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Evaluation of interactions of aripiprazole, the atypical antipsychotic drug, with the selected antidepressants in the rat models of schizophrenia

Ocena interakcji arypiprazolu, atypowego leku przeciwpsychotycznego z wybranymi lekami przeciwdepresyjnymi w szczurzych modelach schizofrenii

Praca doktorska wykonana w Zakładzie Farmakologii
Instytutu Farmakologii im. Jerzego Maja Polskiej Akademii Nauk

Promotor:
dr hab. Zofia Rogóż

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Spis artykułów naukowych stanowiących podstawę rozprawy doktorskiej

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2. **Hereta M**, Kamińska K, Białoń M, Wąsik A, Lorenc-Koci E, Rogóż Z. *Effect of combined treatment with aripiprazole and antidepressants on the MK-801–induced deficits in recognition memory in novel recognition test and on the release of monoamines in the rat frontal cortex.* Behavioural Brain Research 2020, 393, 112769. doi:10.1016/jbbr.2020.112769. 5-letni współczynnik wpływu: 3,518

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Spis treści

1. Wykaz stosowanych skrótów

5-HT - Serotonina, 5-hydroksytryptamina (ang. *5-Hydroxytryptamine)*

ARI - Arypiprazol (ang. *Aripiprazole*)

BDNF - Neurotroficzny czynnik pochodzenia mózgowego (ang. *Brain-derived neurotrophic factor*)

BSO - L-Butionina-(S,R)-sulfoksymina (ang. *L-buthionine-(S,R)-sulfoximine*)

ELISA - Test immunoenzymatyczny (ang. *Enzyme-linked immunosorbent assay*)

ESC - S-citalopram (ang. *Escitalopram*)

GBR 12909 - Wanoxeryna (ang. *Vanoxerine*), 1-[2-Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride

HPLC - Wysokosprawna chromatografia cieczowa (ang. *High-performance liquid chromatography*)

MIR - Mirtazapina (ang. *Mirtazapine*)

MK801 - Dizocylpina (ang. *Dizocilpine*), (5R,10S)-(+)-5-metylo-10,11-dihydro-5H-dibenzo[a,d]cyklohepten-5,10-imina

NaSSA - Leki o działaniu noradrenergicznym i specyficznie serotoninergicznym (ang. *Noradrenergic and specific serotonergic antidepressant*)

NOR - Test rozpoznania nowego obiektu (ang. *Novel Object Recognition Test*)

PPI - Hamowanie przedsygnałowe (ang. *Prepulse Inhibition*)

RT-qPCR - Reakcja łańcuchowa polimerazy w czasie rzeczywistym (ang. *Quantitative polymerase chain reaction*)

SCH 23390 - 7-chloro-3-metylo-1-fenylo-1,2,4,5-tetrahydro-3-benzazepin-8-ol

SERT - Transporter serotoninowy (ang. *The serotonin transporter*)

SSRI - Selektywne inhibitory wychwytu zwrotnego serotoniny (ang. *Selective serotonin reuptake inhibitors*)

WAY 100635 - N-[2-4-(2-metoksyfenylo)-1-piperazynylo]etylo]-
N-(2 pirydylo)cykloheksanokarboskyami

2. Streszczenie w języku polskim i angielskim

2.1. Streszczenie

Schizofrenia jest psychozą endogenną, na którą choruje około 1% populacji. Choroba zaczyna się w wieku młodzieńczym i charakteryzuje się zróżnicowanym przebiegiem. Przyczyny wystąpienia choroby nie są do końca poznane. Istnieje wiele hipotez wyjaśniających genezę pojawiających się objawów. Jest to między innymi hipoteza neurorozwojowa, mówiąca o związku zaburzeń w okresie neurogenezy, a wystąpieniem schizofrenii w przyszłości. Przypuszcza się, że zaburzenia w równowadze redoks we wczesnym okresie postnatalnym mogą przyczyniać się do wystąpienia objawów w życiu dorosłym. Obecnie w klinice terapia farmakologiczna bazuje na chronicznym stosowaniu neuroleptyków starej bądź nowej generacji, w zależności od rodzaju przeważających objawów. Syndromy schizofrenii można podzielić na wytwórcze (pozytywne), negatywne oraz zaburzenia funkcji kognitywnych. U pacjentów schizofrenicznych objawom typowym często towarzyszy depresja. Dlatego zasadne wydaje się zastosowanie terapii farmakologicznej polegającej na łącznym podawaniu niskiej dawki leku przeciwpsychotycznego wraz z niskimi dawkami leków przeciwdepresyjnych.

