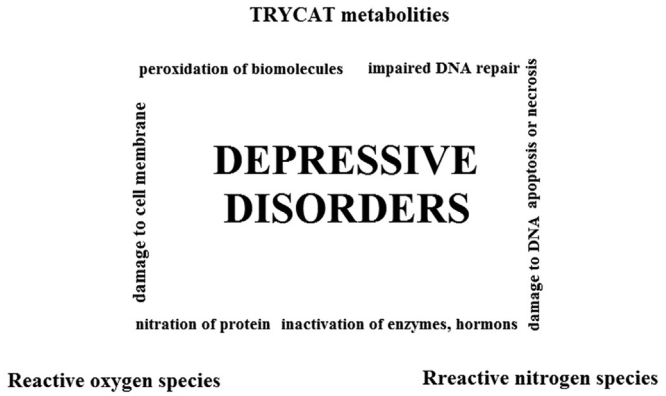

Fig. 1. Mechanisms of development of depressive disorders.

2. Oxidative stress in depression

2.1. Oxidative stress

Oxidative stress is a consequence of life under aerobic conditions (Siciliano et al., 2012) and is associated with the overproduction of free radicals, radical-derived and non-radical reactive species. In the available literary resources, oxidative stress is defined as an imbalance between the systemic production of reactive oxygen species (ROS) and the ability of biological systems to detoxify the reactive components or to repair the resulting damage.

2.2. The level of non-enzymatic antioxidants in depression

Depressive disorders are linked with increased production of ROS and excessively low amounts of antioxidants. Maes et al. (2010) proved that increased plasma levels of peroxides were observed significantly more often in depressed men than in women suffering from DD. Moreover, numerous studies showed that plasma levels of vitamin E are profoundly lowered in patients with depressive disorders than in controls (Gautam et al., 2012; Maes et al., 2011a). On the other hand, studies on vitamin C showed that DD might be associated with an increased level of this vitamin in serum (Kobrosly and van Wijngaarden, 2010). The differences may be due to the properties of vitamins – solubility in water or fat. Glutathione (GSH) is another antioxidant that has been examined in the context of DD. Animal studies confirmed a decreased concentration of glutathione in the chronic mild-stress model of depression in mice (Kumar et al., 2011). Other findings proved that women with depression had lower amounts of GSH in blood and were characterized by increased activity of glutathione peroxidise (GPx) than women without depression (Kodydková et al., 2009; Ozcan et al., 2004). However, other studies revealed that low activity of GPx might also contribute to the development of DD (Galecki et al., 2009a).

Serum concentrations of coenzyme Q_{10} (CoQ_{10}) are dramatically lower in depression patients as compared to control groups (Maes et al., 2009a, 2009b). Subsequent studies involving these animals showed that zinc deficiencies (Zn^{2+}) could lead to the occurrence of DD symptoms. Researchers observed a reduction in the number of progenitor cells and immature nerve cells in the hippocampus of rats which were treated with a diet low in Zn^{2+} (Suh et al., 2009).

Furthermore, a meta-analysis showed that patients with DD were characterized by decreased serum levels of another non-enzymatic antioxidant, i.e. uric acid (Liu et al., 2015). This study also confirmed that the serum level of albumin was significantly lower in patients with DD than in control subjects. Moreover, an antidepressant therapy led to an increase in the concentration of these antioxidants (Liu et al., 2015).

2.3. The role of antioxidants and oxidative enzymes in DD

Abnormal activity of ROS-generating enzymes is yet another element associated with the development of depression. Increased levels of catalase (CAT) may be a risk factor in the case of depressive episodes (Szuster-Ciesielska et al., 2008). Galecki et al. (2009a) confirmed increased activity of the enzyme in patients with depressive disorders as compared to healthy volunteers. Studies on a mouse model of depression showed that the disease was associated with a change in the activity of superoxide dismutase (SOD) (Li et al., 1995). Galecki et al. (2009a) also confirmed significantly higher SOD activity in the patients in comparison to healthy volunteers. Nevertheless, the impact of SOD dysfunction on the development of depression is still not clear. On the one hand, Herken et al. (2007) revealed that SOD levels in serum were lower in patients than in controls. On the other hand, Khanzode et al. (2003) found out that the serum level of SOD was positively associated with the severity of DD. Patients with DD are also characterized by increased activity and high levels of xanthine oxidase (XO) in serum (Maes et al., 2011a; Herken et al., 2007). Morris et al. (2017) confirmed that XO activity in the brain was higher in DD as compared to a control group. In addition, Rybka et al. (2013) showed that the concentration of glutathione reductase (GR) in blood was lower in depressed patients than in healthy volunteers. The discrepancies may be due to differences in the size of the study population or the severity of the disease.

Moreover, studies to date indicate a relationship between polymorphisms in the genes related to oxidative stress processes and the development of depression. The most important single-nucleotide polymorphisms (SNPs), playing a key role in the development of depression, are located in the genes of cyclooxygenase-2, superoxide dismutase, myeloperoxidase, and catalase. Certain SNPs cause conformational changes of enzymes, which affects the stability and activity of proteins. SNPs may also contribute to increased oxidative stress and the development of depressive disorders (Dubois et al., 1998; Galecki et al., 2009b).

2.4. The levels of by-products of cellular macromolecule oxidation in DD

Damage to cellular macromolecules is a consequence of oxidative stress. Studies showed that excessive oxidative stress could cause damage to nucleic acids. Patients with depression are characterized by elevated levels of 8-hydroxyguanine (8-oxoG) in urine, cerebrospinal fluid, plasma, and peripheral blood mononuclear cells (PBMC) (Forlenza and Miller, 2006; Irie et al., 2003; Kupper et al., 2009). 8-oxoG is a repair product of guanine oxidation in DNA. Maes et al. (2009c) also found that the level of 8-oxoG in serum is higher in depressed patients than in healthy volunteers. Furthermore, the severity of the disease is positively correlated with the level of 8-oxoG in serum. Another research – conducted on PBMC obtained from patients with depression – corroborate that depression is accompanied by an increased number of oxidatively modified nucleobases (Czarny et al., 2015).

In addition, the level of malondialdehyde (MDA) in serum is significantly higher in patients with depression compared to control groups (Galecki et al., 2009c). The by-product of peroxidation of saturated fatty acids is most often measured by assessing the reactivity level of thiobarbituric acid reactive substances (TBARS) (Kotan et al., 2011; Stefanescu and Ciobica, 2012; Andreazza et al., 2008). The meta-analysis confirmed that the increased level of TBARS was characteristic of patients with bipolar disorders during a depression phase (Andreazza et al., 2008). Moreover, patients suffering from recurrent depressive disorders are characterized by higher levels of MDA than patients after a single episode of depression. Patients suffering from chronic depression have higher levels of MDA than patients with the first episode of the disease (Rybka et al., 2013).

Moreover, depressed patients have a higher level of 8-iso-prostaglandin F2α as compared to healthy volunteers. 8-iso-prostaglandin

F2α is produced during arachidonic acid oxidation (Dimopoulos et al., 2008; Chung et al., 2013).

2.5. The role of high-density lipoprotein cholesterol in the development of depression

Recent studies showed that the mood disorders, including depression, might be associated with the occurrence of lipid metabolism disorders. DD may increase the risk of somatic diseases, including cardiovascular diseases, hypertension, lipid metabolism disorders, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), and atherosclerosis (deMelo et al., 2017). The study confirmed that mood disorders might be associated with lowered level of HDL-c (high-density lipoprotein cholesterol) and increased triglyceride levels (Bortolasci et al., 2015). Depressed patients are characterized by reduced esterification of serum cholesterol, indicating changes in reverse cholesterol transport, and lowered serum levels of HDL-c (Maes et al., 1994; Parikh et al., 2014). Additionally, oxidative damage of HDL-c directly contributes to lipid reduction. The damage is a result of oxidative and nitrosative stress (deMelo et al., 2017). Another next study showed that insulin resistance could be linked with the occurrence of depressive symptoms (Leboyer et al., 2012). Moreover, depressed patients have lower activity of paraoxonase 1 (PON1) than controls. PON1 is an antioxidant, which is synthesized in liver and secreted into plasma, where it is bound to HDL-c. The PON1-HDL complex protects against lipid peroxidation, including HDL-c and LDL-c (low-density lipoprotein cholesterol). Oxidative modification may also lead to the development of arteriosclerosis (deMelo et al., 2017). Moreover, low activity of PON1 may be correlated with decreased quality of life, higher disability, and amplified severity of depression and anxiety (Bortolasci et al., 2014).

In conclusion, depression may be a consequence of decreased total antioxidant capacity (TAC) in blood. However, there are many discrepancies between studies, which may be due to the different characteristics of patients – the differences included age, BMI, smoking status or methods of previous treatment (Liu et al., 2015). In the future, the compounds mentioned above can be used as biomarkers in early diagnosis of depression (Table 1).

3. Nitrosative stress in depression

3.1. Nitrosative stress

Nitrosative stress causes an imbalance in the generation and elimination of reactive nitrogen species (RNS), which can lead to cellular damage. Nitric oxide (NO) is the main source of RNS in biological systems. NO is produced from L-arginine by nitric oxide synthase (NOS) (Klandorf and Dyke, 2012). Damage caused by increased amounts of RNS can contribute to serious diseases, including diabetes, rheumatoid arthritis, cancer, neurodegenerative disorders, chronic obstructive pulmonary disease, and asthma (Di Meo et al., 2016).

3.2. The role of nitric oxide and nitric oxide synthase in depression

A growing body of evidence suggests that patients with depression may demonstrate elevated levels of nitric oxide in plasma (Selek et al., 2008). Moreover, carcinoma patients suffering from depression are characterized by increased levels of NO as compared to patients without any symptoms of DD (Savaşş et al., 2002). The amount of nitric metabolite is significantly higher in suicidal depressive patients than in non-suicidal patients or control subjects (Kim et al., 2006).

Studies showed that patients with depression had increased expression of cellular NOS in neurons of the nucleus suprachiasmaticus, cornu ammonis area 1, and subiculum regions as compared with a control group (Oliveira et al., 2008). Therefore, there may be links between changes of plasma factors and changes in the brain. These reports suggest that factors involved in nitrosative stress may penetrate the blood-brain barrier. Having penetrated this barrier, they may bring about depressive and neurotoxic activities in the brain (Leonard and Maes, 2012; Moylan et al., 2014). A subsequent research study based on an animal model of depression confirmed that suppression of hippocampal NOS might protect against the development of depression (Wang et al., 2008).

3.3. Excessive nitrosylation and nitration as a cause of depression

However, due to excessive levels of NO and dysregulation of nitrosylation, chronic overproduction of ROS and RNS induces a state of hypernitrosylation and is linked to numerous diseases (Shahani and Sawa, 2011). A nitrosylated compound may be immunogenic as evidenced by elevated levels of IgM-mediated responses against NO-amino acid. A study showed that patients with DD were characterized by the presence of increased IgM-mediated immune responses to NO modified amino-acids, e.g. NO-tyrosine, NO-tryptophan (NOW), and NO-arginine, NO-cysteinylin and NO-albumin (Maes et al., 2011a, 2013). Moreover, hypernitrosylation may cause protein misfolding, synaptic damage, mitochondrial dysfunction, and bioenergetic failure (Nakamura and Lipton, 2011). High levels of nitrosylation may modulate the function of neurons and signal pathways; they may, for example, cause inactivation of protein tyrosine kinases and phosphatases, and lead to nuclear factor (NF)-kβ inactivation of proteins, which may have a negative impact of neural regeneration (including protein kinase B, parkin, and sirtuin 1) and cytokines. In addition, excessive hypernitrosylation may cause degradation of the neuroprotective function of proteins, lysosomal deregulation, and degradation of proteosomic proteins, and – consequently – may lead to apoptosis. A long-lasting process may be a cause of chronic neuroinflammation, impaired neurogenesis, and neurodegeneration. Thus, the volumetric reduction in the basal ganglia, hippocampus, and cortex (orbitofrontal and subgenual prefrontal) was observed in patients with DD (Morris et al., 2017).

On the other hand, peroxynitrite, which causes nitration of biological compounds, including amino acids (the addition of a nitro-group (–NO2) to protein aromatic amino acids, mainly tyrosine and tryptophan), is one of the reactive forms of nitrogen. Nitration of tyrosine and tryptophan may change many properties of amino acids (redox potential, phenol group pKa, hydrophobicity) and may lead to the development of autoimmune responses. The results obtained in patients suffering frombipolar disorders may indicate that this disease may be associated with an increased plasma concentration of 3-nitrotyrosine. Symptoms of depression are periodic in the course of bipolar disorders. Thus, DD can also be associated with excessive nitration and may lead to an increase in 3-nitrotirosine levels (Andreazza et al., 2009).

To sum up, these factors may cause neuroprogression, which is observed in depression, and conjugated NO/NO2 adducts could be used to develop new biomarkers of this disease (Table 2).

4. Tryptophan catabolite (TRYCAT) pathway in depression

4.1. Tryptophan and its metabolism

Tryptophan (2-amino-3-indole propionic acid) is an essential exogenous aromatic amino acid. Tryptophan deficiency is observed in patients suffering from the inflammatory bowel disease and liver damage (Irie et al., 2003). Incorrect lifestyle, i.e. poor diet, stress, may be a result of tryptophan deficiency, which may lead to the development of mood disorders and depression (Keszthelyi et al., 2009; Buczko et al., 2005). About 30% of tryptophan is used for protein synthesis. The remainder of the tryptophan is degraded through the kynurenine pathway and non-protein transformations to serotonin and melatonin. In the periphery, tryptophan undergoes three types of changes: 1. the indole ring opening, which results in the formation of kynurenine; 2.

Table 1
Potential biomarkers involved in oxidative stress.

Potential biomarkers of oxidative damage	**Function**	**Biological specimens**	**Level in depression**	**Laboratory method**	**Author**
Isoprostanes	Compounds produced during non-enzymatic peroxidation of arachidonic acid	Plasma; urine	↑	Competitive immunoassay for the quantitative determination of 8-iso-PGF2α in biological fluids; chromatography/mass spectrometry	Dimopoulos et al. (2008), Chung et al. (2013)
Malondialdehyde (MDA)	By-product of peroxidation of saturated fatty acids	Serum	↑	The method of Misra and Fridovich, Beers and Sizer, Little and O'Brien and Placer et al.	Galecki et al. (2009c)
8-oxoguanine (8-oxoG)	Major products of DNA oxidation	Urine, cerebrospinal fluid, plasma, PBMC, serum	↑	Enzyme-linked immunosorbent assay; high-performance liquid chromatography (HPLC); enzyme-linked immunosorbent assay (ELISA)	Forlenza and Miller (2006), Irie et al. (2003), Kupper et al. (2009),Maes et al. (2009c)
Level of high-density lipoprotein cholesterol (HDL-c)	Lipid	Serum	↓	Automated method in a clinical chemistry system, Dimension® RXL (Siemens Healthcare Diagnostics Inc., Newark, DE, USA)	Bortolasci et al. (2014)
Potential biomarkers involved in antioxidant enzymatic defence	**Function**	**Biological specimens**	**Level in depression**	**Laboratory method**	**Author**
Catalase (CAT)	Enzyme catalysing the decomposition of hydrogen peroxide to water and oxygen	Serum; RBC	↑	The method described by Pifferi et al. as modified by Nowak et al.; assayed using the methods of Misra and Fridovich, Beers and Sizer	Szuster-Ciesielska et al. (2008), Galecki et al. (2009a)
Glutathione peroxidase (GPx)	Antioxidant enzyme inactivating hydrogen and lipid peroxides	RBC	↑	The modified method of Paglia and Valentine using tert-butyl hydroperoxide as a substrate; the method of Paglia and Valentine	Kodydková et al. (2009), Ozcan et al. (2004)
		RBC	↓	The methods of Placer et al.	Galecki et al. (2009a)
Glutathione reductase (GR)	Enzyme catalysing the reduction of glutathione disulphide to the sulfhydryl form of glutathione	RBC	↓	The methods of Paglia and Valentine, Flohé and Günzler, and Misra and Fridovich	Rybka et al. (2013)
Superoxide dismutase (SOD)	Enzyme catalysing the conversion of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide	RBC; serum	↑	The method of Placer et al.; modified method of Nischal et al.	Galecki et al. (2009a), Khanzode et al. (2003)
		serum	↓	The method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by xanthine/xanthine oxidase system	Herken et al. (2007)
Xanthine oxidase (XO)	Enzyme catalysing the oxidation of hypoxanthine to xanthine and generating reactive oxygen species	Serum; brain tissue – hippocampus thalamus, putamen, caudate nucleus	↑	Spectrophotometric method; XO was assessed in the cytosolic fraction of each chosen brain region by means of using a commercially available Kit (Amplex Red Xantine/XO Assay Kit, A22182, Molecular Probes, Invitrogen)	Herken et al. (2007), Michel et al. (2010)
Antioxidants as biomarkers	**Function**	**Biological specimens**	**Level in depression**	**Laboratory method**	**Author**
Coenzyme Q_{10} (CoQ_{10})	Non-enzymatic antioxidants	plasma	↓	High-performance liquid chromatography (HPLC);	Maes et al. (2009b)
Glutathione (GSH)	Non-enzymatic antioxidants	serum; RBC	↓	The modified spectrophotometric method according to Griffith, the method of Paglia and Valentine; the method of Paglia and Valentine	Kodydková et al. (2009), Ozcan et al. (2004)
Vitamin C	Non-enzymatic antioxidants	Serum	↑	Isocratic high-performance liquid chromatography	Kobrosly and van Wijngaarden (2010)
Vitamin E	Non-enzymatic antioxidants	Plasma	↓	Biochemical estimation	Gautam et al. (2012)
Zinc (Zn^{2+})	Non-enzymatic antioxidants	Rat and mice brain	↓	Immunohistochemistry and immunofluorescence staining	Suh et al. (2009)
Uric acid	Non-enzymatic antioxidants	Serum	↓	Meta-analysis	Liu et al. (2015)
Albumin	Non-enzymatic antioxidants	Serum	↓	Meta-analysis	Liu et al. (2015), Huang (2002)
Activity of paraoxonase 1 (PON1)	Antioxidant	Plasma	↓	Activities towards paraoxon (PO) and diazoxon (DZO) with the application of spectrophotometry	Bortolasci et al. (2014)

Table 2
Potential biomarkers involved in nitrosative stress.

Potential biomarkers involved in nitrosative stress	Function	Biological specimens	Level in depression	Laboratory method	Author
Level of 3-nitrotyrosine	Modified amino acids	Plasma	↑	Competitive enzyme-linked immunosorbent assay, as described by Khan and colleagues	Andreazza et al. (2009)
Level of conjugated nitric-oxide (NO) adducts: NO-tryptophan, NO-tyrosine, NO-arginine, and NO-cysteine		Plasma		Enzyme-linked immunosorbent assay (ELISA)	Maes et al. (2013)
Activity of nitric oxide synthases	NO is produced from L-arginine by nitric oxide synthase	Neurons of the nucleus suprachiasmaticus, cornu ammonis area 1, and subiculum regions	↑	Immunohistochemistry	Oliveira et al. (2008)
Level of nitric oxide	The main source of RNS in biological systems	Plasma; serum	↑	Spectrophotometry	Selek et al. (2008), Savaşş et al. (2002)

hydroxylation, which results in the formation of serotonin; 3. decarboxylation, which results in the formation of tryptamine. Tryptophan is a substrate used in the production of melatonin and serotonin in the central nervous system (Buczko et al., 2005).

4.2. Metabolites of tryptophan in depression

Recent findings show that depressed patients are characterized by increased plasma levels of tryptophan and its harmful metabolites, including kynurenine, xanthurenic acid, and quinolinic acid. Latest research confirmed that 2,3-dioxygenase tryptophan (TDO) and indoleamine 2,3-dioxygenase (IDO) activity was greater in patients with DD than in healthy subjects (Maes et al., 2011b, 2011c). Patients with DD have decreased levels of kynurenic acids; thus, this acid may have a neuroprotective effect (Maes et al., 2011c). Specific changes in the TRYCATs pathway may decide on a specific symptom or type of DD (Maes and Rief, 2012; Maes et al., 2011b). Increased activity of IDO is a characteristic feature of patients with somatisation or depressed patients with suicide attempts, or adolescent patients with DD (Sublette et al., 2011; Gabbay et al., 2010).

4.3. The function of serotonin and melatonin in the development of DD

Serotonin deficiency is also associated with the development of depression. Decreased levels of serotonin or their receptors correlate with depressed mood (Albert et al., 2012). Low levels of melatonin are observed in patients with Alzheimer's disease, carcinoma, anorexia and depression (Hardeland, 2012). About 80% of depressed patients exhibit various sleep disorders. Insomnia, which is persistent or deteriorates, may contribute to the risk of recurrence and increase the severity of depression. Sleep disorders, such as trouble falling asleep, waking up frequently during the night or early in the morning, often precede depressed mood. Moreover, some scientists emphasise that insomnia is the main complaint mentioned by patients with depression rather than with depressed mood. In addition, severe insomnia occurs more frequently in patients with depressive suicidal attempts than in patients without suicidal attempts (Heitzman, 2009). The time of the year also determines the occurrence of a depressive episode, as an increased number of patients hospitalized for depression are recorded in the autumn (Gawlik and Nowak, 2006). Old age, low socioeconomic status, and female sex also represent risk factors for sleep disorders and depression (Heitzman, 2009).

4.4. Compensatory (anti)inflammatory reflex system in depression

Furthermore, multiple depressive episodes may cause damage of neural tissue and, in consequence, functional and cognitive sequelae. In DD, patients are characterized by sensitization of immuno-inflammatory pathways, autoimmune responses directed against self-epitope progressive damage by oxidative and nitrosative stress to lipids, proteins, and DNA, and overproduction of TRYCAT metabolites. Previous studies indicated that depression may be associated with activation of the inflammatory response system (IRS). Thus, the occurrence of DD symptoms is associated with increased levels of pro-inflammatory cytokines (PICs), such as interleukin-1 (IL-1), tumour necrosis factor α (TNFα), interleukin-6 (IL-6), PICs and acute phase proteins, including C-reactive protein and haptoglobin. Countering inflammation demands large amounts of energy, which causes reduction of energy consumption by the brain and peripheral organs – energy-consuming processes, such as locomotor, neurocognitive and reproductive activity, are shut down. On the other hand, activation of compensatory (anti)inflammatory reflex system (CIRS) is observed in patients with DD. CIRS controls acute inflammation and limits an overzealous acute inflammatory response. The activity of this system involves: increased synthesis of Il-1 receptor antagonist, which inhibits the action of Il-1; increased level of interleukin-2 receptors (IL-2R),

which limits interleukin-2 (IL-2) concentration needed for immune cell proliferation; increased amount of Il-6, which decreases the production of interleukin-10 (IL-10), IL-1 receptor antagonist and glucocorticoids; and increased production of haptoglobin, which acts as an immunosuppressive factor. Moreover, CIRS may decrease plasma levels of tryptophan. Inflammation in depression may be associated with disorders of TRYCAT pathway. PICs (IL-1 and TNFα) and IFNγ increase the activation of IDO, leading to tryptophan depletion and increased synthesis of toxic tryptophan metabolites (kynurenine, kynurenic acid, xanthurenic acid, and quinolinic acid). Low levels of tryptophan lead to the development of depressive symptoms and are correlated with acute phase reactants, increased cytokine levels, serum neopterin, and sIL-2Rs (Maes et al., 2002; Maes et al., 2012). In addition, animal studies confirmed that cytokine activation caused excessive IDO activation and led to the development of depressive symptoms (O'Connor et al., 2009). As has already been mentioned, CIRS may cause reduction of tryptophan and increase of TRYCAT metabolites, and may, consequently, attenuate the primary immune-inflammatory response by reducing T-cell activation and proliferation. Moreover, depressed patients are characterized by a high degree of anti-5-hydroxytryptamine (anti-5-HT) antibody activity as compared to healthy volunteers. Immune activation may be associated with increased production of IL-1 and TNFα. Additionally, studies showed that previous DD episodes might increase the activity of the anti-5-HT antibody and could elevate the risk of new episodes. This process may lead to the development of chronic inflammation, which may be correlated with the severity of physio-somatic symptoms of depression (Maes et al., 2012).

The elements of the TRYCATs pathway, listed in the text, can be used in diagnostics (Table 3).

5. Oxidative & nitrosative stress and TRYCATs in the treatment of depression

The recovery of well-being and high social activity are the main objectives of an antidepressant therapy is. However, treating a significant group of patients with mood disorders does not necessarily ensure achieving the desired effect. Antidepressant resistance means the lack of response to drugs administered in a suitable dose and for a limited period (Souery et al., 2009).

5.1. Regulation of the "body clock"

Regulation of the so-called "body clock" represents an important element of depression pharmacotherapy. The discovery of the mechanism of melatonin action enabled the development of new effective antidepressants. Being aware of the role of serotonin receptors provides information about the potential for therapeutic targets. Agomelatine (agonist of melatonin receptor type 1 and melatonin receptor type 2, and antagonist of serotonin and 5-hydroxytryptamine receptor 2C) is an example of this type of agents. Additionally, evidence indicates that the use of melatonin increases the effectiveness of depression treatment. Patients treated with a combination of selective reuptake inhibitors of serotonin and melatonin derivatives (agomelatine) demonstrated a superior improvement than a group of patients treated only using selective serotonin reuptake inhibitors (SSRI). Thus, studies showed that melatonin could be used in an adjuvant therapy (Soria and Urretavizcaya, 2009). Maes et al. (1999a, 1999b) revealed that plasma levels of tryptophan might affect the patients resistant to treatment with selective serotonin reuptake inhibitors and tricyclics or heterocyclic antidepressants. Depressed patients with drug-resistance are characterized by a lower plasma level of tryptophan than depressed patients without drug-resistance (Maes et al., 1999a). Studies also showed that polymorphisms in certain genes, including serotonin receptor type 1A, were risk factors for the development of treatment-resistant depression. This type of depression may also be caused by excessive activity of monoamine oxidase A (MAOA) (Smith, 2013).

Table 3
Potential biomarkers involved in the TRYCATs pathway.

Potential biomarkers involved in the TRYCATs pathway	Function	Biological specimens	Level in depression	Laboratory method	Author
Level of kynurenine, xanthurenic acid, and quinolinic acid	Tryptophan metabolites	Plasma	↑	High-performance liquid chromatography (HPLC); spectrophotometric method	Maes et al. (2011b)
Level of kynurenic acid	Tryptophan metabolites	Plasma	↓	High-performance liquid chromatography (HPLC);	Maes et al. (2011b)
Activity of indoleamine 2,3-dioxide (IDO)	Enzyme catalysing O2-dependent oxidation of L-tryptophan to N'-formylkynurenine	Plasma	↑	High-performance liquid chromatography (HPLC); spectrophotometric method	Maes et al. (2011b)
Activity 2,3-dioxygenase tryptophan (TDO)	Enzyme catalysing O2-dependent oxidation of L-tryptophan to N'-formylkynurenine	Fresh brainstem from mice	↑	High-performance liquid chromatography (HPLC);	Kanai et al. (2009)
Level of tryptophan	Exogenous aromatic amino acid	Plasma	↓	High-performance liquid chromatography (HPLC)	Maes et al. (2011b)
Level of pro-inflammatory cytokines (PICs) – for example IL-6, IL-8	Pro-inflammatory factors associated with TRYCATs	Serum	↑	Enzyme-linked immunosorbent assay (ELISA)	Maes et al. (2002)

5.2. The application of omega-3 polyunsaturated fatty acids in the treatment of depression

As has already been mentioned, tryptophan metabolites (quinolinic acid and kynurenine) are highly neurotoxic. Quinolinic acid causes the destruction of postsynaptic structures, induces the destruction of neurons via apoptosis of hippocampal cells, and selective necrosis of granular cells. Moreover, this acid significantly reduces the levels of dopamine, choline, and γ-aminobutyric acid (GABA). Toxic metabolites of tryptophan can also induce the overproduction of proinflammatory cytokines (Maes et al., 1999b; Németh et al., 2005; McNally et al., 2008). Maes et al. (1999b) discovered that omega-3 polyunsaturated fatty acids (ω3PUFAs) could affect neurogenesis through their anti-inflammatory and serotonic effects. ω3PUFAs reduce the level of proinflammatory cytokines (mainly interleukin-1β and tumour necrosis factor α). ω3PUFAs indirectly affect correct serotonin fixation and concentration of neurotrophins, such as the brain-derived neurotrophic factor (BDNF) (Maes et al., 1999b; Beltz et al., 2007; Wu et al., 2004). Furthermore, Nements et al. (2002) demonstrated enhanced efficacy of antidepressant treatment supplemented by the ethyl ester of eicosapentaenoic acid (acid from the omega-3 group). Moreover, modulation of HDL-c level may be used during an antidepressant therapy. Eicosapentaenoic acid (EPA), belonging to ω3 PUFAs, demonstrates antidepressant activity by lowering the levels of triglycerides and increasing HDL-c levels (Lin and Su, 2007; Chaddha and Eagle, 2015). Resveratrol provides a similar action; it decreases plasma triglyceride and LDL-c levels, and increases HDL-c concentration (Bonnefont-Rousselot, 2016).

5.3. SSRIs in the therapy of depression

The high effectiveness of antidepressants, such as SSRIs, indicates the role of monoamines and their receptors in the development of DD. A deficiency of tryptophan can lead to reduced serotonin availability, which contributes to a relapse of the disease in the patients remitted after SSRI treatment (Maes and Meltzer, 1995).

Genetic polymorphisms of tryptophan hydroxylase 2 (TPH-2) could be associated with treatment-resistant depression. TPH-2 deficits are characteristic of patients with symptoms of depression. SNP of the *TPH-2* gene reduces the effectiveness of treatment with SSRIs (Al-Harbi, 2012; Zhang et al., 2005).

5.4. Potential markers of resistance to antidepressants

Decreased levels of CoQ_{10} predispose to the development of depression. Further studies revealed that patients with treatment-resistant depression had significantly lower levels of CoQ_{10} in blood plasma as compared to the patients who were responsive to the therapy applied (Maes et al., 2009b). Maes et al. (1997) found that the plasma levels of Zn^{2+} are associated with depression. Patients with treatment-resistant depression are characterized by a lower serum level of zinc as compared with patients without treatment-resistant depression (Maes et al., 1997).

Moreover, elements of oxidative and nitrosative stress, and the TRYCAT pathway, can be used to assess the efficacy of antidepressant treatment (a measure of response to treatment). The serum SOD decreased after two months of SRRS treatment (Khanzode et al., 2003). In contrast, no significant change in RBC SOD was observed in patients after 3 months of treatment with fluoxetine (Galecki et al., 2009c). Galecki et al. (2009a) discovered that antidepressant treatment had no effect on the plasma concentration of GPx. On the other hand, Bilici et al. (2001) showed that the plasma level of GR and GPx was reduced after three months of therapy using SSRI. Normalization of MDA levels is observed in patients with depression after SSRI-based treatment (Bilici et al., 2001). Other studies showed that a therapy with the addition of NAC decreased the severity of DD in comparison to traditional treatment. Patients undergoing an assisted therapy showed an improvement in the quality of life and general satisfaction (Dean et al., 2011). Studies demonstrated that treatment with antidepressants had no effect on the concentration of kynurenic acid (Myint et al., 2007).

6. Conclusion

Depression is a serious psychosomatic disorder, which affects a growing number of people. A highly individual character of this disease among people has also been pointed out. It is difficult to ensure an early and correct diagnosis of depression, followed by an effective therapy. This is because we still have insufficient knowledge about the pathogenesis of depression. Studies to date suggest that certain factors may affect the risk of depression symptoms and resistance to traditional pharmacotherapy. Interactions between these factors and a disarrangement of different biochemical processes may lead to different responses to a therapy with antidepressants by having an impact on their mechanism of action. Accurate knowledge of the molecular mechanisms accompanying the development of depression would enable an evolution of effective diagnostic biomarkers for early and accurate diagnosis of DD in the future. Besides, this would allow researchers to determine specific processes involved in the mechanism of depression development. As a consequence, scientists can create new drugs and be able to offer more effective and personalized treatment. It is, therefore, important to continue studies to demonstrate the links between the biochemical pathways listed in the article – oxidative and nitrosative stress as well as the pathway of tryptophan catabolites.

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Original article

Variation of genes involved in oxidative and nitrosative stresses in depression

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ABSTRACT

The dominating hypothesis among numerous hypotheses explaining the pathogenesis of depressive disorders (DD) is the one involving oxidative and nitrosative stress. In this study, we examined the association between single-nucleotide polymorphisms of the genes encoding SOD2 (superoxide dismutase 2), CAT (catalase), GPx4 (glutathione peroxidase 4), NOS1 (nitric oxide synthase 1), NOS2 (nitric oxide synthase 2), and the development of depressive disorders. Our study was carried out on the DNA isolated from peripheral blood collected from 281 depressed patients and 229 controls. Using TaqMan probes, we genotyped the following six polymorphisms: c.47T > C (p.Val16Ala) (rs4880) in *SOD2*, c.-89A > T (rs7943316) in *CAT*, c.660T > C (rs713041) in *GPx4*, c.-420-34221G > A (rs1879417) in *NOS1*, c.1823C > T (p.Ser608Leu) (rs2297518), and c.-227G > C (rs10459953) in *NOS2*. We found that the T/T genotype of the c.47T > C polymorphism was linked with an increased risk of depression. Moreover, the T/T genotype and T allele of c.660T > C increased the risk of DD occurrence, while the heterozygote and C allele decreased this risk. On the other hand, we discovered that the A/A genotype of c.-89A > T SNP was associated with a reduced risk of DD, while the A/T genotype increased this risk. We did not find any correlation between the genotypes/alleles of c.-420-34221G > A, c.1823C > T, and c.-227G > C, and the occurrence of DD. In addition, gene-gene and haplotype analyses revealed that combined genotypes and haplotypes were connected with the disease. Moreover, we found that sex influenced the impact of some SNPs on the risk of depression. Concluding, the studied polymorphisms of *SOD2*, *CAT* and *GPx4* may modulate the risk of depression. These results support the hypothesis that oxidative and nitrosative stresses are involved in the pathogenesis of depressive disorders.

1. Introduction

Depression (depressive disorder [DD]) is considered to be the most common mental disorder. Estimations show that 350 million people worldwide suffer from this disease. By the year 2020, it will have become the second most common health problem in the world, only after ischaemic heart disease [1].

The aetiology of this disease has not been examined thoroughly so far and is not completely known [2]. However, certain evidence shows that an imbalance in the generation and elimination of reactive oxygen and nitrogen species (ROS and RNS, respectively) is present during depression [3]. This imbalance leads to increased levels of biomarkers of oxidative and nitrosative process intensification, such as 8-hydroxyguanine (8-oxoG), 8-iso-prostaglandin F2α (8-izo-PGF2α), malondialdehyde (MDA), and nitric oxide (NO) [4–8]. Interestingly, a recent study has shown that increased level of MDA is associated with a reduced ability of the visual-spatial and auditory-verbal working memory and short-term declarative memory, while a high concentration of this biomarker in depressed patients' plasma may be positively correlated with the intensity of the symptoms [9]. Changes in the activity of antioxidant enzymes may be some of the reasons for the imbalance. Accordingly, it has been demonstrated that low activity of glutathione peroxidase (GPx, reduces hydrogen peroxide to

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water and reduces lipid hydroperoxides) may contribute to the development of depression [10]. Moreover, the same researchers have found that GPx activity is correlated with the severity of the disease – the lower the enzyme activity is, the more severe the symptoms are. In addition, activity of the next antioxidant enzyme, i.e. glutathione reductase (GR, reduces glutathione disulphide to the sulfhydryl form of glutathione), decreases in depressed patients as compared to healthy volunteers [9]. On the other hand, increased levels and activity of catalase (CAT, reduces hydrogen peroxide to water and oxygen) may serve as a risk factor for the occurrence of depressive episodes [11,12]. The plasma activity of another antioxidant enzyme, i.e. superoxide dismutase (SOD, catalyses the reaction of superoxide radical dismutation into oxygen or hydrogen peroxide), also increases in the course of depression, which has been proven in various experiments conducted on animal models and during clinical studies [12,13]. However, it has been proven that the over-activation of SOD may lead to the intensification of oxidative stress via H_2O_2 production [9]. A change in SOD levels has been observed in the brain tissue collected from depressed patients. An elevated level of copper/zinc (Cu/Zn) SOD has only been detected in post-mortem prefrontal cortical brain tissue, and not in the hippocampus, while the level of manganese SOD (MnSOD) has not changed in both regions of patients' brain when compared to control subjects. Different locations of these isoforms may serve as an explanation for these differences – Cu/ZnSOD is present primarily in the cytosol of glial cells, while MnSOD is found mainly in neurons and erythrocytes. So far the available results suggest that elevated levels of SOD in peripheral tissues (plasma, erythrocytes, saliva) may be reduced owing to a successful antidepressant therapy [14]. Galecki et al. (2009) found that a combined therapy with the application of fluoxetine (SSRI) and acetylsalicylic acid may lead to a decrease in the activity of Cu/ZnSOD and a reduction in MDA concentration [15]. In addition, antidepressants may also result in the normalisation of serum paraoxonase activity (reduced oxidation of apolipoprotein B containing lipoproteins) [14].

Low amounts of non-enzymatic antioxidants are considered another aspect associated with the risk of depression occurrence. So far, studies have shown that the women with DD have lower amounts of glutathione (GSH) than the women not affected by depression [16]. Moreover, a decreased concentration of GSH has been observed in the chronic mild stress animal model of the disease [17]. Similarly, the level of CoQ_{10} has been dramatically reduced in the serum of the patients suffering from DD as compared to the control group [18]. Moreover, based on an animal model, it has been possible to determine that low-zinc diet reduces the number of progenitor cells and immature nerve cells in the hippocampus of treated rats [19]. Furthermore, decreased levels of yet another group of non-enzymatic antioxidants – vitamins A, C and E – may also play an important part in the aetiology of depression; however, the results are not conclusive in this respect. On the one hand, the plasma amount of ascorbic acid (vitamin C) is reduced in the patients with DD [20]. On the other hand, it has been suggested that increased plasma levels of vitamin C can be associated with the severity of DD [21]. Results of a different study have indicated no difference in the plasma levels of vitamins A, C and E between the patients and the control group [22]. However, Maes et al. [23] found that the plasma level of vitamin E of the affected patients was lower as compared to healthy volunteers. The discrepancies in the results may be due to size differences of the studied groups, the environmental impacts and the severity of the disease. Additionally, the patients with DD are also characterised by decreased levels of other non-enzymatic antioxidants such as albumin and uric acid [9].

Another piece of evidence that supports the hypothesis of ROS and RNS involvement in the pathogenesis of the disease are changes in the level and activity of oxidative and nitrosative enzymes in the patients with DD. It has been revealed that depressed patients have increased serum levels of xanthine oxidase (XO) [3,24]. This enzyme catalyses the oxidation of hypoxanthine to xanthine and then the oxidation of xanthine to uric acid resulting in the generation of superoxide anion and hydrogen peroxide [25]. The patients with depression are characterised by elevated XO activity in the thalamus, the putamen, and the frontal and parietal cortex, the hippocampus and the caudate nuclei; XO activity has been found to be decreased in the temporal and occipital cortex [9,26]. A recent study has revealed that the main symptoms of depression – cognitive dysfunction, anhedonia and melancholia – may be associated with structural or functional neuronal changes of the putamen and the thalamus [9]. Additionally, patients with depression demonstrate increased expression of cellular NOS in the neurons of the suprachiasmatic nucleus, cornu ammonis area 1 (CA1), and subiculum regions as compared with the control group [27]. A growing body of evidence suggests that the factors involved in nitrosative stress may penetrate the blood-brain barrier exhibiting their depressive and neurotoxic activities in the brain. As a result of excessive pro-oxidative enzyme activity (such as XO, NOS), the levels of ROS and RNS are increased, which may lead to the development of neurodegenerative changes [28,29]. A large amount of ROS may induce apoptosis of neural cells by causing damage to DNA or peroxidation of the cell membrane lipid (ROS destroy the lipids of cells, mainly polyunsaturated acids [PUFAs]) [9]. This long-lasting condition may be one of the causes of death of neuronal and glial cells in the central nervous system, observed in neurodegenerative diseases [26]. Interestingly, the study suggests that the patients with DD have a reduced volume of the prefrontal cortex and the hippocampus as compared to healthy volunteers. Furthermore, a post-mortem study confirmed that the patients with DD had a reduced number and density of glial cells [14].

Moreover, the study suggested that RNS (e.g. peroxynitrite) may cause nitration of biological compounds, including amino acids (mainly tyrosine). Additionally, Maes et al. [3] have found increased levels of IgM antibodies to such modified proteins in the blood samples collected from depressed patients.

An imbalance in the production and elimination of ROS and RNS – leading to oxidative and nitrosative stress – may induce various disorders. Oxidative stress is involved in the development of cardio-vascular and neuropsychiatric disorders such as ischaemia, acute respiratory distress syndrome (ARDS), panic disorder [30], preeclampsia [31], autism [32], dementia [33], schizophrenia [34], Parkinson's disease, Alzheimer's disease [35,36], dementia [37], amyotrophic lateral sclerosis, schizophrenia and depression [14,33,34,38–41], and multiple sclerosis [42]. Moreover, a mitochondrial dysfunction can cause overproduction of ROS and – in consequence – may lead to the development of ischaemic heart disease, stroke, atherosclerosis, arterial hypertension, and hypertrophy of the myocardium [43]. On the other hand, nitrosative stress is involved in the development of Parkinson's disease [44], Alzheimer's disease [45], schizophrenia [46], depression [47], cardiomyopathy, heart failure [48], stroke, arthritis, multiple sclerosis, hypercholesterolemia, ischemia [49], and cancer [50–52].

The aforementioned studies indicate that intensification of oxidative and nitrosative stress, caused, among others, by decreased levels and activity of enzymatic antioxidants and/or excessive pro-oxidative enzyme activity, may play an important role in depression aetiology. Therefore, the aim of this study was to investigate the association between the occurrence of *SOD2*, *CAT*, *GPx4*, *NOS1* and *NOS2* polymorphisms and the risk of depression development by means of determining the frequency

Table 1
Characteristics of studied polymorphisms.

Gene	rs number	Polymorphism	Localization
SOD2	rs4880	c.47T > C (p.Val16Ala)	Exon
CAT	rs7943316	c.-89A > T	5′ UTR
GPx4	rs713041	c.660T > C	3′ UTR
NOS1	rs1879417	c.-420-34221G > A	Intron
NOS2	rs2297518	c.1823C > T (p.Ser608Leu)	Exon
	rs10459953	c.-227G > C	5′ UTR

of occurrence of genotypes of selected SNPs in the patients with DD as compared to healthy volunteers in the Polish population.

Table 1 shows the characteristic features of the studied polymorphisms.

2. Materials and methods

2.1. Subjects

The study was conducted on 510 participants, including patients with DD ($n = 281$, 117 women and 114 men; mean age 53.19 ± 12.61) who were hospitalized at the Department of Adult Psychiatry of the Medical University of Lodz (Poland), and healthy controls ($n = 229$, 147 women and 132 men; mean age 49.53 ± 10.175). Detailed characteristics of the patients are shown in Table 2. All the participants were selected randomly without replacement sampling. Qualified patients met the diagnostic criteria for depressive episode and recurrent depressive disorder according to WHO [53]. The inclusion criteria were based on those outlined in ICD-10 (F32.0–7.32.2, F33.0–F33.8). A case history was obtained from each patient using the standardized Composite International Diagnostic Interview (CIDI) [54] prior to the start of the experiment. Depression severity was evaluated and classified using the 21-item Hamilton Depression Rating Scale (HDRS) [55]. Intensity levels of depressive symptoms were measured with the use of the grades presented in the study conducted by Demyttenaere and De Fruyt [56]. Each patient was examined by the same psychiatrist (CIDI and HDRS). A psychiatric evaluation was performed before the patient was included in the study and after antidepressant therapy of selective serotonin reuptake inhibitors (SSRIs). All the subjects were examined during their hospitalisation and no symptoms of concurrent somatic diseases or axis I and II disorders, other than depressive episodes, were diagnosed in them. Inflammatory or autoimmune disorders, central nervous system traumas, and unwillingness to give informed consent were additional exclusion criteria. Patients with familial prevalence of mental disorders other than recurrent depressive disorders were excluded from the examined group. The individuals taking part in the experiment were native Poles from central Poland (not related). They were chosen for the study group at random without replacement sampling. Participation in the study was voluntary. Before making a decision to participate in the study, the subjects were informed of the purpose and assured of the voluntary nature of the experiment, and guaranteed that their personal data would be kept in secret. The patients and healthy volunteers were informed about the details of this experiment and gave their written consent to participate in this study, according to the protocol approved by the Bioethics Committee of the Medical University of Lodz (no. RNN/70/14/KE).

2.2. Selection of single-nucleotide polymorphisms

The studied SNPs in ROS and RNS genes were selected from the database of Single Nucleotide Polymorphisms of the National Center for Biotechnology Information (NCBI dbSNP), available at http://www.ncbi.nlm.nih.gov/snp (Bethesda, MD, USA). The following six polymorphisms were chosen: c.47T > C (p.Val16Ala) (rs4880) in *SOD2*, c.-89A > T (rs7943316) in *CAT*, c.660T > C (rs713041) – *GPx4*, c.-420-34221G > A (rs1879417) in *NOS1*, c.1823C > T (p.Ser608Leu) (rs2297518), and c.-227G > C (rs10459953) in *NOS2*, which a minor allele frequency (MAF) higher than 0.05 in the European population (submitter population ID: HapMap-CEU). All polymorphisms are located in the coding or

Table 2
The detailed characteristic of patients which were qualified the study.

Depression severity (HAMD range of scores)	Percentage of patients before treatment (%)	Percentage of patients after treatment (%)
None (0–7)	0.43	68.65
Mild (8–16)	12.29	30.08
Moderate (17–23)	33.47	1.27
Severe (≥ 24)	53.81	0

Mean age of patients with first episode	30
Mean age of patients during first episode (for other patients)	36
Mean age of patients during first episode (for all patients)	35
Mean age of patients enrolled in the study	49

Duration of disease from the first episode	Percentage of patients (%)
0–10 years	52.12
11–20 years	19.92
21–30 years	17.80
31–40 years	9.32
≥ 41 years	0.84

Number of episodes	Percentage of patients (%)
1	13.56
2	31.36
3	31.78
4	18.64
5	4.24
6	0.42

regulatory regions of genes and may have functional significance for transcription and protein function.

2.3. DNA extraction

Genomic DNA was isolated from venous blood using commercially available Blood Mini Kit (A&A Biotechnology, Gdynia, Poland). Blood samples were collected from each of the patients with DD before antidepressant therapy, and from all healthy volunteers. DNA purity and concentration were measured by comparing the absorbance at 260 and 280 nm; after that, the samples were stored at −20 °C until use.

2.4. Genotyping

The tested SNPs were genotyped using the TaqMan SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 2X Master Mix Takyon for Probe Assay–No ROX (Eurogentec, Liège, Belgium), according to the manufacturer's instructions. Real-time PCRs were carried out in the Bio-Rad CFX96 Real-Time PCR Detection System and analysed in the CFX Manager Software (Bio-Rad Laboratories Inc., Hercules, California, USA).

2.5. Statistical analysis

A statistical analysis of the data was performed using Statistica 12 (Statsoft, Tulsa, OK, USA) and SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). The association between case/control and each studied polymorphism was estimated using an unconditional multiple logistic regression model. The results are shown as odds ratios (ORs) with 95% confidence interval (95% CI). In addition, the OR was adjusted for sex, since women are exposed to a doubled risk of depression in comparison to men [29]. The data presenting the results from the distribution of genotypes by age of the first episode of depression are indicated as the median ± inter-quartile range. The normality of distribution was verified using the Shapiro-Wilk test and then the significance of differences between the studied values was determined, accordingly, by either the Mann-Whitney test or Student's *t* test. The Student's *t* test was used only in the case of the c.1823C > T–*NOS2* (rs2297518) polymorphism; the Mann-Whitney test was used for the remaining polymorphisms.

Table 3
Distribution of genotypes and alleles of c.804-7C > A, c.-1668T > A, c.803 + 221C > A, c.-173A > T, c.-1449C > A and c.-844G > T and the risk of DD.

Genotype/Allele	Control (n = 229)		Depression (n = 281)		Crude OR (95% CI)[a]	P	Adjusted OR (95% CI)[a]	P
	Number	Frequency	Number	Frequency				
c.47T > C (p.Val16Ala)–SOD2 (rs4880)								
T/T	**9**	**0.039**	**29**	**0.103**	**2.524 (1.308–6.096)**	**0.008**	**2.823 (1.308–6.095)**	**0.008**
T/C	150	0.655	170	0.605	0.814 (0.566–1.170)	0.266	0.814 (0.565–1.172)	0.268
C/C	70	0.306	82	0.292	0.925 (0.631–1.354)	0.687	0.922 (0.629–1.353)	0.679
				χ^2 = 2.551; P = 0.110				
T	168	0.367	228	0.406	1.286 (0.943–1.753)	0.111	1.289 (0.945–1.759)	0.109
C	290	0.633	334	0.594	0.778 (0.570–1.060)	0.111	0.776 (0.569–1.058)	0.109
c.-89A > T–CAT (rs7943316)								
A/A	**42**	**0.183**	**33**	**0.117**	**0.595 (0.363–0.975)**	**0.039**	**0.595 (0.363–0.975)**	**0.039**
A/T	**98**	**0.428**	**160**	**0.569**	**1.757 (1.234–2.499)**	**0.002**	**1.762 (1.236–2.511)**	**0.002**
T/T	89	0.389	88	0.313	0.721 (0.500–1.040)	0.080	0.720 (0.498–1.041)	0.080
				χ^2 = 0.0215; P = 0.883				
A	182	0.397	226	0.402	1.020 (0.787–1.321)	0.883	1.019 (0.786–1.321)	0.888
T	276	0.603	336	0.598	0.981 (0.757–1.271)	0.883	0.982 (0.757–1.272)	0.888
c.660T > C–GPx4 (rs713041)								
T/T	**8**	**0.035**	**53**	**0.189**	**6.450 (2.998–13.877)**	**≪ 0.001**	**6.463 (3.003–13.907)**	**≪ 0.001**
T/C	**138**	**0.603**	**141**	**0.603**	**0.659 (0.463–0.939)**	**0.021**	**0.659 (0.463–0.939)**	**0.021**
C/C	83	0.362	87	0.310	0.793 (0.548–1.148)	0.219	0.791 (0.546–1.145)	0.214
				χ^2 = 13.280; P ≪ 0.001				
T	**154**	**0.336**	**247**	**0.440**	**1.678 (1.264–2.226)**	**≪ 0.001**	**1.684 (1.268–2.237)**	**≪ 0.001**
C	**304**	**0.664**	**315**	**0.560**	**0.596 (0.449–0.791)**	**≪ 0.001**	**0.594 (0.447–0.788)**	**≪ 0.001**
c.-420-34221G > A–NOS1 (rs1879417)								
G/G	1	0.004	0	0	–	–	–	–
G/A	156	0.681	204	0.726	1.212 (0.828–1.775)	0.323	1.212 (0.827–1.775)	0.324
A/A	72	0.314	77	0.274	0.827 (0.564–1.213)	0.331	0.827 (0.564–1.213)	0.331
				χ^2 = 0.889; P = 0.641				
G	158	0.345	204	0.363	1.161 (0.794–1.696)	0.442	1.161 (0.794–1.696)	0.442
A	300	0.655	358	0.637	0.835 (0.572–1.218)	0.348	0.835 (0.572–1.218)	0.348
c.1823C > T (p.Ser608Leu)–NOS2 (rs2297518)								
C/C	15	0.066	14	0.050	0.751 (0.355–1.590)	0.454	0.745 (0.350–1.585)	–
C/T	67	0.293	78	0.278	0.934 (0.634–1.374)	0.728	0.934 (0.635–1.376)	0.324
T/T	147	0.642	189	0.673	1.140 (0.789–1.646)	0.485	1.140 (0.789–1.647)	0.331
				χ^2 = 0.720; P = 0.396				
C	97	0.212	106	0.189	0.881 (0.657–1.181)	0.396	0.880 (0.656–1.180)	0.393
T	361	0.788	456	0.811	0.881 (0.657–1.181)	0.396	1.136 (0.848–1.524)	0.393
c.-227G > C–NOS2 (rs10459953)								
G/G	78	0.341	118	0.420	1.401 (0.976–2.012)	0.067	1.401 (0.976–2.012)	0.068
G/C	114	0.498	120	0.427	0.752 (0.529–1.068)	0.111	0.991 (0.530–1.068)	0.111
C/C	37	0.162	43	0.153	0.938 (0.581–1.513)	0.792	0.938 (0.581–1.514)	0.793
				χ^2 = 1.990; P = 0.158				
G	270	0.590	356	0.633	1.197 (0.932–1.536)	0.159	1.197 (0.932–1.537)	0.159
C	188	0.410	203	0.367	0.836 (0.651–1.073)	0.159	0.836 (0.651–1.073)	0.159

$P \ll 0.05$ along with corresponding ORs are in bold.
[a] OR adjusted for sex.

3. Result

3.1. Single nucleotide polymorphism of the SOD2, CAT, GPx4, NOS1 and NOS2 gene and depression occurrence

Table 3 shows the distribution of genotypes and alleles of the studied polymorphisms of the *SOD2*, *CAT*, *GPx4*, *NOS1* and *NOS2* gene in the patients with DD and in healthy volunteers. The distribution of the genotypes in all groups was in agreement with the Hardy-Weinberg equilibrium. The results demonstrated that the T/T genotype of the c.47T > C (rs4880) polymorphism of the *SOD2* gene was associated with an increased risk of depression. In the case of c.-89A > T (rs7943316) – *CAT*, the A/A genotype was linked with an increased risk of DD occurrence, while the A/T genotype of the same polymorphism reduced this risk. Moreover, genotype T/T and allele T of c.660T > C (rs713041) – *GPx4* increased the risk of DD development, while the T/C heterozygote and C allele diminished this risk.

No correlation was found between genotypes/alleles of the c.-420-34221G > A (rs1879417)–*NOS1*, c.1823C > T (rs2297518), and c.-227G > C (rs10459953)–*NOS2* polymorphisms, and DD development.

3.2. Single-nucleotide polymorphisms of genes encoding enzymes of oxidative and nitrosative stress, and the age of the first episode of depression and the severity classification on the Hamilton Depression Rating Scale

A difference was found in the age distribution of the first depressive episode between the T/T and T/C genotypes of the c.47T > C–*SOD2* (rs4880) polymorphism. Moreover, a difference between the A/A and A/T genotypes of the c.-89A > T–*CAT* (rs7943316) polymorphism was detected. However, no association was found for the remaining polymorphisms studied (Fig. 1 and Supplementary Fig. 1). Moreover, no significant differences in the distribution of the severity classification on the Hamilton Depression Rating Scale and the genotypes of studied SNPs (data unpublished) were revealed.

3.3. Single-nucleotide polymorphisms of genes encoding enzymes of oxidative and nitrosative stress, and depression occurrence in male and female population

Previous studies have suggested that women have been exposed to a doubled risk of depression development in comparison to men [57]. Therefore, we decided to investigate the association between the prevalence of DD in male/female population and all studied polymorphisms (Table 4). In the case of the c.47T > C (p.Val16Ala)–*SOD2* (rs4880) polymorphism, the T/T genotype and T allele increased the risk of DD in men, while the C allele of the same SNP reduced this risk in the population. However, no correlation between the studied polymorphisms and the risk of DD in female population was detected and confirmed. Furthermore, the A/A genotype of c.-89A > T–*CAT* (rs7943316) decreased the risk of depression in men, while the A/T and T/T genotypes of the same polymorphism reduced the risk in women. The T/T genotype and the T allele of the c.660T > C (rs713041)–*GPx* polymorphism are associated with the occurrence of depression in both groups studied. Moreover, the T/C heterozygote of the same SNP caused an increase of DD risk only in the male population. On the other hand, the C allele of the same polymorphism was associated with a decreased risk in the group of males and females. In the case of c.-227G > C–*NOS2* (rs10459953), the G/C genotype was associated with a reduced risk of depression development in women, while the G/G genotype of the same polymorphism increased this risk in the population. However, no correlation between the polymorphism and the appearance of depressive disorders was found in the male population.

Moreover, the differences between the distribution of genotypes and alleles and the sex of the patients with depression were investigated. It was revealed that the C/C genotype of the c.660T > C–*GPx4* polymorphism occurred about two times more seldom in women than in men (Crude OR [95% CI] = 0.574 [0.343–0.963], $P = 0.035$). This association was not found for the remaining polymorphisms studies (data not published).

3.4. Haplotypes and DD prevalence

Our team also studied the correlation between the occurrence of depressive disorders and haplotypes of the c.1823C > T (rs2297518) and c.-227G > C (rs10459953) polymorphisms of the *NOS2* gene. Supplementary Table 1 shows the association between depression and haplotypes of the studied polymorphisms. No correlation between the studied haplotypes of the two polymorphisms and the risk of DD was found.

3.5. Gene-gene interactions and the risk of depression

We also investigated the correlation between the occurrence of DD and combined genotypes of the studied polymorphisms. The distribution of the combined genotypes is presented in Table 5 and Supplementary Table 2. We observed that the A/T-T/T combined genotype of the c.-89A > T (rs7943316)–*CAT* and c.47T > C (rs4880)–*SOD2* polymorphisms was associated with an increased risk of DD. Moreover, the T/T-T/T combined genotypes of c.47T > C–*SOD2* (rs4880) and c.1823C > T–*NOS2* (rs2297515) caused a nearly five-fold increase of the risk in the Polish population. The link between a reduced risk of depression and the frequency of the T/C-

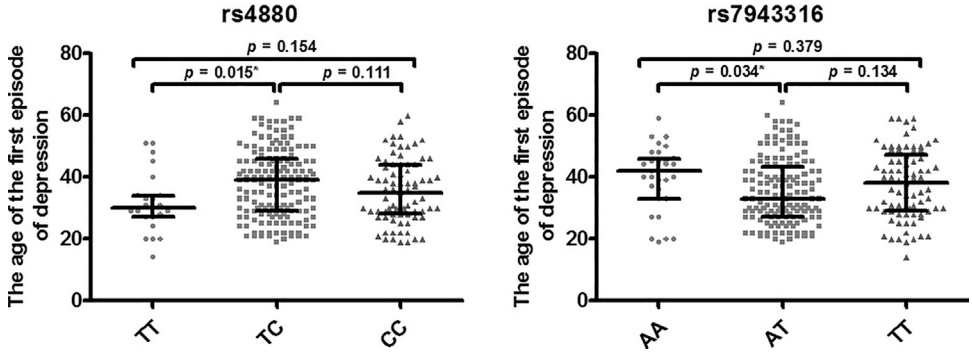

Fig. 1. Distribution of the age of the first episode of depression and single-nucleotide polymorphisms of genes encoding SOD2 and CAT. The horizontal lines denote the median, while the whiskers show the inter-quartile range.

Table 4
Distribution of genotypes and alleles of c.804-7C > A, c.-1668T > A, c.803 + 221C > A, c.-173A > T, c.-1449C > A and c.-844G > T and the risk of DD in male and female population.

Genotype/Allele	Men (n = 268)				Women (n = 242)			
	Control (n = 120)	Depression (n = 148)	Crude OR (95% CI)[a]	P	Control (n = 109)	Depression (n = 133)	Crude OR (95% CI)[a]	P
	N (Freq.)	N (Freq.)			N (Freq.)	N (Freq.)		
c.47T > C (p.Val16Ala)–SOD2 (rs4880)								
T/T	4 (0.033)	17 (0.115)	**3.763 (1.231–11.505)**	**0.020**	5 (0.046)	12 (0.090)	2.063 (0.704–6.048)	0.187
T/C	71 (0.592)	86 (0.581)	0.957 (0.587–1.561)	0.861	79 (0.725)	84 (0.632)	0.561 (0.376–1.127)	0.125
C/C	45 (0.375)	45 (0.304)	0.728 (0.438–1.212)	0.222	25 (0.229)	37 (0.278)	1.295 (0.721–2.327)	0.387
		χ^2 = 4.465; P = 0.035				χ^2 = 0.004; P = 0.949		
T	79 (0.329)	120 (0.405)	**1.561 (1.028–2.369)**	**0.037**	89 (0.408)	108 (0.406)	0.985 (0.616–1.574)	0.949
C	161 (0.671)	176 (0.595)	**0.641 (0.422–0.973)**	**0.037**	129 (0.592)	158 (0.594)	1.015(0.635–1.623)	0.949
c.-89A > T–CAT (rs7943316)								
A/A	25 (0.208)	14 (0.095)	**0.397 (0.196–0.804)**	**0.010**	17 (0.156)	19 (0.143)	0.902 (0.444–1.834)	0.776
A/T	58 (0.483)	89 (0.601)	1.613 (0.991–2.622)	0.054	40 (0.367)	71 (0.534)	**1.975 (1.177–3.314)**	**0.010**
T/T	37 (0.308)	45 (0.304)	0.980 (0.581–1.652)	0.940	52 (0.477)	43 (0.323)	**0.524 (0.311–0.883)**	**0.015**
		χ^2 = 1.868; P = 0.172				χ^2 = 2.474; P = 0.116		
A	108 (0.450)	117 (0.395)	0.773 (0.533–1.120)	0.173	74 (0.339)	109 (0.410)	1.343 (0.928–1.945)	0.118
T	132 (0.550)	179 (0.605)	1.194 (0.893–1.877)	0.173	144 (0.661)	157 (0.590)	0.744 (0.514–1.078)	0.118
c.660T > C–GPx (rs713041)								
T/T	3 (0.025)	27 (0.182)	**8.702 (2.570–29.464)**	**$\ll$ 0.001**	5 (0.046)	26 (0.195)	**5.054 (1.870–13.662)**	**0.001**
T/C	72 (0.600)	67 (0.453)	**0.551 (0.339–0.898)**	**0.017**	66 (0.606)	74 (0.556)	0.817 (0.489–1.367)	0.442
C/C	45 (0.375)	54 (0.365)	0.957 (0.582–1.576)	0.864	3 (0.349)	33 (0.248)	0.617 (0.353–1.076)	0.089
		χ^2 = 9.683; P = 0.002				**χ^2 = 9.683; P = 0.002**		
T	78 (0.325)	121 (0.409)	**1.508 (1.028–2.211)**	**0.035**	76 (0.349)	126 (0.474)	**1.930 (1.262–2.952)**	**0.002**
C	162 (0.675)	175 (0.591)	**0.663 (0.452–0.972)**	**0.035**	142 (0.651)	140 (0.526)	**0.518 (0.339–0.792)**	**0.002**
c.1823C > T (p.Ser608Leu)–NOS2 (rs2297518)								
C/C	12 (0.100)	10 (0.068)	0.652 (0.272–1.566)	0.339	3 (0.280)	4 (0.030)	1.096 (0.240–5.004)	0.906
C/T	35 (0.292)	35 (0.236)	0.752 (0.435–1.299)	0.307	32 (0.294)	4 (0.323)	1.150 (0.664–1.992)	0.619
T/T	73 (0.608)	103 (0.696)	1.474 (0.888–2.447)	0.134	74 (0.679)	86 (0.647)	0.865 (0.506–1.480)	0.598
		χ^2 = 0.251; P = 0.616				χ^2 = 0.004; P = 0.949		
C	59 (0.246)	55 (0.186)	0.746 (0.511–1.088)	0.128	38 (0.174)	51 (0.192)	1.128 (0.703–1.809)	0.617
T	181 (0.754)	241 (0.814)	1.341 (0.919–1.956)	0.128	180 (0.826)	215 (0.808)	0.886 (0.553–1.422)	0.617
c.-420-34221G > A–NOS1 (rs1879417)								
G/G	0 (–)	0 (–)	–	–	1 (0.009)	0 (–)	–	–
G/A	82 (0.068)	108 (0.730)	1.251 (0.737–2.123)	0.406	74 (0.679)	96 (0.722)	1.227 (0.706–2.133)	0.468
A/A	38 (0.317)	40 (0.270)	0.799 (0.471–1.356)	0.406	34 (0.312)	37 (0.278)	0.850 (0.488–1.481)	0.567
		χ^2 = 0.889; P = 0.641				χ^2 = 0.169; P = 0.681		
G	82 (0.342)	108 (0.365)	1.251 (0.737–2.123)	0.406	76 (0.349)	96 (0.361)	1.121 (0.649–1.938)	0.681
A	158 (0.658)	188 (0.635)	0.799 (0.471–1.356)	0.406	142 (0.651)	170 (0.639)	0.892 (0.516–1.541)	0.681
c.-227G > C–NOS2 (rs10459953)								
C/C	19 (0.158)	21 (0.142)	0.879 (0.448–1.724)	0.707	18 (0.165)	22 (0.165)	1.002 (0.507–1.981)	0.995
G/C	54 (0.450)	68 (0.459)	1.039 (0.641–1.685)	0.877	60 (0.550)	52 (0.391)	**0.524 (0.314–0.876)**	**0.014**
G/G	47 (0.392)	59 (0.399)	1.030 (0.629–1.685)	0.907	31 (0.284)	59 (0.444)	**2.006 (1.171–3.438)**	**0.011**
		χ^2 = 0.075; P = 0.784				χ^2 = 3.066; P = 0.080		
C	92 (0.383)	110 (0.372)	0.953 (0.674–1.347)	0.784	96 (0.440)	96 (0.361)	0.724 (0.503–1.041)	0.082
G	148 (0.617)	186 (0.628)	1.050 (0.743–1.483)	0.784	122 (0.560)	170 (0.639)	1.381 (0.960–1.987)	0.082

$P \ll 0.05$ along with corresponding ORs are in bold.
[a] OR adjusted for sex.

C/G genotype of the c.47T > C (rs4880) – *SOD2* and c.-227G > C (rs10459953) – *NOS2* polymorphisms was confirmed in our study. Additionally, the presence of the T/C-T/T combined genotype of the c.47T > C (rs4880)–*SOD2* and c.660T > C (rs713041)–*GPx4* polymorphisms elevated the risk of depression development more than 11 times. On the other hand, the T/C-T/C and T/C-C/C genotypes of the same combination of polymorphisms were associated with a diminished risk of DD occurrence. The A/T-T/T and T/T-T/T genotypes of c.-89A > T (rs7943316)–*CAT* and c.660T > C (rs713041)–*GPx4* caused an increase of the risk of DD by just about six times, whereas the A/T-T/C and T/T-C/C genotypes of the same combined polymorphisms brought about a reduction of this risk. In the case of c1823C > T (rs2297518) and c. 660T > C (rs713041), combined genotypes T/T–T/T were associated with a twelvefold increase of the DD risk. Moreover, we found that the G/A-T/T genotype of c.-420-3422G > A (rs187944)–*NOS1* and c.660T > C (rs713041)–*GPx4* increased the risk of depression nearly sevenfold. However, the G/C-T/T and G/G-T/T genotypes of the c.-227G > C (rs10459953)–*NOS2* and c.660T > C (rs713041)–*GPx4* combined polymorphisms were positively correlated with depression, while genotype G/C-C/C of the same SNP combination was negatively correlated with the disease. On the other hand, the T/T-C/T genotype of the c.-89A > T (rs7943316)–*CAT* and c.1823C > T (rs2297518)–*NOS2* combined polymorphisms increased the risk of depression, while the T/T-T/T genotype of the same combination reduced the risk. In the case of c.-89A > T (rs7943316)–*CAT* and c.-227G > C (rs10459953)–*NOS2*, combined genotype A/T-G/G was associated with occurrence of DD. Moreover, the A/T-G/A genotype of the c.-89A > T (rs7943316)–*CAT* and c.-420-34221G > A (rs1879417)–*NOS1* combined polymorphisms increased the risk of depression occurrence, while the A/A-A/A genotype of the same polymorphisms combination reduced this risk.

No statistical correlation was found between combined genotypes of c.47T > C (rs4880)–*SOD2* and c.-420-34221G > A (rs1879417)–*NOS1*, c.1823C > T (rs2297518)–*NOS2* and c.-420-34221G > A (rs1879417)–*NOS1*, c.-420-34221G > A (rs1879417)–*NOS1* and c.-227G > C (rs10459953)–*NOS2* SNPs and the development of depressive disorders.

Table 5
Distribution of the combined genotype of the studied polymorphisms and risk of the depression.

Combined genotype	Control ($n = 229$)		Depression ($n = 281$)		Crude OR (95% CI)[a]	P	Adjusted OR (95% CI)[a]	P
	Number	Frequency	Number	Frequency				
c.-89A > T (rs7943316)–CAT and c.47T > C (p.Val16Ala)–SOD2 (rs4880)								
A/A-T/T	3	0.013	3	0.011	0.813(0.163–)4.067	0.801	0.813 (0.163–4.068)	0.801
A/A-T/C	26	0.114	20	0.071	0.598 (0.325–1.102)	0.099	0.598 (0.325–1.102)	0.099
A/A-C/C	13	0.057	10	0.036	0.613 (0.264–1.425)	0.256	0.613 (0.264–1.425)	0.256
A/T-T/T	**2**	**0.009**	**13**	**0.046**	**5.506 (1.229–24.654)**	**0.026**	**5.506 (1.229–24.661)**	**0.026**
A/T-T/C	68	0.297	96	0.342	1.229 (0.844–1.789)	0.283	1.229 (0.844–1.789)	0.283
A/T-C/C	28	0.122	51	0.181	1.592 (0.967–2.620)	0.068	1.599 (0.968–2.639)	0.067
T/T-T/T	4	0.017	13	0.046	2.729 (0.877–8.485)	0.083	2.728 (0.877–8.485)	0.083
T/T-T/C	56	0.245	54	0.192	0.735 (0.481–1.122)	0.153	0.733 (0.479–1.122)	0.153
T/T-C/C	29	0.127	21	0.075	0.557 (0.308–1.006)	0.052	0.557 (0.308–1.006)	0.052
c.47T > C (p.Val16Ala)–SOD2 (rs4880) and c.1823C > T (p.Ser608Leu)–NOS2 (rs2297518)								
T/T-C/C	2	0.009	5	0.018	2.056 (0.395–10.698)	0.392	2.057 (0.394–10.748)	0.393
T/T-C/T	4	0.017	7	0.025	1.437 (0.415–4.971)	0.567	1.438 (0.416–4.977)	0.566
T/T-T/T	**3**	**0.013**	**17**	**0.060**	**4.851 (1.404–16.766)**	**0.013**	**4.853 (1.404–16.774)**	**0.013**
T/C-C/C	7	0.031	7	0.025	0.810 (0.280–2.344)	0.698	0.809 (0.279–2.343)	0.696
T/C-C/T	43	0.188	52	0.185	0.982 (0.628–1.537)	0.937	0.983 (0.628–1.540)	0.940
T/C-T/T	100	0.437	111	0.395	0.842 (0.591–1.200)	0.342	0.842 (0.591–1.201)	0.343
C/C-C/C	6	0.026	2	0.007	0.266 (0.053–1.333)	0.107	0.263 (0.052–1.321)	0.105
C/C-C/T	20	0.087	19	0.068	0.758 (0.394–1.457)	0.406	0.758 (0.394–1.457)	0.405
C/C-T/T	44	0.192	61	0.217	1.166 (0.755–1.800)	0.489	1.166 (0.754–1.801)	0.490
c.47T > C (p.Val16Ala)–SOD2 (rs4880) and c.-227G > C–NOS1 (rs10459953)								
T/T-C/C	0	–	5	0.018	–	–	–	–
T/T-C/G	4	0.017	12	0.043	2.509 (0.798–7.888)	0.115	2.509 (0.798–7.892)	0.116
T/T-G/G	5	0.022	12	0.043	1.999 (0.694–5.758)	0.200	1.998 (0.693–5.758)	0.200
T/C-C/C	23	0.100	25	0.089	0.875 (0.482–1.586)	0.659	0.875 (0.482–1.588)	0.661
T/C-C/G	**76**	**0.332**	**63**	**0.224**	**0.582 (0.393–0.862)**	**0.007**	**0.581 (0.392–0.861)**	**0.007**
T/C-G/G	51	0.223	82	0.292	1.438 (0.960–2.154)	0.078	1.439 (0.961–2.154)	0.078
C/C-C/C	14	0.061	13	0.046	0.745 (0.343–1.618)	0.457	0.744 (0.342–1.617)	0.455
C/C-C/G	34	0.148	45	0.160	1.094 (0.674–1.775)	0.717	1.093 (0.673–1.774)	0.719
C/C-G/G	22	0.096	24	0.085	0.879 (0.479–1.612)	0.676	0.877 (0.478–1.611)	0.673
c.47T > C (p.Val16Ala)–SOD2 (rs4880) and c.660T > C–GPx4 (rs713041)								
T/T-T/T	1	0.004	5	0.018	4.130 (0.479–35.608)	0.197	4.129 (0.479–35.613)	0.197
T/T-T/C	6	0.026	15	0.053	2.096 (0.800–5.492)	0.132	2.101 (0.801–5.508)	0.131
T/T-C/C	2	0.009	9	0.032	3.756 (0.803–17.558)	0.093	3.762 (0.803–17.619)	0.093
T/C-T/T	**3**	**0.013**	**36**	**0.128**	**11.069 (3.362–36.444)**	**≪ 0.001**	**11.222 (3.403–37.006)**	**≪ 0.001**
T/C-T/C	**85**	**0.371**	**80**	**0.285**	**0.674 (0.464–0.979)**	**0.038**	**0.672 (0.462–0.978)**	**0.038**
T/C-C/C	**62**	**0.271**	**54**	**0.192**	**0.641 (0.423–0.971)**	**0.036**	**0.639 (0.421–0.970)**	**0.035**
C/C-T/T	4	0.017	12	0.043	2.509 (0.798–7.888)	0.115	2.512 (0.798–7.908)	0.115
C/C-T/C	47	0.205	46	0.164	0.758 (0.483–1.189)	0.228	0.756 (0.482–1.188)	0.225
C/C-C/C	19	0.083	24	0.085	1.032 (0.550–1.936)	0.921	1.032 (0.550–1.935)	0.922
c.-89A > T (rs7943316)–CAT and c.660T > C–GPx4 (rs713041)								
A/A-T/T	1	0.004	5	0.018	4.130 (0.479–35.608)	0.197	4.138 (0.479–35.775)	0.197
A/A-T/C	**28**	**0.122**	**14**	**0.050**	**0.376 (0.193–0.733)**	**0.004**	**0.376 (0.193–0.733)**	**0.004**
A/A-C/C	13	0.057	14	0.050	0.871 (0.401–1.893)	0.728	0.871 (0.401–1.892)	0.727
A/T-T/T	**4**	**0.017**	**29**	0.103	**6.473 (2.421–18.698)**	**≪ 0.001**	**6.493 (2.247–18.762)**	**≪ 0.001**
A/T-T/C	60	0.262	80	0.285	1.121 (0.757–1.660)	0.568	1.121 (0.757–1.660)	0.569
A/T-C/C	34	0.148	51	0.181	1.272 (0.792–2.043)	0.320	1.273 (0.791–2.049)	0.321
T/T-T/T	**3**	**0.013**	**19**	**0.068**	**5.463 (1.596–18.701)**	**0.007**	**5.475 (1.599–18.747)**	**0.007**
T/T-C/T	50	0.218	47	0.167	0.719 (0.462–1.120)	0.145	0.718 (0.460–1.120)	0.145
T/T-C/C	**36**	**0.157**	**22**	**0.078**	**0.455 (0.260–0.799)**	**0.006**	**0.455 (0.260–0.799)**	**0.006**
c.1823C > T (p.Ser608Leu)–NOS2 (rs2297518) and c.660T > C–GPx4 (rs713041)								
C/C-T/T	1	0.004	1	0.004	0.814 (0.051–13.090)	0.885	0.810 (0.050–13.079)	0.882
C/C-T/C	10	0.044	7	0.025	0.559 (0.210–1.494)	0.246	1.226 (0.341–4.403)	0.755
C/C-C/C	4	0.017	6	0.021	1.227 (0.342–4.402)	0.753	1.226 (0.341–4.403)	0.755
C/T-T/T	4	0.017	12	0.043	2.509 (0.798–7.888)	0.115	2.520 (0.800–7.932)	0.114
C/T-T/C	34	0.148	40	0.142	0.952 (0.580–1.561)	0.845	0.953 (0.580–1.566)	0.849
C/T-C/C	29	0.127	26	0.093	0.703 (0.401–1.232)	0.218	0.702 (0.400–1.231)	0.217
T/T-T/T	**3**	**0.013**	**40**	**0.142**	**12.503 (3.814–40.985)**	**≪ 0.001**	**12.508 (3.816–41.000)**	**≪ 0.001**
T/T-T/C	94	0.410	94	0.335	0.722 (0.503–1.036)	0.077	0.722 (0.503–1.037)	0.078
T/T-C/C	50	0.218	55	0.196	0.871 (0.567–1.340)	0.530	0.871 (0.566–1.339)	0.528
c.-89A > T–CAT (rs7943316) and c.1823C > T (p.Ser608Leu)–NOS2 (rs2297518)								
A/A-C/C	5	0.022	2	0.007	0.321 (0.062–1.671)	0.177	0.320 (0.061–1.666)	0.176
A/A-C/T	5	0.22	2	0.007	0.321 (0.062–1.671)	0.177	0.318 (0.061–1.661)	0.174
A/A-T/T	5	0.022	10	0.036	1.653 (0.557–4.907)	0.365	1.654 (0.556–4.921)	0.366
A/T-C/C	14	0.061	10	0.036	0.567 (0.247–1.301)	0.180	0.567 (0.247–1.302)	0.181
A/T-C/T	31	0.135	44	0.157	1.186 (0.722–1.949)	0.501	1.185 (0.721–1.949)	0.503
A/T-T/T	22	0.096	24	0.085	0.879 (0.479–1.612)	0.676	0.879 (0.478–1.618)	0.679
T/T-C/C	23	0.100	21	0.075	0.723 (0.389–1.344)	0.305	0.723 (0.389–1.343)	0.305
T/T-C/T	**62**	**0.271**	**114**	**0.406**	**1.839 (1.262–2.679)**	**0.002**	**1.840 (1.262–2.682)**	**0.002**
T/T-T/T	**62**	**0.271**	**54**	**0.192**	**0.641 (0.423–0.971)**	**0.036**	**0.640 (0.422–0.971)**	**0.036**
c.-420-34221G > A–NOS1 (rs1879417) and c.660T > C–GPx4 (rs713041)								
G/G-T/T	0	–	0	–	–	–	–	–
G/G-T/C	0	–	0	–	–	–	–	–
G/G-C/C	1	0.004	0	–	–	–	–	–

Table 5 (*Continued*)

Combined genotype	Control ($n = 229$)		Depression ($n = 281$)		Crude OR (95% CI)[a]	P	Adjusted OR (95% CI)[a]	P
	Number	Frequency	Number	Frequency				
G/A-T/T	**6**	**0.026**	**43**	**0.153**	**6.715 (2.804–16.084)**	**≪ 0.001**	**6.718 (2.804–16.090)**	**0.001**
G/A-T/C	94	0.410	97	0.345	0.757 (0.528–1.085)	0.130	0.757 (0.528–1.086)	0.130
G/A-C/C	56	0.245	64	0.228	0.911 (0.604–1.374)	0.657	0.910 (0.603–1.373)	0.653
A/A-T/T	2	0.009	10	0.036	4.188 (0.908–19.311)	0.066	4.199 (0.910–19.371)	0.066
A/A-T/C	44	0.192	44	0.157	0.781 (0.493–1.236)	0.291	0.781 (0.493–1.237)	0.292
A/A-C/C	26	0.114	23	0.082	0.696 (0.386–1.256)	0.229	0.695 (0.385–1.255)	0.228
c.-227G > C–NOS2 (rs10459953) and c.660T > C–GPx4 (rs713041)								
C/C-T/T	1	0.004	9	0.032	7.544 (0.949–59.993)	0.056	7.569 (0.951–60.223)	0.056
C/C-T/C	5	0.109	21	0.075	0.659 (0.359–1.211)	0.179	0.659 (0.359–1.211)	0.179
C/C-C/C	11	0.048	13	0.045	0.961 (0.422–2.188)	0.925	0.962 (0.422–2.191)	0.927
G/C-T/T	**2**	**0.009**	**24**	**0.085**	**10.599 (2.478–45.344)**	**0.001**	**10.605 (2.479–45.372)**	**0.001**
G/C-T/C	67	0.293	61	0.27	0.670 (0.449–1.002)	0.051	0.670 (0.448–1.002)	0.051
G/C-C/C	**45**	**0.197**	**35**	**0.125**	**0.582 (0.360–0.941)**	**0.027**	**0.580 (0.358–0.939)**	**0.027**
G/G-T/T	**5**	**0.022**	**20**	**0.071**	**3.433 (1.268–9.295)**	**0.015**	**3.433 (1.268–9.296)**	**0.015**
G/G-T/C	46	0.201	59	0.210	1.057 (0.686–1.629)	0.801	1.058 (0.686–1.630)	0.800
G/G-C/C	27	0.118	39	0.139	1.206 (0.713–2.038)	0.485	1.205 (0.712–2.040)	0.486
c.-89A > T–CAT (rs7943316) and c.-227G > C–NOS2 (rs10459953)								
A/A-C/C	6	0.026	6	0.021	0.811(0.258–2.549)	0.720	0.812 (0.258–2.558)	0.722
A/A-C/G	19	0.083	14	0.050	0.580 (0.284–1.183)	0.134	0.580 (0.284–1.184)	0.134
A/A-G/G	17	0.074	13	0.046	0.602 (0.286–1.271)	0.183	0.602 (0.286–1.270)	0.183
A/T-C/C	13	0.057	23	0.082	1.481 (0.733–2.994)	0.274	1.481 (0.733–2.993)	0.274
A/T-C/G	55	0.240	68	0.241	1.010 (0.672–1.519)	0.962	1.009 (0.670–1.519)	0.965
A/T-G/G	**30**	**0.131**	**69**	**0.246**	**2.159 (1.349–3.455)**	**0.001**	**2.159 (1.349–3.456)**	**0.001**
T/T-C/C	18	0.079	14	0.050	0.615 (0.299–1.265)	0.186	0.615 (0.299–1.265)	0.186
T/T-C/G	40	0.175	38	0.135	0.739 (0.456–1.198)	0.219	0.738 (0.455–1.199)	0.220
T/T-G/G	31	0.135	36	0.128	0.939 (0.560–1.571)	0.809	0.939 (0.561–1.573)	0.811
c.-89A > T–CAT (rs7943316) and c.-420-34221G > A–NOS1 (rs1879417)								
A/A-G/G	0	–	0	–	–	–	–	–
A/A-G/A	26	0.114	29	0.103	0.899 (0.513–1.574)	0.708	0.898 (0.513–1.574)	0.708
A/A-A/A	**16**	**0.070**	**4**	**0.014**	**0.192 (0.063–0.583)**	**0.004**	**0.192 (0.063–0.583)**	**0.004**
A/T-G/G	0	–	0	–	–	–	–	–
A/T–G/A	**67**	**0.293**	**115**	**0.409**	**1.675 (1.156–2.427)**	**0.006**	**1.677 (1.157-2.432)**	**0.006**
A/T–A/A	31	0.135	45	0.160	1.218 (0.742–1.998)	0.435	1.218 (0.742-1.998)	0.436
T/T-G/G	1	0.004	0	–	–	–	–	–
T/T-G/A	63	0.275	60	0.214	0.715 (0.476–1.075)	0.107	0.715 (0.475–1.075)	0.107
T/T-A/A	25	0.109	28	0.100	0.903 (0.511–1.597)	0.726	0.904 (0.511–1.598)	0.727

$P \ll 0.05$ along with corresponding ORs are in bold.
[a] OR adjusted for sex.

3.6. Single-nucleotide polymorphisms of genes encoding oxidative and nitrosative stress enzymes, and effectiveness of depression treatment

We also studied the impact of single-nucleotide polymorphisms of genes encoding enzymes, generating ROS and NOS, on the effectiveness of antidepressant treatment with the administration of SSRI. The patients were divided into two groups–those who received a maximum of 7 points on the Hamilton Rating Scale for Depression after treatment (marked as effectiveness of antidepressant therapy), and those whose total score after treatment was more than 7 points (marked as ineffective antidepressant therapy). No impact of single-nucleotide polymorphisms of genes encoding enzymes, generating of ROS and NOS, on the effectiveness of the SSRIs therapy was found (data not shown). Moreover, we investigated the distribution of genotypes of the studied polymorphism and the percentage of the Hamilton Rating Scale for Depression (Supplementary Fig. 2), yet no difference in the percentage dispersion of the Hamilton Rating Scale for Depression between genotypes of the studied polymorphism was confirmed.

4. Discussion

Previous studies suggest that the intensification of oxidative and nitrosative stress processes may play a crucial role in depression development [58]. As mentioned in the Introduction, these abnormalities may be a result of the irregular functioning of the enzymes involved in the generation and elimination of ROS and RNS, such as SOD2, CAT, GPx4, NOS1 and NOS2. This is the first study to show that the chosen SNPs of genes encoding these proteins may modulate the risk of DD.

One of the most effective intracellular antioxidants is manganese superoxide dismutase (encoded by the *SOD2* gene which is located on chromosome 6q25), which is a key mitochondrial enzyme and protects the cell against ROS [59]. In our study, we found the association between depression development and the occurrence of c.47T > C SNP (rs4880)–*SOD2*. The studied SNP brings about an transformation of amino acid from valine (Val) to alanine (Ala) at position 16, which – as a result – leads to a conformational change in the target sequence of SOD2 and decreases its antioxidant potential in mitochondria [60,61]. Moreover, it has been suggested that the T allele may be associated with lower enzymatic efficiency and the risk of higher ROS levels [62]. In addition, another study confirmed that the Val variant may induce a 30 to 40% increase in SOD2 activity in mitochondria [63]. Accordingly, our results suggest that the T/T homozygotes increase the risk of DD (Table 3). However, this genotype is associated with the development of depression only in Polish men (Table 4). Such results may reflect the differences between sexes in the regulation of enzymatic activity. So far, the polymorphism has been studied in somatic diseases and a previous study revealed that the T/T genotype was positively correlated with the occurrence of migraine symptoms in Caucasian population [64]. Another study demonstrated that the T/T and T/C genotypes were correlated with the development of medulloblastoma in children [65].

The next important antioxidant enzyme, which converts hydrogen peroxide into water and oxygen, is CAT. Its gene is located on chromosome 11p13, while the studied c.89A > T polymorphism (rs7943316) of the gene is present in its promoter region and may cause a decrease of its expression and enzyme activity [66]. So far, the CAT polymorphism has not been studied in mental disorders, but it has been shown that the T/T genotype and T allele are more frequent in the patients with vitiligo as compared to controls in the population of Gujarat [67]. Similarly, we found that the A/A genotype of the polymorphism was associated with a decreased risk of depression development in Polish population, while the A/T genotype elevated this risk (Table 3). However, we found that the A/T and T/T genotypes were linked with a reduced risk of depression development in females, while the A/A genotype was associated with a decreased risk in males (Table 4). Such a discrepancy may be a result of differences in the regulation of enzymatic activity between men and women.

The next studied gene, *GPx4*, is located on chromosome 19p13.3 and encodes selenoprotein, which catalyses the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by means of glutathione oxidation [68–70]. We were the first to detect that polymorphism c.660T > C (rs713041) of *GPx4* may modulate the risk of depression development. The studied SNP is located near the insertion sequence element in the gene's 3′ untranslated region (3′UTR) and alters the protein-binding site to this region. Studies suggest that the C variant determines higher expression of *GPx* and protein activity than the T variant [71,72]. Accordingly, we found that the T/T homozygote increased the risk of DD (Table 3). In view of that, we demonstrated that the C/C homozygote was less common in women with DD than in depressed men (Table 4.). Similar results were obtained in the case of the patients with hypertension; the T/T genotype of the studied *GPx4* SNP was associated with the occurrence of cerebral stroke in the patients with essential hypertension [73]. On the other hand, it was indicated that the heterozygote was associated with an increased risk of death in the patients with breast cancer, and correlated with the occurrence of colorectal cancer [74,75]; our results indicate the T/C carriers reduce the risk of DD.

The next three polymorphisms studied are located in the gene encoding nitric oxide synthase which catalyses conversion of L-arginine to nitric oxide. So far, three isoforms of this enzyme have been described, i.e. the neuronal type I (NOS1) and the endothelial type (eNOS), which are constitutively expressed and regulated by calmodulin and Ca^{2+}, and the inducible type (iNOS or NOS2). NOS1 generates NO in the nervous tissue, while iNOS produces large quantities of this compound upon stimulation by proinflammatory cytokines [3,76]. Literature suggests that immune system deregulation, including abnormal levels of interleukin 1beta (IL)-1β, interleukin 6 (IL-6), interleukin 11 (IL-11), tumour necrosis factor-alpha (TNF-α), and C-reactive protein (CRP), may increase the production of NO and, as a consequence, may lead to the development of DD [77]. Additionally, animal studies confirmed that administration of IL-1β may cause behavioural alterations and the occurrence of symptoms similar to those observed in major depression, such as anhedonia, anorexia, weight loss, social withdrawal, psychomotor retardation, irritability, and sleep disturbances. On the other hand, increased levels of regulatory T cells (CD4(+)CD25(hi) Tregs) during an antidepressant therapy can be the reason for a decrease in cytokine production and recovery from depression. Thus, regulatory T cells can indirectly reduce the production of reactive nitrogen forms and may improve mental health.