Opracowano dwa modele badawcze z wykorzystaniem samców szczurów rasy Sprague-Dawley: krótkotrwały model symptomatyczny, gdzie objawy charakterystyczne dla schizofrenii wywołano podaniem dizocylpiny (MK-801) oraz model neurorozwojowy, gdzie w okresie od 5 do 16 dnia po urodzeniu oseskom podawano chronicznie L-Butionina- (S,R)-sulfoksyminę (BSO) oddzielnie lub w kombinacji z wanoxeryną (GBR 12909). Zastosowanie tych związków miało na celu obniżenie poziomu glutationu w komórkach, a tym samym zaburzyć prawidłową równowagę redoks w rozwijającym się mózgu. Zaburzenie to przedkłada się na wystąpienie typowych syndromów schizofrenii w dorosłości. U szczurów zastosowano terapię chronicznych podań leku przeciwpsychotycznego- arypiprazolu (ARI) wraz z lekiem przeciwdepresyjnym S-citalopramem (ESC) lub mirtazapiną (MIR). Aby sprawdzić opracowane modele, wykonano testy behawioralne oceniające występowanie negatywnych objawów schizofrenii oraz zaburzeń funkcji kognitywnych (interakcji socjalnej, test rozpoznania nowego obiektu (NOR), otwartego pola). Następnie wykonano badania biochemiczne (qRT-PCR i ELISA) oceniające amplifikację genu dla neurotroficznego czynnika pochodzenia mózgowego (BDNF) oraz ilość tego białka w korze czołowej i hipokampie dorosłego szczura.

Wyniki uzyskane w artykułach stanowiących rozprawę doktorską sugerują, że zarówno ostre podanie związku MK-801 jak i obniżenie poziomu glutationu na wczesnym etapie neurorozwojowym przyczynia się do wystąpienia deficytów zachowania charakterystycznych dla objawów schizofrenii w badanych testach. W modelu neurorozwojowym zaobserwowano również redukcję ekspresji genu i ilości białka dla BDNF w dorosłości w wybranych strukturach mózgu. Wyniki badań pokazują, że odwrócenie wyżej wymienionych deficytów następuje po podaniu wysokiej dawki ARI lub niskiej dawki ARI łącznie z niską dawką MIR lub ESC.

Przedstawione w rozprawie wyniki sugerują słuszność zastosowanej terapii farmakologicznej oraz potwierdzają zgodność wybranych modeli badawczych z założeniami charakterystycznymi dla zwierzęcych modeli schizofrenii. Opublikowane badania mogą przyczynić się do zastosowania w klinice nowej terapii farmakologicznej, co może skutkować polepszeniem stanu zdrowia i jakości życia pacjentów schizofrenicznych.

2.2. Summary

Schizophrenia is an endogenous psychosis affecting approximately 1% of the population. The disease begins in adolescence and has a varied course. The causes of the disease are not fully understood. There are many hypotheses explaining the genesis of the symptoms. The neurodevelopmental hypothesis, which says about the relationship between disorders in the period of neurogenesis and the occurrence of schizophrenia in the future. It is hypothesized that redox imbalances in the early postnatal period may contribute to the onset of symptoms in adulthood. Currently, in the clinic pharmacological therapy is based on the chronic use of neuroleptics, depending on the type of prevailing symptoms. Schizophrenia syndromes can be divided into positive, negative and cognitive disorders. Typical symptoms are often associated with depression in schizophrenic patients. Therefore, it seems justified to use pharmacological therapy consisting of the combined administration of a low dose of an antipsychotic with low doses of antidepressants.

In the present study, two research models were developed using male Sprague-Dawley rats. At the first a short-term symptomatic model, where symptoms characteristic of schizophrenia were induced by the administration of dizocilpine (MK-801). Next a neurodevelopmental model, where puppies were chronically administered L- Butionin- (S, R) -sulfoximin (BSO) alone or in combination with vanoxerine (GBR 12909). The use of these compounds was aimed at lowering the level of glutathione in the cells and thus disrupting the normal redox balance in the developing brain. This disorder translates into the typical syndromes of schizophrenia in adulthood.

Rats were treated with chronic administration of the antipsychotic drug aripiprazole (ARI) together with the antidepressant S-citalopram (ESC) or mirtazapine (MIR). In order to validate the developed models, behavioral tests were performed to assess the presence of negative symptoms of schizophrenia and cognitive disorders (social interaction test, new object recognition test (NOR), open field test). Then, biochemical tests (qRT-PCR and ELISA) were performed to assess the gene amplification of brain-derived neurotrophic factor (BDNF) and the amount of this protein in the frontal cortex and hippocampus in adulthood.

The results obtained in the articles constituting the doctoral dissertation suggest that both acute administration of MK-801 and the reduction of glutathione levels at the early neurodevelopmental stage contribute to the behavioral deficits characteristic of the symptoms of schizophrenia in tests. In the neurodevelopmental model, a reduction in gene expression and

protein amount for BDNF was also observed in selected brain structures in adulthood. Research results show that the reversal of the above-mentioned deficits occurs after administration of a high dose of ARI or a low dose of ARI together with a low dose of MIR or ESC.