NOS1 is located on chromosome 12q24 [78], while *NOS2* is located at 17q11.2–q12 [79]. The previous study showed that c.-420-34221G > A–*NOS1* and c.1823C > T–*NOS2* were linked with the longevity phenotype. Moreover, the C allele of the former SNP was associated with weakened cognitive performance in geriatric patients. In addition, this allele was associated with a lower probability of survival until very old age [77]. The latter polymorphism is located at exon 16 of *NOS2*, which partly encodes the reductase domain [80], and thus may alter the structure or function of NOS2 (F-SNP database). We were the first to study the association between NOS1 and NOS2 polymorphisms and the development of DD. No correlation between these three studied polymorphisms of genes encoding NOS and the risk of depression was found in this paper (Table 3). On the other hand, the gene-gene analysis demonstrated that genotypes of combined SNPs may strongly modulate the risk of DD occurrence (Table 5). Moreover, we confirmed that the G/C and G/G genotypes of the c.-227G > C–*NOS2* (rs10459953) polymorphism were associated with the development of depression in Polish women, while no such association was found for the male population (Table 4). So far, it has been shown that *NOS2* SNP is not correlated with the male infertility risk in Chinese population [81]. However, the T allele of the same polymorphism increases the risk of nephritis in Henoch-Schönlein purpura children [81]. Moreover, the C/C genotype and the C allele bring about an increased risk of recurrent aphthous stomatitis, whereas such a correlation has not been found in the case of the c.-227G > C polymorphism [82]. On the other hand, the T/T genotype of c.1823C > T SNP increases the risk of benign prostatic hyperplasia in Korean men, while c.-227G > C does not modulate this risk [83]. However, a recent study has shown that the c.-227G > C polymorphism of *NOS2* is linked with susceptibility to Type 2 Diabetes Mellitus and Diabetic Nephropathy in the Chinese Han population [84].

5. Conclusion

We have demonstrated that the selected SNPs of the genes involved in oxidative and nitrosative stress may have an impact on the risk of developing depressive disorders. We have found that the studied SNPs of the *SOD2* and *GPx4* gene may modulate depression occurrence. Therefore, these polymorphisms may be considered an independent marker of depression. Our study supports the hypothesis that oxidative and nitrosative stress may be involved in the development of depression.

Disclosure of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eurpsy.2017.10.012.

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Association between single nucleotide polymorphisms of TPH1 and TPH2 genes, and depressive disorders

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Abstract

Tryptophan catabolites pathway disorders are observed in patients with depression. Moreover, single nucleotide polymorphisms of tryptophan hydroxylase genes may modulate the risk of depression occurrence. The objective of our study was to confirm the association between the presence of polymorphic variants of TPH1 and TPH2 genes, and the development of depressive disorders. Six polymorphisms were selected: c.804-7C>A (rs10488682), c.-1668T>A (rs623580), c.803+221C>A (rs1800532), c.-173A>T (rs1799913)—*TPH1*, c.-1449C>A (rs7963803), and c.-844G>T (rs4570625)—*TPH2*. A total of 510 DNA samples (230 controls and 280 patients) were genotyped using TaqMan probes. Among the studied polymoorphisms, the G/G genotype and G allele of c.804-7C>A—*TPH1*, the T/T homozygote of c.803+221C>A—*TPH1*, the A/A genotype and A allele of c.1668T>A—*TPH1*, the G/G homozygote and G allele of c.-844G>T—*TPH2*, and the C/A heterozygote and A allele of c.-1449C>A—*TPH2* were associated with the occurrence of depression. However, the T/T homozygote of c.-1668T>A—*TPH1*, the G/T heterozygote and T allele of c.-844G>T—*TPH2*, and the C/C homozygote and C allele of c.-1449C>A—*TPH2* decreased the risk of development of depressive disorders. Each of the studied polymorphisms modulated the risk of depression for selected genotypes and alleles. These results support the hypothesis regarding the involvement of the pathway in the pathogenesis of depression.

Keywords: depression • tryptophan catabolites pathways • tryptophan hydroxylase • single nucleotide polymorphism

Introduction

Although the pathogenesis of depression (depressive disorder—DD) is not fully understood, studies suggest that disturbances in the TRYCATs pathway may play a key role in the development of this disease. A reduced level of tryptophan in plasma may lead to mood disorders in patients [1, 2]. Moreover, increased plasma levels of harmful tryptophan metabolites—*i.e.* kynurenine, xanthurenic acid and quinolinic acid—were found in depressed patients [1, 2]. Quinolinic acid may cause the destruction of postsynaptic structures and neurons *via* apoptosis of hippocampal cells and selective necrosis of granular cells. Additionally, it reduces the levels of dopamine, choline and γ-aminobutyric acid (GABA) [3–5]. On the other hand, studies showed that some tryptophan metabolites, for example kynurenic acid, may exhibit neuroprotective and antidepressant properties [2].

Recent findings have revealed that the patients with DD are characterized by greater activity of 2,3-dioxygenase tryptophan (TDO) and 2,3-dioxygenase indoleamine (IDO) as compared to healthy volunteers. Both are rate-limiting enzymes in tryptophan metabolism [6]. IDO/TDO converts tryptophan into kynurenine, which may be later metabolized into neurotoxic compounds, such as quinolinic acid. As a result, depressed patients are characterized by an increased kynurenine/tryptophan ratio and decreased serotonin/tryptophan ratio [1, 2].

Additionally, toxic TRYCATs can bring about increased production of reactive oxygen species (ROS). For example, 3-hydroxykynurenine can induce neuronal apoptosis *via* overproduction of ROS [7]. Moreover, kynurenine may penetrate the blood–brain barrier and exhibits toxic effects in the brain. As a consequence, it can lead to spreading cortical and subcortical atrophy [8].

Decreased levels of serotonin (5-TH) or its receptors are also associated with depressed mood [9]. This neurotransmitter is synthesized by tryptophan hydroxylase (TPH) [10]. TPH is an enzyme which

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is involved in the initial and rate-limiting step in the synthesis of serotonin and melatonin. It is responsible for the addition of a hydroxyl group to tryptophan and for the creation of amino acid 5-hydroxytryptophan. There are two distinct genes encoding TPH in humans—*TPH1* and *TPH2*. Studies show that different expression levels of TPH genes may be related to aggression, schizophrenia, alcoholism, drug abuse, suicidality and depression [11–15]. Placidi *et al.* [16] demonstrated that a low level of 5-hydroxyindoleacetic acid (5-HIAA) (the main metabolite of serotonin) in cerebrospinal fluid may be associated with suicidal attempts in DD. Moreover, a recent study has suggested that TPH2 expression in the midbrain is implicated in the antidepressant action of selective serotonin reuptake inhibitors (SSRI) [17]. TPH2 may be a good candidate for a biomarker in pharmacogenetic studies of SSRI efficacy. Low levels of melatonin, which is also created in the TRYCATs pathway, are observed in patients suffering from Alzheimer's disease, carcinoma, anorexia and depression [18]. About 80% of depressed patients exhibit different sleep disturbances. Persistent or worsening insomnia may contribute to the risk of depression recurrence and may increase its severity. In addition, severe insomnia occurs more frequently in the patients with depressive suicidal attempts than in the patients without such attempts [19]. Therefore, in this article, we have decided to examine the relationship between six SNPs in the following genes responsible for encoding key TRYCAT enzymes: *TPH1* and *TPH2*.

Materials and methods

Volunteers

The study was carried out on a group of 280 patients suffering from DD, hospitalized at the Department of Adult Psychiatry of the Medical University of Lodz (Poland), and 230 healthy volunteers, randomly selected without replacement sampling. The volunteers taking part in the experiment were native Poles from central Poland (not related). The characteristics of the patients are presented in Table 1. The inclusion criteria were based on those outlined in ICD-10 and APA (F32.0-7.32.2, F330-F33.8) [20, 21]. The exclusion criteria included the presence of axis I and axis II disorders, other than DD, severe and chronic somatic diseases, injuries of the central nervous system, inflammatory or autoimmune disorders, and unwillingness to give informed consent. Additionally, volunteers with familial prevalence of mental disorders, other than recurrent depressive disorders, were excluded from the examined group. Medical history for all cases was obtained in accordance with the Standardized Composite International Diagnostic Interview (CIDI) prior to the start of the experiment [22]. The 21-item Hamilton Depression Rating Scale (HDRS-21) was used to evaluate and classify depression severity [23]. The scores presented in a study conducted by Demyttenaere and De Fruyt [24] were used in the measurements of intensity levels of DD symptoms. Each patient was examined by the same psychiatrist (CIDI and HDRS); psychiatric evaluation was performed before the patient was enrolled to take part in the study. Participation in the study was voluntary, and the volunteers were informed of the purpose, assured of the voluntary character of the experiment, and guaranteed that their personal data would be kept in secret before deciding to participate in the study. According to the protocol approved by the Bioethics Committee of the Medical University of Lodz (no. RNN/70/14/KE), all the volunteers consented to participate in the study.

Table 1 Characteristics of the investigated controls and patients

Characteristics	Controls (n = 230)	Patients (n = 280)
Sex (male/female)	114/116	148/132
Age (mean ± S.D.)	53.19 ± 12.61	49.53 ± 10.175
Age of onset (mean ± S.D.)	–	36.64 ± 10.89
HDRS-21 (mean ± S.D.)	–	23.50 ± 6.14

Selection of SNPs

The public domain of the database for SNPs of the National Center for Biotechnology Information (NCBI dbSNP), available at http://www.ncbi.nlm.nih.gov/snp (Bethesda, Montgomery County, MD, USA), was used to choose the studied polymorphisms. The selection criteria regarding SNPs were as follows: Their minor allele frequency had to be larger than 0.05 (submitter population ID: HapMap-CEU), and they had to be localized either in the coding or regulatory region of the genes (Table 2).

DNA extraction

Genomic DNA was isolated from venous blood according to the Blood Mini Kit protocol (A&A Biotechnology, Gdynia, Poland). Blood samples were collected from the patients suffering from DD before commencement of the antidepressant therapy. The purity of the DNA samples was measured spectrophotometrically by calculating the ratio between absorbance at 260 and 280 nm; after that, the samples were stored at −20°C.

Genotyping

The chosen SNPs were genotyped using the TaqMans SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, MA, USA) and 2× Master Mix Takyon for Probe Assay—No ROX (Eurogentec, Liège, Belgium).

Table 2 Characteristics of studied polymorphisms

Gene	rs number	Polymorphism	Localization
TPH1	rs1799913	c.804-7C>A	near gene 5′
	rs623580	c.-1668T>A	
	rs1800532	c.803+221C>A	Intron
	rs10488682	c.-173A>T	
TPH2	rs7963803	c.-1449C>A	near gene 5′
	rs4570625	c.-844G>T	

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Reactions were performed according to the manufacturers' instructions and recommendations. Real-time PCRs were carried out in the Bio-Rad CFX96 Real-Time PCR Detection System and analysed in the CFX Manager Software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis

A statistical analysis of data was performed in Statistica 12 (Statsoft, Tulsa, OK, USA) and SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). An unconditional multiple logistic regression model was used to calculate the association between case/control and each polymorphism. The results are shown as odds ratios (ORs) with 95% confidence interval (95% CI). Additionally, the OR was adjusted for gender, as women are exposed to doubled risk of depression development in comparison to men [25]. The data presenting the results from the distribution of genotypes in terms of the age of depression episode onset are shown as mean ± S.D. Distribution normality was examined using the Shapiro–Wilk test, and then, significance of the difference between studied values was determined based on the Mann–Whitney test or Student's *t*-test.

Results

SNPs of the genes encoding TRYCATs enzymes (TPH1 and TPH2) as the risk of depressive disorders

The distribution of genotypes and alleles, as shown in Table 3, was in agreement with the Hardy–Weinberg equilibrium. Among the studied SNPs, the C/C genotype and the C allele of c.804-7C>A—*TPH1* (rs1799913), the homozygote A/A of c.803+221C>A—*TPH1* (rs1800532), the T/T genotype and T allele of c.-173A> T—*TPH1* (rs10488682) were significantly associated with an increased risk of DD. The T/T genotype of c.-1668T>A—*TPH1* (rs623580) was negatively correlated with depression, while genotype A/A and allele A of the same SNP were positively correlated with the disease. In addition, the G/G genotype and G allele of c.-844G>T—*TPH2* (rs4570625) were positively correlated with depression, whereas the G/T heterozygote and T allele of the same SNP were negatively correlated with the disease. The C/C genotype and C allele of c.-1449C>A—*TPH2* (rs7963803) were negatively correlated with DD, while heterozygote C/A and allele A of the same SNP were positively correlated with the disease.

SNPs of the genes encoding TRYCATs enzymes and the age of the first episode of depression, and the severity classification on the Hamilton Depression Rating Scale

We only found one difference in the age distribution of the first depressive episode between the G/G and G/T genotypes of the c.-844G>T—*TPH2* (rs4570625) polymorphism (Figs 1 and S1). We did not find any significant differences in the distribution of genotypes and the severity classification on the Hamilton Depression Rating Scale (data unpublished).

Gene–gene interactions and the risk of depression

We also studied whether the combined genotypes of the studied polymorphisms are associated with the occurrence of depression, and the results are presented in Table 4. We observed that the C/C-G/G, C/A-G/G, A/A-G/G genotypes of c.804-7C>A—*TPH1* and c.-844G>T—*TPH2* (rs1799913 *versus* rs4570625) were associated with an increased risk of DD occurrence, while the C/A-G/T, A/A-G/T genotypes of the same polymorphism combination reduced this risk. We also found that the C/A-C/C combined genotype of the c.804-7C>A—*TPH1* and c.-1449C>A—*TPH2* (rs1799913 *versus* rs7963803) was related to a decreased risk of the disease, but the C/A-C/A combined genotype of the same polymorphism may increase this risk.

The C/C-G/G, C/A-G/G, A/A-G/G combined genotype of c.803+221C>A—*TPH1* and c.-844G>T—*TPH2* (rs180532 *versus* rs4570625) was linked with an increased risk of DD occurrence, while the C/C-G/T, C/A-G/T genotype of the same polymorphism combination decreased this risk. The C/A-C/A and A/A-C/A combined genotypes of the c.803+221C>A—*TPH1* and c.-1449C>A—*TPH2* (rs1800532 *versus* rs7963803) may lead to a development of depression, and the C/A-C/C combined genotype of the same SNPs combination reduce the risk of developing the disease.

The T/T-G/G and T/A-G/G combined genotypes of c.-173A>T—*TPH1* and c.-844G>T—*TPH2* (rs10488682 *versus* rs4570625) were associated with DD development, but the T/T-G/T and the T/A-G/T genotype of the same SNPs combination decreased the risk. Additionally, the increased risk of DD occurrence was associated with the T/T-C/A combined genotype of c.-173A>T—*TPH1* and c.-1449C>A—*TPH2* (rs10488682 *versus* rs7963803); however, the T/A-C/C genotype decreased the risk depression occurrence.

The T/T-G/G, T/A-G/G, A/A-G/G combined genotypes of c.-1668T>A—*TPH1* and c.-844G>T—*TPH2* (rs623580 *versus* rs4570625) contributed to the development of DD, while T/T-G/T and T/A-G/G of the same SNP-SNP combinations decreased this risk. In case of c.-1668T>A—*TPH1* and c.-1449C>A—*TPH2* (rs623580 *versus* rs7963803), combined T/A-C/A and A/A-C/A genotypes were associated with the occurrence of DD, while the T/T-C/C genotype of the same polymorphism combination decreased this risk.

In summary, we found that the A/A-G/G combined genotype of c.804-7C>A—*TPH1* and c.-844G>T—*TPH2* (rs1799913 and rs4570625) was associated with a five-time higher risk of DD occurrence ($P < 0.001$). In the case of c.803+221C>A—*TPH1* (rs1800532) and c.-844G>T—*TPH2* (rs4570625), the C/A-G/G combined genotypes were associated with the risk of depression higher by nearly four times ($P < 0.001$), while the C/A-G/T combined genotypes of the same polymorphism combination decreased this risk by more than three times ($P < 0.001$). Moreover, the T/T-G/G combined genotypes of c.-173A>T—*TPH1* (rs10488682) and c.-844G>T—*TPH2* (rs4570625) caused a five-time greater risk among the Polish

Table 3 Distribution of genotypes and alleles of c.804-7C>A, c.-1668T>A, c.803+221C>A, c.-173A>T, c.-1449C>A and c.-844G>T and the risk of DD

Genotype/Allele	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)*	P	Adjusted OR (95% CI)*	P
	Number	Frequency	Number	Frequency				
c.804-7C>A - *TPH1* (rs1799913)								
C/C	**65**	**0.283**	**100**	**0.357**	**1.473 (1.013–2.141)**	**0.042**	**1.413 (0.969–2.060)**	**0.073**
C/A	118	0.513	127	0.454	0.865 (0.613–1.222)	0.411	0.787 (0.555–1.117)	0.180
A/A	47	0.204	53	0.189	0.964 (0.623–1.492)	0.868	0.908 (0.586–1.408)	0.666
χ^2 = 2.131; P = 0.345								
C	**248**	**0.539**	**327**	**0.584**	**1.288 (1.012–1.639)**	**0.040**	**1.196 (0.935–1.531)**	**0.154**
A	212	0.461	233	0.416	0.911 (0.715–1.160)	0.448	0.836 (0.653–1.070)	0.154
c.803+221C>A - *TPH1* (rs1800532)								
C/C	66	0.287	93	0.331	1.319 (0.905–1.922)	0.150	1.212 (0.831–1.769)	0.318
C/A	151	0.657	158	0.564	0.772 (0.543–1.098)	0.149	**0.676 (0.471–0.969)**	**0.033**
A/A	**11**	**0.048**	**29**	**0.104**	**2.416 (1.180–4.947)**	**0.016**	**2.308 (1.126–4.730)**	**0.022**
χ^2 = 0.249; P = 0.883								
C	283	0.615	344	0.614	1.160 (0.872–1.544)	0.307	0.970 (0.719–1.309)	0.843
A	173	0.376	216	0.386	1.170 (0.871–1.571)	0.298	1.058 (0.784–1.429)	0.712
c.-173A>T - *TPH1* (rs10488682)								
T/T	**119**	**0.517**	**167**	**0.596**	**1.515 (1.070–2.145)**	**0.019**	1.377 (0.968–1,958)	0.075
A/T	99	0.430	98	0.35	0.772 (0.541–1.102)	0.154	0.713 (0.498–1.020)	0.064
A/A	12	0.052	15	0.054	1.080 (0.495–2.355)	0.846	1.032 (0.473–2.252)	0.937
χ^2 = 2.221; P = 0.329								
T	**337**	**0.733**	**432**	**0.771**	**1.444 (1.095–1.904)**	**0.009**	**1.242 (0.927–1.664)**	**0.147**
A	123	0.267	128	0.229	0.860 (0.644–1.149)	0.309	0.805 (0.601–1.079)	0.147
c.-1668T>A - *TPH1* (rs623580)								
T/T	**121**	**0.267**	**116**	**0.414**	**0.701 (0.496–0.992)**	**0.045**	**0.638 (0.449–0.908)**	**0.012**

Table 3. Continued

Genotype/Allele	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)*	*P*	Adjusted OR (95% CI)*	*P*
	Number	Frequency	Number	Frequency				
T/A	95	0.733	132	0.471	1.371 (0.967–1.943)	0.077	1.264 (0.888–1.798)	0.193
A/A	**14**	**0.061**	**32**	**0.114**	**2.092 (1.089–4.021)**	**0.027**	**1.996 (1.037–3.839)**	**0.038**
χ^2 = 8.468; ***P* = 0.014**								
T	337	0.413	364	0.65	0.800 (0.617–1.039)	0.094	**0.667 (0.505–0.881)**	**0.004**
A	**123**	**0.526**	**196**	**0.35**	**1.598 (1.212–2.107)**	**<0.001**	**1.500 (1.136–1.981)**	**0.004**
c.-844G>T - *TPH2* (rs4570625)								
G/G	**48**	**0.209**	**167**	**0.596**	**5.942 (3.999–8.831)**	**<0.001**	**5.647 (3.790–8.413)**	**<0.001**
G/T	**179**	**0.778**	**111**	**0.396**	**0.223 (0.153–0.324)**	**<0.001**	**0.186 (0.125–0.275)**	**<0.001**
T/T	3	0.013	2	0.007	0.571 (0.0946–3.444)	0.541	0.546 (0.0905–3.299)	0.510
χ^2 = 78.662; ***P* < 0.001**								
G	**275**	**0.598**	**445**	**0.794**	**5.496 (3.764–8.027)**	**<0.001**	**5.213 (3.632–7.695)**	**<0.001**
T	**185**	**0.402**	**115**	**0.205**	**0.230 (0.159–0.333)**	**<0.001**	**0.192 (0.130–0.283)**	**<0.001**
c.-1449C>A - *TPH2* (rs7963803)								
C/C	**114**	**0.496**	**96**	**0.343**	**0.581 (0.408–0.828)**	**0.003**	**0.529 (0.370–0.759)**	**<0.001**
C/A	**106**	**0.461**	**178**	**0.636**	**2.223 (1.563–3.160)**	**<0.001**	**2.054 (1.438–2.934)**	**<0.001**
A/A	10	0.043	6	0.021	0.506 (0.181–1.413)	0.193	0.478 (0.171–1.338)	0.160
χ^2 = 7.447; ***P* = 0.024**								
C	**334**	**0.726**	**370**	**0.661**	**0.823 (0.609–1.111)**	**0.203**	**0.641 (0.463–0.886)**	**0.007**
A	**126**	**0.274**	**190**	**0.339**	**1.694 (1.228–2.335)**	**0.001**	**1.561 (1.128–2.159)**	**0.007**

*OR adjusted for sex.
$P < 0.05$ along with corresponding ORs are in bold.

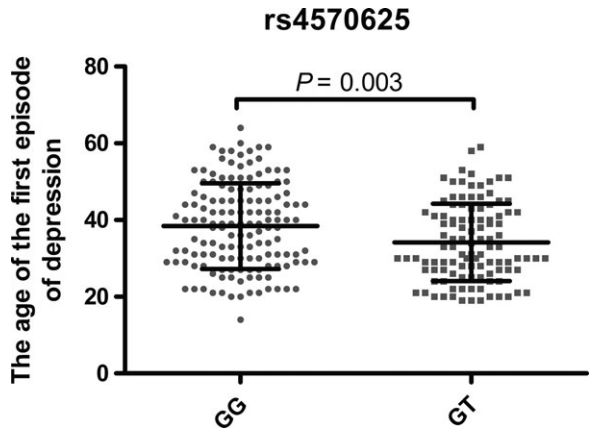

Fig. 1 Distribution of single nucleotide polymorphisms of genes encoding TPH2 and the age of the first episode of depression. Horizontal lines denote the average, while whiskers show the S.D. The distribution of the T/T genotype is not shown because this group included only two patients.

population ($P < 0.001$). The risk of DD occurrence decreased by just four times in case of the T/T-G/G combined genotypes of c.-1668T>A—*TPH1* (rs623580) and c.-844G>T—*TPH2* (rs4570625) ($P < 0.001$), while the A/A-G/G genotypes of the same gene–gene combination were linked to a six-time higher risk ($P = 0.004$). An increase by seven times of the risk of depression was confirmed for the A/A-C/A combined genotypes of c.-1668T>A—*TPH1* (rs623580) and c.-1449C>A—*TPH2* (rs7963803) ($P = 0.011$).

Haplotypes and the risk of depression

The association between depression and haplotypes of the studied polymorphisms of the *TPH1* or *TPH2* genes was also assessed (Table 5.). The presence of CC and CA haplotypes of c.804-7C>A—*TPH1* (rs1799913) and c.803+221C>A—*TPH1* (rs1800532) resulted in an increased risk of DD occurrence. We also noticed a link between the CA haplotype of c.804-7C>A—*TPH1* (rs1799913) and c.-1668T>A—*TPH1* (rs623580), and the increased rate of depression. In case of c.-173A>T—*TPH1* (rs10488682) and c.803+221C>A—*TPH1* (rs1800532), the TA haplotype increased the risk of DD development, while the AA haplotype of the same SNPs combination reduced this risk. The TA, AC and AA haplotypes of c.-1668T>A—*TPH1* (rs623580) and c.803+221C>A—*TPH1* (rs1800532) were responsible for an increased risk of depression development, while the TC haplotype of the same polymorphism combination decreased this risk. In case of c.-173A>T (rs10488682) and c.-1668T>A—*TPH1* (rs623580), the TA haplotype was associated with the occurrence of the disease. The CG and AG haplotypes of c.-1449C>A—*TPH2* (rs7963803) and c.-844G>T—*TPH2* (rs4570625) were connected with an increased risk of the disease, while the CT haplotype of the same SNPs combination increased the risk.

In summary, the haplotype analysis revealed that the AC haplotype of c.-1668T>A—*TPH1* (rs623580) and c.803+221C>A—*TPH1* (rs1800532) nearly doubled the risk ($P < 0.001$). In case of c.-1449C>A—*TPH2* (rs7963803) and c.-844G>T—*TPH2* (rs4570625), the CT haplotype increased risk of DD by almost two times ($P < 0.001$), while the AG haplotype of the same combination decreased the risk almost twice ($P < 0.001$).

Discussion

A growing body of evidence and data suggests that when impaired the TRYCATs pathway may play an essential role in the development of depression [1, 2]. As mentioned in the Introduction, these abnormalities may be linked with the irregular functioning of pathway enzymes, such as TPH. Two isoforms of TPH—*i.e. TPH1* and *TPH2*, can be found in humans and in other mammals. The human *TPH1* and *TPH2* are highly homologous proteins which exhibit 71% of amino acid identity. The human *TPH1* is located on chromosome 11p15.3-14, comprises 11 exons and covers a region of 29 kb; the human TPH2 gene is located on chromosome 12q15, comprises 11 exons and covers a region of 97 kb [26]. TPH1 and TPH2 are expressed in almost equal amounts in only a few regions of the brain such as the frontal cortex, thalamus, hippocampus, hypothalamus and amygdale, whereas TPH1 is also expressed in peripheral tissues such as the heart, lung, kidney, duodenum and the adrenal gland [27]. TPH1 and TPH2 are essential enzymes for the correct metabolism of tryptophan. Both are also considered to be the rate-limiting enzymes in serotonin biosynthesis [10]. TPH converts L-tryptophan into L-5-hydroxytryptophan (serotonin precursor) by means of adding an -HO group (hydroxylation) to position 5 of L-tryptophan. Disturbances in their amount or activity may lead to deficiencies of neuroprotective compounds such as kynurenic acid and, consequently, to the occurrence of mood disorders. Polymorphic variants or altered expression levels of TPH genes may be related to depression, schizophrenia, alcoholism, drug abuse, aggression and suicidality [13–15, 28].

During this experiment, we genotyped six SNPs: four in *TPH1* and two in *TPH2*. We confirmed that the selected genotypes and alleles of four SNPs localized in *TPH1* modulated the risk of depression (Table 3). One of them, that is c.804-7C>A—*TPH1* (rs1799913), is localized at intron 7 of the TPH1 gene and also at the polypyrimidine stretch immediately upstream of the 3′acceptor splice site. Although substitution of pyrimidine for purine in the polypyrimidine consensus sequence has been shown to decrease the fidelity of splicing, sequencing of TPH1 cDNA revealed no evidence of exon skipping or aberrant splicing [28]. The studies showed that the c.804-7C>A polymorphism was associated with 5-HIAA concentrations in CSF [29]. Moreover, the meta-analysis confirmed that the link between TPH1 and bipolar disorders was not clear [30]. Thus far, it has been confirmed that the polymorphism is associated with depression treatment through an assessment of the harm avoidance and novelty seeking [31]. Andre *et al.* [31] found a greater effect of interactions between the CC genotype and remission status as compared to A-allele carriers. Other studies demonstrated that the TPH1

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Table 4 Gene–gene interactions of studied polymorphisms and the risk of DD

Combined genotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P	Adjusted OR (95% CI)*	P
	Number	Frequency	Number	Frequency				
c.804-7C>A—*TPH1* (rs1799913) and c.-844G>T—*TPH2* (rs4570625)								
C/C-G/G	**17**	**0.074**	**60**	**0.214**	**3.594 (2.033–6.353)**	**<0.001**	**3.428 (1.936–6.070)**	**<0.001**
C/C-G/T	48	0.209	39	0.139	0.651 (0.410–1.034)	0.069	**2.181 (1.089–4.366)**	**0.028**
C/C-T/T	0	0	1	0.004	–	0.986	*	0.986
C/A-G/G	**25**	**0.109**	**74**	**0.264**	**3.104 (1.898–5.076)**	**<0.001**	**2.947 (1.1800–4.824)**	**<0.001**
C/A-G/T	**92**	**0.4**	**52**	**0.186**	**0.369 (0.248–0.550)**	**<0.001**	**0.342 (0.229–0.511)**	**<0.001**
C/A-T/T	1	0.004	1	0.004	0.860 (0.0535–13.827)	0.915	0.821 (0.0511–13.199)	0.889
A/A-G/G	**6**	**0.026**	**33**	**0.118**	**5.233 (2.153–12.717)**	**<0.001**	**4.988 (2.051–12.127)**	**<0.001**
A/A-G/T	**39**	**0.170**	**20**	**0.071**	**0.398 (0.225–0.704)**	**0.002**	**0.377 (0.213–0.667)**	**<0.001**
A/A-T/T	2	0.009	0	0	–	–	–	–
c.804-7C>A—*TPH1* (rs1799913) and c.-1449C>A—*TPH2* (rs7963803)								
C/C-C/C	30	0.130	41	0.146	1.207 (0.727–2.001)	0.467	1.143 (0.688–1.899)	0.605
C/C-C/A	32	0.139	55	0.196	1.597 (0.993–2.566)	0.053	1.513 (0.940–2.434)	0.088
C/C-A/A	3	0.013	4	0.014	1.150 (0.255–5.189)	0.856	1.096 (0.243–4.949)	0.905
C/A-C/C	**62**	**0.269**	**39**	**0.139**	**0.467 (0.299–0.729)**	**<0.001**	**0.438 (0.280–0.685)**	**<0.001**
C/A-C/A	**52**	**0.226**	**86**	**0.307**	**1.611 (1.082–2.400)**	**0.019**	**1.517 (1.017–2.264)**	**0.041**
C/A-A/A	4	0.017	2	0.007	0.426 (0.0774–2.348)	0.327	0.406 (0.0733–2.246)	0.302
A/A-C/C	22	0.096	16	0.057	0.603 (0.309–1.177)	0.138	0.571 (0.292–1.119)	0.102
A/A-C/A	22	0.096	37	0.132	1.516 (0.867–2.649)	0.144	1.440 (0.822–2.522)	0.202
A/A-A/A	3	0.013	0	0	–	–	–	–
c.803+221C>A—*TPH1* (rs1800532) and c.-844G>T—*TPH2* (rs4570625)								
C/C-G/G	**17**	**0.074**	**55**	**0.196**	**3.221 (1.813–5.721)**	**<0.001**	**3.063 (1.723–5.446)**	**<0.001**
C/C-G/T	**50**	**0.217**	**37**	**0.132**	**0.582 (0.365–0.926)**	**0.022**	**0.548 (0.343–0.874)**	**0.012**

Table 4. Continued

Combined genotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P	Adjusted OR (95% CI)*	P
	Number	Frequency	Number	Frequency				
C/C-T/T	0	0	1	0.004	–	–	–	–
C/A-G/G	**26**	**0.113**	**93**	**0.332**	**4.113 (2.553–6.626)**	**<0.001**	**3.903 (2.420–6.294)**	**<0.001**
C/A-G/T	**125**	**0.543**	**64**	**0.229**	**0.275 (0.189–0.401)**	**<0.001**	**0.248 (0.169–0.363)**	**<0.001**
C/A-T/T	1	0.004	1	0.004	0.860 (0.0535–13.827)	0.915	0.821 (0.0511–13.199)	0.889
A/A-G/G	**5**	**0.022**	**19**	**0.068**	**3.436 (1.263–9.347)**	**0.016**	**3.284 (1.205–8.950)**	**0.020**
A/A-G/T	4	0.017	10	0.034	2.194 (0.679–7.089)	0.189	2.096 (0.648–6.778)	0.216
A/A-T/T	2	0.009	0	0	–	–	–	–
c.803+221C>A—*TPH1* (rs1800532) and c.-1449C>A—*TPH2* (rs7963803)								
C/C-C/C	33	0.143	38	0.136	0.990 (0.599–1.635)	0.968	0.937 (0.566–1.590)	0.799
C/C-C/A	32	0.139	53	0.189	1.525 (0.946–2.458)	0.083	1.445 (0.895–2.331)	0.132
C/C-A/A	2	0.009	2	0.007	0.860 (0.120–6.150)	0.880	0.820 (0.115–5.870)	0.844
C/A-C/C	**77**	**0.335**	**50**	**0.179**	**0.463 (0.308–0.697)**	**<0.001**	**0.430 (0.285–0.650)**	**<0.001**
C/A-C/A	**67**	**0.291**	**104**	**0.371**	**1.535 (1.058–2.226)**	**0.024**	1.438 (0.989–2.089)	0.057
C/A-A/A	8	0.035	4	0.014	0.422 (0.126–1.420)	0.163	0.402 (0.119–1.354)	0.141
A/A-C/C	4	0.017	8	0.029	1.743 (0.518–5.860)	0.369	1.662 (0.494–5.592)	0.412
A/A-C/A	**7**	**0.030**	**21**	**0.075**	**2.710 ()1.132–6.492**	**0.025**	**2.584 (1.078–6.196)**	**0.033**
A/A-A/A	0	0	0	0	–	–	–	–
c.-173A>T—*TPH1* (rs10488682) and c.-844G>T—*TPH2* (rs4570625)								
T/T-G/G	**22**	**0.096**	**101**	**0.361**	**5.617 (3.401–9.276)**	**<0.001**	**5.337 (3.228–8.822)**	**<0.001**
T/T-G/T	**96**	**0.417**	**65**	**0.232**	**0.457 (0.313–0.667)**	**<0.001**	**0.422 (0.288–0.618)**	**<0.001**
T/T-T/T	1	0.004	1	0.004	0.860 (0.0535–13.827)	0.915	0.821 (0.0511–13.199)	0.889
A/T-G/G	**25**	**0.109**	**58**	**0.207**	**2.257 (1.362–3.740)**	**0.002**	**2.143 (1.292–3.555)**	**0.003**
A/T-G/T	**72**	**0.313**	**39**	**0.139**	**0.380 (0.245–0.588)**	**<0.001**	**0.355 (0.299–0.550)**	**<0.001**
A/T-T/T	2	0.009	1	0.004	0.428 (0.0386–4.753)	0.490	0.408 (0.0367–4.529)	0.465

Table 4. Continued

Combined genotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P	Adjusted OR (95% CI)*	P
	Number	Frequency	Number	Frequency				
A/A-G/G	1	0.004	8	0.029	7.059 (0.877–56.847)	0.066	6.746 (0.837–54.350)	0.073
A/A-G/T	11	0.048	7	0.025	0.536 (0.205–1.405)	0.205	0.509 (0.194–1.336)	0.170
A/A-T/T	0	0	0	0	–	–	–	–
c.-173A>T—*TPH1* (rs10488682) and c.-1449C>A—*TPH2* (rs7963803)								
T/T-C/C	58	0.252	59	0.211	0.842 (0.558–1.272)	0.414	0.792 (0.523–1.198)	0.269
T/T-C/A	**52**	**0.226**	**104**	**0.371**	**2.148 (1.453–3.176)**	**<0.001**	**2.023 (1.366–2.996)**	**<0.001**
T/T-A/A	9	0.039	4	0.014	0.374 (0.114–1.229)	0.105	0.356 (0.108–1.172)	0.089
A/T-C/C	**50**	**0.217**	**29**	**0.104**	**0.441 (0.269–0.724)**	**0.001**	**0.416 (0.253–0.683)**	**<0.001**
A/T-C/A	48	0.209	67	0.239	1.265 (0.832–1.922)	0.272	1.192 (0.783–1.815)	0.412
A/T-A/A	1	0.004	2	0.007	1.727 (0.156–19.160)	0.656	1.651 (0.149–18.346)	0.683
A/A-C/C	6	0.026	8	0.0289	1.152 (0.394–3.368)	0.796	1.098 (0.375–3.210)	0.865
A/A-C/A	6	0.026	7	0.025	1.004 (0.333–3.030)	0.994	0.956 (0.317–2.888)	0.937
A/A-A/A	0	0	0	0	–	–	–	–
c.-1668T>A—*TPH1* (rs623580) and c.-844G>T—*TPH2* (rs4570625)								
T/T-G/G	**23**	**0.1**	**72**	**0.257**	**3.281 (1.977–5.444)**	**<0.001**	**3.119 (1.877–5.182)**	**<0.001**
T/T-G/T	**96**	**0.417**	**42**	**0.15**	**0.267 (0.176–0.405)**	**<0.001**	**0.246 (0.162–0.375)**	**<0.001**
T/T-T/T	2	0.009	2	0.007	0.860 (0.120–6.150)	0.880	0.820 (0.115–5.870)	0.844
T/A-G/G	**22**	**0.096**	**74**	**0.264**	**3.576 (2.142–5.970)**	**<0.001**	**3.398 (2.033–5.679)**	**<0.001**
T/A-G/T	**72**	**0.313**	**58**	**0.207**	**0.613 (0.411–0.914)**	**0.016**	**0.573 (0.383–0.856)**	**0.007**
T/A-T/T	1	0.004	0	0	–	0.986	–	0.985
A/A-G/G	**3**	**0.013**	**21**	**0.075**	**6.432 (1.894–21.841)**	**0.003**	**6.145 (1.808–20.884)**	**0.004**
A/A-G/T	11	0.048	11	0.039	0.855 (0.364–2.009)	0.719	0.814 (0.346–1.914)	0.638
A/A-T/T	0	0	0	0	–	–	–	–
c.-1668T>A—*TPH1* (rs623580) and c.-1449C>A—*TPH2* (rs7963803)								

Table 4. Continued

Combined genotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P	Adjusted OR (95% CI)*	P
	Number	Frequency	Number	Frequency				
T/T-C/C	**65**	**0.283**	**37**	**0.132**	**0.412 (0.263–0.645)**	**<0.001**	**0.386 (0.246–0.605)**	**<0.001**
T/T-C/A	55	0.239	78	0.279	1.306 (0.877–1.945)	0.189	1.228 (0.823–1.833)	0.314
T/T-A/A	1	0.004	1	0.004	0.860 (0.0535–13.827)	0.915	0.816 (0.0505–13.188)	0.886
T/A-C/C	40	0.174	44	0.157	0.937 (0.587–1.496)	0.785	0.886 (0.554–1.417)	0.613
T/A-C/A	**49**	**0.213**	**84**	**0.3**	**1.679 (1.120–2.517)**	**0.012**	**1.583 (1.055–2.376)**	**0.027**
T/A-A/A	6	0.026	4	0.014	0.568 (0.158–2.036)	0.385	0.540 (0.150–1.948)	0.346
A/A-C/C	9	0.039	15	0.054	1.459 (0.627–3.397)	0.381	1.390 (0.597–3.239)	0.445
A/A-C/A	**2**	**0.009**	**16**	**0.057**	**7.242 (1.648–31.827)**	**0.009**	**6.913 (1.572–30.398)**	**0.011**
A/A-A/A	3	0.013	1	0.004	0.284 (0.0294–2.752)	0.278	0.270 (0.0279–2.618)	0.259

*OR adjusted for sex.
$P < 0.05$ along with corresponding ORs are in bold.

polymorphism in the Swedish population may lead to unipolar disorders, suicidal behaviour and substance abuse [32]. Gizatullin *et al.* [33] discovered that the c.804-7C>A polymorphism was associated with depression in the Caucasian population of the North European descent. Our study, conducted among the Polish population, confirmed the results recorded by Gizatullin's team that selected genotypes and alleles of c.-173A>T (rs1799913) could contribute to genetic predisposition for DD (Table 3). However, Gizatullin *et al.* analysed only haplotypes for six TPH1 SNPs, whereas our team showed also that the gene–gene combination of c.-173A>T (rs1799913)—*TPH1* and c.-844G>T (rs4570625)—*TPH2* or c.-1449C>A (rs7963803)—*TPH2* may also modulate the risk of depression development. Moreover, haplotype analyses performed in both studies confirmed that the examined haplotypes may be associated with the occurrence of DD.

Another TPH1 polymorphism studied during this experiment is c.-173A>T (rs10488682). The SNP is localized in the promoter region of TPH1 [34] (Cote *et al.*, 2002). It may decrease the activity of the promoter, affecting the transcription level of TPH1. TPH is an initial enzyme in the TRYCATs pathway, and its low expression may lead to stopping the pathway. The SNP was associated with the occurrence of adolescent idiopathic scoliosis. Patients with the A allele of this SNP are prone to be resistant to brace treatment [35]. In turn, we confirmed that the G/G genotype and G allele of c.804-7C>A—*TPH1* (rs10488682) may lead to the development of DD (Table 3).

The c.-1668T>A SNP (rs623580) is localized in the exon 1c/intron 1 region, but the polymorphism is within the 5′UTR and therefore does not result in the substitution of amino acids [36]. Studies involving the c.-1668T>A polymorphism revealed the negative results linked with affective disorders and suicide-related behaviour [37, 38]. Ching-Lopes *et al.*, similarly to our team, discovered that the c.-1668T>A polymorphism of TPH1 may increase the risk of depression [39], but an earlier study involving this polymorphism showed negative results accompanied by affective disorders [40]. Moreover, Ching-Lopes *et al.* (2015) found that the polymorphism of the corticotropin-releasing hormone receptor 1 and 5-hydroxytryptamine receptor 2A genes was also associated with depressive disorders [39]. This emphasizes that other stages of the TRYCATs pathway are also important in the development of DD.

The next studied SNP was the c.803+221C>A polymorphism of TPH1 (rs18005832) localized at intron 7. The site is a potential GATA transcription factor-binding site. The GATA transcription binding factors allow the initiation of transcription. Studies of the TPH1 polymorphism showed that the SNP affects expression of the gene. The genotypic and allelic distribution of the SNP was not associated with the occurrence of schizophrenia in Asian populations [41]. However, we showed that the T/T genotype of the SNP may induce the development of DD in Polish patients. In addition, Jun *et al.* [42] revealed that the same SNP was also associated with the quality of life in women suffering from the irritable bowel syndrome.

In this paper, besides the SNP localized in *TPH1*, we also studied *TPH2* polymorphisms. 844G>T (rs4576025) was one of them. It may alter DNA–protein interactions, ultimately affecting transcription of the TPH2 gene, as the presence of the T allele is associated with a reduced TPH2 promoter activity [43, 44]. As a consequence,

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Table 5 Distribution of haplotypes of the studied polymorphisms of the *TPH1 or TPH2* genes and risk of the depression

Haplotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P
	Number	Frequency	Number	Frequency		
c.804-7C>A—*TPH1* (rs1799913) and c.803+221C>A—*TPH1* (rs1800532)						
CC	**359**	**0.39**	**467**	**0.42**	**1.320 (1.101–1.581)**	**0.003**
CA	**137**	**0.15**	**187**	**0.17**	**1.283 (1.008–1.633)**	**0.043**
AC	213	0.23	221	0.20	0.918 (0.742–1.137)	0.433
AA	211	0.23	245	0.22	1.062 (0.861–1.311)	0.574
c.804-7C>A—*TPH1* (rs1799913) and c.-173A>T –TPH1 (rs10488682)						
CT	339	0.37	478	0.43	0.970 (0.807–1.1652)	0.743
CA	157	0.17	176	0.16	1.013 (0.800–1.284)	0.912
AT	335	0.36	386	0.34	1.063 (0.884–1.279)	0.651
AA	89	0.10	80	0.07	0.795 (0.579–1.090)	0.154
c.804-7C>A—*TPH1* (rs1799913) and c.-1668T>A - TPH1 (rs623580)						
TT	353	0.38	384	0.34	0.838 (0.699–1.005)	0.056
CA	**143**	**0.16**	**270**	**0.24**	**1.726 (1.378–2.161)**	**<0.001**
AT	321	0.35	344	0.31	0.950 (0.787–1.146)	0.540
AA	103	0.11	122	0.11	1.078 ()0.815–1.424)	0.599
c.-173A>T—*TPH1* (rs10488682) and c.803+221C>A—*TPH1* (rs1800532)						
TC	403	0.44	491	0.44	1.191 (0.996–1.424)	0.056
TA	**271**	**0.29**	**373**	**0.33**	**1.381 (1.141–1.671)**	**<0.001**
AC	169	0.18	197	0.188	1.060 (0.844–1.331)	0.500
AA	**77**	**0.08**	**59**	**0.05**	**0.672 (0.473–0.955)**	**0.027**
c.-1668T>A—*TPH1* (rs623580) and c.803+221C>A—*TPH1* (rs1800532)						
TC	**413**	**0.45**	**393**	**0.35**	**0.770 (0.642–0.922)**	**0.005**
TA	**261**	**0.28**	**335**	**0.30**	**1.583 (1.308–1.916)**	**<0.001**
AC	**159**	**0.17**	**295**	**0.26**	**1.948 (1.566–2.422)**	**<0.001**
AA	**87**	**0.09**	**97**	**0.09**	**1.006 (0.742–1.364)**	**0.040**
c.-173A>T (rs10488682) and c.-1668T>A—*TPH1* (rs623580)						
TT	469	0.51	518	0.46	0.992 (0.830–1.186)	0.932
TA	**205**	**0.22**	**346**	**0.31**	**1.791 (1.463–2.192)**	**<0.001**
AT	205	0.22	210	0.19	0.904 (0.728–1.124)	0.364
AA	41	0.04	46	0.04	1.013 (0.658–1.558)	0.955

Table 5. Continued

Haplotype	Control (n = 230)		Depression (n = 280)		Crude OR (95% CI)	P
	Number	Frequency	Number	Frequency		
c.-1449C>A—*TPH2* (rs7963803) and c.-844G>T—*TPH2* (rs4570625)						
CG	**389**	**0.42**	**588**	**0.53**	**1.858 (1.551–2.225)**	**<0.001**
CT	**279**	**0.30**	**152**	**0.14**	**0.402 (0.322–0.503)**	**<0.001**
AG	**161**	**0.18**	**302**	**0.27**	**1.983 (1.596–2.463)**	**<0.001**
AT	91	0.10	78	0.07	0.754 (0.550–1.035)	0.081

$P < 0.05$ along with corresponding ORs are in bold.

serotonin synthesis is inhibited [45, 46]. Moreover, studies showed that the A allele of the SNP was related to an increased risk of multiple sclerosis in the progressive subtypes of the disease among Finnish patients [47]. The results of an earlier study showed that the TPH2 SNP was characteristic for depressed patients with suicidal attempts [48]. The homozygous G allele (G/G genotype) frequency was higher in suicidal depressed patients when compared to control volunteers. Similarly, our study proved that the G/G genotype and allele G of c.-844G>T—*TPH2* increased the risk of DD, whereas the G/T heterozygote and allele T of the same SNP were negatively correlated with the disease. However, a study on the Chinese Han population showed no association between SNP and depression. These discrepancies may result from demographic properties of the research groups—the difference in the number of women and men in the cited study [49]. Furthermore, in another study on the Chinese population, the GG genotype of the same SNP carriers was more likely to enable depressive and anxiety symptom remission following escitalopram treatment compared with T allele carriers [50].

The last studied polymorphism presented in this paper is c.-1449C>A (rs7963803) of *TPH2*. Yi *et al.* [51] found that the polymorphism was not correlated with occurrence of paranoid schizophrenia. Previous studies showed that two polymorphisms localized in the intron of *TPH2* (c.608+9108T>C—rs1386494 and rs1843809—c.608+5263G>T) were associated with major depression [52]. During our experiment, we found a significant correlation between the studied TPH2 polymorphisms (rs4570625 and rs7963803) and the development of depression.

We are the first to investigate the association between combined genotypes of studied polymorphisms and DD. We also found a significant correlation between depressive disorders and haplotypes of the studied polymorphisms. Moreover, we were the first to demonstrate that three of six examined polymorphisms— *i.e.* rs1800532, rs10488682 and rs7963803—were associated with the risk of depression occurrence. The other three SNPs (previously examined by other researchers) were also found to strongly modulate DD development. For example, the AA genotype of c.-1668T>A (rs623580) doubled the risk of DD ($P < 0.001$). In turn, the G/G genotype and G allele increased the risk of the disease in the Polish population by five times, while the G/T genotype and T allele decreased this risk fivefold. In summary, the c.804-7C>A, c.-1668T>A, c.803+221C>A and c.-173A>T polymorphisms of the TPH1 gene and the c.-1449C>A, c.-844G>T polymorphism of the TPH2 gene may be associated with depression in the Polish population. At the end of our article, we indicated and recommended that the relationship between the genes of TRYCATs enzymes and DD should be investigated further.

Conclusion

We confirmed that SNPs of the genes involved in tryptophan metabolism, particularly the TRYCATs pathway, may have an impact on the risk of depressive disorders occurrence. We demonstrated that every studied SNP may modulate the development of depression. Therefore, these gene polymorphisms may be considered independent markers of depression. Our study supports the hypothesis that the TRYCATs pathways may be involved in the development of depression.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Distribution of single-nucleotide polymorphisms of genes encoding TPH1 and TPH2 and the age of the first episode of depression. Horizontal lines denote the average, while whiskers show the S.D.

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Original article

Variation of genes encoding KAT1, AADAT and IDO1 as a potential risk of depression development

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ABSTRACT

Numerous data suggests that the disorders of tryptophan catabolites (TRYCATs) pathway, including a decreased level of tryptophan or evaluated concentration of harmful TRYCATs –kynurenine, quinolinic acid, 3-hydroxyanthranilic acid, 3-hydroxytryptophan – may cause the occurrence of DD symptoms. In this work, we assessed the relationship between single-nucleotide polymorphisms (SNPs) of KAT1, KAT2 and IDO1 gene encoding, and the risk of depression development. Our study was performed on the DNA isolated from peripheral blood of 281 depressed patients and 236 controls. We genotyped, by using TaqMan probes, four polymorphisms: c.*456G > A of *KAT1* (rs10988134), c.975-7T > C of *AADAT* (rs1480544), c.-1849C > A (rs3824259) and c.-1493G > C(rs10089084)of *IDO1*. We found that only the A/A genotype of c.*456G > A – *KAT1* (rs10988134) increased the risk of depression occurrence. Interestingly, when we stratified the study group according to gender, this relationship was present only in male population. However, a gene–gene analysis revealed a link between the T/T-C/C genotype of c.975-7T > C – *AADAT* (rs1480544)or c.-1493G > C – *IDO1* (rs10089084) and C/C-C/A genotype of c.975-7T > C – *AADAT* (rs1480544)and c. –1849C > A – *IDO1* (rs3824259) and the disease. Moreover, we found, that the c.975-7T > C – *AADAT* and c. *456G > A *KAT1* (rs10988134) polymorphisms may modulate the effectiveness of selective serotonin reuptake inhibitors therapy. Concluding, our results confirm the hypothesis formulated in our recently published article that the SNPs of genes involved in TRYCATs pathway may modulate the risk of depression. This provides some further evidence that the pathway plays the crucial role in development of the disease.

1. Introduction

Depression (a depressive disorder, DD) is the most common mental disorder. All around the world, 300 million people suffer from DD [1]. Moreover, by 2020, depression will have become the second health and economic problem, second only to the ischemic heart disease [2]. Additionally, about 1/3 patients does not respond to the conventional therapy, and untreated or inappropriately treated depression may lead to suicide attempts [3]. Previous studies showed that the DD is regarded as multi-casual disease and its pathology remains unclear [4–7]. Additionally, depression is associated with numerous somatic disease – it increases the risk of developing the atherosclerotic heart disease, type 2 diabetes mellitus, cancer – and increases mortality rates [8–10]. Furthermore, recent studies showed, that DD occurrence is also strongly associated with obesity [11–14]. Interestingly, obese patients, similarly to depressed patients, were characterized by an imbalance of tryptophan catabolites (TRYCATs) pathway [15]. Our earlier study of single nucleotide polymorphisms (SNPs) of genes encoding tryptophan hydroxylase suggests that the tryptophan metabolism may play the key role in the pathophysiology of DD [16]. Another study showed that a decreased level of tryptophan or an elevated concentration of harmful TRYCATs, i.e. kynurenine, quinolinic acid, 3-hydroxyanthranilic acid, 3-hydroxytryptophan-may cause the DD symptoms to surface [6]. Although tryptophan is converted into toxic kynurenine, it also is a precursor of serotonin (5-HT) and melatonin – a main neurotransmitter that regulates the mood [17]. The first step of TRYCATs pathway, which is degradation

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of tryptophan to N-formylkynurenine, is catalysed by tryptophan 2,3-dioxygenase (TDO) or indoleamine-pyrrole-2,3-dioxygenase (IDO). There are rate-limiting enzymes of this pathway. TDO is expressed in liver and converts tryptophan only, whereas IDO is expressed in placenta, lungs, brain, blood – and, aside from tryptophan, it also metabolizes melatonin and serotonin [18,19]. Furthermore, the activity of IDO may be regulated by cytokines, whereas TDO does not depend on this regulation. Pro-inflammatory cytokines – interferon-γ (INFγ), interferon-α (INFα), and tumour necrosis factor-α (TNFα) – may act as potent activators of IDO. On the other hand, the anti-inflammatory cytokines may be inhibitors of the enzyme [20–22]. Clinical trials showed that the activity of IDO may be assessed by the examination of kynurenine/tryptophan ratio or by expression level of *IDO* [5,6]. The previous studies suggest that an increased activity of IDO and TDO may be an associated with occurrence of the depressive-like behaviours [23,24,5,6]. Moreover, an elevated kynurenine/tryptophan ratio may cause a development of anhedonia, which is the primary symptom of depression [25]. Animal studies confirmed that depression may be associated with an increased IDO expression/activity and levels of kynurenine, 5-hydroxykynurenine, quinolinic acid in brain areas, i.e. hippocampus, hypothalamus and amygdala [26–29]. Moreover, the IDO activation and serum level of the harmful TRYCATs were associated with the onset and severity of the disease symptoms [30–33]. Interestingly, the study showed that the female patients with the DD were characterized by a higher serum concentration of IDO, TNFα, INFγ but by a lower level of serotonin when compared to healthy volunteers. Moreover, the levels of IDO and TNFα were decreased in patients after antidepressant therapy [34,35]. In the same study, Zoga et al. [34] found a strong positive correlation between the concentration of IDO and INFγ. Moreover, transcription of *IDO1,* a gene encoding one of the protein isoform, is strongly controlled by cytokines. The gene promoter contains multiple sequence elements that induction of responsiveness to type I (INFα and –β) and type II (INFγ) interferons [36,37]. Induction of the expression by the latter one is mediated by a signal transducer and activator of transcription 1 (STAT1) and INF-regulatory factor 1 [38]. Moreover, IDO metabolizes the serotonin degradation into N-formyl-5-hydroxykynurenine; thus its over-expression results in a deficiency of the neurotransmitter [39]. Accordingly, the increased activity of the IDO and an impaired central serotonin system may lead to development of depression in patients with inflammatory disease [40,41]. The same study proved that glial cells secrete interleukin-6 (IL-6), interleukin-1 beta (IL-1β), TNF-α, and INF-γin response to injury and infection. Furthermore, it was found that DD could be potentially attributed to a hippocampal depletion of tryptophan, degradation of serotonin and increased level of kynurenine derived from serotonin degradation [6].

The next important step of TRYCATs pathway includes a conversion of kynurenine into kynurenic acid (enzyme – kynurenine formamidase) or, alternatively, into 3-hydroxykynurenine (3-HK), enzyme – kynurenine aminotransferase, up to date, its four isoforms have been described: KAT1/glutamine transaminase K (GTK)/cysteine conjugate beta-lyase (CCBL) 1, KAT2/aminoadipate aminotransferase (AADAT), KAT3/CCBL2 and KAT4/glutamicoxaloacetic transaminase (GOT) 2/mitochondrial aspartate aminotransferase (ASAT)or anthranilic acid (enzyme – kynurenine hydroxylase) [42]. Subsequently, 3-HK can be metabolized by kynureninase to form 3-hydroxyanthranilic acid (3-HAA), which can be further metabolized to form the quinolinic acid (QUIA) [43]. Although QUIA is neurotoxic, it cannot penetrate the blood-brain barrier. On the other hand, kynurenine is not neuroactive; however it may cross the blood-brain barrier and it generates free-radical-producing 3-HK or 3-HAA or is converted to the glutamatergically-active QUIA [43]. The harmful actions of TRYCATs may be related to an oxidative damage, inflammation, mitochondrial dysfunction, cytotoxicity, excitotoxicity, neurotoxicity and lowered neuroplasticity in central nervous system, e.g. 3-hydroxykynurenine may initiate neuronal apoptosis [44,45,6,7]. In contrast, some TRYCATs induce begin effects, e.g. kynurenic acid manifests antioxidant and neuroprotective properties which based on block N-methyl-D-aspartate (NMDA) receptors [44,45,5]. This acid is converted from kynurenine by kynurenineaminotransferase (KAT1 and KAT2/AADAT) Stone and Darlingtone, 2002. Therefore, the kynurenine/kynurenic acid (KYN/KA) ratio indicates a KAT activity (the ratio increase in inverse proportion to the KAT activity) and was found to be higher in depressed patients than controls [7]. Accordingly, an elevated expression of kynurenine aminotransferase in skeletal muscles can protect from depression [46]. Interestingly, the same study showed that physical exercise may be used as an alternative treatment of DD and that endurance exercise led to an increased expression of KAT in muscles, while the patients who were practising some sports/taking the training were characterized by an increased level of kynurenic acid in plasma. Another piece of evidence supporting involvement of TRYCATs pathway in depression development is the fact that abnormal activity of IDO may lead to impairment of serotonin metabolism and a decrease of melatonin level, and, in consequence, may trigger sleep disorders, all of these symptoms often present in the course of the disease [34]. Moreover, patients with depressive suicidal attempts suffer more frequently from severe insomnia than patents without the attempts [47].

The exact pathogenesis of the DD is unclear, genetic, environmental, and behavioral factors as well as interaction between them may be involved. Although the DD is recognized as a multifactorial disease, genetic factors may play the crucial role in its development, as was confirmed by segregation analyses, genetic epidemiological data and gene mapping studies. Several gene *loci*/chromosomal regions for DD have been mapped by genome-wide linkage analysis, including 12q23.3–q24.11 and 13q31.1–q31.3, 15q25.2 McGuffin et al., 2005, 3p21.1 Sullivian et al., 2013,19q12., 11p14.2, 8q22.2, 8q12.1, 8q23.3, 3p26.1, 2p25.1, 11p14.3, 6p22.3, 1q32.1, 3q26.1 (Shyn et al. 2011). These findings show a heterogeneous and complex genetic nature of the DD. Moreover, according to the study report, the genomic regions significantly associated with the DD were localized on chromosome 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14,15, 16, 17, 18, 22 Wray et al., 2017. Therefore, in this paper, we examine the link between single nucleotide polymorphisms (SNPs) of enzymes involved in TRYCATs pathway: c.*456G > A of *KAT1* (rs10988134, is located on 9q34.11), c.975-7T > C of *AADAT* (rs1480544, is located on 4q33), c.-1849C > A (rs3824259) and c.-1493G > C (rs10089084)of *IDO1* (is located on 8p11.21) and incidence of depression. Moreover, we detected differences between male or female groups in examined polymorphisms frequency. Interesting results were also obtained for the analysis an impact the single-nucleotide polymorphisms of genes encoding TRYCATs enzymes on effectiveness of antidepressant therapy.

2. Materials and methods

2.1. Subjects

281 patients with depression (age 49.53 ± 10.18, n male = 148, n female = 133) hospitalized at the Department of Adult Psychiatry of the Medical University of Lodz (Poland) and 236 healthy volunteers (age 53.19 ± 12.61, n male = 121, n female = 115) participated in the study. The Table 1. shows a detailed characteristic of depressed patients. All subjects in the control and depressed groups were native Poles from central Poland (not related), randomly selected without replacement sampling. All the participants had to meet

Table 1
Detailed characteristic of patients taking part in the study.

Depression severity (HAMD range of scores)	Number of patients before treatment	Number of patients after treatment
None (0–7)	1	193
Mild (8–16)	35	85
Moderate (17–23)	94	3
Severe (≥24)	151	0
Mean age of patients with first episode		30
Mean age of patients with a further episode		50
Mean age of first episode (for all patients)		36
Duration of disease from the first episode		Number of patients
0-10 Years		147
11–20 Years		56
21–30 Years		50
31–40 years		26
≥41 years		2
Number of episodes		Number of patients
1		38
2		88
3		89
4		53
5		12
6		1

the inclusion criteria outlined in ICD-10 [48]. The axes I and II disorders, other than the DD, severe and chronic somatic diseases, injuries of the central nervous system, inflammatory or autoimmune disorders and unwillingness to give informed consent were exclusion criteria. Moreover, familial prevalence of mental disorders other than recurrent depressive disorders was also a factor of exclusion from the examined groups. Standardized Composite International Diagnostic Interview (CIDI) served as the guide to conducting a medical history for all cases before the study began. Evaluation and classification of depression severity were based on the Hamilton Depression Rating Scale (HDRS) [49]. The intensity of DD symptoms was measured in the study according to the grades proposed by Demyttenaere and De Fruyt [50] before and after antidepressant therapy with a selective serotonin reuptake inhibitor (SSRI). The same psychiatrist examined each patient before the start of the study and after 8 weeks of pharmacotherapy. Participation in this study was voluntary. Its subjects were informed about the details of the study and assured of their voluntary participation in the experiment. Moreover, patients were guaranteed that their personal data would be kept secret before making a decision to participate in the study. During hospitalization, all the participants were treated according to antidepressant treatment standards. According to the protocol approved by the Bioethics Committee of the Medical University of Lodz (no. RNN/70/14/KE), all the subjects consented to participation in the study.

2.2. Selection of single nucleotide polymorphism

The public domain of the National Center for Biotechnology Information the Single Nucleotide Polymorphisms database (NCBI dbSNP) at http://www.ncbi.nlm.nih.gov/snp was used to identify potentially functional polymorphisms in genes encoding IDO and KAT. The four polymorphisms were chosen with a minor allele frequency (MAF) higher than 0.05 in the European population, respectively (submitter population ID: HapMap-CEU for both; http://www.ncbi.nlm.nih.gov/snp). The selection of studied polymorphisms was mainly determined by a potential biological significance – the localization of the SNPs was in the coding or regulatory regions of genes and might have functional meaning for transcription and protein function. The c.*456G > A(rs10988134) polymorphism of *KAT1* is localized in 3′ untranslated region and thec.975-7T > C (rs1480544) SNPis localized in intron of*AADAT*. The c.-1849C > A (rs3824259) and c.-1493G > C(rs10089084) polymorphisms are located near 5′ end of *IDO1*.

2.3. DNA extraction

We used the commercially available Blood Mini Kit (A&A Biotechnology, Gdynia, Poland) to extract genomic DNA from venous blood of patients with depression and from all controls. DNA purity and concentration were determined by measuring e absorbance at 260 and 280 nm. The sample of purified isolated DNA was stored at −20C until further analysis.

2.4. Genotyping

The TaqMans SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 2X Master Mix Takyon for Probe Assay – No ROX (Eurogentec, Liège, Belgium) were used to genotype the studied SNPs in the Bio-Rad CFX96 Real-Time PCR Detection System and analysed in the CFX Manager Software (Bio-Rad Laboratories Inc., Hercules, California, USA). Theywere carried out the real-time polymerase chain reactions.

2.5. Statistical analysis

The SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA, USA), a statistical software package was used to the statistical analyses. The unconditional multiple logistic regression model was used to the evaluation of the correlation between case-control and each studied SNPs. This association was measured by the odds ratio (OR) with 95% confidence interval (95% CI). Additionally, the OR was adjusted for sex, since women have doubled risk of depression in comparison to men [51]. We also evaluated the correlation between the cases and controls for each studied polymorphisms in male/female population by used to unconditional logistic regression model. Distribution of genotypes according to the year of the first episode of depression and severity of actual depressed episodes are showed as the median ± inter-quartile range. The Shapiro-Wilks test was used to evaluate normality of the distribution. Next, the Mann-Whitney test or Student's *t* test was used to estimate the significance of the difference between the analysed values.

3. Results

3.1. Single nucleotide polymorphisms of the gene encoding TRYCATs enzymes (KAT1, AADAT, IDO1) as the risk of the depressive disorder

Table 2 shows the distribution of genotypes and alleles in patients with the DD and in controls, which is in agreement with the Hardy-Weinberg equilibrium. We only found that the A/A genotype of the c.*456G > A – *KAT1* (rs10988134) may increase the risk of depression occurrence. No correlation was found between depressed patients and healthy volunteers in terms of distribution of genotypes of the left-over studied polymorphisms.

3.2. Single-nucleotide polymorphisms of the genes encoding TRYCATs enzymes and the age of the first episode of depression, and the severity classification on the Hamilton Depression Rating Scale

No significant differences was found between distribution of genotypes and the severity classification on the Hamilton

Table 2
Distribution of genotypes and alleles of c.*456G > A of *KAT1* (rs10988134), c.975-7T > C of*AADAT*(rs1480544), c.-1849C > A (rs3824259) and c.-1493G > C(rs10089084) of *IDO1* and incidence of depression.

Genotype/Allele	Control (n = 236)		Depression (n = 281)		Crude OR (95% CI)[a]	P	Adjusted OR (95% CI)[a]	P
	Number	Frequency	Number	Frequency				
c.[a]456G > A – *KAT1* (rs10988134)								
A/A	**9**	**0.038**	**23**	**0.082**	**2.266 (1.027–4.998)**	**0.043**	**2.269 (1.029–5.007)**	**0.042**
A/G	77	0.326	95	0.338	1.066 (0.738–1.540)	0.733	1.065 (0.737–1.539)	0.736
G/G	150	0.636	163	0.580	0.782 (0.548–1.117)	0.176	0.783 (0.548–1.117)	0.177
$\chi^2 = 3.659$; $p = 0.056$								
A	95	0.201	141	0.251	1.323 (0.991–1.766)	0.058	1.323 (0.991–1.766)	0.058
G	377	0.799	421	0.749	0.756 (0.566–1.009)	0.058	0.756 (0.566–1.009)	0.058
c.975-7T > C – *AADAT* (rs1480544)								
C/C	67	0.284	62	0.221	0.709 (0.475–1.059)	0.093	0.710 (0.475–1.060)	0.094
T/C	116	0.492	142	0.505	1.065 (0.753–1.506)	0.723	1.063 (0.751–1.504)	0.732
T/T	53	0.225	77	0.274	1.299 (0.868–1.945)	0.204	1.301 (0.869–1.949)	0.201
$\chi^2 = 3.283$; $p = 0.070$								
C	250	0.530	266	0.473	0.796 (0.622–1.020)	0.071	0.796 (0.622–1.019)	0.071
T	222	0.470	296	0.527	1.255 (0.981–1.607)	0.071	1.256 (0.981–1.607)	0.071
c.-1849C > A – *IDO1* (rs3824259)								
C/C	53	0.225	79	0.281	1.340 (0.896–2.003)	0.154	1.336 (0.893–1.999)	0.158
C/A	115	0.487	131	0.466	0.918 (0.649–1.299)	0.629	0.920 (0.650–1.303)	0.640
A/A	68	0.288	71	0.253	0.843 (0.571–1.245)	0.392	0.843 (0.571–1.245)	0.390
$\chi^2 = 1.920$; $p = 0.166$								
C	221	0.468	289	0.514	1.185 (0.932–1.507)	0.167	1.184 (0.931–1.506)	0.169
A	251	0.532	273	0.486	0.844 (0.664–1.073)	0.167	0.845 (0.664–1.074)	0.169
c. −1493G > C – *IDO1* (rs10089084)								
G/G	39	0.165	46	0.164	0.997 (0.625–1.591)	0.991	0.996 (0.624–1.589)	0.986
G/C	125	0.530	136	0.484	0.833 (0.588–1.178)	0.301	0.833 (0.589–1.179)	0.303
C/C	72	0.305	99	0.352	1.233 (0.852–1.786)	0.267	1.233 (0.851–1.786)	0.268
$\chi^2 = 0.592$; $p = 0.442$								
G	203	0.430	228	0.406	0.905 (0.703–1.167)	0.442	0.905 (0.702–1.166)	0.440
C	269	0.570	334	0.594	1.105 (0.857–1.423)	0.442	1.105 (0.857–1.424)	0.440

$p<0.05$ along with corresponding Ors are in bold.
[a] OR adjusted for sex.

Depression Rating Scale. Neither did we observe the defferences in the distribution of genotypes and the age distribution of the first depressive episode (Supplementary Fig. 1 and Supplementary Fig. 2).

3.3. Gene-gene interactions and the risk of depression

Gene-gene interactions as a risk of depressive disorder is showed in Table 3. We observed that the T/T-C/C combined genotype of the c.975-71T > C – *AADAT* (rs1480544) and the c.-1493G > C – *IDO1* (rs10089084) more than doubled the risk of the DD occurrence, while the C/C-C/A genotype of the c.975-7T > C – *AADAT* (rs1480544) and the c. C > A – *IDO1*(rs3824259) polymorphism combination reduced this risk. No statistical correlation was found between the combined genotypes of c. *456G > A (rs10988134) – *KAT1* and c.-1849C > A – *IDO1* (rs3824259), c.*456G > A (rs10988134) – *KAT1* and – *IDO1* (rs10089084), c.975-7T > C – *AADAT*(rs1480544) and c.*456G > A – *KAT1* (rs10988134) and development of depression.

3.4. Haplotypes and the risk of occurrence of depression

We also analysed if the haplotypes of the studied polymorphisms is associated with occurrence of depression and the results are presented in Table 4. We found that the CC haplotype of the c.-1849C > A – *IDO1* (rs3824259) and c.-1493G > C – *IDO1* (rs10089084) polymorphism increased the risk of depression occurrence. However, we did not observe a connection between depression and other haplotypes of the studied polymorphisms of*IDO1*gene.

3.5. Single-nucleotide polymorphisms of genes encoding enzymes of TRYCATs pathway depression occurrence in male and female population

Kessler [51] observed that women were exposed to a doubled risk of depression when compared to men. Therefore, we studied the connection between the depression occurrence in male or female groups and all examined polymorphisms (Table 5). On the one hand, we found that the A/A genotype and A allele of the c. *456G > A – *KAT1* (rs10988134) were association with an increased risk of depression development in male population, while in female population we did not observe this correlation. On the other hand, the G/G genotype and the G allele of the same polymorphism were linked to a lower risk in male population, while this dependence was not observed in females. Moreover, we investigated the relationship between the distribution of genotypes or alleles and gender in patients with depression but this association was not found for all the studied polymorphisms (data not published).

3.6. Single-nucleotide polymorphisms of genes encoding TRYCATs enzymes of and effectiveness of treatmentof the depression

We also study an impact the single-nucleotide polymorphisms of genes encoding TRYCATs enzymes on the effectiveness of

Table 3
Gene-gene interactions of studied polymorphisms and the risk of DD.

Combined genotype	Control (n = 236)		Depression (n = 281)		Crude OR (95% CI)[a]	p	Adjusted OR (95% CI)[a]	p
	Number	Frequency	Number	Frequency				
c.[a]456G > A (rs10988134) – *KAT1* and c. –1849C > A – *IDO1* (rs3824259)								
A/A-C/C	1	0.004	7	0.025	6.004 (0.733–49.151)	0.095	5.953 (0.726–48.807)	0.097
A/A–C/A	3	0.013	11	0.039	3.164 (0.872–11.478)	0.080	3.185 (0.877–11,561)	0.078
A/A-A/A	5	0.021	5	0.18	0.837 (0.239–2.927)	0.781	0.843 (0.241–2.949)	0.789
G/A-C/C	13	0.055	28	0.100	1.898 (0.960–3.755)	0.065	1.890 (0.955–3.744)	0.068
G/A-C/A	40	0.169	44	0.157	0.910 (0.570–1.453)	0.692	0.911 (0.570–1.455)	0.696
G/A-A/A	24	0.101	23	0.082	0.787 (0.432–1.435)	0.435	0.789 (0.433–1.438)	0.439
G/G-C/C	39	0.165	44	0.157	0.938 (0.586–1.501)	0.789	0.939 (0.586–1.503)	0.792
G/G-C/A	72	0.305	76	0.270	0.844 (0.576–1.238)	0.386	0.846 (0.577–1.240)	0.390
G/G-A/A	39	0.165	43	0.153	0.913 (0.569–1.464)	0.705	0.910 (0.567–1.461)	0.696
c.[a]456G > A (rs10988134) – *KAT1* and c. –1493G > C – *IDO1* (rs10089084)								
G/G-G/G	1	0.004	5	0.018	4.257 (0.494–36.697)	0.187	4.205 (0.486–36.349)	0.192
G/G-G/C	6	0.025	10	0.036	1.415 (0.506–3.951)	0.498	1.428 (0.510–3.995)	0.497
G/G-C/C	2	0.008	8	0.028	3.429 (0.721–16.304)	0.121	3.433 (0.722–16.330)	0.121
G/A-G/G	11	0.047	17	0.060	1.317 (0.604–2.870)	0.488	1.319 (0.605–2.874)	0.486
G/A-G/C	39	0.165	46	0.164	0.989 (0.620–1.577)	0.962	0.987 (0.618–1.574)	0.955
G/A-C/C	27	0.114	32	0.114	0.995 (0.577–1.714)	0.985	0.996 (0.578–1.716)	0.987
A/A-G/G	27	0.114	24	0.085	0.723 (0.405–1.290)	0.272	0.722 (0.405–1.290)	0.271
A/A-G/C	80	0.339	80	0.285	0.776 (0.534–1.128)	0.184	0.777 (0.535–1.129)	0.186
A/A-C/C	43	0.182	59	0.210	1.193 (0.770–1.848)	0.430	1.192 (0.769–1.847)	0.431
c.975-7T > C – *AADAT* (rs1480544) and c. –1493G > C – *IDO1* (rs10089084)								
C/C-G/G	10	0.042	9	0.032	0.748 (0.299–1.872)	0.535	0.742 (0.296–1.861)	0.525
C/C-G/C	39	0.165	30	0.107	0.604 (0.362–1.007)	0.053	0.606 (0.363–1.012)	0.055
C/C-C/C	18	0.076	23	0.082	1.080 (0.568–2.053)	0.815	1.076 (0.565–2.046)	0.824
T/C-G/G	19	0.081	27	0.096	1.214 (0.657–2.244)	0.536	1.219 (0.659–2.255)	0.527
T/C-G/C	56	0.237	68	0.242	1.026 (0.684–1.539)	0.901	1.021 (0.679–1.533)	0.922
T/C-C/C	41	0.174	47	0.167	0.955 (0.603–1.513)	0.845	0.957 (0.604–1.515)	0.850
T/T-G/G	10	0.042	10	0.036	0.834 (0.341–2.039)	0.691	0.827 (0.338–2.025)	0.678
T/T-G/C	30	0.127	38	0.135	1.074 (0.643–1.794)	0.786	1.078 (0.645–1.803)	0.773
T/T-C/C	**13**	**0.055**	**29**	**0.103**	**1.974 (1.002–3.891)**	**0.049**	**1.977 (1.003–3.897)**	**0.049**
c.975-7T > C – *AADAT* (rs1480544) and c.*456G > A – *KAT1* (rs10988134)								
C/C-A/A	2	0.008	6	0.021	2.553 (0.510–12.768)	0.254	2.527 (0.504–12.664)	0.260
C/C-G/A	24	0.102	19	0.068	0.641 (0.342–1.201)	0.165	0.640 (0.341–1.200)	0.164
C/C-G/G	41	0.174	37	0.132	0.721 (0.445–1.169)	0.184	0.723 (0.446–1.173)	0.189
T/C-A/A	5	0.021	7	0.025	1.180 (0.370–3.768)	0.780	1.194 (0.373–3.819)	0.765
T/C-G/A	32	0.136	56	0.199	1.587 (0.988–2.548)	0.056	1.584 (0.986–2.545)	0.057
T/C-G/G	79	0.335	79	0.281	0.777 (0.534–1.131)	0.188	0.775 (0.532–1.128)	0.183
T/T-A/A	2	0.008	10	0.036	4.317 (0.937–19.903)	0.061	4.324 (0.938–19.935)	0.060
T/T-G/A	21	0.089	20	0.071	0.785 (0.414–1.485)	0.456	0.786 (0.415–1.488)	0.460
T/T-G/G	30	0.127	47	0.167	1.379 (0.841–2.262)	0.203	1.381 (0.842–2.265)	0.202
c.975-7T > C – *AADAT* (rs1480544) and c. –1849C > A – *IDO1* (rs3824259)								
C/C-C/C	12	0.051	20	0.071	1.430 (0.684–2.991)	0.342	1.423 (0.680–2.978)	0.349
C/C-C/A	**40**	**0.169**	**26**	**0.093**	**0.500 (0.295–0.847)**	**0.010**	**0.501 (0.295–0.850)**	**0.010**
C/C-A/A	15	0.064	16	0.057	0.890 (0.430–1.840)	0.752	0.886 (0.428–1.834)	0.745
T/C-C/C	29	0.123	40	0.142	1.185 (0.709–1.978)	0.517	1.181 (0.707–1.973)	0.526
T/C-C/A	53	0.225	63	0.224	0.998 (0.659–1.511)	0.992	0.997 (0.658–1.509)	0.988
T/C-A/A	34	0.144	39	0.139	0.957 (0.583–1.573)	0.864	0.958 (0.583–1.574)	0.866
T/T-C/C	12	0.051	19	0.068	1.354 (0.643–2.850)	0.425	1.360 (0.646–2.864)	0.419
T/T-C/A	22	0.093	42	0.149	1.709 (0.988–2.956)	0.055	1.710 (0989–2.958)	0.055
T/T-A/A	19	0.081	16	0.057	0.690 (0.346–1.373)	0.290	0.690 (0.346–1.374)	0.291

$p < 0.05$ along with corresponding Ors are in bold.
[a] OR adjusted for sex.

Table 4
Haplotypes of *IDO1* and the risk of depression.

Haplotype	Control (n = 236)		Depression (n = 281)		Crude OR (95% CI)	p
	Number	Frequency	Number	Frequency		
c.-1849C > A – *IDO1* (rs3824259) and c. –1493G > C – *IDO1* (rs10089084)						
CG	132	0.14	158	0.14	1.006 (0.784–1.291)	0.967
CC	**310**	**0.33**	**420**	**0.37**	**1.215 (1.013–1.457)**	**0.036**
AG	274	0.29	298	0.27	0.882 (0.727–1.070)	0.203
AC	228	0.24	248	0.22	0.889 (0.724–1.092)	0.261

$p < 0.05$ along with corresponding ORs are in bold.

Table 5
Distribution of genotypes and alleles of the c.*456G > A of *KAT1* (rs10988134), c.975-7T > C of*AADAT*(rs1480544), c.-1849C > A (rs3824259) and c.-1493G > C (rs10089084) of *IDO1* and the risk of DD in male and female population.

Genotype/Allele	MEN (n = 169)				WOMEN (n = 248)			
	Control (n = 121)	Depression (n = 148)	Crude OR (95% CI)*	p	Control (n = 115)	Depression (n = 133)	Crude OR (95% CI)*	p
	N (Freq.)	N (Freq.)			N (Freq.)	N (Freq.)		
c.*456G > A – *KAT1* (rs10988134)								
A/A	3 (0.025)	**13 (0.088)**	**3.816 (1.061–13.718)**	**0.040**	6 (0.052)	10 (0.075)	1.489 (0.524–4.232)	0.455
A/G	37 (0.306)	54 (0.365)	1.318 (0.790–2.199)	0.290	40 (0.348)	41 (0.308)	0.845 (0.496–1.438)	0.534
G/G	**81 (0.669)**	**81 (0.547)**	**0.590 (0.358–0.971)**	**0.038**	69 (0.600)	82 (0.617)	1.059 (0.635–1.766)	0.827
$\chi^2 = 5.228$; $p = 0.022$						$\chi^2 = 0.386$; $p = 0.534$		
A	**43 (0.178)**	**80 (0.270)**	**1.705 (1.124–2.589)**	**0.012**	52 (0.226)	48 (0.180)	1.027 (0.683–1.542)	0.899
G	**199 (0.822)**	**216 (0.730)**	**0.586 (0.386–0.890)**	**0.012**	178 (0.774)	182 (0.684)	0.974 (0.648–1.463)	0.899
c.975-7T > C – *AADAT* (rs1480544)								
C/C	33 (0.273)	32 (0.216)	0.742 (0.424–1.299)	0.296	34 (0.296)	30 (0.226)	0.701 (0.396–1.240)	0.222
T/C	59 (0.488)	80 (0.541)	1.221 (0.754–1.977)	0.417	57 (0.496)	62 (0.466)	0.901 (0.546–1.487)	0.684
T/T	29 (0.240)	36 (0.243)	1.029 (0.587–1.804)	0.921	24 (0.208)	41 (0.308)	1.649 (0.920–2.954)	0.093
$\chi^2 = 2.552$; $p = 0.110$						$\chi^2 = 2.877$; $p = 0.090$		
C	125 (0.517)	144 (0.486)	0.883 (0.625–1.248)	0.481	125 (0.543)	107 (0.402)	0.729 (0.513–1.036)	0.078
T	117 (0.483)	152 (0.514)	1.132 (0.801–1.601)	0.481	105 (0.457)	123 (0.462)	1.372 ()0.966–1.951	0.078
c. –1849C > A – *IDO1* (rs3824259)								
C/C	32 (0.264)	42 (0.284)	1.112 (0.649–1.908)	0.699	21 (0.183)	37 (0.278)	1.679 (0.913–3.085)	0.095
C/A	55 (0.455)	67 (0.453)	0.978 (0.603–1.586)	0.927	60 (0.522)	64 (0.481)	0.863 (0.523–1.423)	0.563
A/A	34 (0.281)	39 (0.264)	0.924 (0.539–1.585)	0.774	34 (0.296)	32 (0.241)	0.762 (0.433–1.341)	0.346
$\chi^2 = 0.150$ $p = 0.698$						$\chi^2 = 2.844$ $p = 0.092$		
C	119 (0.492)	151 (0.510)	1.070 (0.773–1.481)	0.685	102 (0.443)	121 (0.455)	1.337 (0.934–1.914)	0.112
A	123 (0.508)	145 (0.490)	0.935 (0.675–1.294)	0.685	128 (0.557)	109 (0.410)	0.748 (0.522–1.070)	0.112
c.-1493G > C – *IDO1* (rs10089084)								
G/G	20 (0.165)	26 (0.176)	1.076 (0.568–2.041)	0.822	19 (0.165)	20 (0.150)	0.902 (0.455–1.789)	0.768
G/C	63 (0.521)	71 (0.480)	0.849 (0.525–1.375)	0.504	62 (0.539)	65 (0.489)	0.829 (0.503–1.369)	0.464
C/C	38 (0.314)	51 (0.345)	1.148 (0.688–1.917)	0.596	34 (0.296)	48 (0.361)	1.317 (0.771–2.252)	0.314
$\chi^2 = 0.051$ $p = 0.822$						$\chi^2 = 0.739$; $p = 0.390$		
G	103 (0.426)	123 (0.416)	0.959 (0.677–1.358)	0.812	100 (0.435)	105 (0.395)	0.850 (0.587–1.232)	0.391
C	139 (0.574)	173 (0.584)	1.043 (0.737–1.477)	0.812	130 (0.565)	161 (0.605)	1.176 (0.812–1.704)	0.391

$p < 0.05$ along with corresponding ORs are in bold.

antidepressant therapy (Table 6). Therefore, we divided the patients into two groups – those with the total score of Hamilton Rating Scale for Depression after treatment at the maximum of 7 points (marked as the effectiveness of antidepressant therapy) and those with the total score after treatment above 7 points (marked as an ineffective antidepressant therapy). We observed that the T/T genotype and the T allele of c.975-7T > C – *AADAT*(rs1480544) were related to a low effectiveness of the antidepressant therapy, while the C allele of the same polymorphism was positively correlated with a response to the applied SSRIs treatment. In the remaining cases, no correlation between SNP's occurrence and effectiveness of antidepressant treatment was found. Moreover, we showed some significant differences in the distribution of studied polymorphism genotypes and the percentage of Hamilton Rating Scale for Depression (Fig. 1). We found a difference in the percentage dispersion of Hamilton Rating Scale for Depression between the A/A and G/A, G/A and G/G, A/A and G/G genotypes of the c. *456G > A *KAT1* (rs10988134) polymorphism. Moreover, the disturbances were also observed between the C/C and T/C, T/C and T/T, C/C and T/T genotypes of the c.975-7T > C – *AADAT* (rs1480544) polymorphism.

4. Discussion

Among various hypotheses explaining the pathogenesis of depression, impairments in TRYCATs pathway are now considered the major contributor to the development of DD. Elevated TRYCATs markers, i.e.quinolinic acid and kynurenine, were found in depressed patients and may be associated with some incorrect actions of the pathway enzymes [6,7]. In addition, our recent study demonstrated that SNPs of genes encoding two isoforms of tryptophan hydroxylase – an enzyme involved in the initial and rate-limiting step in the synthesis of serotonin and melatonin – modulated the risk of depression Wigner et al., 2017b. In this paper, we reconfirmed that the TRYCATs pathway may be involved in pathogenesis of depression and we studied the relationship between four SNPs in *KAT1*,*AADAT* and *IDO1* (two polymorphisms) genes and the risk of DD occurrence.

One of such key enzymes in TRYCATs pathway is kynurenine aminotransferase encoded by gene localized in 9q34.11.The enzyme plays many important roles, including, its cysteine conjugating beta-lyase activity, transaminase activity towards many amino acids and is involved in salvaging of α-keto acids derived from essential amino acids [52]. As mentioned in the introduction, KAT catalyzes formation of kynurenic acid, which shows neuroprotective potency [7]. Hence, previous studies showed that the change of KAT level may be associated with neurodegenerative disorders [52]. The patients with Alzheimer's disease, Parkinson's disease, multiple sclerosis and Huntington's disease were characterized by a lower concentration of KAT in the central nervous system, while patients with HIV infection, Down's syndrome, amyotrophic lateral sclerosis, schizophrenia and epilepsy had increased level of the enzyme [52]. Up to date, no evidence has been presented to prove a relationship existing between polymorphisms of *KAT1* and the development of neurodegeneration; however, it was established that the SNP analysed in the study causes a transition in the 3′UTR region which may affect a *KAT1* transcript stability [53], http//:genome.UCSC.edu). Additionally, other studies showed that the SNPs in this region of gene may affect mRNA half-life and degradation and, consequently, may lead to an increase or a decrease of gene expression [54,55]. Accordingly,

Table 6
The impact of the single-nucleotide polymorphisms of genes encoding enzymes on the effectiveness of depression treatment.

Genotype/Allele	Control (n = 236)		Depression (n = 281)		Crude OR (95% CI)*	p
	Number	Frequency	Number	Frequency		
c.*456G > A – *KAT1* (rs10988134)						
A/A	13	0.081	6	0.083	1.020 (0.372–2.798)	0.970
A/G	53	0.329	25	0.342	1.061 (0.592–1.904)	0.842
G/G	94	0.588	43	0.581	0.941 (0.537–1.648)	0.832
$\chi^2 = 0.032$; $p = 0.859$						
A	79	0.245	37	0.253	1.040 (0.677–1.597)	0.858
G	241	0.753	111	0.750	0.962 (0.626–1.477)	0.858
c.975-7T > C – *AADAT* (rs1480544)						
C/C	38	0.236	12	0.164	0.637 (0.311–1.305)	0.218
T/C	88	0.547	34	0.466	0.723 (0.415–1.259)	0.252
T/T	**34**	**0.213**	**28**	**0.378**	**2.113 (1.154–3.870)**	**0.015**
$\chi^2 = 5.363$; $p = 0.021$						
C	**164**	**0.509**	**58**	**0.397**	**0.618 (0.408–0.934)**	**0.023**
T	**156**	**0.488**	**90**	**0.608**	**1.619 (1.070–2.448)**	**0.023**
c. −1849C > A – *IDO1* (rs3824259)						
C/C	51	0.317	17	0.233	0.655 (0.347–1.237)	0.192
C/A	68	0.425	35	0.473	1.162 (0.667–2.026)	0.596
A/A	41	0.255	22	0.301	1.263 (0.684–2.330)	0.456
$\chi^2 = 1.534$; $p = 0.215$						
C	170	0.531	69	0.466	0.791 (0.545–1.148)	0.217
A	150	0.468	79	0.534	1.264 (0.871–1.834)	0.217
c. −1493G > C – *IDO1* (rs10089084)						
G/G	29	0.180	12	0.164	0.895 (0.428–1.873)	0.769
G/C	77	0.481	34	0.459	0.878 (0.504–1.529)	0.646
C/C	54	0.335	28	0.384	1.233 (0.694–2.189)	0.475
$\chi^2 = 0.416$; $p = 0.519$						
G	135	0.422	58	0.392	0.878 (0.591–1.305)	0.520
C	185	0.578	90	0.608	1.139 (0.767–1.691)	0.520

$p < 0.05$ along with corresponding ORs are in bold.

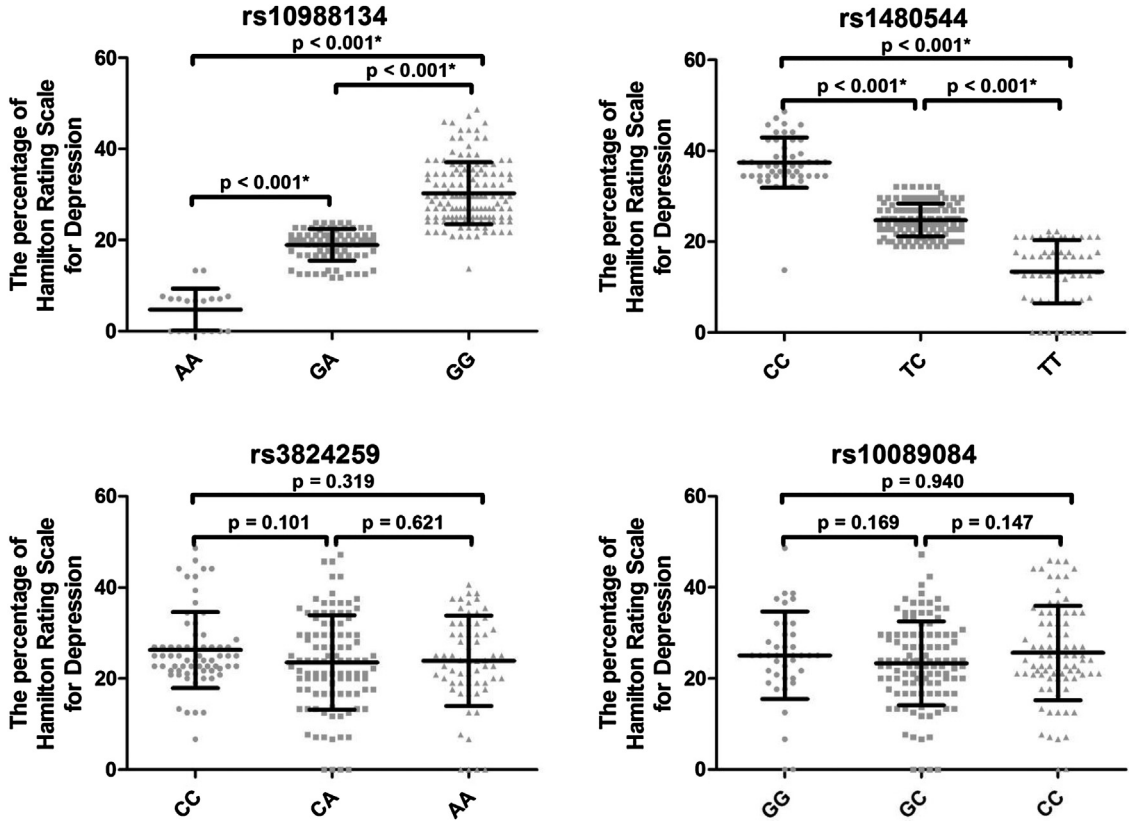

Fig. 1. Distribution of single-nucleotide polymorphisms of genes encoding KAT1, KAT2 and IDO1 and the percentage of the Hamilton Rating Scale for Depression after an antidepressant therapy. Horizontal lines represent the median, while whiskers show the inter-quartile range.

we were the first to show that the A/A genotype of c.*456G > A – *KAT1* (rs10988134) may increase the risk of depression occurrence ($p < 0.05$)(Table 2). Thus, the A/A genotype may be associated with a decreased concentration or activity of KAT1 and may lead to an increase the level of neurotoxic kynurenine. Interestingly, when we stratified the study group by gender, this association was valid only for the male population ($p < 0.05$) (Table 5.). Although the consequence of this status is unknown, we may speculate that this differences may result from a different enzymes activity or concentration in men and in women group. The next studied polymorphism is located in locus 4q33 encoding AADAT (KAT2). The enzyme demonstrates the activity towards aminoadipate and α-ketoglutarate and catalyzes transamination for a number of amino acids [52]. The c.975-71T > C – *AADAT* (rs1480544) SNP is in a putative exonic splicing silencers (ESSs), thus it may lead to some quantitative changes in the production of canonical mRNAs and peptide [56]. So far, only one study, carried out on the Brazilian population, showed that this SNP may have an impact on the phenotype – the C/T genotype of this SNP affected the host's expression of markers of the immune response to bacterial meningitis [42]. On the other hand, in our study we did not find any significant differences between depressed patients and healthy volunteers (Table 2). However, in gene–gene analyses, we found that the combined genotype of c.975-71T > C – *AADAT* (rs1480544) and c.-1849C > A (rs3824259) of *IDO1* or c.-1493G > C of *IDO1* (rs10089084) may modulate the risk of the depression development ($p < 0.05$) (Table 3). Additionally, we showed that the T/T genotype is associated with poorer outcome of the SSRIs treatment ($p < 0.05$) (Table 6).This discovery can contribute to choosing the right personalized antidepressant treatment.

Lastly, we evaluated distribution of two SNPs encodes indoleamine 2,3-dioxygenase, which catalyzes the first and rate-limiting step in the TRYCATs pathway, leading to initiation of N-formylkynurenine. Both polymorphism present near 5′ (regulatory region) end of *IDO1*–gene located on chromosome 8p11. [57–59]. The previous studies suggested that the SNP in noncoding regions, including introns and regulatory regions, may cause an altered mRNA stability, degradation and expression, resulting in some changes in the activity of the final protein product [60–62]. Despite the potential changes caused by these polymorphisms, we did not detect the link between the SNPs occurrence and the development of depression (Table 2). Similarly, in a study of these polymorphisms frequency in patients with interferon-alpha-related depression in hepatitis C as compared to controls [63] its authors did not find any statistically significant results concerning thesetwo polymorphisms. On the other hand, as mentioned earlier, the combined genotype of either of these SNPs and c.975-71T > C – *AADAT* (rs1480544) may be associated with occurrence of depression ($p < 0.05$) (Table 3).

The present study had some potential limitations. First of all, the sample size was relatively small and consequently of low power, which could lead to both false negative as well as false positive findings. Therefore these results should be interpreted with caution and considered preliminary. Second, the studied population was from Poland only, which reduces the possibility of confounding from ethnicity, so it does not permit any extrapolation of the results to other ethnic groups.

The findings of this work cast a new light on the pathogenesis of depression; however, some additional larger case-control studies on different population groups and functional experiments are necessary before the final resolution about/findings as to the role of the TRYCATs pathway in the development of this disease.

5. Conclusion

In the current work,we showed that the chosen four SNPs of genes involved in tryptophan catabolites pathway may influence the risk of depression occurrence. Therefore, these polymorphisms may be considered as independent depression markers.

Conflict of interest

None.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eurpsy.2018.05.001.

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ORIGINAL ARTICLE

Effects of venlafaxine on the expression level and methylation status of genes involved in oxidative stress in rats exposed to a chronic mild stress

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Abstract

Recent human and animal studies indicate that oxidative and nitrosative stress may play a role in the aetiology and pathogenesis of depression. This study investigates the effect of chronic administration of the serotonin-norepinephrine reuptake inhibitor, venlafaxine, on the expression and methylation status of *SOD1*, *SOD2*, *GPx1*, *GPx4*, *CAT*, *NOS1* and *NOS2* in the brain and blood of rats exposed to a chronic mild stress (CMS) model of depression. Separate groups of animals were exposed to CMS for 2 or 7 weeks; the second group received saline or venlafaxine (10 mg/kg/d, IP) for 5 weeks. After completion of both stress conditions and drug administration, the mRNA and protein expression of selected genes and the methylation status of their promoters were measured in peripheral mononuclear blood cells (PBMCs) and in brain structures (hippocampus, amygdala, hypothalamus, midbrain, cortex, basal ganglia) with the use of TaqMan Gene Expression Assay, Western blot and methylation-sensitive high-resolution melting techniques. CMS caused a decrease in sucrose consumption, and this effect was normalized by fluoxetine. In PBMCs, *SOD1*, *SOD2* and *NOS2* mRNA expression changed only after venlafaxine administration. In brain, *CAT*, *Gpx1*, *Gpx4* and *NOS1* gene expression changed following CMS or venlafaxine exposure, most prominently in the hippocampus, midbrain and basal ganglia. CMS increased the methylation of the Gpx1 promoter in PBMCs, the second *Gpx4* promoter in midbrain and basal ganglia, and *SOD1* and *SOD2* in hippocampus. The CMS animals treated with venlafaxine displayed a significantly higher CAT level in midbrain and cerebral cortex. CMS caused an elevation of Gpx4 in the hippocampus, which was lowered in cerebral cortex by venlafaxine. The results indicate that CMS and venlafaxine administration affect the methylation of promoters of genes involved in oxidative and nitrosative stress. They also indicate that peripheral and central tissue differ in their response to stress or antidepressant treatments. It is possible that that apart from DNA methylation, a crucial role of expression level of genes may be played

by other forms of epigenetic regulation, such as histone modification or microRNA interference.

These findings provide strong evidence for thesis that analysis of the level of mRNA and protein expression as well as the status of promoter methylation can help in understanding the pathomechanisms of mental diseases, including depression, and the mechanisms of action of drugs effective in their therapy.

KEYWORDS

brain structures and blood cells, chronic mild stress model, gene expression and methylation, oxidative and nitrosative stress, rat, venlafaxine

1 | INTRODUCTION

Depression is the most common of all mental disorders, with around 350 million sufferers Worldwide. According to WHO estimation, depression will become the second most common health problem in the World by 2020.[1,2] Furthermore, patients with depression are characterized by an increased risk of the development of somatic disease, including cardiovascular disease,[3] obesity,[4] diabetes,[5] cancer[6] and cognitive impairment.[7] Despite intensive research, the ethology and pathogenesis of depression remain unclear.[8] Moreover, around 30% of depressed patients do not benefit from antidepressant drug treatment.[9,10] Unfortunately, despite extensive studies, the pathophysiology of depression is not fully known, because the condition is not associated with any gross alternations of brain.[11]

On the other hand, a rapidly growing body of evidence suggests the involvement of various factors in the pathophysiology of depression, including inflammation, DNA damage and impairment of tryptophan transformation. There are also reports suggesting that affective disorders, including depression, are characterized by disturbances of the oxidant/antioxidant balance, that in consequence lead to damage of cellular macromolecules.[12-17] Reactive oxygen and nitrogen species can cause changes in cell membranes, receptor and enzymes functions, as well as activity of genes, and these imbalances can be influenced by antidepressant treatments.[14,18,19] Among the signs of antioxidant defects, increased levels of 8-F2-isoprostane (8-iso-PGF2α), malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine have been found in depressed patients and are also believed to be markers of the effectiveness of an antidepressant treatment.[20-23] For example, an enhanced concentration of MDA is normalized after 3 months of SSRI treatment.[24,25] Similarly, chronic mild stress (CMS) model was associated with increased protein peroxidation—carbonyl—level in prefrontal, hippocampus, striatum and cortex as compared to control rats.[26] Moreover, MDA level was increased in cerebellum and striatum of stressed rats.[26] However, in another study, bupropion or sertraline increased 8-iso-PGF2α in patients with major depression and the severity of the disease was strongly correlated with an increased level of F2 isoprostane.[27]

Decreased levels of enzymatic and non-enzymatic antioxidants can also play a crucial role in the mechanisms of depression. For example, a low glutathione peroxidase (Gpx) and superoxide dismutase (SOD) activity have been observed in depressed patients, with this decrease being correlated with the severity of the disease[28-31]; however, opposite effects have also been reported.[25,32,33] Additionally, antidepressant therapy may lead to partly improve of antioxidant response.[33] Similarly, previous studies found that chronic ozone inhalation induced depression-like symptoms, including anxiety, and reduced cortical and hippocampal SOD and CAT activity.[34,35] Interestingly, next study showed that the antidepressant therapy caused a reduction myeloperoxidase activity in the amygdala, hippocampus, amygdala and prefrontal cortex while SOD and CAT activity is believed to increase in nucleus accumbens of rat brain after antidepressant therapy.[36]

Another important oxidative enzyme associated with depression is xanthine oxidase (XO); a post-mortem study had shown an increased level of XO in serum and the thalamus.[37,38] In addition, patients with depression have been characterized by decreased levels of vitamin E, vitamin C, zinc, uric acid, coenzyme Q10 and glutathione (GSH), although the latter did not change following electroconvulsive therapy.[19,32,39–44] Moreover, an animal study showed that the level of GSH was reduced in cerebral cortex, hypothalamus and brain stem regions of stressed mice, although it was not changed in cerebellum.[45]

Nitric oxide (NO) is another important chemical associated with the mechanisms of depression. Low levels of NO help facilitate the release of dopamine and noradrenalin, but high concentrations lead to nitration and hypernitrosylation of amino acids and proteins. This results in the generation of highly reactive substances: NO-tyrosine, NO-tryptophan and NO-arginine.[46,47] It was found that the levels of IgM against NO-aspartate, NO-phenylalanine and NO-tyrosine are increased in serum of depressed patients and the inhibition of NO synthesis can induce an antidepressant-like effect.[48] Thus, a NO inhibitor can increase the effectiveness of serotonergic antidepressants (SNRIs) and can be applied to patients with treatment-resistant depression.[48] Furthermore, an animal study suggests that the depression may be associated with increased activity of endothelial NOS (eNOS) and increased nNOS (neuronal NOS; NOS1) protein and mRNA expression in the hippocampus.[49] However, Yoshino et al[50] found that antidepressant treatment increased *nNOS* mRNA

expression in hippocampus, midbrain, cerebellum and olfactory bulb, and iNOS (inducible NOS, NOS2) mRNA expression in frontal cortex and midbrain, and decreased *eNOS* mRNA expression in most brain regions.

The above data suggest that the mechanisms of depression can be associated with disturbances in the balance between oxidants and antioxidants. Thus, antioxidant agents may be an effective antidepressant therapy. Molecular hydrogen has antioxidative activities, and the mice after inhalation of hydrogen were characterized by decreased pathological damage, neuronal apoptosis and BBB disruption and reversed the cognitive decline.[51] Similarly, Gao et al[52] found that that repeated inhalation of hydrogen-oxygen mixed gas decreased both the acute and chronic stress-induced depressive- and anxiety-like behaviours of mice. The next antioxidant compound—vanillin—inhibits the protein oxidation and lipid peroxidation in hepatic mitochondria. Thus, many previous studies showed that the vanillin relieved symptoms of CMS and it may be a potential antidepressant.[53-55] Moreover, Amira et al[55] found that CMS procedure caused an increase of lipid peroxidation and a decrease of GSH and serotonin in the brain. Sesamol is another antioxidant agent, which exerted antidepressant-like effects, since it reversed the unpredictable chronic stress-induced behavioural, including increased immobility period and reduced sucrose preference and biochemical parameters (increased lipid peroxidation and nitrite levels; decreased GSH levels, SOD and catalase activities) in stressed mice.[41] Human studies also confirmed that antioxidants, including N-acetylcysteine, may relieve symptoms of depression.[56] On the other hand, a growing body of evidence suggests that antidepressants, including SSRIs, serotonin norepinephrine reuptake inhibitors (SNRIs) and tricyclic antidepressants (TCAs), may have antioxidant action.[57] Therefore, a chronic treatment of imipramine increased SOD and CAT activity and decreased lipid and protein damage in prefrontal cortex and hippocampus of rats.[58] Similarly, Zafir et al[59] found that the activities of SOD, CAT, GST, GR and GSH levels in the rat brain increased after fluoxetine and venlafaxine administration. Additionally, the therapy prevented lipid and protein oxidative damage induced by stress.

Therefore, this study aimed to investigate whether: (a) the CMS procedure, used as an validated animal model of depression[59–61] changes the expression of *SOD1*, *SOD2*, *GPx1*, *GPx4*, *CAT*, *NOS1* and *NOS2* at the mRNA and protein levels in peripheral blood mononuclear cells (PBMCs) and is selected brain structures (hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia); (b) chronic administration of serotonin-norepinephrine reuptake inhibitor, venlafaxine, affects the expression of these genes; (c) the CMS procedure and venlafaxine administration cause epigenetic changes, that is methylation level of these gene promoters; (d) a degree to which these changes in methylation affect the genes expression; and (e) the changes observed in PBMCs can serve as markers of similar changes in the brain. The last point has an important clinical implication, as there is a great need for peripheral markers that would allow earlier diagnosis, more precise prognosis of pharmacotherapy outcome, and more personalized therapies of the mood disorders. All of the genes analysed in our study are located on chromosomes significantly associated with depression (Table S1).

2 | MATERIALS AND METHODS

2.1 | Animals

The study was carried out on male Wistar Han rats (Charles River), brought into the laboratory for adaptation to housing conditions 1 month before the start of the experiment. Except as described below, the animals were singly housed with freely available food and water, and maintained on a 12-hour light/dark cycle (lights on at 8.00) in a constant temperature (22 ± 20°C) and humidity (50 ± 5%) conditions. All procedures used in this study conform to the rules and principles of Directive 86/609/EEC and have been approved by the Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland.

2.1.1 | Chronic mild stress procedure

The CMS procedure was conducted as previously described.[60–62] Briefly, the animals (200-220 g) were first trained to consume a 1% sucrose solution in seven once weekly baseline tests, in which sucrose solution was presented for 1 hour following 14-hour food and water deprivation in the home cage. Subsequently, sucrose consumption was monitored once weekly, under similar conditions, until the end of the study. On the basis of their sucrose intakes in the final baseline test, the animals were divided into two matched groups. One group was exposed to the stress procedure for a period of 2 or 7 weeks. Each week of the stress regime consisted of two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (light on and off every 2 hours), two periods of soiled cage (250 mL water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. The duration of all stressors was 10-14 hours, and they were used individually and continuously, day and night. The control, non-stressed animals were housed in separate rooms, with no contact with the stressed animals. They were deprived of food and water for 14 hours before each weakly sucrose test, but otherwise food and water were available ad libitum. In all CMS studies conducted in our laboratory, the stressed animals display a gradual decrease in the consumption of the sucrose solution to approximately 40% of pre-stress values. When this effect stabilized, that is after initial 2 weeks of stress, the animals were either decapitated (see below) or further divided into matched subgroups, and for subsequent 5 weeks, they received daily administration of vehicle (1 mL/kg, IP) or venlafaxine (10 mg/kg, IP). The drug was administered to all animals (ie control and stressed) at approximately 10.00, and the weekly sucrose tests were carried out 24 hours after the last dose.

Twenty-four hours after the sucrose test conducted following initial 2 weeks of stress, that is before venlafaxine administration was commenced (groups: Control and Stressed) and after the final sucrose test conducted following 7 weeks of stress, that is after completion of 5 weeks administration (groups: Control/Venla, Stressed/Saline, Stressed/Venla), the animals were decapitated and the blood and brain samples were collected (see below).

2.2 | Specimen collection and separation of peripheral blood mononuclear cells

Blood samples were collected into 5 mL vacutainers containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated based on differential migration of cells during centrifugation, that is mixed with equal volumes of PBS, layered on top of Gradisol L (Aqua-Med) and centrifuged. The interfacial layer (lymphocyte coat) was transferred to a new tube and centrifuged. The supernatant was removed and PBMCs stored as pellets at −20°C until required.

2.3 | Isolation of RNA and DNA from PBMCs

RNA and DNA were extracted from PBMCs using the commercial spin column methods and eluted in RNAse-Free water (GenElute Mammalian Total RNA Miniprep Kit; Sigma-Aldrich; QIAamp DNA Mini Kit; Qiagen, respectively), in accordance with the manufacturer's protocols. Total concentrations of RNA and DNA were determined by spectrophotometer. The purity of the RNA and DNA samples was determined as a 260/280 nm OD ratio with expected values between 1.8 and 2.0. Finally, the RNA and DNA samples were stored at −20°C until required for further analysis.

2.4 | Specimen collection and RNA/DNA isolation from animal brain

After blood harvesting, hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia were removed, rapidly frozen in liquid nitrogen and stored at −80°C until further analysis. A sufficient volume of PBS was added to each samples, which were then homogenized by a FastGene®Tissue Grinder (Nippon Genetics Europe). The homogenized samples were sonicated, centrifuged and rinsed with PBS using a commercial kit (ISOLATE II RNA/DNA/Protein Kit; Bioline), according to the manufacturer's instructions. Finally, the total concentration and purity of the RNA and DNA isolated from each sample was determined using a spectrophotometer as a 260/280 nm OD ratio with expected values between 1.8 and 2.0. Finally, the RNA and DNA samples were stored at −20°C until further analysis.

2.5 | Reverse transcription and gene expression

The reverse transcription reaction was performed using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, the following components were mixed to form a 20 μL reaction volume: nuclease-free water; 10xRT Buffer; 10xRT Random Primers; 25xdNTP Mix (100 mM); total RNA (0.5 ng/μL) and MultiScribe® Reverse Transcriptase. The reaction tubes were incubated for 10 minutes at 25°C, then for 120 minutes at 37°C, and then finally for 5 minutes at 85°C to inactivate reverse transcriptase. PCR was performed in a C1000™ programmed Thermal Cycler (Bio-Rad Laboratories Inc). After the reverse transcription, the cDNA samples were stored at −20°C until further analysis.

Expression of SOD1 (assay ID: Rn00566938_m1), SOD2 (assay ID: Rn00690588_g1), Gpx1 (assay ID: Rn00577994_g1), Gpx4 (assay ID: Rn00820818_g1), CAT (assay ID: Rn00560930_m1), NOS1 (assay ID: Rn00583793_m1) and NOS2 (assay ID: Rn00561646_m1) genes was performed on a TaqMan Gene Expression Assay in a CFX96™ Real-Time PCR Detection System Thermal Cycler (Bio-Rad Laboratories Inc). The house-keeping gene for human 18S ribosomal RNA gene (18S) was used as an internal control (reference gene) for all reverse transcription-quantitative polymerase chain reactions (RT-qPCRs), as it normalizes RNA input measurement errors and variations in reverse transcriptase-PCR efficiency.

For the PCR process, a 10 μL mixture was used consisting of total cDNA samples, a TaqMan Universal Master Mix, no UNG (Applied Biosystems), TaqMan Probe (Thermo Fisher Scientific) and RNase-free water. Standard PCR conditions were as follows: 10 minutes at 95°C (enzyme activation), followed by 60 cycles of 30 seconds at 95°C (denaturation), and 1 minute at 60°C (for annealing/extension). All samples were run in duplicate. Negative controls containing no cDNA were included in each RT-qPCR run. The cycle threshold (C_t) values were calculated automatically by a CFX96 Real-Time PCR Detection System Software System (Bio-Rad Laboratories Inc). For each sample, the gene expression of the target mRNA was calculated relative to a reference gene ($\Delta C_{t\,\text{sample}} = C_{t\,\text{target gene}} - C_{t\,\text{reference gene}}$). Levels of gene expression are given as a normalization ratio calculated as fold = $2^{-\Delta Ct}$ $^{\text{sample}}$. Fold change in the expression caused by venlafaxine administration was calculated using the 2 ^ ($-\Delta\Delta C_t$) method.[63]

2.6 | Methylation and HRM analysis

The methylation status of gene promoters was specified by methylation-sensitive high-resolution melting.[64,65] Of the gene promotors studied in this assay, only the NOS2 promoter did not contain any CpG islands. In the case of genes expression regulated by many promoters, primers were designed for promoters containing CpG islands. Next, we designed primers using the Methyl Primer Express™ Software v 1.0 (Thermo Fisher Scientific). Bisulphite modification was performed using 200 ng of DNA with a CiTi Converter DNA Methylation Kit (A&A Biotechnology), according

to the manufacturer's protocols. Methylated DNA (CpGenome™ Rat Methylated Genomic DNA Standard; Merck Millipore) and unmethylated DNA (CpGenome™ Rat Unmethylated Genomic DNA Standard; Merck Millipore), were used as controls for the MS-HRM experiments; these were prepared following the same procedure as the blood and brain DNA samples.

In order to control for the sensitivity of methylation detection, serial dilutions were performed: non-methylated, 10% methylated, 25% methylated, 50% methylated, 75% methylated and 100% methylated DNA (Figure S1). These methylation analyses were performed using the Bio-Rad CFX96 Real-Time PCR Detection System and analysed in HRM Powered by Precision Melt Analysis™ Software (Bio-Rad Laboratories Inc). Each PCR contained 5× HOT FIREPol® EvaGreen® HRM Mix (no ROX) (Solis BioDyne), 500 nM of each primer and 10 ng of DNA after bisulphite modification (theoretical calculation) and was performed as three replicates. The parameters for amplification and HRM analyses included initial activation for 12 minutes at 95°C, 45 cycles of 95°C for 15 seconds; annealing at optimal primer temperatures (tested experimentally) for 20 seconds (see Table 1 for characteristics of the primers), and elongation at 72°C for 20 seconds. The HRM analysis consisted of denaturation at 95°C for 15 seconds, reannealing at 60°C for 1 minute and melting from 60 to 95°C at a ramp rate of 0.2°C.

2.7 | Protein samples isolated from animal brains and the Western blot procedure

For the analysis, we selected proteins that are crucial for the regulation of oxidative stress, occurred in various cell locations (cytoplasm, mitochondria and cell nucleus) and have not been investigated in animal models of depression so far. The protein fractions isolated from the tissue of the rat brains were used for Western blotting.[98] Frozen samples of the brain parts were homogenized using a FastGene®Tisue Grinder (Nippon Genetics Europe), in a RIPA buffer (10 mM Tris-HCl PH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 10 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, serine protease inhibitor). The homogenates were then sonicated twice for 15 seconds each in an ice bath and centrifuged at 2500 *g* for 5 minutes at 4°C. The supernatant was then collected. The protein content of the homogenate tissue fractions was estimated by means of the Lowry procedure,[71] using bovine serum albumin (BSA). The samples (50 μg/lane) of homogenates were resolved with 10% polyacrylamide gel. Electrophoresis was conducted in a Tris/glycine/SDS buffer (25 mM Tris, 190 mM glycine, 0.2% SDS, pH 8.3), for 2.5 hours at 100 mA in OmniBlot Mini (Cleaver Scientific).

After electrophoresis, the samples were transferred onto a nitrocellulose membrane of Immobilon-P (Millipore), in 25 mM Tris-HCl containing glycine (190 Mm) and methanol (20%), and run at 55 V overnight.[99] Then, the membranes were blocked with a blocking solution (5% non-fat dry milk in a 0.1% TBST buffer—Tris buffered saline with Tween-20) for 1 hour at room temperature. The blots were then incubated with primary antibodies: overnight at 4°C with mouse monoclonal antibodies specific to catalase (Santa Cruz Biotechnology Inc) and rabbit monoclonal antibodies specific to glutathione peroxidase 4 (Abcam). Primary mouse monoclonal antibodies specific to β-actin (a reference protein) and superoxide dismutase 1 (Santa Cruz Biotechnology Inc) were incubated at room temperature for 1 and 2 hours, respectively. Dilutions of the antibodies were prepared according to the manufacturer's protocols. After being washed three times with 0.1% TBST, the membranes were incubated for 1 hour at room temperature with secondary goat anti-rabbit or antimouse antibodies conjugated with horseradish peroxidase (Cell Signalling Technologies Inc), added in a 1:6000 dilution. Then, the

TABLE 1 The characteristics of primers used for analysis of methylation levels in the promoter regions of the studied genes

Gene	Starter sequence (5' -> 3')	Product size [bp]	Tm [°C]	Number of CpG islands in promotor region
CAT	F:TTTGAGATTATTGTGTTTGAAA R:TACCTACACCCAAAAAAAAATA	148	59	1
Gpx1	F:GTTGTTTTAGGTTTTGTTGTTG R:AAAACTAAAATCCTCCAACTCT	102	65	1
Gpx4 (promotor 2)	F:AGGTTGGAGGTTTAGAGGTTTA R:TCCCCTAAATACAAAAATCTCT	118	59	1
Gpx4 (promotor 3)	F:AGGTTGGAGGTTTAGAGGTTTA R:AAAACATAACAAAATCATCTCCC	147	65	1
SOD1	F: AAGGAGGTGTGTTTAATTGGTA R: AACCCCTCTCACAAATTTCTAA	144	65	1
SOD2	F: GGGGAAGGTTATTTAGGGTATA R: CCTTTTCCATTCCTAATTCTAAA	133	59	1
NOS1 (promotor 3)	F: GGGTTTTTAATTTTTTTATTGTG R: CAACCCTCATTAAAAAAACC	124	59	1
NOS1 (promotor 7)	F: GTTTGAGATTGGAATTTTTTGG R: CCAAAACATCCAAAAATACACA	124	59	1

blots were again washed six times with 0.1% TBST and incubated with peroxidase substrate solution. For chemiluminescent reaction, the membranes were incubated for 3 minutes in a stable peroxide solution, an enhanced luminol solution and water in a 1:1:1 proportion (Thermo Fisher Scientific). The proteins were visualized on X-ray film by enhanced chemiluminescence. Using Gel-Pro® Analyzer Software (Media Cybernetics Inc), the integrated optical density (IOD) of the immunoreactivity bands was measured from digital images. The level of protein expression was normalized using the reference protein—beta-actin (ACTB; IOD_{gene}/IOD_{ACTB}).

2.8 | Drugs

Venlafaxine HCl (Carbosynth Ltd) was dissolved in 0.9% sterile saline, which was used for vehicle administration, and administered IP in a volume of 1 mL/kg of body weight, at the dose of 10 mg/kg, as used previously.[61,62]

2.9 | Statistical analysis

The effect of initial 2 weeks of CMS on sucrose consumption was analysed by test *t* when the data were normally distributed or Mann-Whitney rank-sum Test when the data were not normally distributed. The sucrose intakes, gene expression and methylation data were analysed using an one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's test, where *F* ratios were significant for the following groups: Controls/Vehicle, Stressed/Vehicle and Stressed/Venlafaxine when the data were normally distributed. Otherwise, the data were analysed using a Kruskal-Wallis one-way analysis of variance on ranks followed by post hoc Student-Newman-Keuls test, where *H* ratios were significant for the studied groups. Student's *t* test was used to analyse differences between blood and brain samples. *P* values <.05 were considered significant. Statistics were calculated using Statistica 12 (StatSoft), SigmaPlot 11.0 (Systat Software Inc) and GraphPad Prism 5.0 (GraphPad Software, Inc).

3 | RESULTS

3.1 | Sucrose intakes and body weights of animals exposed to CMS and venlafaxine

As shown in Table 2, before the stress was initiated (Week 0), the consumption of 1% sucrose solution was comparable in all groups. Following initial 2 weeks of stress (Week 2), the intakes decreased by approximately 40% ($F = 6.76$, fd = 2, $P < .01$, Tukey's test $P = .01$) and remained low until the end of experiment (Week

TABLE 2 Sucrose intakes in animals exposed to chronic mild stress (CMS) for 2 wk (Week 2) and in animals exposed to CMS for 7 wk (Week 7) and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk

Weeks of CMS	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Week 0	12.6 ± 1.6	11.0 ± 0.7	11.7 ± 0.7	11.9 ± 0.7	11.4 ± 0.5
Week 2	15.6 ± 1.9	6.8 ± 1.0$^{\#\#}$	4.9 ± 0.6$^{@@@}$	13.9 ± 0.9	5.8 ± 0.5$^{\&}$
Week 7			6.1 ± 0.7	13.3 ± 1.3	12.6 ± 1.0**

The data represent means ± SEM. N = 6.

$^{\#\#}P < .01$; relative to Week 2 in the Stressed group.

$^{\&}P < .05$; relative to Week 2 in the Stressed/Venla group.

$^{**}P < .01$; relative to Week 2 in the Stressed/Venla group.

$^{@@@}P < .001$; relative to Week 0 in the Control/Venlafaxine group.

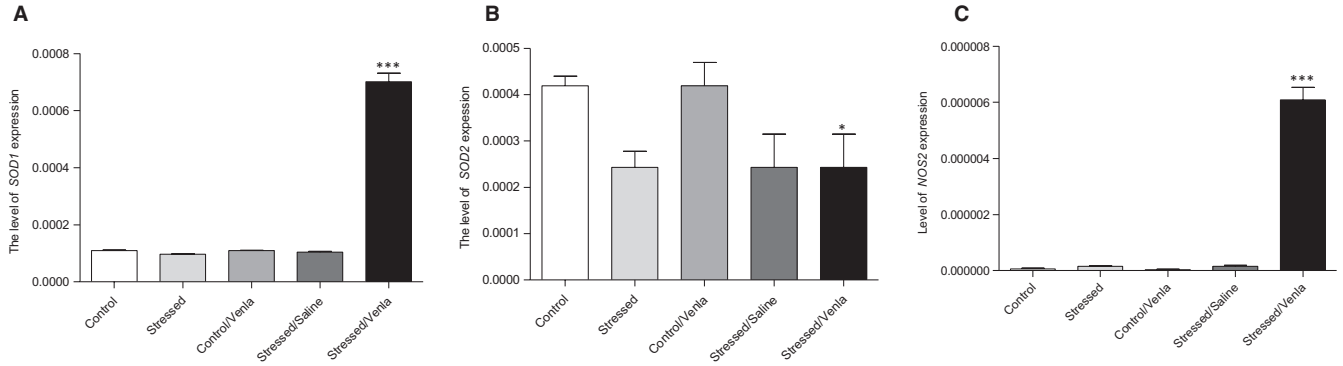


FIGURE 1 mRNA expression of *SOD1* (A), *SOD2* (B) and *NOS2* (C) genes in PBMCs of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). Relative gene expression levels were estimated using a $2^{-\Delta Ct\ (Ctgene-Ct18S)}$ method. Data represent means ± SEM. N = 6. $^{*}P < .05$; $^{***}P < .001$; relative to Stressed/Saline group

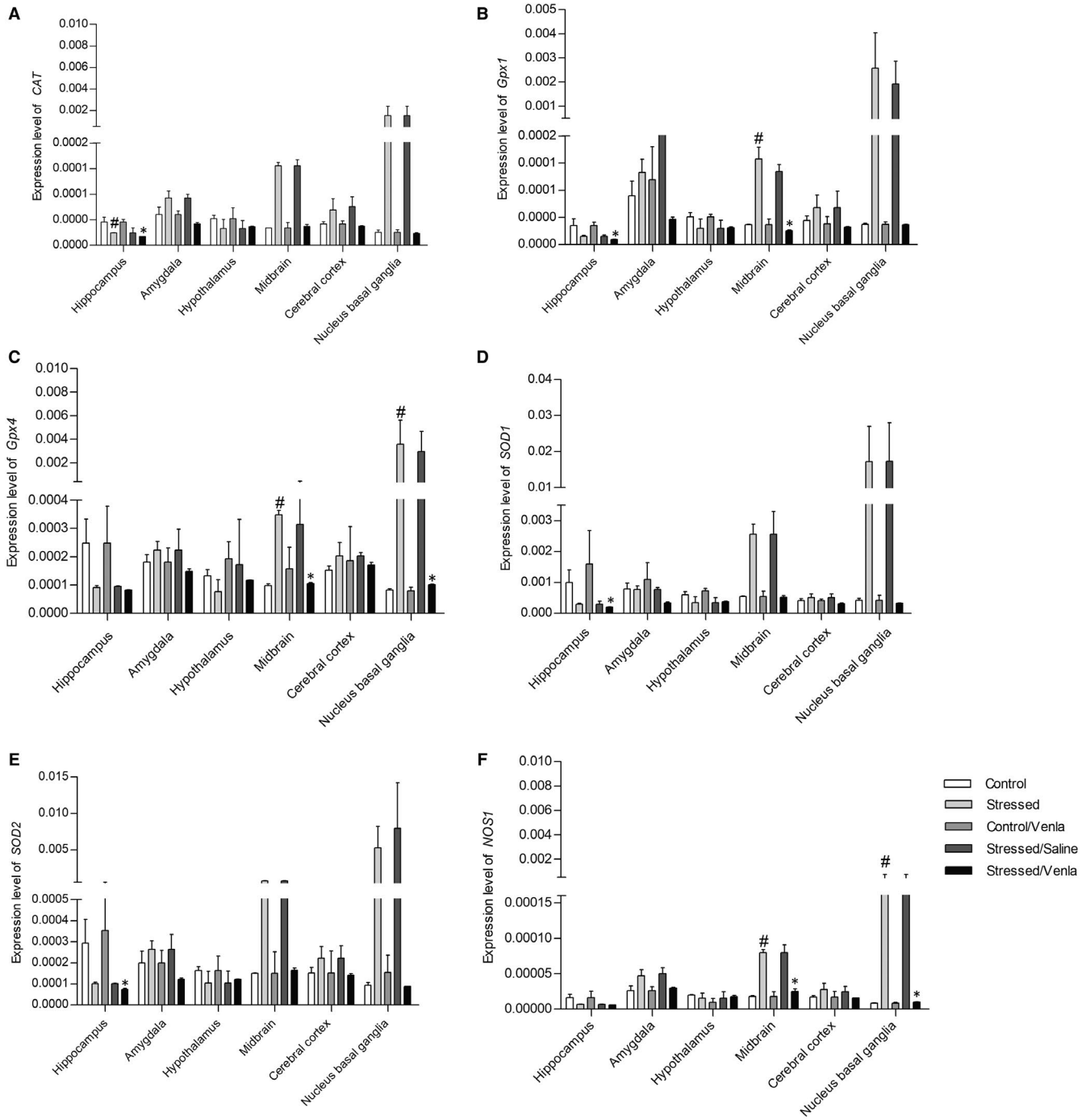


FIGURE 2 mRNA expression of *CAT*, *Gpx1*, *Gpx4*, *SOD1*, *SOD2* and *NOS1* in brain structures of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). Relative gene expression levels were estimated using a $2^{-\Delta Ct\,(Ctgene-Ct18S)}$ method. Data represent means ± SEM. N = 6. $^{*}P < .05$; relative to Stressed/Saline group. $^{\#}P < .05$; relative to Control group

7) in stressed animals treated with vehicle. Chronic (5 weeks) administration of venlafaxine had no effect in control animals but normalized the intakes in stressed rats ($F = 6.76$, fd = 2, $P < .01$, Tukey's test $P = .01$). Neither stress nor venlafaxine had any significant effect on body weights of control or stressed animals (data not shown).

3.2 | Gene expression at the mRNA level

3.2.1 | Gene expression in PBMCs

The expression of *Gpx1*, *Gpx4* and *CAT* mRNA in PBMCs did not differ between the two groups exposed to the control and stress

conditions for 2 weeks (Table S2), as well as between the control and stressed animals treated with saline (Figure 1.). Chronic (5 weeks) administration of venlafaxine had no effect in control animals but significantly increased the expression of *SOD1* ($F = 6.49$, $df = 2$, $P < .001$, Tukey's test $P < .001$), *SOD2* ($F = 7.20$, $df = 2$, $P < .01$, Tukey's test $P < .05$) and *NOS2* ($F = 6.49$, $df = 2$, $P < .001$) Tukey's test $P < .001$) genes in the stressed rats after venlafaxine therapy.

3.2.2 | Gene expression in brain

As shown in Figure 2, the effect of CMS and venlafaxine on the mRNA expression of the studied genes was clearly dependent on the structure, while CMS decreased the hippocampal expression of *CAT* ($F = 7.20$, $df = 2$, $P < .01$, Tukey's test $P < .05$), which was further down-regulated after venlafaxine administration ($P < .05$). On the other hand, the procedure increased the expression of *Gpx1* in midbrain ($F = 7.20$, $df = 2$, $P < .01$, Tukey's test $P < .05$), and *Gpx4* and *NOS1* expression in both midbrain and nucleus basal ganglia, all of which returned to control levels after administration ($F = 7.20$, $df = 2$, $P < .01$, Tukey's test $P < .05$). Moreover, after treatment with venlafaxine, the stressed animals were characterized by down-regulated hippocampal expression of *SOD1* ($F = 6.49$, $df = 2$, $P < .05$, Tukey's test $P < .05$), *SOD2* ($F = 5.96$, $df = 2$, $P < .05$, Tukey's test $P < .05$) and Gpx1 ($F = 5.96$, $df = 2$, $P < .05$, Tukey's test $P < .05$). Additionally, no differences in mRNA expression were found between the non-stressed group and the control animals after venlafaxine administration, or between the CMS group and the CMS group after vehicle administration.

3.2.3 | Comparison of gene expressions in PMBCs and brain

In order to compare effects of CMS and venlafaxine on the expression of *CAT*, *Gpx1*, *Gpx4*, *SOD1*, *SOD2* and *NOS1* genes in the brain and in blood, all data measured in the brain structures were pooled and compared with the changes detected in PMBCs. As shown in Figure 3, in all groups the expression of Gpx1 was higher, and the expression of *GPx4* lower, in the PBMCs than in the brain structures ($P < .01$ and $P < .05$, respectively). The expression of *CAT* and *SOD2* were lower in the brain tissue of the stressed rats treated with venlafaxine ($P < .001$). In addition, the expression of *SOD1* in brain tissue was higher than that observed in PBMCs of the stressed rats and the stressed rats treated with saline and venlafaxine ($P < .01$).

3.2.4 | Effect of venlafaxine on the expression of genes in PMBCs and brain structures

Venlafaxine significantly decreased the expression of *CAT* and *Gpx4* in amygdala ($P < .05$) and midbrain ($P < .01$), when compared to the expression of these genes in the PMBCs (Figure 4). Administration of venlafaxine caused a down-regulation of *Gpx1* and *SOD1* expression (Figure 4) in hippocampus ($P < .01$, $P < .05$, respectively), amygdala ($P < .01$), midbrain ($P < .001$, $P < .01$, respectively) and cerebral cortex ($P < .05$, $P < .01$, respectively), as compared to its effects in PBMCs. No differences in the effect of venlafaxine on SOD2 expression was found between the blood and brain tissue.

3.3 | Methylation of the studied gene promoters

3.3.1 | Methylation in PBMCs

The only significant change that was found in PBMCs was an increased methylation of *Gpx1* promoter region in animals exposed to the CMS procedure for 2 weeks ($H = 12.83$, $df = 2$, $P < .05$, Tukey's test $P < .05$; Figure 5).

No significant differences between groups were observed for promoters of other genes in PBMCs (Table S3).

3.3.2 | Methylation in brain

As shown in Figure 6, the CMS procedure significantly increased methylation level of the second promoter of *Gpx4* in midbrain ($F = 28.19$, $df = 2$, $P < .001$, Tukey's test $P < .001$) and basal ganglia ($H = 6.76$, $df = 2$, $P < .05$, Tukey's test $P < .05$) and methylation levels of *SOD1* and *SOD2* promoters in hippocampus ($H = 5.14$, $df = 2$, $P < .05$, Tukey's test $P < .05$; $H = 5.96$, $df = 2$, $P < .05$, Tukey's test $P < .01$, respectively). No other significant differences between groups were observed for the other promoters in the brain structures (Table S4).

3.3.3 | Comparison of the methylation status of gene promoters in PBMCs and brain

In order to compare the effects of CMS and venlafaxine on the brain and peripheral expression of *CAT*, *Gpx1*, *Gpx4*, *SOD1*, *SOD2* and *NOS1* genes, all results obtained for the brain structures were pooled and compared with changes detected in the PBMCs (Figure 7).

The untreated and venlafaxine-treated control animals showed an increased methylation in the promoter region of *CAT* and the third *Gpx4* promoter in brain tissue, compared to PBMCs ($P < .05$). In the stressed animals treated with venlafaxine, the methylation level of the second *Gpx4* promoter was lower in the brain than in PBMCs ($P < .01$). In all groups, the third promoter of NOS1 demonstrated lower methylation status in brain tissue than in PBMCs ($P < .001$). No other significant differences were observed for other promoters (Table S5).

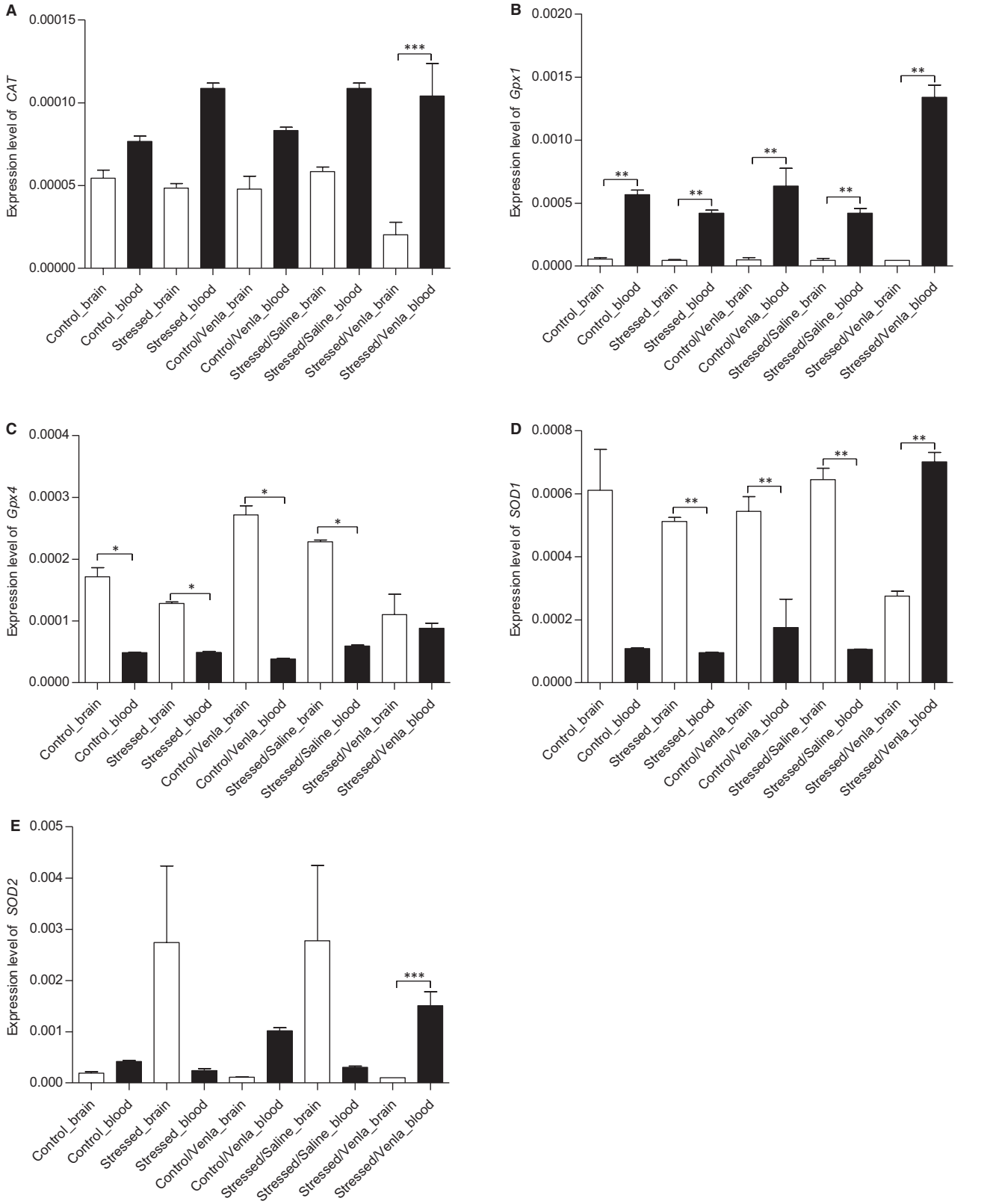
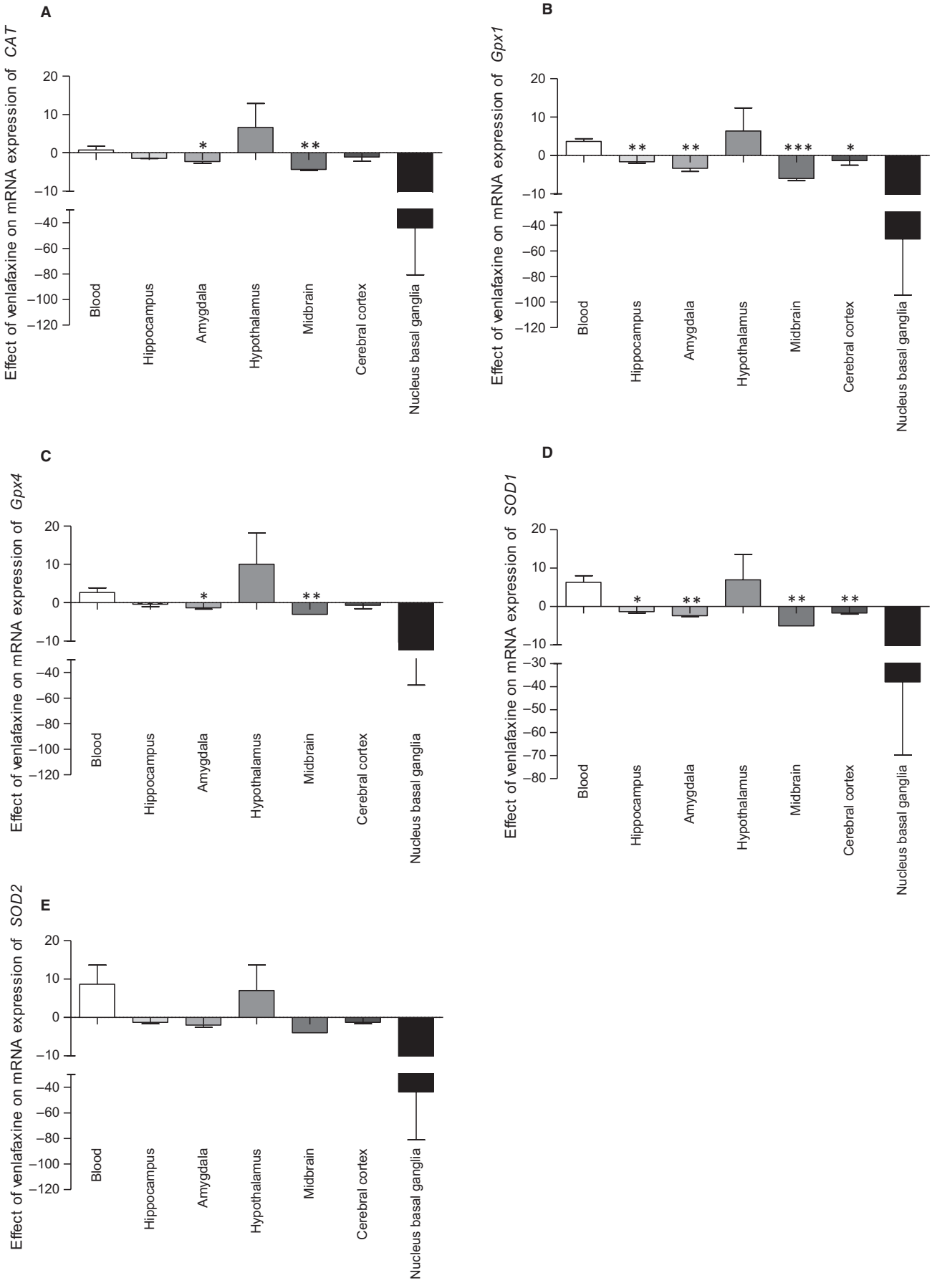


FIGURE 3 Differences in *CAT* (A), *Gpx1* (B), *Gpx4* (C), *SOD1* (D) and *SOD2* (E) gene expression between brain tissue and PBMCs of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). The mRNA relative expression levels of all five genes measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia were pooled and compared with the data detected in PBMCs. Relative gene expression levels were estimated using a $2^{-\Delta Ct\ (Ct\ gene - Ct\ 18S)}$ method. Data represent means ± SEM. N = 6. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$


A
Effect of venlafaxine on mRNA expression of CAT
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−120
*
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Blood
Hippocampus
Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia
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Effect of venlafaxine on mRNA expression of Gpx1
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Hippocampus
Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia
C
Effect of venlafaxine on mRNA expression of Gpx4
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−10
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−60
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Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia
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Effect of venlafaxine on mRNA expression of SOD1
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Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia
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Effect of venlafaxine on mRNA expression of SOD2
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Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia

FIGURE 4 mRNA expression of *CAT* (A), *Gpx1* (B), *Gpx4* (C), *SOD1* (D) and *SOD2* (E) in PBMCs and in brain structures of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). The effects are presented as fold change ($2^{-\Delta\Delta Ct}$ method[51]). Data represent means ± SEM. N = 6. **P* < .05, ***P* < .01, ****P* < .001; relative to PBMCs

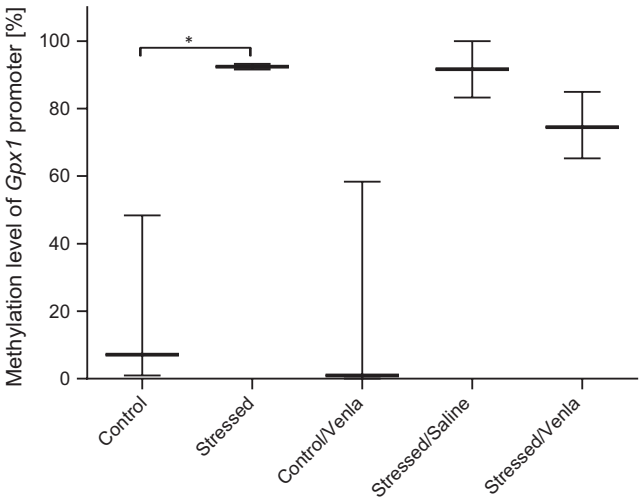

FIGURE 5 Methylation level of *Gpx1* promoter in PBMCs of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). Data represent median and maximum-minimum values. N = 6. **P* < .05

3.3.4 | Effect of venlafaxine on the methylation status of gene promoters in PBMCs and brain structures

Compared to the effect of venlafaxine in PBMCs, the drug caused a stronger increase in the methylation level of *CAT* promoter in amygdala and hippocampus (*P* < .05) and the NOS1 seventh promoter in hippocampus and midbrain (*P* < .01) (Figure 8).

3.4 | Gene expression on the protein level

As shown in Figure 9, venlafaxine significantly elevated the level of CAT protein in midbrain and cerebral cortex ($F = 7.62$, $df = 2$, $P < .01$, Tukey's test $P < .01$; $F = 15.22$, $df = 2$, $P < .001$, Tukey's test $P < .001$, respectively). The level of Gpx4 protein was elevated in hippocampus of the stressed animals ($F = 7.45$, $df = 2$, $P < .01$, Tukey's test $P < .05$) (Figure 10), and this effect was further enhanced following venlafaxine treatment ($F = 7.45$, $df = 2$, $P < .01$, $P < .05$). In cerebral cortex, the Gpx4 protein level was decreased in stressed animals ($F = 6.16$, $df = 2$, $P < .05$, Tukey's test $P < .05$), and again, this effect was enhanced by venlafaxine treatment ($F = 6.16$, $df = 2$, $P < .05$, Tukey's test, $P < .05$). No significant effects were observed in the other proteins (Table S6).

4 | DISCUSSION

The present study is the first to investigate effects of repeated administration of venlafaxine on the expression of selected genes in the PBMCs of rats exposed to the CMS model of depression. Our results also demonstrate the CMS- and venlafaxine-induced changes in the genes involved in oxidative and nitrosative stress in PBMCs and in six regions of the brain (hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia) with regard to their promoter methylation status and their expression of the mRNA and protein levels. In this study, genes expression were measured, rather than, activity of the antioxidant enzymes to highlight the role of epigenetic changes, that is promoter methylation, which is possible only when DNA, RNA and proteins are isolated from the same sample. There are reports showing that the expression of antioxidant enzymes is correlated with their activity[66,67] so the proteins selected in this study were not enzymatically active and, therefore, could only be quantified using either Western blot or immunosorbent assays.

The first studied gene encodes catalase (CAT), an important antioxidative enzyme that decomposes hydrogen peroxide into water and oxygen.[68] It is believed to be involved in the mechanisms of depression but the exact nature of this involvement remains unclear. Our findings indicate that *CAT* expression in hippocampus was lowered in animals exposed to the CMS procedure, and this finding is in line with other studies showing that chronic stress causes a reduction in cortical and hippocampal catalase activity.[34,35] Similar reductions in enzyme activity were observed in the erythrocytes of depressed patients.[69] However, CAT activity was also found to be increased in the serum of these patients,[70] a finding inconsistent with our observation that the CMS procedure did not affect *CAT* expression in PBMCs. Interestingly, we found that administration of venlafaxine to stressed animals significantly elevated the level of CAT protein in their midbrain and cerebral cortex. Similarly, catalase activity increased in the nucleus accumbens of rat brains after administration of quetiapine, an atypical antipsychotic used also in the pharmacotherapy of depression.[36] In addition, a study employing a model of behavioural despair in mice showed that venlafaxine restored reduced CAT activity in the brain.[41] However, our present findings do not suggest that the peripheral expression of CAT was affected by venlafaxine, which is in line with report by Ozcan et al[69] that the treatment with venlafaxine did not change the CAT activity in erythrocytes.

Another antioxidant enzyme believed to be associated with mechanisms of depression is GSH peroxidase (Gpx), which reduces lipid hydroperoxides and free hydrogen peroxide.[72] Gpx1 and Gpx4 have been shown to be selenium-containing enzymes. While Gpx1

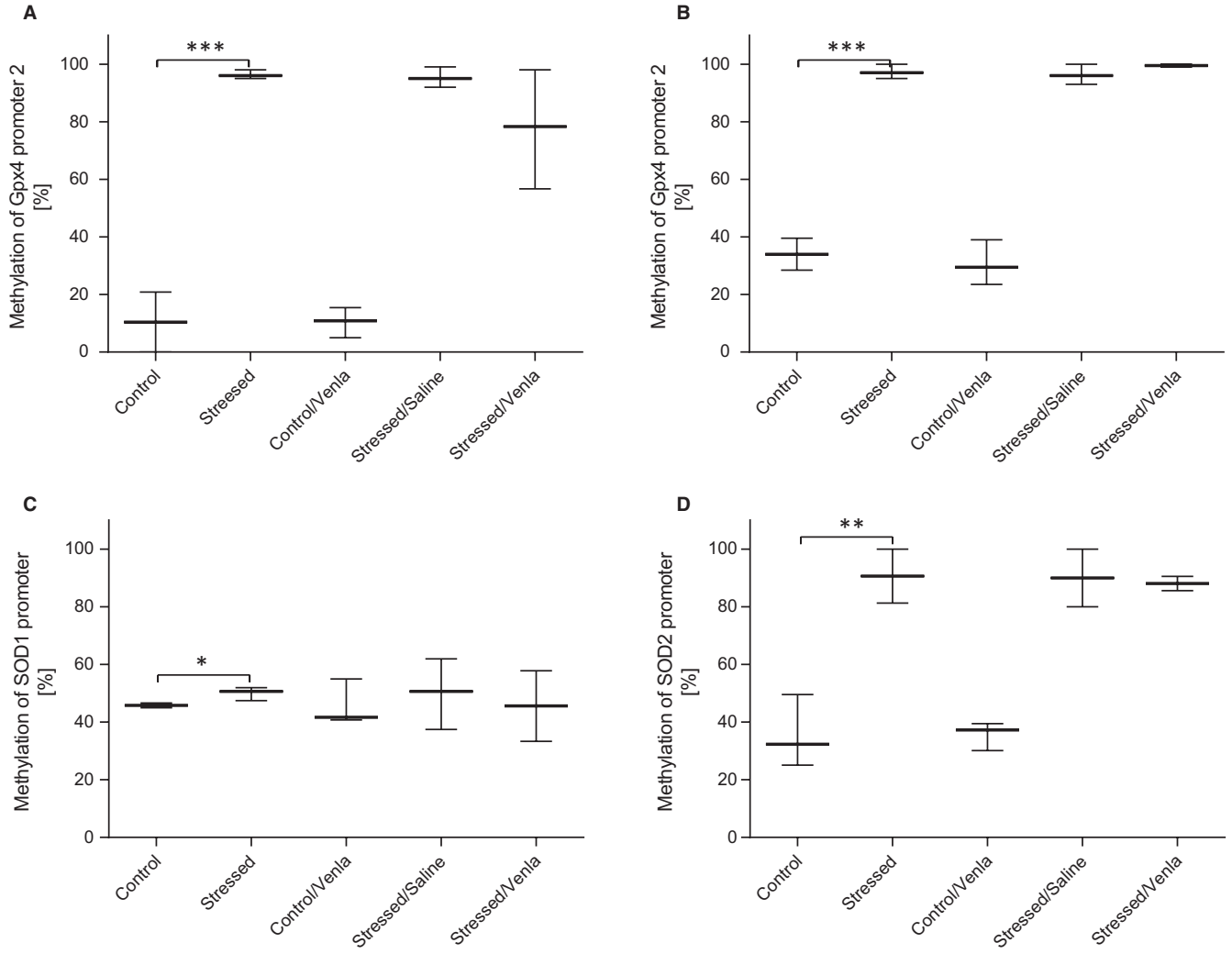

FIGURE 6 Methylation levels of *Gpx4* promoter 2 in midbrain (A) and basal ganglia (B), *SOD1* (C) and *SOD2* (D) promoter in hippocampus of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). Data represent means ± SEM (A) or median and minimum-maximum values (B–D). N = 6. **P* < .05, ***P* < .01, ****P* < .001

is present only in the cytosol, Gpx4 is located in the membrane and mitochondria.[73] Our findings indicate that the mRNA expression of Gpx1 was increased in midbrain of the CMS rats. In contrast, Eren et al[74] found that Gpx activity in the cortex is lowered in rats subjected to the CMS procedure and that venlafaxine administration normalized this effect. In humans, Gpx1 activity was found to be decreased in the haemolysed erythrocytes of depressed women [32] and increased in the erythrocytes of patients with melancholia.[75] Moreover, the changes in the Gpx1 concentration have been found to be negatively correlated with the severity of depressive symptoms.[76] Our present findings indicate that the midbrain and hippocampal expression of *Gpx1* mRNA was significantly lower in the venlafaxine-treated stressed animals than in the vehicle-treated stressed rats. Accordingly, the activity of Gpx in serum was found to decrease over the course of 3-month administration of different SSRIs.[75] In the present study, higher *Gpx1* mRNA expression was observed in PBMCs than in brain tissue in all of the stress and treatment conditions, and the methylation of the Gpx1 promoter in PBMCs was lower in the control than the CMS rats: a key novel finding of other crucial regulation of mRNA gene expression.

This study is also the first to examine the changes in the level of Gpx4 isoform in an animal model of depression. The CMS procedure increased the expression of Gpx4 mRNA in midbrain and basal ganglia, and this effect was normalized by administration of venlafaxine. Interestingly, the methylation levels of the second Gpx4 promoter in these two brain regions were also higher in the stressed group. This however did not affect the level of mRNA expression, because the *Gpx4* gene contains six promoters and only one of them was found to have higher methylation status in animals exposed to the CMS procedure. CMS also increased the Gpx4 protein level in hippocampus, and venlafaxine caused a further increase in Gpx4 protein expression while the opposite trend was observed in cerebral cortex. The differences between the expression of *Gpx4* mRNA and protein could be a result of epigenetic or posttranscriptional modifications, for example the influence of microRNA. Moreover, the presence of a high level of *Gpx4* mRNA expression and a high methylation status

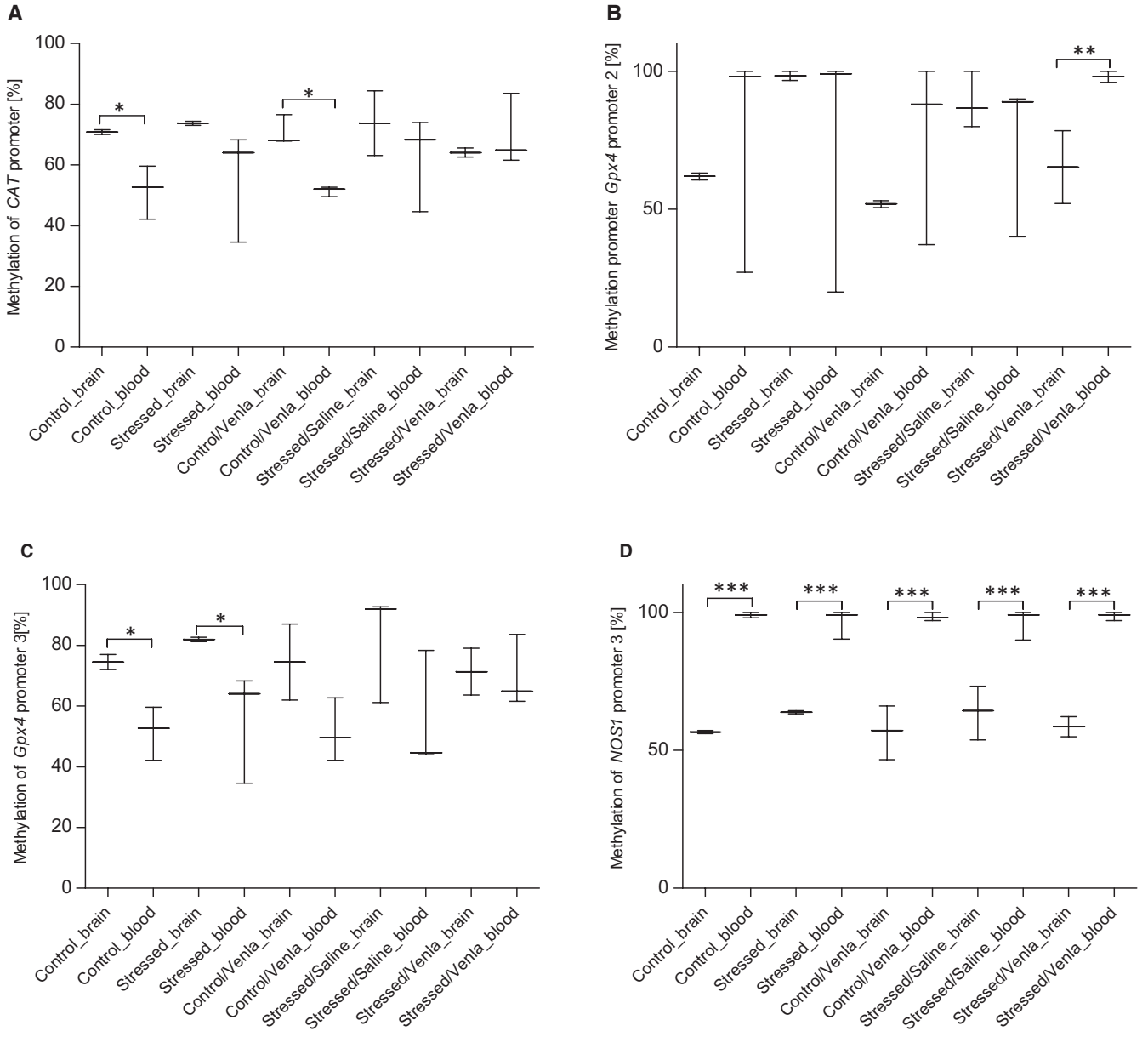

FIGURE 7 Differences in methylation level of *CAT* (A), *Gpx4* promoter 2 (B), *Gpx4* promoter 3 (C) and *NOS1* promoter 3 (D) between brain tissue and PBMCs of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 weeks (Control/Venla, Stressed/Saline, Stressed/Venla). The methylation level of all four genes measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia were pooled and compared with the data detected in PBMCs. Data represent median and maximum-minimum values. N = 6. **P* < .05, ***P* < .01, ****P* < .001

of the *Gpx4* promoter could suggest that gene expression is subject to other forms of regulation than the methylation of promoter regions. Other epigenetic modifications that alter DNA accessibility and chromatin structure, thereby regulating patterns of gene expression, could also be considered, that is histone modification (such as methylation and acetylation) and nucleosome positioning. These processes are crucial to the normal development and functioning of cells.[77] The results may also indicate the involvement of other, post-transcriptional epigenetic regulation, that is RNA silencing by microRNA, as the mRNA expression of the studied genes was found to differ from their respective protein levels. As such, the role of these processes should be addressed in future studies.

The next antioxidant enzyme associated with the mechanism of depression is SOD. In post-mortem clinical studies, increases in SOD1 and SOD2 levels were found in frontal cortex of patients with schizophrenia and in prefrontal cortex of depressive subjects.[78] A greater erythrocyte activity of SOD1 was also found in patients with depression[75]; however, there are reports showing opposite effects: lower serum[31,37] and erythrocyte SOD1 activity[79] in depressed patients. In the present study, we did not observe any significant effect of the CMS procedure on the PBMCs level of SOD1 protein.

The concentration of SOD1 in serum of depressed patients is decreased during the course of citalopram and fluoxetine therapy[24]; this finding is consistent with our observation of increased *SOD1*

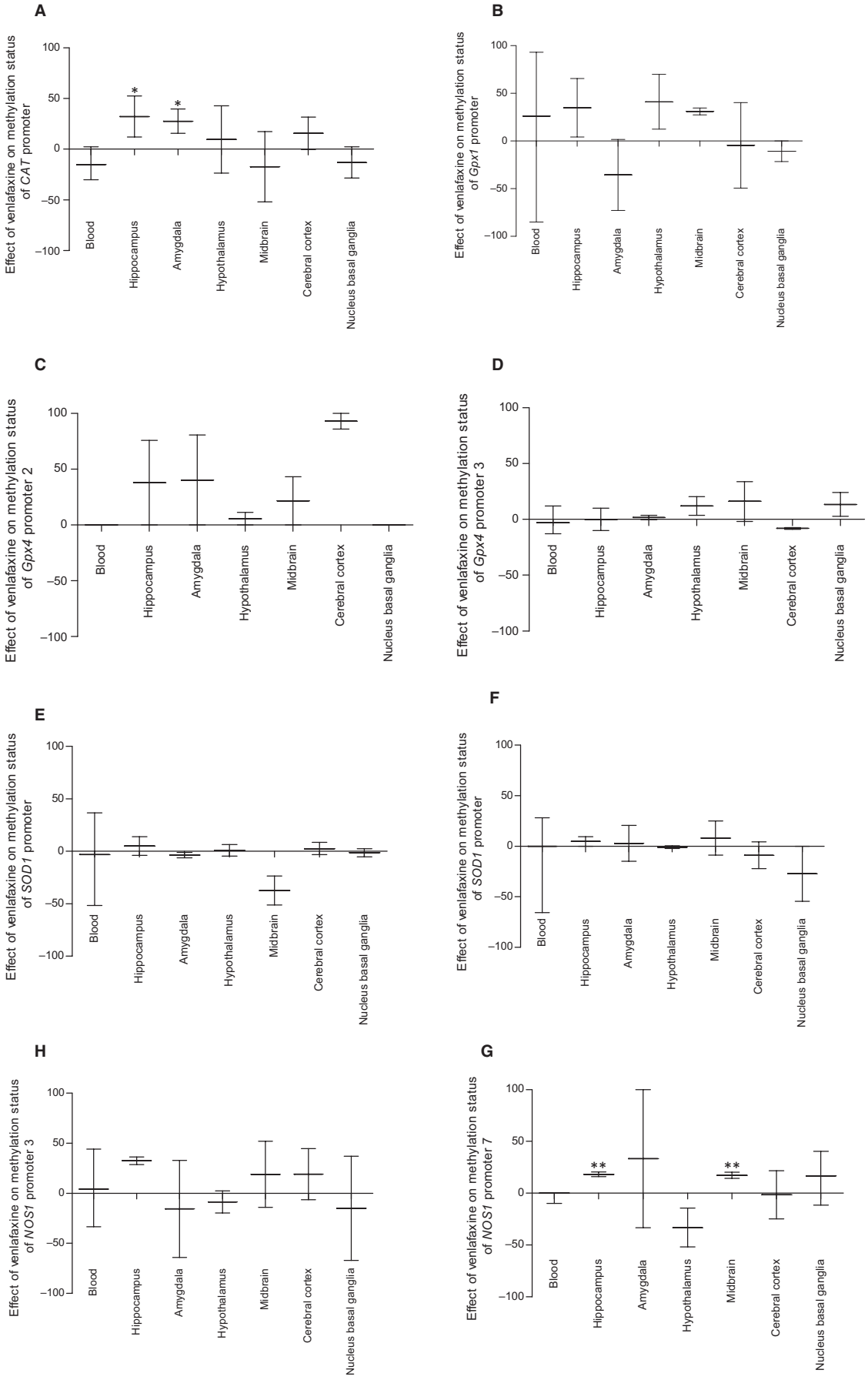
A
Effect of venlafaxine on methylation status of CAT promoter
100
50
0
−50
−100
*
*
Blood
Hippocampus
Amygdala
Hypothalamus
Midbrain
Cerebral cortex
Nucleus basal ganglia
B
Effect of venlafaxine on methylation status of Gpx1 promoter
C
Effect of venlafaxine on methylation status of Gpx4 promoter 2
D
Effect of venlafaxine on methylation status of Gpx4 promoter 3
E
Effect of venlafaxine on methylation status of SOD1 promoter
F
Effect of venlafaxine on methylation status of SOD1 promoter
H
Effect of venlafaxine on methylation status of NOS1 promoter 3
G
Effect of venlafaxine on methylation status of NOS1 promoter 7
**
**

FIGURE 8 Differences in methylation level of *CAT* (A), *Gpx1* (B), *Gpx4* promoter 2 (C), *Gpx4* promoter 3 (D), *SOD1* (E), *SOD2* (F), *NOS1* promoter 3 (G) and *NOS1* promoter 7 (H) between brain tissue and PBMCs of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). The methylation level of all eight genes measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia were pooled and compared with the data detected in PBMCs. Data represent means ± SEM. N = 6. $^{*}P < .05$, $^{**}P < .01$; relative to PBMCs

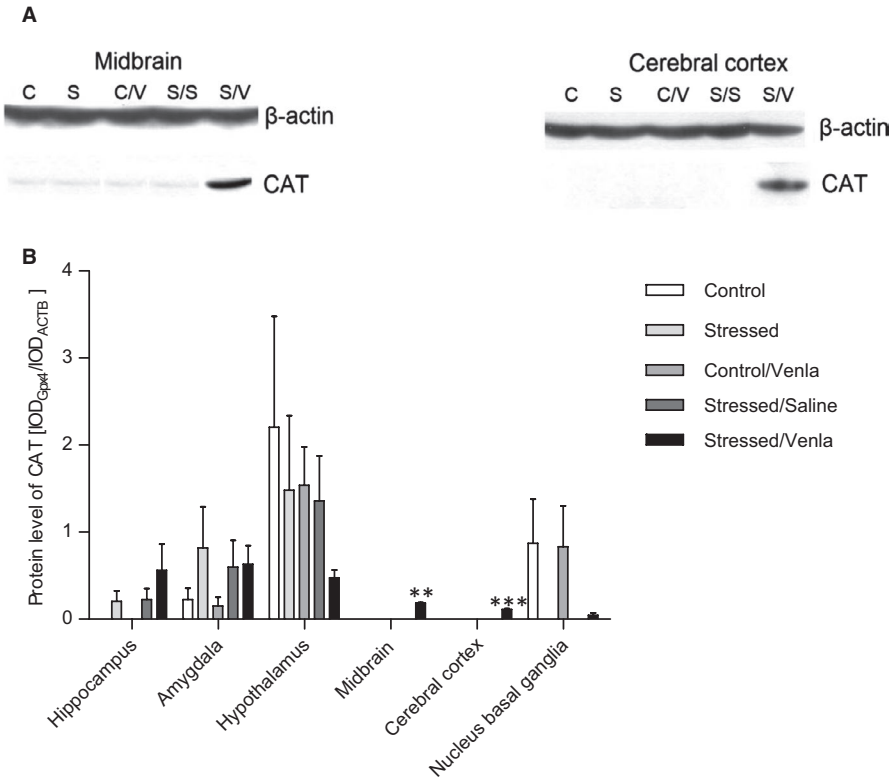

FIGURE 9 mRNA expression of CAT in brain structures of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). A, Representative Western blot analysis of the effects in midbrain and cerebral cortex. C—controls, S—stressed for 2 wk, C/V—Control/Venlafaxine, S/S—Stressed/Saline, S/V—Stressed/Venlafaxine. B, Level of CAT proteins measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia. Samples containing 25 μg of proteins were resolved by SDS-PAGE. The intensity of bands corresponding to CAT was analysed by densitometry. Integrated optical density (IOD) was normalized by protein content and a reference sample (see the Methods section for details). The graphs show the mean IODs of the bands from all analysed samples. The IOD$_{gene}$/IOD$_{ACTB}$ method was used to estimate the relative protein expression levels in the analysed samples. Data represent means ± SEM. N = 6. $^{**}P < .01$, $^{***}P < .001$; relative to Stressed/Saline group

mRNA level in hippocampus of stressed animals administered venlafaxine. Similarly, Galecki et al[80] reported decreased SOD1 activity in the red blood cells (RBCs) of patients treated with combined fluoxetine and acetylsalicylic acid therapy; however, our present findings indicate that SOD1 mRNA expression in PBMCs of the CMS rats was increased by venlafaxine. *SOD2* mRNA expression and protein activity is lower in depressed patients,[81] and the RBCs activity of SOD2 is positively associated with severity of the disease.[82] Our present animal data show that *SOD2* mRNA expression in PBMCs was greater in CMS rats after venlafaxine therapy compared to the CMS group. Similarly, while SOD2 activity was previously found to be elevated in brain tissue of animals treated with venlafaxine,[59] our findings indicate that hippocampal *SOD2* mRNA expression is decreased after venlafaxine therapy. There is evidence that overexpression of *SOD2* could play a crucial role in reducing oxidative stress and preventing neurodegenerative disease[83] and that reduced *SOD2* expression in the cerebral cortex, cerebellum and basal ganglia may lead to neurodegeneration.[84] The differences between blood and brain in rats after therapy may be result of different tissue response to treatment. Probably, enzyme activity might have been more susceptible to antidepressant therapy in blood than in the brain. Moreover, these changes in SOD2 concentration may be responsible for the reductions in the volume of prefrontal cortex and hippocampus that are characteristic of patients with depression.[85] Therefore, the reduction in brain structure volume may cause irreversible changes in antioxidant capacity, resulting in no response to treatment in brain. Our present results show that the *SOD2* promoter region displayed higher methylation status in the stressed groups, suggesting that

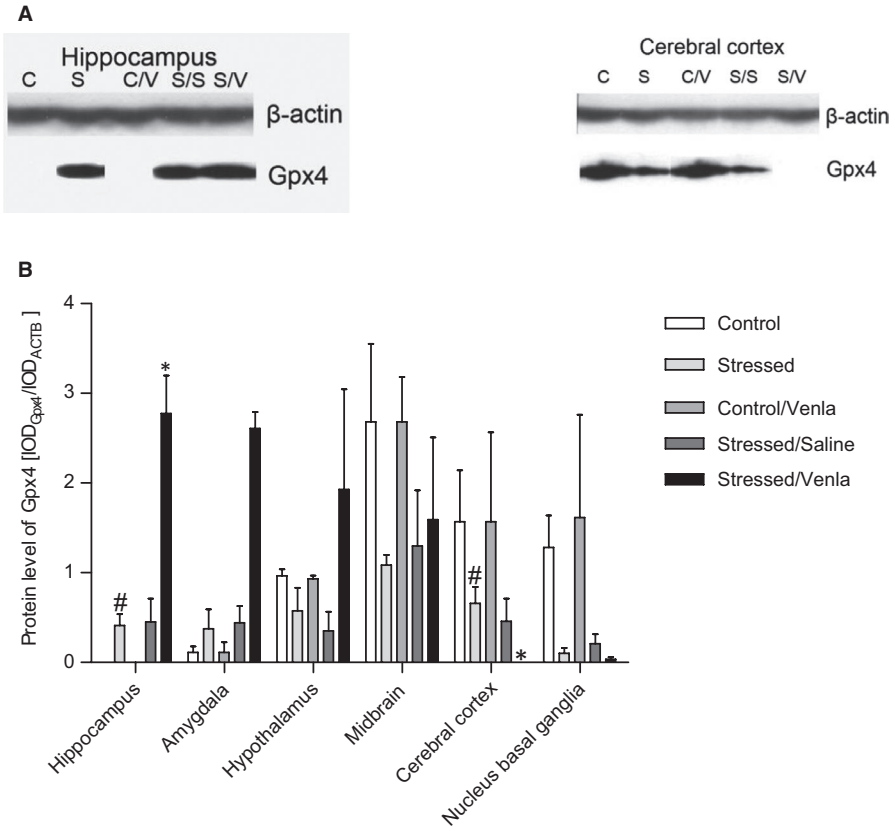

FIGURE 10 mRNA expression of Gpx4 in brain structures of animals exposed to chronic mild stress (CMS) for 2 wk (Control, Stressed) and in animals exposed to CMS for 7 wk and administered vehicle (1 mL/kg) or venlafaxine (10 mg/kg) for 5 wk (Control/Venla, Stressed/Saline, Stressed/Venla). A, Representative Western blot analysis of the effects in hippocampus and cerebral cortex. C—controls, S—stressed for 2 wk, C/V—Control/Venlafaxine, S/S—Stressed/Saline, S/V—Stressed/Venlafaxine. B, Level of Gpx4 proteins measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia. Samples containing 25 μg of proteins were resolved by SDS-PAGE. The intensity of bands corresponding to CAT was analysed by densitometry. Integrated optical density (IOD) was normalized by protein content and a reference sample (see the Methods for details). The graphs show the mean IODs of the bands from all analysed samples. The IOD_{gene}/IOD_{ACTB} method was used to estimate the relative protein expression levels in the analysed samples. Data represent means ± SEM. N = 6. $^{**}P < .01$, $^{***}P < .001$; relative to Stressed/Saline group. $^{**}P < .01$, $^{***}P < .001$; relative to Stressed/Saline group. $^{\#}P < .05$; relative to Control group

the antioxidant defence in the stressed animals was indeed less efficient. It should be however noted that only one of the two *SOD2* promoters was characterized by high methylation status, and no differences in SOD2 protein level were observed.

NOS generates NO, which plays a key role in many biological processes and in regulation of cognitive and emotional functions. Overproduction of NO can lead to oxidative and antioxidative imbalance, which is believed to be involved in pathomechanisms of neurodegenerative diseases, including anxiety and depression.[86,87] Previous studies confirmed that depressed patients are characterized by an increased level of NO in plasma.[88,89] In line with this, the CMS rats and mice showed an increased level of *NO* in cortex.[74,90] Our results also indicate that the *NOS1* mRNA expression in midbrain and basal ganglia was higher in rats exposed to the CMS procedure. However, no changes were observed in the stressed animals administered venlafaxine, which is inconsistent with the results obtained by Yoshino et al[50] who reported that antidepressant treatment increased *nNOS* (*NOS1*) mRNA expression in the hippocampus, midbrain, cerebellum and olfactory bulb,[50] *iNOS* (*NOS2*) mRNA expression in the frontal cortex and midbrain, and decreased *eNOS* mRNA expression in most brain regions. Interestingly, in the study which used a similar CMS paradigm, Wang et al reported that the suppression of hippocampal NOS can protect against the development of depressive-like symptoms in this model of depression.[92]

The presence of elevated NO levels in depressed patients could be a result of an increased expression of cellular *NOS1* in suprachiasmatic nucleus, cornu ammonis area 1 and subiculum regions.[92] The serum NO level was significantly decreased in depressed patients after 8 weeks of SSRI treatment,[37] while an earlier animal study indicated that CMS caused a deformation of neurons in the hippocampus, while fluoxetine therapy led to-normalization of these neurons by inhibiting NOS1.[93] Hence, it is likely that CMS could cause a nitrosative stress that is inhibited by venlafaxine-induced reduction in NO generation. However, the level of NO in cortex of the CMS animals was found to be reduced following venlafaxine treatment.[85] In the present study, no significant differences were detected between any groups with regard to NOS1 protein level in the brain. Talarowska et al (2015) found

no significant difference in NOS2 levels between patients with recurrent depressive disorders and those in a first episode of depression.[95] However, our findings indicate that in the CMS animals venlafaxine did increase mRNA expression of NOS2 in PBMCs compared to the untreated stressed group.

This study is the first to report the effect of CMS and antidepressant treatment on the expression and methylation status of genes involved in oxidative and nitrosative stress in both the blood and the brain in the same experimental setting. Such comparative analysis is not possible to carry out in humans, because it would require too many subjects, and the brain tissue would be available post-mortem only. Hence, the use of a validated animal model of depression, which can pinpoint most promising genes that can be then verified in depressed patients, seems to be most reasonable for identification peripheral markers of the central nervous system pathology. We found that the mRNA expression of *Gpx1* was significantly higher, and that of *Gpx4* significantly lower, in PBMCs than the brain structures in all groups. Also, the expression of SOD1 mRNA was higher in the brains of all control and stressed groups, except for the stressed animals successfully treated with venlafaxine, which showed higher expression of this gene in the PBMCs. Similar enhanced expression in the PBMCs of the venlafaxine-treated stressed animals was observed for the *CAT* and *SOD2* genes; however, in other groups these genes either did not change (*CAT*) or changed regardless of the conditions (*SOD2*).

The present findings and those of previously reported by others [28–31,50,95] suggest that the lack of sufficient enzymatic antioxidant defence in PBMCs and brain are involved in effects observed in animals exposed to the CMS procedure, a conclusion that could be cautiously extrapolated to the mechanisms of human depression. We can speculate that this inadequate antioxidant defence leads to increased production of toxic free radicals which, in excessive amounts, imply damage to biomolecules. In consequence, prolonged exposure to free radicals may cause a cell death, neuronal and glia atrophy leading to decreased grey matter volume, reduced cell numbers and low glucose metabolism observed in depressed patients.[96,97] On the other hand, antidepressant therapy may modulate the activity of enzymes restoring this antioxidant defence system to its proper functioning. Accordingly, our findings confirm that venlafaxine reduces the mRNA expression of the genes encoding the SOD1, SOD2, Gpx1, Gpx4, CAT and NOS1 enzymes in the brain, but increases that of SOD1, SOD2 and NOS2 in PBMCs. Only brain expression of *Gpx1*, *Gpx4* and *NOS1* mRNA was reduced to control levels by venlafaxine therapy. The decrease in the level of antioxidant enzyme gene expression may indicate reduced production of free radicals after venlafaxine therapy, and the decreased expression of genes involved in oxidative stress may, in turn, indicate effectiveness of the antidepressant treatment.

Unfortunately, our results were characterized by a large variability. Depression is a heterogeneous disease and the clinical symptoms vary among patients. The distinct symptoms may be either present or absent in individual cases of depression, or even go in opposite directions. The opposing symptoms are also seen in patients with depression—excessive or lack of appetite, insomnia or excessive sleepiness Thus, the developing a stable model of this disease is very difficult. Moreover, a many symptoms of human depression, including feelings of worthlessness, guilt, or thoughts of death, cannot be modelled in animals. The animal species, strain and sex are also important in preparation of a valid animal model of depression. Additionally, the disease genes for depression are unknown, and thus, many transgenic models and models based on behavioural selection might not represent valid models of the disease.[11]

5 | CONCLUSION

Our findings indicate that oxidative and nitrosative stress are involved in the effects of CMS and venlafaxine administration, more specifically, (a) the CMS procedure changed the expression of *CAT*, *Gpx1*, *Gpx4* and *NOS1* at the mRNA level only in the brain; (b) chronic administration of venlafaxine affected the mRNA expression of *SOD1*, *SOD2* and *NOS1* both in the PBMCs and in the brain, and *CAT*, *Gpx1* and *Gpx4* only in the brain; (c) changes in promoter methylation was caused only by the CMS procedure in both the PBMCs and the brain; (d) contradictory results were found concerning the influence of promoter methylation on mRNA expression, that is an increased methylation status of the *Gpx4* promoter was associated with increased expression, showing that other epigenetic factors, like histone modifications, can affect the expression of the studied genes; and (e) the results obtained using peripheral tissue could predict the condition of the brain; however, this was dependent on brain structure. These findings provide strong evidence for thesis that analysis of the level of mRNA and protein expression as well as the status of promoter methylation can help in understanding the pathomechanisms of mental diseases, including depression, and the mechanisms of action of drugs effective in their therapy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Tomasz Śliwiński and Mariusz Papp conceived and planned the experiments; Paulina Wigner, Ewelina Synowiec, Piotr Czarny and Piotr Gruca contributed to sample preparation; Paulina Wigner, Ewelina Synowiec, Piotr Czarny, Michal Bijak, Paweł Jóźwiak, Piotr Gruca and Janusz Szemraj carried out the experiment; Paulina Wigner, Ewelina Synowiec, Mariusz Papp and Tomasz Śliwiński analysed the data; Paulina Wigner, Mariusz Papp and Tomasz Śliwiński wrote the manuscript and designed the figures and tables. All authors provided critical feedback and helped shape the research, analysis and manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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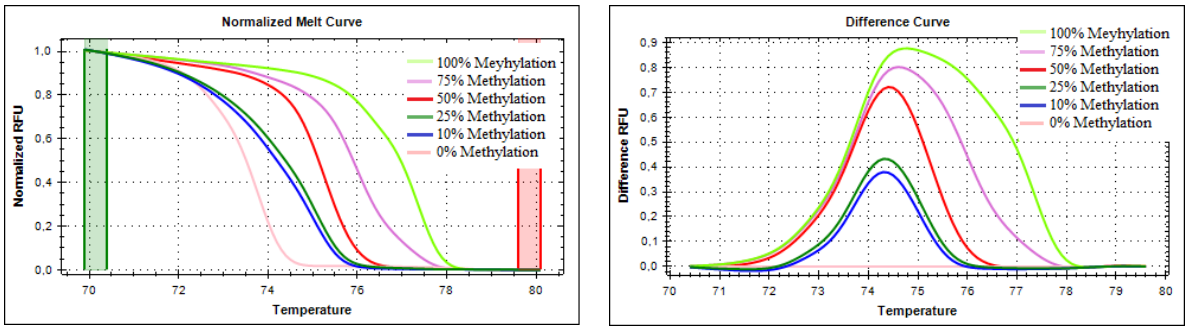
14. Supplementary information



Supplementary Figure 1. An example of HRM reaction results for the Gpx1 gene in the depressed patient group. Representative standard curves are shown for the determination of methylation level in DNA samples. Standard samples were 0%, 10%, 25%, 50%, 75% and 100% methylated genomic DNA standard of rat.

Supplementary Table 1. Characteristics of the genes studied (All data contained in the table were compiled with the help of Genomatix Software Suite, Intrexon Bioinformatics Germany GmbH, Munich, Germany, 2019).

Gene name	Function of protein encoding the gene	Chromosomal location	alternative transcripts	promotors	SNPs affecting transcription factor binding sites	SNPs affecting protein sequence	5' UTR	3' UTR	repeat regions	exons
Catalase (CAT)	hydrogen peroxide reductase	3	1	3	6	2	1	1	8	13
Glutathione peroxidase 1 (Gpx1)	Catalyses reduction of organic hydroperoxides and hydrogen peroxide by glutathione and thereby protect cells from oxidative damage	8	1	1	0	1	1	1	0	2
Glutathione peroxidase 4 (Gpx4)	Catalyses reduction of hydrogen peroxide, organic hydroperoxides and lipid hydroperoxides, and thereby protect cells from oxidative damage	7	4	6	8	1	2	11	2	22
superoxide dismutase 1 (SOD1)	Catalyzes conversion of supeoxide to hydrogen peroxide	11	1	2	2	0	2	2	1	5

	and molecular oxygen, involved in response to oxidative stress									
Superoxide dismutase 2 (SOD2)	Intramitochondrial free radical scavenging enzyme	1	1	2	2	0	1	1	1	5
Nitric oxide synthase 1 (NOS1)	Catalyzes production of nitric oxide	12	5	13	1	3	19	23	22	170
Nitric oxide synthase 2 (NOS2)	Cytokine-inducible enzyme involved in nitric oxide production	10	52	5	1	1	55	27	85	1383

Supplementary Table 2.

mRNA expression of Gpx1, Gpx4, CAT genes in PBMCs of animals exposed to CMS for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (Control/Venla, Stressed/Saline, Stressed/Venla). Relative gene expression levels were estimated using the $2^{-\Delta Ct}$ (Ct_{gene}–Ct_{18S}) method. The $2^{-\Delta Ct}$ values were re-calculated into relative copy number values (number of gene mRNA copies per 1 000 copies of 18S mRNA). Data represents means ± SEM. N = 6. No significant changes were found between any groups.

Group	Gpx1	Gpx4	CAT
Control	$5.7*10^{-4} \pm 2.0*10^{-5}$	$4.9*10^{-5} \pm 4.5*10^{-7}$	$7.7*10^{-5} \pm 1.7*10^{-6}$
Stressed	$4.2*10^{-4} \pm 1.3*10^{-5}$	$4.9*10^{-5} \pm 8.7*10^{-7}$	$1.1*10^{-4} \pm 1.7*10^{-6}$
Control/Venla	$5.9*10^{-4} \pm 1.9*10^{-5}$	$4.3*10^{-5} \pm 4.1*10^{-7}$	$7.5*10^{-5} \pm 1.9*10^{-6}$
Stressed/Saline	$6.2*10^{-4} \pm 2.6*10^{-5}$	$5.3*10^{-5} \pm 3.2*10^{-7}$	$7.9*10^{-5} \pm 1.9*10^{-6}$
Stressed/Venla	$4.4*10^{-4} \pm 5.3*10^{-5}$	$8.8*10^{-5} \pm 4.5*10^{-7}$	$1.0*10^{-4} \pm 1.1*10^{-5}$

Supplementary Table 3.

Methylation level of CAT promoter, Gpx4 promoter 2, Gpx4 promoter 3, SOD1 promoter, SOD2 promoter, NOS1 promoter 3 and NOS1 promoter 7 in PBMCs of animals exposed to CMS for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (Control/Venla, Stressed/Saline, Stressed/Venla). Data represents means ± SEM. N = 6. No significant changes were found between any groups.

Group	CAT	Gpx4	Gpx4	SOD1	SOD2	NOS1 3	NOS1 7
Control	51.50 ± 5.10	75.72 ± 24.28	51.50 ± 7.16	45.86 ± 4.50	32.65 ± 23.36	99.00 ± 0.58	61.52 ± 22.30
Stressed	55.65 ± 10.60	66.67 ± 33.33	55.65 ± 8.65	30.66 ± 13.37	13.88 ± 8.17	96.73 ± 3.27	66.34 ± 17.10
Control/Venla	53.62 ± 6.89	72.86 ± 23.47	52.11 ± 11.68	40.89 ± 9.82	29.18 ± 15.21	98.77 ± 2.11	62.15 ± 16.68
Stressed/Saline	61.02 ± 11.02	61.89 ± 27.89	65.72 ± 9.21	33.56 ± 11.65	19.21 ± 9.85	97.82 ± 3.82	65.48 ± 19.75
Stressed/Venla	70.06 ± 16.86	98.67 ± 0.88	70.15 ± 15.60	36.73 ± 12.45	26.31 ± 16.51	98.67 ± 0.89	61.21 ± 15.79

Supplementary Table 4.

Methylation level of CAT, Gpx1, Gpx4 promoter 2, Gpx4 promoter 3, SOD1, SOD2 promoter, NOS1 promoter 3 and NOS1 promoter 7 in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia of animals exposed to CMS for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (Control/Venla, Stressed/Saline, Stressed/Venla). Data represents means ± SEM. N = 6. No significant changes were found between any groups.

Methylation level of CAT promoter					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	65.13 ± 8.40	85.07 ± 2.21	55.13 ± 18.40	64.06 ± 7.10	52.89 ± 18.80
Amygdala	92.32 ± 0.85	93.30 ± 2.98	82.32 ± 10.23	63.49 ± 14.07	65.64 ± 6.82
Hypothalamus	61.73 ± 15.16	76.08 ± 16.08	65.51 ± 12.11	62.28 ± 0.21	66.48 ± 10.99
Midbrain	54.20 ± 23.04	51.78 ± 6.33	63.25 ± 18.15	70.47 ± 7.40	69.18 ± 21.99
Cerebral cortex	60.57 ± 8.02	74.94 ± 12.97	50.66 ± 12.22	37.57 ± 24.26	59.29 ± 14.01
Basal ganglia	91.38 ± 2.99	61.50 ± 5.31	81.22 ± 12.89	71.78 ± 7.87	74.52 ± 7.33

Methylation level of Gpx1 promoter					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	88.65 ± 3.31	94.32 ± 9.42	75.34 ± 13.13	96.10 ± 0.75	59.38 ± 27.30
Amygdala	60.07 ± 22.01	57.68 ± 20.73	59.11 ± 6.71	73.85 ± 5.58	93.14 ± 17.21
Hypothalamus	12.27 ± 6.11	93.85 ± 11.97	15.42 ± 5.89	58.66 ± 8.59	52.61 ± 17.60
Midbrain	49.02 ± 11.98	86.96 ± 5.91	52.32 ± 9.63	93.95 ± 3.49	55.94 ± 23.98
Cerebral cortex	72.59 ± 14.26	55.33 ± 23.12	65.89 ± 17.38	56.09 ± 13.27	59.85 ± 12.75
Basal ganglia	67.15 ± 9.61	82.48 ± 10.11	71.42 ± 12.96	69.71 ± 13.92	93.21 ± 14.56

Methylation level of Gpx4 promoter 2					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	82.17 ± 14.56	49.12 ± 11.85	76.08 ± 19.53	51.97 ± 21.59	52.12+39.09

Amygdala	59.15 ± 33.36	52.42 ± 9.49	61.64 ± 19.71	54.43 ± 15.63	59.75 ± 32.86
Hypothalamus	85.60 ± 11.76	79.71 ± 16.87	87.07 ± 10.56	82.74 ± 19.83	89.37 ± 9.87
Cerebral cortex	99.00 ± 0.82	98.56 ± 0.94	99.60 ± 0.32	98.02 ± 0.89	98.75 ± 0.92

Methylation level of Gpx4 promoter 3					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	69.18 ± 12.77	90.54 ± 3.26	72.51 ± 9.87	86.20 ± 0.24	86.31 ± 6.00
Amygdala	76.37 ± 6.93	73.28 ± 5.77	79.63 ± 9.37	77.31 ± 2.22	75.57 ± 3.38
Hypothalamus	63.43 ± 14.70	85.21 ± 9.56	70.31 ± 11.02	77.50 ± 12.32	65.53 ± 11.21
Midbrain	86.29 ± 8.06	70.26 ± 9.16	79.11 ± 9.16	67.70 ± 7.11	51.70 ± 13.12
Cerebral cortex	74.07 ± 2.20	88.08 ± 24.11	71.51 ± 8.27	78.68 ± 1.22	86.82 ± 10.75
Basal ganglia	77.89 ± 11.81	84.23 ± 16.32	79.21 ± 9.81	75.58 ± 11.68	62.20 ± 15.21

Methylation level of SOD1 promoter					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Amygdala	40.74 ± 7.05	55.47 ± 12.63	39.99 ± 11.32	56.38 ± 9.21	59.06 ± 13.43
Hypothalamus	25.95 ± 8.26	51.13 ± 1.34	35.11 ± 17.22	46.91 ± 3.82	50.26 ± 1.14
Midbrain	44.25 ± 5.01	18.65 ± 5.62	33.11 ± 11.89	23.39 ± 19.10	56.13 ± 3.93
Cerebral cortex	56.18 ± 7.85	52.29 ± 15.23	51.85 ± 18.21	47.12 ± 0.70	49.62 ± 4.07
Basal ganglia	43.72 ± 11.02	57.19 ± 5.81	51.21 ± 10.99	51.78 ± 1.46	58.48 ± 5.59

Methylation level of SOD2 promoter					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Amygdala	37.31 ± 9.96	68.57 ± 21.84	41.15 ± 11.02	47.11 ± 24.55	65.69 ± 15.38
Hypothalamus	15.72 ± 12.84	43.02 ± 24.28	18.64 ± 9.85	39.56 ± 9.54	43.96 ± 23.19
Midbrain	27.96 ± 22.83	66.28 ± 27.53	31.69 ± 19.83	69.96 ± 31.09	75.09 ± 16.24
Cerebral cortex	34.35 ± 5.39	29.57 ± 14.80	32.35 ± 7.99	30.81 ± 14.07	38.51 ± 25.65
Basal ganglia	67.47 ± 15.83	74.26 ± 29.18	71.89 ± 21.08	90.96 ± 7.38	91.56 ± 6.89

Supplementary information

Methylation level of NOS1 promoter 3					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	70.09 ± 4.09	75.21 ± 0.91	69.09 ± 9.91	74.67 ± 5.76	72.64 ± 8.99
Amygdala	80.70 ± 25.75	55.40 ± 16.99	79.89 ± 21.32	44.10 ± 1.93	71.01 ± 22.64
Hypothalamus	51.07 ± 15.82	69.08 ± 3.31	49.09 ± 17.21	42.60 ± 12.09	77.63 ± 22.32
Midbrain	31.17 ± 0.85	64.16 ± 29.26	39.81 ± 10.89	41.68 ± 3.03	45.19 ± 17.30
Cerebral cortex	67.06 ± 26.90	59.80 ± 23.02	59.09 ± 25.32	37.23 ± 2.75	40.68 ± 9.14
Basal ganglia	39.37 ± 15.55	58.96 ± 21.82	41.71 ± 17.71	86.76 ± 30.10	73.97 ± 20.67

Methylation level of NOS1 promoter 7					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	45.03 ± 9.11	53.43 ± 12.31	49.11 ± 11.21	43.57 ± 10.11	55.15 ± 11.70
Amygdala	51.87 ± 10.52	83.21 ± 38.99	49.78 ± 9.23	58.96 ± 16.87	49.9 ± 18.27
Hypothalamus	38.07 ± 12.79	27.94 ± 9.58	41.71 ± 13.99	25.16 ± 9.21	51.15 ± 19.76
Midbrain	57.51 ± 9.21	59.78 ± 15.63	51.41 ± 8.72	43.79 ± 17.01	42.48 ± 15.16
Cerebral cortex	68.04 ± 26.72	45.75 ± 12.29	59.01 ± 16.85	41.69 ± 11.99	37.28 ± 16.72
Basal ganglia	41.93 ± 13.97	55.71 ± 10.99	39.32 ± 15.22	54.01 ± 12.17	35.23 ± 19.36

Supplementary Table 5.

Differences in the methylation level of SOD1, SOD2 (A), NOS1 promoter 7 (B) between brain tissue and PBMCs of animals exposed to CMS for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (Control/Venla, Stressed/Saline, Stressed/Venla). Data represents means ± SEM. N = 6. No significant changes were found between any groups.

Group	SOD1 promoter		SOD2 promoter		NOS1 promoter 7	
	Brain	Blood	Brain	Blood	Brain	Blood
Control	42.76 ± 1.69	45.86 ± 4.50	27.27 ± 8.95	32.65 ± 23.36	44.58 ± 5.53	61.52 ± 22.30
Stressed	47.56 ± 1.92	30.66 ± 13.37	21.43 ± 9.76	13.88 ± 8.17	53.52 ± 11.96	66.34 ± 17.10
Control/Venla	42.65 ± 5.36	40.89 ± 9.82	35.25 ± 8.42	29.18 ± 15.21	55.86 ± 6.85	62.15 ± 16.68
Stressed/Saline	35.87 ±7.99	33.56 ± 11.65	30.18 ± 6.43	19.21 ± 9.85	61.95 ± 7.65	65.48 ± 19.75
Stressed/Venla	53.18 ± 5.71	36.73 ± 12.45	35.52 ± 6.78	26.31 ± 16.51	53.53 ± 4.71	61.21 ± 15.79

Supplementary Table 6.

Expression of NOS2 and SOD1 proteins in animals exposed to CMS for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (Control/Venla, Stressed/Saline, Stressed/Venla). Samples containing 25 μg of proteins were resolved by SDS-PAGE. The intensity of the bands corresponding to NOS1 and SOD1 was analysed by densitometry, and integrated optical density (IOD) was normalized by protein content and a reference sample (see the Methods for details). The data show mean IODs of the bands from all analysed samples. The IOD_{gene}/IOD_{ACTB} method was used to estimate the relative protein expression levels in the analysed samples.

N = 6. No significant changes were found between any groups.

Expression of NOS2 proteins					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	3.008 ± 1.004	3.231 ± 1.347	1.908 ± 0.604	1.542 ± 0.905	2.335 ± 0.318
Amygdala	1.556 ± 0.673	2.933 ± 1.028	2.156 ± 0.503	2.607 ± 0.330	2.422 ± 0.668
Hypothalamus	3.680 ± 0.809	2.034 ± 0.695	2.990 ± 0.511	2.843 ± 0.663	2.123 ± 0.748
Midbrain	1.461 ± 0.682	1.917 ± 0.992	1.531 ± 0.786	2.015 ± 0.734	2.618 ± 0.634
Cerebral cortex	3.843 ± 1.613	1.509 ± 0.761	2.949 ± 1.010	1.798 ± 0.709	1.262 ± 0.301
Basal ganglia	2.165 ± 0.574	2.208 ± 0.120	1.991 ± 0.891	3.008 ± 0.723	2.001 ± 0.221

Expression of SOD1 proteins					
Structures	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	1.595 ± 0.604	2.811 ± 1.535	2.192 ± 0.711	2.430 ± 1.403	2.851 ± 0.958
Amygdala	1.618 ± 0.831	1.381 ± 0.719	1.337 ± 0.639	1.569 ± 0.168	2.254 ± 0.733
Hypothalamus	2.523 ± 1.281	1.325 ± 0.689	2.113 ± 0.963	1.496 ± 0.448	2.320 ± 0.769
Midbrain	3.566 ± 1.892	1.779 ± 0.928	2.986 ± 0.889	1.218 ± 0.619	1.279 ± 0.563
Cerebral cortex	4.178 ± 2.287	2.642 ± 1.461	3.178 ± 1.028	1.192 ± 0.990	1.985 ± 0.586
Basal ganglia	3.485 ± 1.895	1.830 ± 0.935	3.221 ± 1.002	1.375 ± 0.722	1.974 ± 0.672

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The Effect of Chronic Mild Stress and Venlafaxine on the Expression and Methylation Levels of Genes Involved in the Tryptophan Catabolites Pathway in the Blood and Brain Structures of Rats

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Abstract

A growing body of evidence suggests that depression may be associated with impairment of the tryptophan catabolites (TRYCATs) pathway. The present study investigated the effects of the chronic administration of venlafaxine on the expression and methylation status of *Katl*, *Tph1/2*, *Ido1*, *Kmo* and *Kynu* in the brain and blood of rats exposed to the CMS model of depression. The rats were subjected to the CMS procedure for 2 or 7 weeks and administered venlafaxine (10 mg/kg/day, IP) for 5 weeks. mRNA and protein expression and the methylation status of gene promoters in PBMCs and six brain structures were evaluated and analysed using the TaqMan Gene Expression Assay and Western blotting, and methylation-sensitive high-resolution melting (MS-HRM), respectively. We found that the CMS procedure increased *KatI* expression in the midbrain and *KatII* expression in the midbrain and the amygdala, while venlafaxine administration decreased *KatII* expression in the hypothalamus and the cerebral cortex. The methylation status of the *Tph1* and *Kmo* promoters in peripheral blood mononuclear cells (PBMCs) was significantly increased in the stressed group after antidepressant therapy. The protein levels of Tph1 and Ido1 were decreased following venlafaxine administration. Our results confirmed that CMS and venlafaxine modulate the expression levels and methylation status of genes involved in the TRYCATs pathway.

Keywords Chronic mild stress model of depression · Venlafaxine · Tryptophan catabolites pathwayGene expression and methylation

Introduction

According to a World Health Organization (WHO) report, 350 million people globally suffer from depression, and 800,000 people commit suicide every year (James et al. 2018; Wang et al. 2007). Moreover, it is estimated that by 2020, depression will be the main cause of significant health, economic and social burdens (Murray and Lopez 1997). Unfortunately, despite extensive studies, the pathogenesis of depression is not fully known. A growing body of evidence has suggested that the mechanisms of this disease are associated with disorders of the tryptophan catabolites (TRYCATs) pathway (Maes et al. 2011a, b). A detailed description of the TRYCATs pathway is presented in previous studies have confirmed that depression is associated with reduced levels of tryptophan and neuroprotective kynurenic acid and elevated concentrations of neurotoxic metabolites, including 3-hydroxykynurenine, quinolinic acid and anthranilic acid, as well as with disorders of enzymes involved in tryptophan metabolism, including increased activity of indoleamine 2,3-dioxygenase 1 (IDO 1) and tryptophan 2,3-

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dioxygenase 2 (TDO 2), and decreased activity of kynurenine aminotransferase 1 and 2 (KATI and KATII) (Maes et al. 2011a; Ogawa et al. 2014; Kwidzinski and Bechmann 2007). IDO 1 and TDO 2 are rate-limiting enzymes which catalyse the oxidation of L-tryptophan to *N*-formylkynurenine. TDO 2 is expressed in the liver, whereas IDO 1 is expressed in the placenta, lungs, brain and blood (Hayaishi 1976; Watanabe et al. 1980). Kynurenic acid is a product of the reaction catalysed by KATI or KATII from kynurenine. The next important enzymes associated with depression are tryptophan hydroxylase 1 and 2 (TPH1, TPH2). TPH is involved in the initial and rate-limiting step in the synthesis of serotonin and melatonin. The enzyme catalyses the monooxygenation of tryptophan to 5-hydroxytryptophan (Wigner et al. 2018a). In turn, kynurenine-3-monooxygenase (KMO) catalyses the hydroxylation of L-kynurenine to neurotoxic 3-hydroxykynurenine (Breton et al. 2000). In previous studies, polymorphisms in the genes that encode TPH1, TPH2, IDO1 and KMO have been found to modulate the risk of depression development (Wigner et al. 2018a, b; Lezheiko et al. 2016). Thus, studying TRYCATs pathway in depression can provide new diagnostic biomarkers of this disease and may allow for the development of promising new personalized antidepressive drugs in the future (Smith 2013). This is of particular importance, as antidepressant treatment is not effective in approximately 30% of depressed patients (Joffe et al. 1996), and recent studies have shown that a critical drop in the level of tryptophan is associated with the development of drug-resistant depression (Smith 2013). Interestingly, the occurrence of *TPH1*, *TPH2* and *KATI* polymorphisms may be associated with a lack of response to traditional therapy based on the application of selective serotonin reuptake inhibitors (SSRIs). The c.-173A>T (rs10488682) polymorphism is localized in the promoter region of *TPH1* and may decrease the activity of the promoter, affecting the transcription level of *TPH1*. Moreover, the 844G>T (rs4576025) *TPH2* polymorphism may alter DNA–protein interactions and may affect the transcription level. The presence of the T allele is associated with reduced *TPH2* promoter activity (Smith 2013; Zhang et al. 2005; Wigner et al. 2018a, b).

In addition to SSRIs, serotonin-norepinephrine reuptake inhibitors (SNRIs), including venlafaxine, are the first line of depression therapy. Venlafaxine is approved by the US Food and Drug Administration (FDA) to treat and manage symptoms of depression, general anxiety disorder, social phobia and panic disorder (Safarova et al. 2018). Venlafaxine is a bicyclic phenylethylamine compound and works by blocking the transporter "reuptake" proteins for key neurotransmitters, including serotonin and norepinephrine, thereby leaving more active neurotransmitters in the synapse. Moreover, venlafaxine is a more potent inhibitor of serotonin reuptake than norepinephrine reuptake (Saad et al. 2019). Additionally, methylation status and mRNA expression level may be modulated by stress and antidepressant therapy. Previous studies showed that the expression and activity of DNA methyltransferases were increased in patients with depression. In the case of animals exposed to stress stimuli, studies confirmed that the level of DNA methyltransferases was increased in the prefrontal cortex and hippocampus (Nagy et al. 2018; Webb et al. 2020). The effectiveness of antidepressants may also be associated with differential methylation of the CYP450 enzymes of the liver which metabolize antidepressant drugs. Elevated methylation status of the promoter region was found to correlate with low or no transcription. Methylation levels may also differ between different tissue types and between normal cells and diseased cells from the same tissue (Suzuki and Bird 2008; Habano et al. 2015; Tili et al. 2015). Thus, this study aimed to investigate whether (i) the chronic mild stress (CMS) procedure changes the expression of genes involved in the TRYCATs pathway at the mRNA and protein levels and causes epigenetic changes, i.e. methylation level of these gene promoters in peripheral blood mononuclear cells (PBMCs) and in selected brain structures (hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia); and (ii) chronic administration of the serotonin-norepinephrine reuptake inhibitor venlafaxine affects the expression and methylation status of these genes. The latter point has important clinical implications, since there is a great need for peripheral markers that would enable earlier diagnosis, more precise prognosis of pharmacotherapy outcome, and more personalized therapies for mood disorders. All of the genes analysed in our study are located on chromosomes significantly associated with depression (Supplementary Table 1). Buczko et al. (2005) presented the correct course of the TRYCATs.

Materials and Methods

Animals

Male Wistar Han rats were obtained from Charles River (Germany). The animals were singly housed with free access to food and water and kept on a 12-h light/dark cycle (lights on at 8:00) at a controlled temperature (22 ± 2.0 °C) and humidity (50 ± 5%). All procedures and tests used in this study were approved by the Bioethical Committee of the Institute of Pharmacology of the Polish Academy of Sciences in Krakow (Poland) and were conducted in compliance with the rules and principles of the 86/609/EEC directive.

Chronic Mild Stress Procedure

CMS experiments were performed according to the method described previously (Papp 2012). Briefly, the animals (approximately 220 g at the start of the procedure) were first trained to consume 1% sucrose solution in 7 weekly baseline

tests, in which they received the solution for 1 h following 14 h of food and water deprivation. The animals were then divided into two matched groups. The first group included animals subjected to the stress procedure for 2 or 7 weeks. Each week of the stress procedure involved two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (lights on and off every 2 h), two periods of soiled cage (250 ml water in sawdust bedding), one period of paired housing, two periods of low-intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. The animals from the control group had free access to food and water, except for the period of food and water deprivation before the sucrose consumption tests. They were kept in a separate room and had no contact with the stressed rats. After the initial 2 weeks of stress, i.e., when the decrease in sucrose intake stabilized, the animals were either decapitated or further divided into subgroups and administered vehicle (1 ml/kg, IP) or venlafaxine (10 mg/kg, IP) daily for 5 weeks. Finally, the animals were decapitated 24 h after the last sucrose test, and samples of blood and brain structures were collected.

Drugs

Venlafaxine HCl (Carbosynth Ltd., Compton, Berkshire, UK) was dissolved in 0.9% sterile saline, which was used for vehicle injection, and was administered intraperitoneally (IP) in a volume of 1 ml/kg of body weight at a dose of 10 mg/kg, as used previously (Papp et al. 2017; Papp et al. 2019).

Specimen Collection

After decapitation, blood samples were collected in 5-ml Vacutainer tubes containing EDTA and stored at −20 °C. Next, differential migration of cells during centrifugation with Gradisol L (Aqua-Med, Lodz, Poland) was applied to isolate peripheral blood mononuclear cells (PBMCs). Finally, after centrifugation (400×*g*, 30 min, 4 °C), the PBMC pellet was stored at −20 °C until further analysis. The hippocampus, amygdala, midbrain, hypothalamus, cerebral cortex and basal ganglia were also isolated, rapidly frozen in liquid nitrogen and stored at −80 °C until further analysis. A FastGene® tissue grinder (Nippon Genetics Europe, Düren, Germany) was then used to homogenize the tissues and prepare DNA, RNA and protein specimens.

RNA Isolation, cDNA Synthesis and mRNA Expression Levels

Commercial spin column methods (GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich, and ISOLATE II RNA/DNA/Protein Kit, Bioline) were used to isolate RNA from the PBMCs and frozen brain structures in accordance with the manufacturers' instructions. The total concentration and quality of the RNA samples were determined by comparing the absorbance values at 260 nm and 280 nm, after which the samples were stored at −20 °C until use. The next step involved the synthesis of cDNA products from total RNA using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Foster City, CA, USA) according to the manufacturer's instructions. Briefly, the reverse transcription reaction consisted of MultiScribe® Reverse Transcriptase, 10× RT random primers, 25× dNTP Mix (100 mM), nuclease-free water, 10× RT buffer and total RNA (0.5 ng/μl). The conditions of the cDNA synthesis reaction executed in a C1000™ programmed thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were as follows: 10 min at 25 °C (enzyme activation), 37 °C for 120 min (proper synthesis of cDNA), and 85 °C for 5 min (enzyme inactivation). The level of mRNA expression was measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using commercially available TaqMan Universal Master Mix, no UNG, and species-specific TaqMan Gene Expression Assay (Thermo Fisher Scientific, Waltham, MA, USA). The target genes included *KatI* (assay ID Rn01439192_m1), *KatII* (assay ID Rn00567882_m1), *Tph1* (assay ID Rn00598017_m1), *Tph2* (assay ID Rn01476867_m1), *Ido1* (assay ID Rn01482210_m1), *Kmo* (assay ID Rn01411937_m1) and *Kynu* (assay ID Rn01449532_m1), As an internal mRNA control, we used 18S ribosomal RNA (18S, Applied Biosystems, CA, USA), and the mRNA expression of 18S ribosomal RNA was used to normalize the target gene expression levels. A quantitative RT-PCR reaction was carried out using a CFX96™ Real-Time PCR Detection System Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The two-step amplification conditions were as follows: 10 min at 95 °C followed by 60 cycles at 95 °C for 30 s and 1 min at 60 °C. The experiments were performed in duplicate for each sample. Gene expression was calculated in relation to that of the reference gene ($\Delta C_{t\ sample} = C_{t\ target\ gene} - C_{t\ reference\ gene}$). Next, the levels of gene expression were given as a the ratio calculated as fold = $2^{-\Delta Ct\ sample}$. The fold change in expression caused by venlafaxine administration was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

DNA Isolation and Methylation and HRM Analysis

Genomic DNA was isolated from the PBMCs and brain structures according to the manufacturer's instructions supplied with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and ISOLATE II RNA/DNA/Protein Kit (Bioline, Alvinston, Canada), and stored at −20 °C until use. The quantity and quality of the isolated DNA samples were measured by a spectrophotometer. Methylation-sensitive high-resolution melting (MS-HRM) was used to assess the methylation level of the gene promoter region (Wojdacz and Dobrovic 2007; Wojdacz et al. 2008). Therefore, the EMBOSS

Cpgplot bioinformatics tool (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/, Settings: Window: 100, Shift: 1, Obs./Exp.: 0.6, GC content: 50%) was used to predict CpG islands in the promoter regions of all the studied genes. The next step involved designing primers in MethPrimer 2 (http://www.urogene.org/methprimer2/) according to the recommendations provided by Wojdacz et al. (2009). Supplementary Table 2 provides the specifications of the designed primers. Bisulfite conversion was then performed using the CiTi Converter DNA Methylation Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocols. Real-time PCR amplification was carried out on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following thermal cycling conditions: initial activation for 12 min at 95 °C, 45 cycles at 95 °C for 15 s; annealing at the optimal primer temperature (tested experimentally) for 20 s (see Supplementary Table 1 for the characteristics of the primers), and elongation at 72 °C for 20 s. The HRM analysis involved denaturation at 95 °C for 15 s, re-annealing at 60 °C for 1 min, and melting from 60 to 95 °C at a ramp rate of 0.2 °C every 2 s. Each PCR was composed of 5x HOT FIREPol® EvaGreen® HRM Mix (no ROX) (Solis BioDyne, Tartu, Estonia), 500 nM of each primer and 10 ng of DNA after bisulfite modification (theoretical calculation). Finally, Bio-Rad Precision Melt Analysis Software was used to analyse the obtained data. In addition, unmethylated and methylated bisulfite-converted control DNA (CpGenome™ Rat Methylated Genomic DNA Standard, Merck Millipore, Burlington, MA, USA, and CpGenome™ Rat Unmethylated Genomic DNA Standard, Merck Millipore) was used in different ratios for HRM calibration (0%, 10%, 25%, 50%, 75% and 100% methylated controls).

Western Blot Analysis

Protein expression levels in the structures of rat brain tissues were estimated using Western blot analysis as described previously (Laemmli 1970). A FastGene® tissue grinder (Nippon Genetics Europe, Düren, Germany) was used for the homogenization of frozen brain samples in RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 10 Mm NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, serine protease inhibitor). Following double sonication and centrifugation (5000 rpm, 5 min, 4 °C), the supernatant containing the protein was collected. The protein concentration was measured based on the modified Lowry procedure using bovine serum albumin (BSA) as a standard (Lowry et al. 1951). Samples of brain homogenates (50 μg/lane) were resolved by 10% SDS-PAGE and then electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA) as previously described (Towbin et al. 1979). After blocking at room temperature for 1 h and washing three times with TBST (Tris-buffered saline with Tween-20), the blots were incubated overnight at 4 °C with primary antibodies (incubation for 2 h at room temperature except for the anti-β-actin antibody) diluted according to the manufacturer's protocol. The antibody specifications are presented in Supplementary Table 3. After incubation, the blots were washed three times with TBST and then incubated for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase (1:6000 dilution). Finally, the membranes were again washed with TBST and incubated with peroxidase substrate solution (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were visualized on X-ray film by enhanced chemiluminescence. Densitometry analysis of protein bands was performed with Gel-Pro® Analyzer Software (Media Cybernetics, Inc., Rockville, MD, USA). The protein expression levels were normalized to the reference protein, i.e., beta-actin (ACTB; IOD_{gene}/IOD_{ACTB}).

Statistical Analysis

All data are presented as the means ± standard error of means. The normality of the data was confirmed using the Shapiro–Wilk test. Differences between samples with normal distribution were verified using analysis of variance (ANOVA), whereas the Kruskal–Wallis test combined with a multiple comparison of average ranks was used to determine differences between samples with non-normal distribution. Subsequently, the Tukey test was used as the post hoc test. In the case of differences in mRNA expression and methylation levels between the blood and brain, a *t* test was used. *P* values <0.05 were considered statistically significant. The results were analysed using Statistica 12 (StatSoft, Tulsa, OK, USA), SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The Effect of CMS and Venlafaxine on Sucrose Intake

Before the stress was initiated (week 0), the consumption of 1% sucrose solution was comparable in all groups. As shown in Fig. 1, after 2 weeks of initial stress, the rats showed an approximately 40% decrease in sucrose intake ($p < 0.01$), whereas in vehicle-treated stressed animals, sucrose intake remained at a similar level until the end of the experiment (week 7). The chronic administration of venlafaxine (10 mg/kg, IP) normalized the decreased intake in the stressed rats ($p < 0.01$) and had no effect on the behaviour of non-stressed control animals. Neither the stress procedure nor venlafaxine treatment had a significant effect on the body

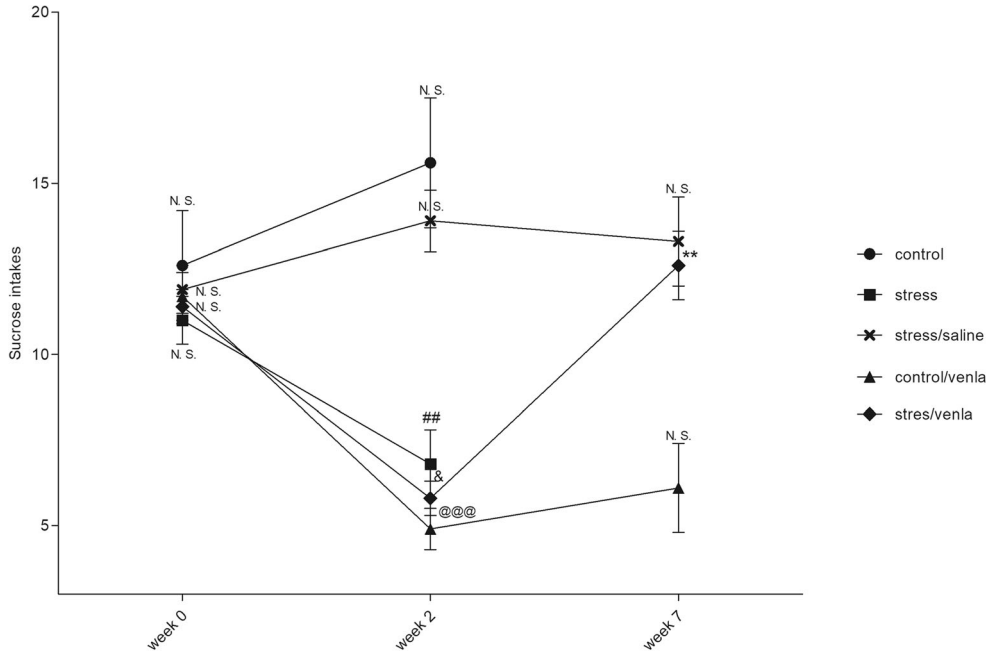

Fig. 1 Sucrose intake in animals exposed to CMS for 2 weeks (week 2) and in animals exposed to CMS for 7 weeks (week 7) and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks. The data represent means ± SEM. $N = 6$. $^{\#\#}p < 0.01$; relative to week 2 in the stressed group. $^{\&}p < 0.05$; relative to week 2 in the stressed/venla group. $^{**}p < 0.01$; relative to week 2 in the stressed/venla group. $^{@@@}p < 0.001$; relative to week 0 in the control/venla group. *N.S.* no significant differences between studied groups

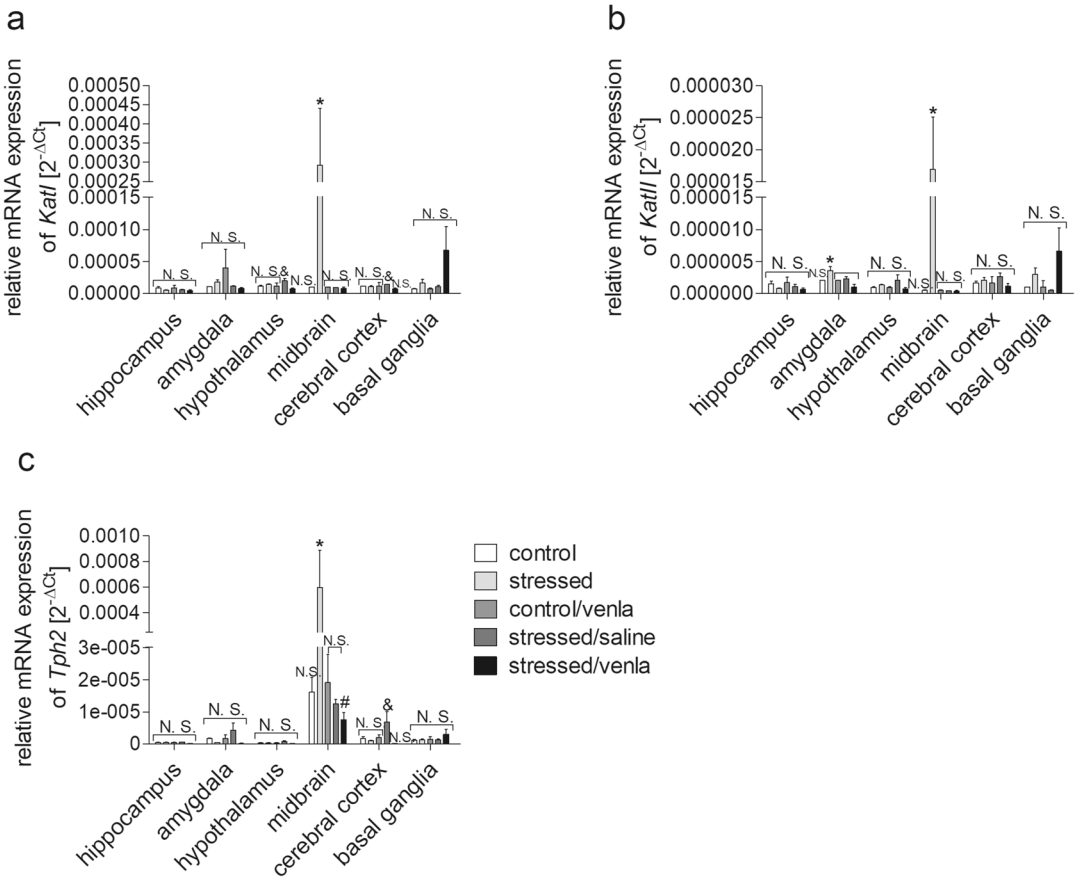

Fig. 2 mRNA expression of *KatI* (**a**), *KatII* (**b**) and *Tph2* (**c**) genes in brain structures of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). The expression of either gene was normalized to the 18S gene, and relative gene expression levels were estimated using a $2^{-\Delta Ct\ (Ct_{gene} - Ct_{18S})}$ method. N = 6. $^{\#}p < 0.05$ for differences between stressed and stressed/venla group, $^{*}p < 0.05$ stressed and control groups, $^{\&}p < 0.05$ for differences between stressed/saline and stressed/venla groups, and $^{\#}p$ for differences between controls and stressed groups. *N.S.* no significant differences between studied groups

weight of the animals from any of the studied groups (data not shown).

mRNA Expression

Gene Expression in Brain Structures and PBMCs

As shown in Fig. 2 and Supplementary Fig. 1, the effect of the CMS procedure and venlafaxine treatment on *Tph1*, *Tph2*, *KatI*, *KatII*, *Ido1*, *Kmo* and *Kynu* mRNA expression was dependent on the tissue and brain structure. The CMS procedure increased *KatI* expression in the midbrain ($p < 0.05$), while venlafaxine decreased it in the hypothalamus ($p < 0.05$) and the cerebral cortex ($p < 0.05$) in stressed rats. The amygdala ($p < 0.05$) and midbrain ($p < 0.05$) of the stressed rats exhibited elevated *KatII* expression. mRNA expression of the *Tph2* gene was elevated in the midbrain of the stressed group as compared with controls, while reduced expression was observed following administration of venlafaxine in stressed rats ($p < 0.05$). *Tph2* expression in the cerebral cortex was lower after venlafaxine administration than after saline treatment ($p < 0.05$). On the other hand, neither stress nor venlafaxine had a significant effect on the expression of any of the studied genes in PBMCs (Supplementary Fig. 2).

The Effect of Venlafaxine on Gene Expression in PBMCs and Brain Structures

As shown in Supplementary Fig. 3, venlafaxine caused an increase in *KatII* and *Tph2* expression in the hippocampus amygdala, hypothalamus, midbrain and cerebral cortex ($p < 0.001$) and a decrease in *Kmo* expression in the hypothalamus of stressed rats ($p < 0.01$).

Methylation of the Studied Gene Promoters

Methylation in PBMCs and Brain Structures

As shown in Fig. 3 and Supplementary Fig. 4, the methylation status of the *Tph1* ($p < 0.001$) and *Kmo* ($p < 0.05$) promoters in PBMCs was significantly increased in the venlafaxine-treated stressed group as compared with the stressed group, while the CMS procedure led to an elevated level of methylation of the *Ido1* promoter in the midbrain (Fig. 4 and Supplementary Table 4) ($p < 0.05$).

The Effect of Venlafaxine on the Methylation Status of Gene Promoters in PBMCs and Brain Structures

As shown in Supplementary Fig. 5, in stressed group venlafaxine decreased the methylation level of the *Tph1* promoter and *Kmo* promoter in all brain structures compared with the blood ($p < 0.001$, $p < 0.01$, respectively).

Gene Expression at the Protein Level

The protein levels of Tph2, KatII and Kynu did not differ between the studied groups (Supplementary Fig. 6). Only the protein expression level of Tph1 ($p < 0.05$) and Ido1 ($p < 0.05$) (Fig. 5) was reduced in stressed animals administered venlafaxine as compared with the stressed group after saline treatment ($p < 0.05$).

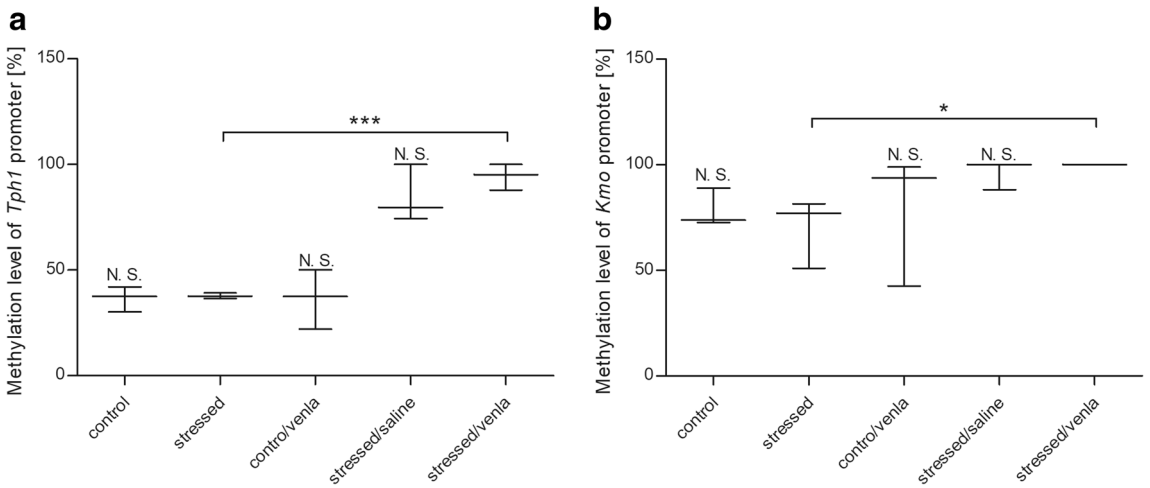

Fig. 3 Methylation level of *Tph1* promoter (**a**) and *Kmo* promoter (**b**) in PBMCs of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and treated with vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). Data represent means ± SEM. $N = 6$. $^{*}p < 0.05$ and $^{***}p < 0.001$ for differences between stressed and stressed/venla groups. *N.S.* no significant differences between studied groups

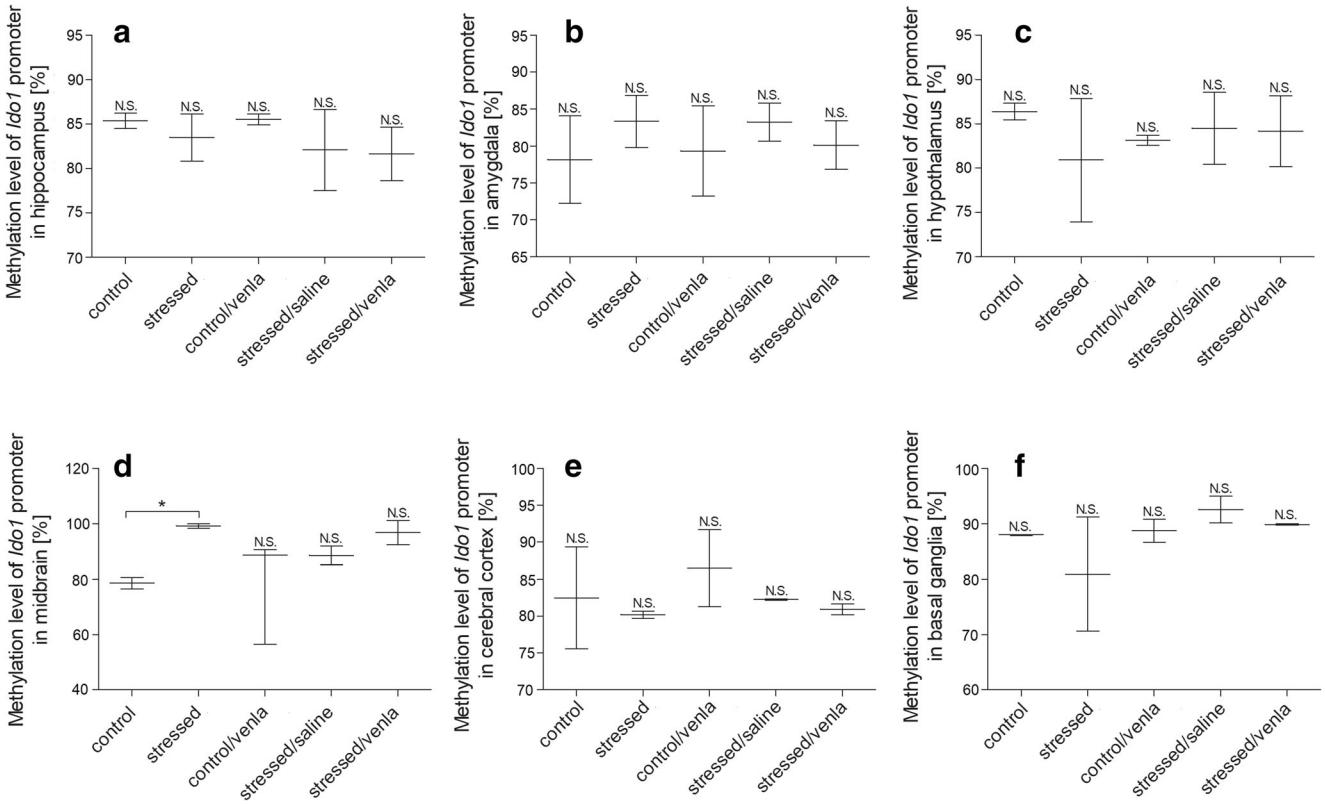

Fig. 4 Methylation levels of *Ido1* promoter in hippocampus (**a**), amygdala (**b**), hypothalamus (**c**), midbrain (**d**), cerebral cortex (**e**) and basal ganglia (**f**) of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and treated with vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). Data represent means ± SEM. $N = 6$. *$p < 0.05$ for differences between stressed and stressed/venla groups. *N.S.* no significant differences between studied groups

Discussion

The present study demonstrates the effect of the CMS model of depression and repeated administration of venlafaxine on the expression and methylation status of genes involved in the TRYCATs pathway in PBMCs and in six regions of the brain (hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia). In this study, genes expression were measured, rather than, activity of the antioxidant enzymes to highlight the role of epigenetic changes, i.e. promoter methylation, which is possible only when DNA, RNA and proteins are isolated from the same sample. All testes were done on tissue from animals after 5 weeks of CMS and venlafaxine, a point at which venlafaxine normalized behavioural impact of CMS.

Previous studies have shown that the CMS procedure leads to the development of depression-like behaviour, including anhedonia (Gamaro et al. 2003; Bekris et al. 2005, Papp 2012, Papp et al. 2017, 2019). The present study confirmed that CMS caused a reduction in the consumption of 1% sucrose solution, indicating a generalized deficit in sensitivity to reward, which is a characteristic symptom of depression, in stressed animals; this effect was normalized by the chronic administration of venlafaxine. Moreover, our results suggested that the CMS procedure and venlafaxine administration modulated the level of mRNA and protein expression and the status of the promoter methylation of genes that encode enzymes involved in the TRYCATs pathway in PBMCs and various brain structures, i.e., the hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia. Earlier animal studies confirmed that tryptophan insufficiency and serotonin disorders contribute to the development of depression-like symptoms (Tanke et al. 2008; Jacobsen et al. 2012). Likewise, clinical studies have also indicated that the TRYCATs pathway is dysfunctional in patients with depression (Myint 2012).

In our study, we focused on genes encoding enzymes involved in the TRYCATs pathway. The first of these is the gene encoding kynurenine aminotransferase (Kat). Kat catalyses the synthesis of kynurenic acid, which has neuroprotective properties and protects against neurodegenerative changes (Danzter 2017). Clinical studies have shown that the hippocampal and amygdala volume is reduced in depressed patients, which confirms the contribution of kynurenic acid generation imbalance to dendritic atrophy and anhedonia (Savitz et al. 2015). On the other hand, we found that the mRNA

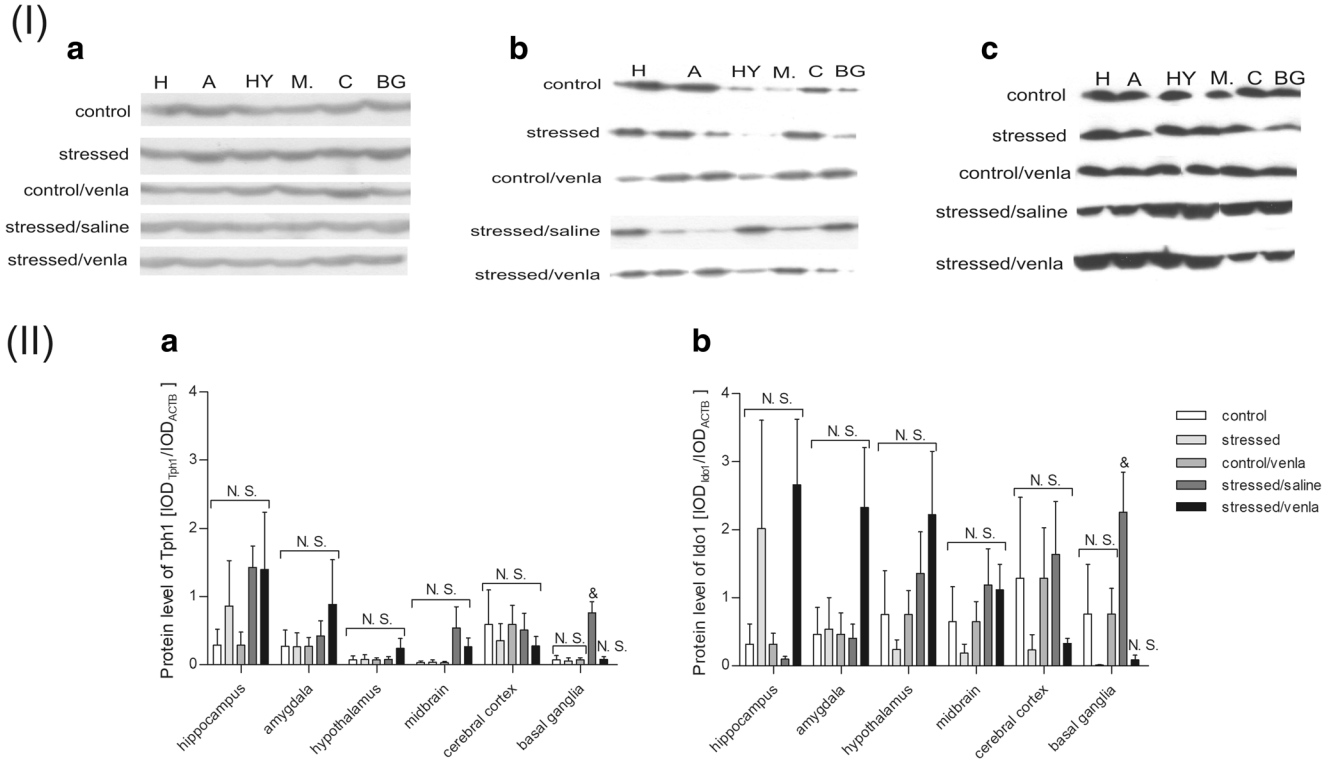

Fig. 5 Protein expression of Tph1 (**a**) and Ido1 (**b**) in brain structures of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). (**I**) Representative western blot analysis in hippocampus (H), amygdala (A), hypothalamus (HY), midbrain (M), cerebral cortex (C) and basal ganglia (BG). A = β-actin, B = Tph1, C = Ido1. **(II)** Levels of Tph1 (**a**) and Ido1 (**b**) proteins measured in hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia. Samples containing 25 μg of proteins were resolved by SDS-PAGE. The intensity of bands corresponding to Gpx4 was analysed by densitometry. Integrated optical density (IOD) was normalized by protein content and a reference sample (see the Methods section for details). The graphs show the mean IODs of the bands from all analysed samples. The IOD_{gene}/IOD_{ACTB} method was used to estimate the relative protein expression levels in the analysed samples. Data represent means ± SEM. $N = 6$. $^{\&}p < 0.05$ for the difference between stressed/saline and stressed/venla groups. *N.S.* no significant differences between studied groups

expression of *KatI* was lower in the hypothalamus and the cerebral cortex in the stressed animals administered venlafaxine, whereas stressed rats exhibited increased mRNA expression of *KatI* in the midbrain. We also found that the CMS procedure caused an elevated level of *KatII* mRNA expression in the amygdala and midbrain. However, Laugeray et al. (2010) observed that mice subjected to unpredictable CMS exhibit a reduced level of kynurenic acid in the amygdala and striatum. Similarly, CMS caused a downregulation of *KatII* mRNA expression in the cortex (Duda et al. 2019). These differences may be related to the fact that the analysed studies used a variety of biological material, including human, mouse and rat tissues. Additionally, the stress procedures used in the analysed studies were different. Thus, we cannot exclude the possibility that depression-like behaviour may be associated with the neurotoxic direction of kynurenine metabolism.

The next main finding of our study was that chronic venlafaxine administration in stressed rats decreased Tph1 protein expression in the basal ganglia and decreased the methylation status of the *Tph1* promoter region in PBMCs; however, no changes were observed in the brain. On the other hand, Chen et al. (2017) observed that stressed rats showed increased levels of *Tph1/Tph2* promoter methylation, which was normalized by paroxetine (an SSRI), in the brain, liver and kidney. These differences may be related to the fact that the stressors used in the above study were much more severe and that the animal breeds that were used differed between studies. Moreover, we found that the CMS procedure increased the mRNA expression of *Tph2* in the midbrain and that these effects were normalized by chronic venlafaxine administration. These findings are in line with clinical observations that an excess of *TPH2* may lead to tryptophan depletion and the development of depression-like symptoms (Jacobsen et al. 2012). On the other hand, TPH is involved in the initial and rate-limiting step of the synthesis of the neurotransmitter serotonin, and a low level of TPH may lead to the development of depression (McKinney et al. 2001; Cowen and

Browning 2015). Thus, our results showed that antidepressant treatment in stressed rats, compared with placebo, caused an increase in the mRNA expression of *Tph2* in the cerebral cortex. Similarly, Jiao et al. (2019) showed that chronic immobilization stress (CIS) decreased the mRNA and protein expression of *Tph2* in the hippocampus, whereas fluoxetine normalized these effects.

The next studied gene encodes indoleamine 2,3-dioxygenase. A previous study confirmed that an increased level of *Ido1* leads to the overproduction of neurotoxic metabolites and a deficit in tryptophan. Additionally, high protein expression of *Ido1* may contribute to the inhibition of serotonin production (Myint et al. 2007; Wichers et al. 2005). Jiao et al. (2019) found that CIS increases the level of mRNA and protein expression in the hippocampus and that these effects are normalized by fluoxetine. In turn, our results showed that venlafaxine treatment in stressed rats caused a reduction in Ido1 protein expression in the basal ganglia. Moreover, citalopram therapy (an SSRI) also has the ability to inhibit the action of Ido1 as well as increase the turnover of serotonin via Ido1 inhibition in the hippocampus, amygdala and hypothalamus of stressed rats (Ara and Bano 2012). A similar effect associated with reduced depressive symptoms was observed in a group of animals administered Ido1 inhibitors and in *Ido1* knockout mice (O'Connor et al. 2009; Lawson et al. 2013; Liu et al. 2015, Salazar et al. 2012). These results may indicate the ability of antidepressant treatment to modulate the expression of enzymes involved in the TRYCATs pathway. Specifically, the studied drugs may limit the production of kynurenine and thus may reduce the levels of harmful metabolites and inhibit the neurodegenerative processes associated with the development of depression.

The next studied gene was *Kmo*. The only change we found was an increase in the methylation status of the *Kmo* promoter region in the PBMCs of stressed animals administered venlafaxine. Since the activity of the *Kmo* gene is associated with the production of neurotoxic 3-hydroxykynurenine and quinolinic acid and the generation of reactive oxygen species, the observed elevated methylation level might contribute to the reduction in Kmo activity and the impairment of neurons after venlafaxine administration (Guillemin 2012; Colin-Gonzalez et al. 2013). Moreover, the CMS procedure increased the mRNA expression level of *Kmo* in the cortex and may be associated with a higher level of the excitotoxic compound (Duda et al. 2019). However, Wang et al. (2018) found that rats subjected to the chronic unpredictable mild stress procedure exhibited diminished concentrations and reduced activity of *Kmo* in the plasma. These differences may be related to the fact that the stressors used in the above-mentioned study were much more severe and that the animal breeds used in the two studies were different. Additionally, all analysed studies were based on different biological materials, including PBMCs, cortex and plasma.

The present findings and those previously reported by others suggest that disorders of functioning enzymes involved in the TRYCATs pathway in PBMCs and the brain are involved in the effects observed in animals exposed to the CMS procedure. Antidepressant therapy, including venlafaxine, may modulate the activity of enzymes to restore tryptophan metabolism to its proper functioning. Moreover, our results and those of previous studies suggest that the stress stimuli and antidepressant therapy may modulate the methylation of promoter regions, with the effect that access of transcription factors to regulatory regions is reduced. However, depending on the type of regulatory agent, different changes are observed. If the regulatory binding site is for enhancers, DNA methylation is associated with transcriptional repression, whereas if the site is for repressors, DNA methylation will have the opposite effect on transcription (Murgatroyd et al. 2010; Jaenisch and Bird 2003). A traumatic and stressful event may cause epigenetic modification, including methylation, which may determine gene expression (Tsankova et al. 2007; Levenson and Sweatt 2005; Mill and Petronis 2007). We found that the CMS caused an increase in methylation status of the *Ido1* promoter. Previous studies have also confirmed that antidepressants, including tricyclic antidepressants (TCA) and SSRIs, may reduce DNA methylation in rat primary astrocytes by decreased activity of DNA methyltransferase 1 (Perisic et al. 2010; Zimmermann et al. 2012) Valproate, used as a mood stabilizer, may also cause a global reduction of DNA methylation level (Alonso-Aperte et al. 1999; Detich et al. 2003). Activation of DNA demethylation was also observed in the frontal cortex and striatum after treatment with the antipsychotics sulpiride and clozapine (Dong et al. 2008). On the other hand, we observed that venlafaxine therapy was associated with increased methylation levels of the *Tph1* and *Kmo* promoter regions. Interestingly, the DNA methylation level may determine the response to antidepressant treatment (Lopez et al. 2013; Domschke et al. 2014; Uher et al. 2009).

Epigenetic profiling may also be used for disease diagnosis and prognosis of disease progression and therapy effectiveness (Heyn and Esteller 2012). As previously noted, reduced gene expression is traditionally associated with elevated levels of methylation. However, the changes are dependent on binding of the enhancers or repressors to the affected sequence (Murgatroyd et al. 2010; Portela and Esteller 2010; Shukla et al. 2011; Mehta et al. 2013). The modification may be observed in DNA from the peripheral tissue (PBMCs) and brain (Provencal et al. 2012; Klengel et al. 2013; Suderman et al. 2012; Perroud et al. 2011). The epigenetic changes may be tissue-specific or may overlap. Thus, extrapolation of changes observed in one tissue to others should be made only with caution. Moreover, the epigenetic changes also include modification of histones and microRNAs (Mehler 2008). Therefore, we observed changes in expression levels without methylation modification.

Conclusion

The results presented in this study confirm the hypothesis that the tryptophan catabolites pathway is involved in the pro-depressive effects of the CMS procedure. Moreover, disorders of tryptophan metabolites can be alleviated by venlafaxine. We also found that expression and methylation levels depend on the type of tissue (i.e., blood vs brain) as well as specific brain structure. In general, our results suggest that venlafaxine may prevent the overproduction of neurotoxic metabolites by inhibiting the protein expression of *Ido1* in the basal ganglia. We also confirmed that CMS causes a deficit in tryptophan through the mRNA expression of *Tph2* in the midbrain; however, this effect can be reversed by venlafaxine administration. Additionally, the hypermethylation of the *Kmo* promoter region may reduce the activity of the enzyme and limit the generation of neurotoxic 3-hydroxykynurenine and quinolinic acid.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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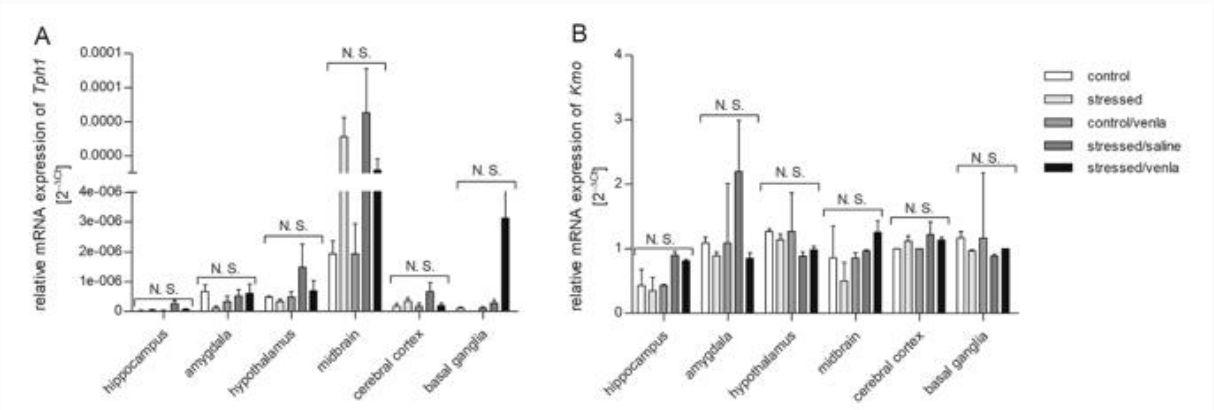
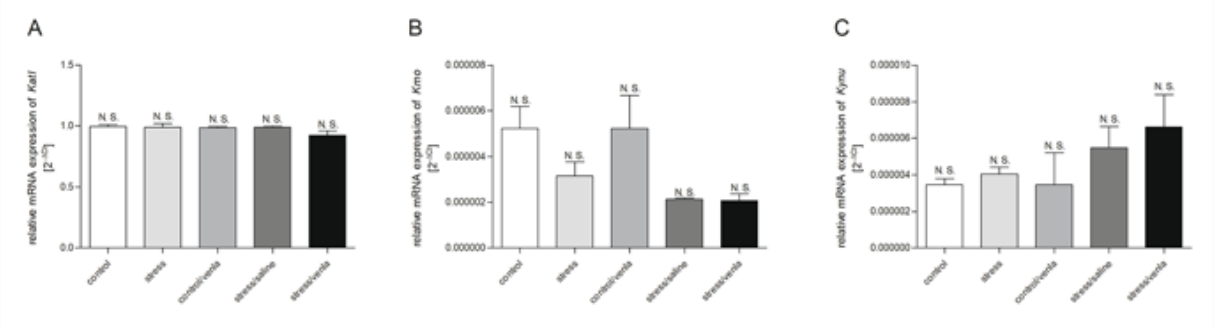
Electronic Supplementary Material

ESM 1



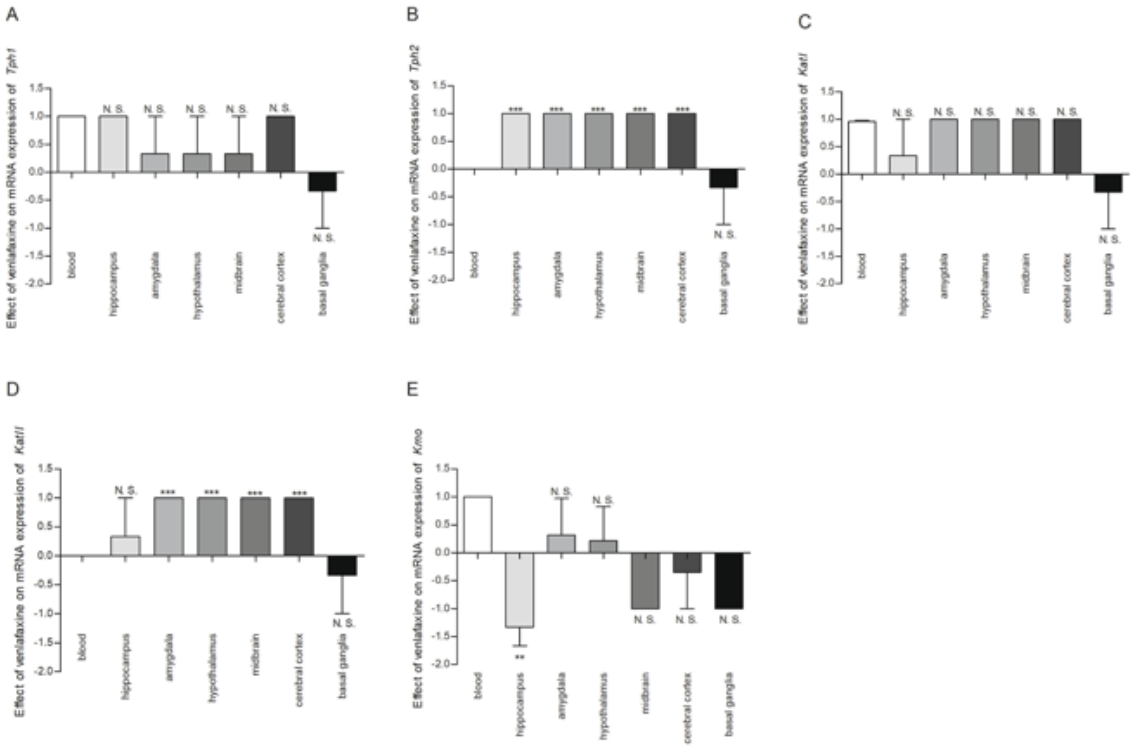
Supplementary Figure 1. mRNA expression *Tph1* (A) and *Kmo* (B) genes in brain structures of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). The expression of either gene was normalized to the 18S gene, and relative gene expression levels were estimated using a 2-ΔCt (Ct_{gene}–Ct_{18S}) method. *N* = 6; *N.S.* no significant differences between studied groups.

ESM 2



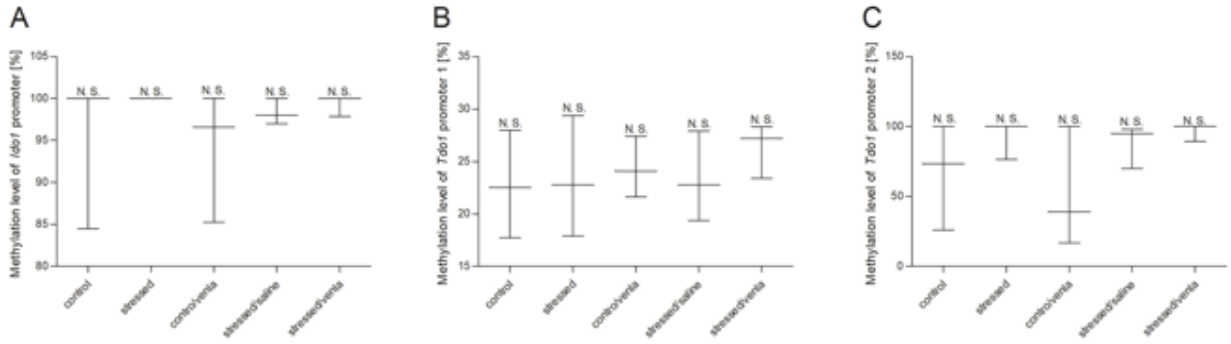
Supplementary Figure 2. mRNA expression of KatI (A), Kmo (B) and Kynu (C) genes in PBMCs of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). Relative gene expression levels were estimated using a 2-ΔCt (Ctgene–Ct18S) method. Data represent means ± SEM. N = 6; N.S. no significant differences between studied groups.

ESM 3



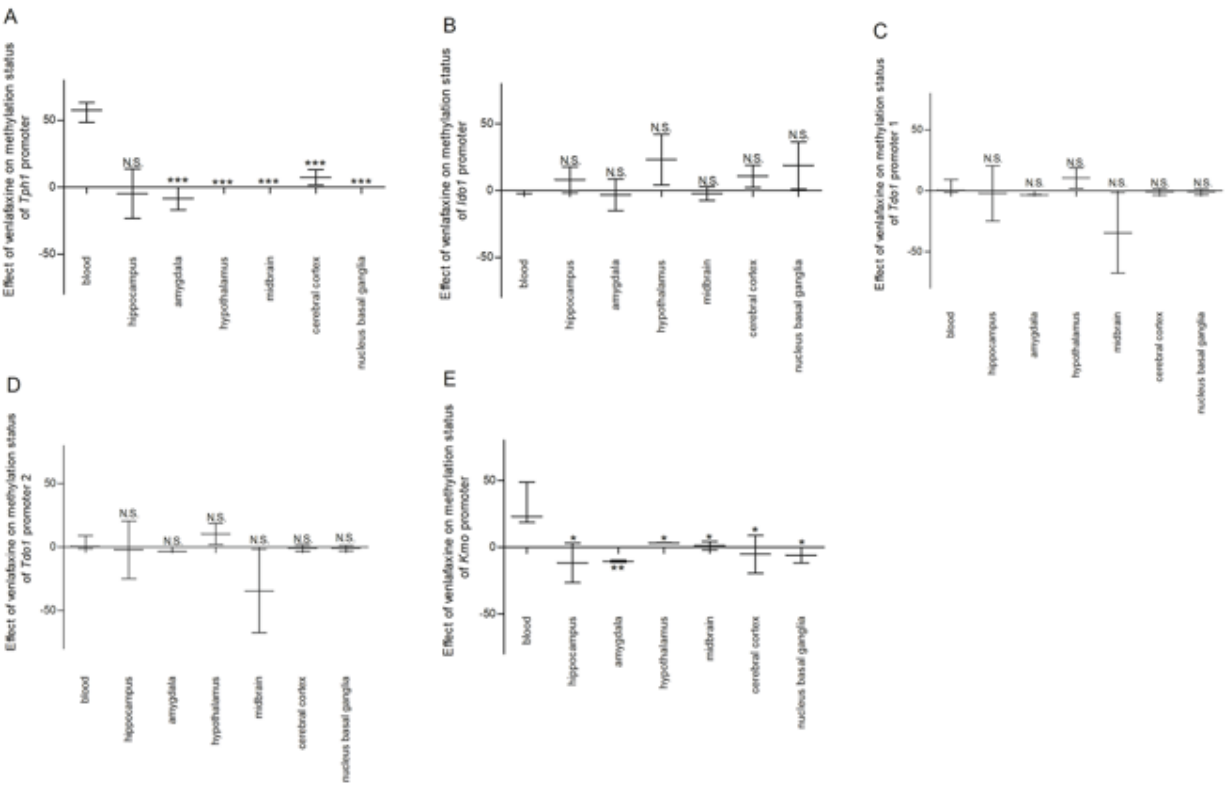
Supplementary Figure 3. mRNA expression of *Tph1* (A), *Tph2* (B), *KI* (C), *KATII* (D), *Kmo* (E) in PBMCs and in brain structures of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). The effects are presented as fold change ($2^{-\Delta\Delta Ct}$ method; Schmittgen and Livak 2008). Data represent means ± SEM. $N = 6$. $^{***}p < 0.001$ and $^{**}p < 0.01$ for differences between blood and all studied brain structures.

ESM 4



Supplementary Figure 4. Methylation level of *Ido1* promoter (A), *Tdo2* promoter 1 (B) and *Tdo2* promoter 2 (C) in PBMCs of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and treated with vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). Data represent means ± SEM. *N* = 6; *N.S.* no significant differences between studied groups. (PNG 2004 kb)

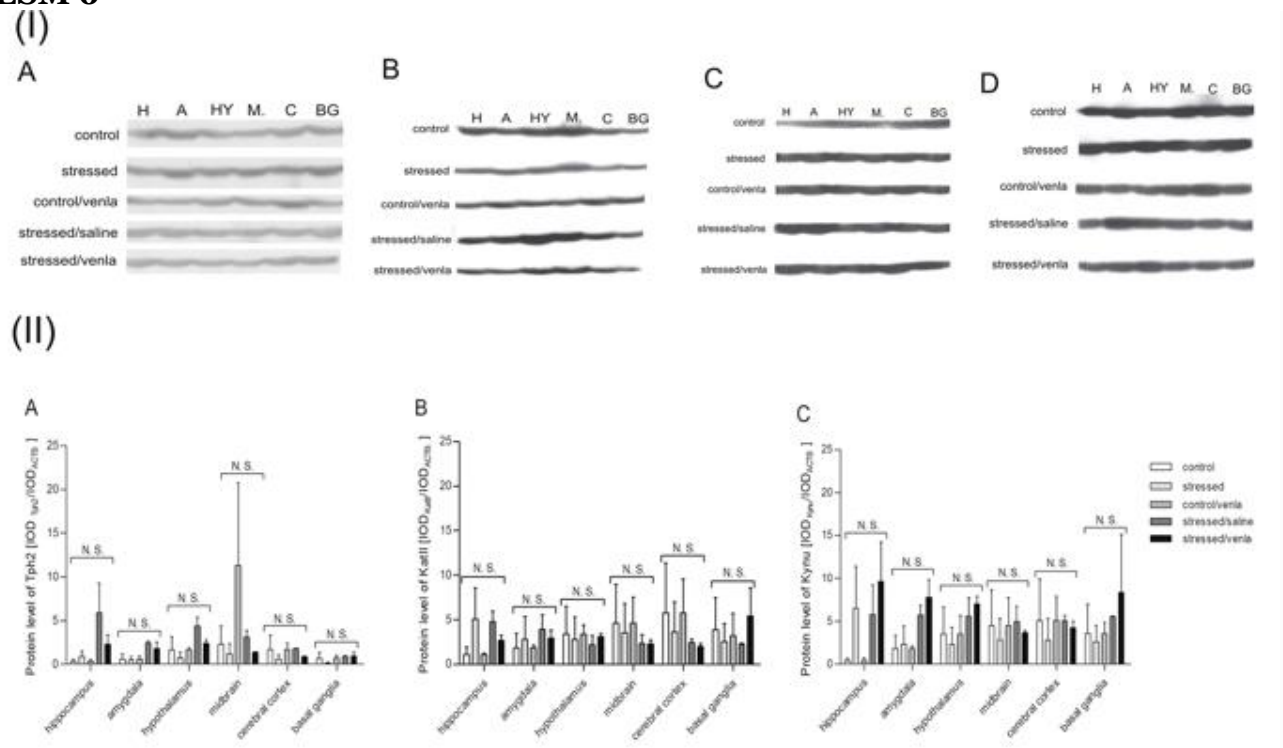
ESM 5



Supplementary Figure 5. The methylation level of *Tph1* (A), *Ido1* (B), *Tdo2* promoter 1 (C), *Tdo2* promoter 2 (D) and *Kmo* (E) between brain structures and PBMCs of animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and treated with vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). Data represent means ± SEM. *N* = 6. **p* < 0.05, ***p* < 0.01,

****p* < 0.001 for differences between blood and all studied brain structures; *N.S.* no significant differences between studied groups.

ESM 6



Supplementary Figure 6. Expression of Tph2 (A), KatII(B) and Kynu (C) proteins in animals exposed to CMS for 2 weeks (control, stressed) and in animals exposed to CMS for 7 weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for 5 weeks (control/venla, stressed/saline, stressed/venla). (I) Representative western blot analysis in hippocampus (H), amygdala (A), hypothalamus (HY), midbrain (M), cerebral cortex (C) and basal ganglia (BG). A = β-actin, B = Tph2, C = KatII, D = Kynu. (II) Levels of Tph2 (A), KatII (B) and Kynu (C) proteins measured in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia. Samples containing 25 μg of proteins were resolved by SDS-PAGE. The intensity of the bands corresponding to Tph2, KATII and Kynu was analysed by densitometry, and integrated optical density (IOD) was normalized by protein content and a reference sample (see the Methods for details). The data show mean IODs of the bands from all analysed samples. The IOD_{gene}/IOD_{ACTB} method was used to estimate the relative protein expression levels in the analysed samples. *N* = 6; *N.S.* no significant differences between studied groups.

ESM7

Supplementary Table 1. Characteristics of the genes studied (All data contained in the table were compiled with the help of Genomatix Software Suite, Intrexon Bioinformatics Germany GmbH, Munich, Germany, 2019).

Gene name	Function of protein encoding the gene	Chromosomal location	Biased expression
Cysteine conjugate-beta lyase 1 (*Katl*)	Catalyzes the irreversible transamination of the L-tryptophan metabolite L-Kynurenine to form Kynurenic acid	3p12	Adrenal gland, brain, heart, kidney, liver, lung, muscle, spleen, thymus, testes, uterus
2-aminoadipate aminotransferase (*KatII*)	Endogenous modulator of glutamatergic neurotransmission with Kynurenine aminotransferase (KAT) activity	16p12	Kidney, liver
Tryptophan hydroxylase 1 (*Tph1*)	Catalyzes the conversion of l-tryptophanof to 5-hydroxy-l-tryptophan, member of pterin-dependent aromatic amino acid hydroxylase family	1q22	Thymus, brain, kidney, muscle, testes
Tryptophan hydroxylase 2 (*Tph2*)	Isoform of tryptophan hydroxylase, may play a role in serotonin biosynthesis	7q22	Adrenal, brain, testes
Indoleamine-2,3-dioxygenase (Ido1)	Enzyme which catalyses degradation of L-tryptophan to N-formylkynurenine; human homolog is induced by gamma-interferon and is belived to be involved in that agent's tumor antiproliferative effects	16q12.5	Testes, thymus, heart, lung
Tryptophan 2,3-dioxygenase (*Tdo2*)	Rate-limiting in the catabolism of tryptophan	2q34	Liver, lung
Kynurenine 3-hydroxylase (*Kmo*)	Catalyses the hydroxylation of L-Kynurenine to form L-3-hydroxyKynurenine	13q25	Kidney, liver, lung, spleen, thymus, uterus
Kynureninase (*Kynu*)	Enzyme involved in the biosynthesis of NAD cofactors from tryptophan	3q12	Kidney, liver, spleen

ESM8

Supplementary table 2. The characteristics of primers used for analysis of methylation levels in the promoter regions of the studied genes.

Gene	Starter sequence	Product size	Tm
Tph1 (promoter 2)	F:GGGAGTTTTGTTTTGGTTTTTA R:TCCTCAACCACAAAAAATCTAA	132	55
Ido1 (promoter 2)	F:TTTGAGTTTTAGTGATTTTGGG R:TTAATATCTAATCCCAATCTCTAAAAC	100	59
Tdo2 (promoter 1)	F:GATGATTTAGGTGGTTTGAGGT R:CAAAAAAAACAAAATTCATCCA	123	59
TDO2 (promoter 2)	F:ATGATTTAGGTGGTTTGAGGTT R:ACCCAATCTACCTAACTAACAAC	187	61.4
Kmo (promoter 7)	F: TTGGTTTAGGGAAGGAAAT R: ATAAAAAACTAAACCCAAAACAC	150	55.7

ESM 9

Supplementary table 3. Conditions of the antibodies used in the Western blot analysis.

	Primary antibody	Secondary antibody
β-actin (a reference protein)	mouse, 1:1000, (Santa Cruz Biotechnolgy Inc), 1 hour at room temperature	anti-mouse, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature
Tryptophan hydroxylase 1	rabbit, 1:1000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), overnight at 4°C	anti-rabbit, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature
Tryptophan hydroxylase 2	rabbit, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), overnight at 4°C	anti-rabbit, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature
Indoleamine 2,3-dioxygenase	mouse, 1:1000, (Santa Cruz Biotechnolgy Inc), overnight at 4°C	anti-mouse, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature
Kynurenine aminotransferases	mouse, 1:1000, (Santa Cruz Biotechnolgy Inc), overnight at 4°C	anti-mouse, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature
Kynureninase	mouse, 1:1000, (Santa Cruz Biotechnolgy Inc), overnight at 4°C	anti-mouse, 1:6000, (Cell Signalling Technologies Inc., Danvers, Massachusetts, USA), 1 hour at room temperature

ESM 10

Supplementary table 4. Methylation level of, *Tph1* promoter (A), *TDO2* promoter 1 (B), *TDO2* promoter 2 (C) and *Kmo* (D) in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia of animals exposed to CMS for two weeks (control, stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 ml/kg) or venlafaxine (10 mg/kg) for five weeks (control/venla, stressed/saline, stressed/venla). Data represents means ± SEM. N = 6. No significant changes were found between any groups.

(A)

Methylation level of *Tph1* promoter

Part of brain	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	95.90 ± 2.37	93.11 ± 3.98	96.62 ± 3.17	99.60 ± 0.20	88.29 ± 6.76
Amygdala	98.76 ± 0.71	99.15 ± 0.54	99.73 ± 0.12	100.00 ± 0.00	91.54 ± 4.88
Hypothalamus	98.01 ± 0.51	99.23 ± 0.43	99.01 ± 0.66	96.07 ± 2.27	99.76 ± 0.11
Midbrain	91.34 ± 0.38	98.54 ± 0.43	90.99 ± 0.21	99.65 ± 0.21	99.65 ± 0.31
Cerebral cortex	99.01 ± 0.31	92.57 ± 3.34	99.52 ± 0.41	96.09 ± 2.26	98.54 ± 0.15
Basal ganglia	98.65 ± 0.76	99.54 ± 0.11	97.21 ± 0.77	99.54 ± 0.15	99.01 ± 0.75

(B)

Methylation level of *TDO2* promoter 1

Part of brain	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	61.99 ± 21.95	12.45 ± 7.19	55.66 ± 16.53	23.58 ± 0.05	10.36 ± 5.98
Amygdala	9.21 ± 8.21	25.02 ± 0.74	11.16 ± 5.98	24.10 ± 0.21	21.61 ± 0.81
Hypothalamus	11.65 ± 6.73	12.14 ± 7.01	8.87 ± 6.87	12.19 ± 7.04	22.55 ± 2.09
Midbrain	24.12 ± 0.50	58.12 ± 17.62	27.76 ± 6.75	25.10 ± 0.26	23.57 ± 1.50
Cerebral cortex	12.12 ± 7.00	24.28 ± 0.41	21.77 ± 9.21	0.00 ± 0.00	23.45 ± 1.10
Basal ganglia	25.70 ± 0.45	24.52 ± 0.15	20.12 ± 2.54	24.83 ± 0.02	23.66 ± 1.49

(C)

Methylation level of *TDO2* promoter 2

Part of brain	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	0.00 ± 0.00	12.14 ± 7.01	0.00 ± 0.00	0.00 ± 0.00	9.84 ± 5.68
Amygdala	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hypothalamus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.11 ± 7.57
Midbrain	0.00 ± 0.00	16.33 ± 9.43	0.00 ± 0.00	0.00 ± 0.00	30.96 ± 2.74
Cerebral cortex	0.00 ± 0.00	11.56 ± 6.67	0.00 ± 0.00	0.00 ± 0.00	2.26 ± 1.30
Basal ganglia	0.93 ± 0.54	0.00 ± 0.00	0.83 ± 0.45	0.00 ± 0.00	29.84 ± 17.23

(D)

Methylation level of *Kmo* promoter

Part of brain	Control	Stressed	Control/Venla	Stressed/Saline	Stressed/Venla
Hippocampus	96.43 ± 1.81	88.65 ± 2.96	94.66 ± 1.43	89.49 ± 3.27	76.83 ± 5.60
Amygdala	98.40 ± 0.92	96.75 ± 1.88	96.51 ± 1.27	97.07 ± 7.89	86.31 ± 2.35
Hypothalamus	94.32 ± 0.96	88.66 ± 3.57	96.76 ± 1.05	86.76 ± 0.28	91.85 ± 3.54
Midbrain	97.10 ± 1.67	96.77 ± 1.87	93.09 ± 3.87	97.79 ± 1.27	98.00 ± 0.01
Cerebral cortex	91.50 ± 3.20	75.10 ± 5.11	94.65 ± 4.76	67.24 ± 10.19	69.82 ± 13.31
Basal ganglia	100.00 ± 0.00	99.76 ± 0.11	99.53 ± 0.32	99.91 ± 0.05	94.08 ± 3.42

Oświadczenia współautorów

Łódź, 29.04.2020

mgr Paulina Wigner
Katedra Genetyki Molekularnej
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Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy **Wigner P**, Czarny P, Galecki P, Su KP, Sliwinski T. The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression. Psychiatry Res. 2018; 262: 566-574 mój udział wynosił 60% i obejmował planowanie prac, realizację części doświadczalnej, przygotowanie rycin i tabel, opracowanie wyników i ich interpretację oraz przygotowanie manuskryptu.

Oświadczam, że w pracy **Wigner P**, Czarny P, Synowiec E, Bijak M, Białek K, Talarowska M, Galecki P, Szemraj J, Sliwinski T. Association between single nucleotide polymorphisms of TPH1 and TPH2 genes, and depressive disorders. J Cell Mol Med. 2018; 22(3): 1778-1791 mój udział wynosił 60% i obejmował planowanie prac, realizację części doświadczalnej, przygotowanie rycin i tabel, opracowanie wyników i ich interpretację oraz przygotowanie manuskryptu.

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Paulina Wigner

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Paulina Wigner

Łódź, 29.04.2020

Prof. dr hab. Tomasz Śliwiński
Pracownia Genetyki Medycznej
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Oświadczenie o udziale w publikacjach

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Oświadczam, że w pracy Wigner P, Czarny P, Synowiec E, Bijak M, Białek K, Talarowska M, Galecki P, Szemraj J, Sliwinski T. Association between single nucleotide polymorphisms of TPH1 and TPH2 genes, and depressive disorders. J Cell Mol Med. 2018; 22(3): 1778-1791 mój udział wynosił 10% i obejmował współudział w projektowaniu badań, uczestnictwo w dyskusji nad uzyskanymi wynikami, merytoryczne konsultacje, pomoc w redakcji pracy.

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KIEROWNIK
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prof. dr hab. Tomasz Śliwiński

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ADIUNKT
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Oświadczam, że w pracy Wigner P, Synowiec E, Jóźwiak P, **Czarny P**, Bijak M, Białek K, Szemraj J, Gruca P, Papp M, Sliwinski T. The effect of chronic mild stress and venlafaxine on the expression and methylation levels of genes involved in the tryptophan catabolites pathway in the blood and brain structures of rats. J Mol Neurosci. 2020; doi 10.1007/s12031-020-01563-2 mój udział wynosił 5% i obejmował współudział w projektowaniu badań i wykonywaniu eksperymentów.

ADIUNKT
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Oświadczenie o udziale w publikacjach

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Ewelina Synowiec

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30.04.2020

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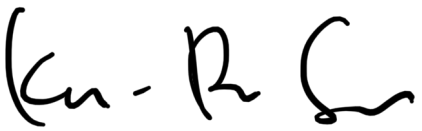
Declaration of participation in publications

I declare that in the publication Wigner P, Czarny P, Galecki P, **Su KP**, Sliwinski T. The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression. Psychiatrists Res. 2018; 262: 566-574 my share was 5% and included substantive consultation, help in editing and proofreading.



2020/4/7

Łódź, 29.04.2020

dr Paweł Jóźwiak
Katedra Cytobiochemii
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Oświadczenie o udziale w publikacjach

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Paweł Jóźwiak

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Zagrożeniom Biologicznym
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Prof. dr hab. n. med. Janusz Szemraj

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Katarzyna Białek