The results presented in the dissertation suggest the correctness of the pharmacological therapy used and confirm the compliance of used research models with the assumptions characteristic of animal models of schizophrenia. Published studies may contribute to the application of a new pharmacological therapy in the clinic and may have an impact on the improvement of the health condition and quality of life of patients with schizophrenia.

3. Wprowadzenie

3.1. Charakterystyka schizofrenii - etiologia i objawy

Schizofrenia jest zaburzeniem psychicznym zaliczanym do psychoz endogennych. Stan psychiczny organizmu jest chorobowo zmieniony i cechuje się nieadekwatnym postrzeganiem i oceną rzeczywistości. Etymologia tej choroby wywodzi się z języka starogreckiego, gdzie σχίζειν, *schizein*- znaczy rozszczepić, natomiast drugi człon φρεν-, *phrēn*- umysł. Schizofrenia często nazywana jest chorobą wieku młodzieńczego, gdyż pierwsze epizody psychozy pojawiają się w okresie młodzieńczym, tj. 15-20 lat. Znane są także przypadki, gdzie pierwsze objawy pojawiały się w okresie dzieciństwa. Przebieg schizofrenii charakteryzuje się dużą nawrotowością epizodów, szczególnie gdy pozostaje ona nieleczona. Po pierwszym epizodzie w wieku młodzieńczym, choroba może pozostać w tzw. uśpieniu i objawić się dopiero po kilku miesiącach czy latach (Pearlson, 2000).

Objawy charakterystyczne dla schizofrenii cechuje naprzemienność występowania. Można podzielić je na trzy główne grupy: objawy pozytywne (wytwórcze), objawy negatywne oraz zaburzenia funkcji poznawczych (Neill i wsp., 2010). Do pierwszej grupy objawów możemy zaliczyć zachowania takie jak: nadpobudliwość ruchowa i psychiczna, gonitwa myśli, urojenia, omamy wzrokowe i słuchowe, halucynacje, a także agresje słowną lub fizyczną. Osoby posiadające przewagę tych objawów mogą stanowić zagrożenie dla siebie lub otoczenia, natomiast obecna nauka pozwala na leczenie tych objawów i minimalizację ich nawrotów (Roy i DeVriendt, 1994).

Syndromy negatywnie to przede wszystkim zubożenie emocjonalne, wycofanie z życia społecznego i rodzinnego, obniżenie aktywności zarówno fizycznej jak i ogólnej aktywności umysłowej, obniżenie zainteresowania, a także może występować otępienie. Objawy z tej grupy często występują niezauważalne przez bliskich chorego, a niestety są główną przyczyną samobójstw wśród osób chorych. Dodatkowo obecnie nie ma dobrego sposobu leczenia tego typu objawów. U około 50% pacjentów, z przewagą objawów negatywnych występuje dodatkowo depresja. Stany depresyjne utrudniają leczenie, psychiczną stabilizację pacjentów oraz zwiększają prawdopodobieństwo nawrotu objawów (Conley i wsp., 2007). Ostatnia grupa objawów, jest bardzo ważna w kontekście pojawiania się schizofrenii wśród ludzi młodych. Zaburzenia funkcji poznawczych to głównie: zaburzenia koncentracji, nauki, pamięci, przetwarzania informacji, reakcji na bodźce oraz ograniczenie działania na różnych płaszczyznach (Nuechterlein i wsp., 2004).

Etiologia schizofrenii jest bardzo złożona. Pomimo tego, że około 0,5-1% populacji zmaga się z tą chorobą, nie można określić jednego czynnika zapoczątkowującego chorobę. Jako główny czynnik przyjmuje się genetykę i dziedziczność tej choroby. Najwyższy stopień dziedziczenia występuje po rodzicach, a także wśród bliźniąt zarówno dwujajowych jak i jednojajowych. Jednakże czynniki genetyczne mogą zostać zaktywowane poprzez czynniki środowiskowe takie jak: cywilizacyjne, społeczne, ekonomiczne. Istotną rolę spełniają także czynniki prenatalne i nieprawidłowości w trakcie ciąży, infekcje podczas jej trwania, a także na wczesnym etapie życia postnatalnego. Wiadomym jest, że stan zapalny, występujący w trakcie ciąży, a także po urodzeniu może przyczyniać się do rozwinięcia schizofrenii. Zauważalne jest wśród pacjentów, że większość osób chorych urodziła się w okresie jesienno-zimowym, czyli bardziej narażonym na infekcje wirusowe i bakteryjne, a co za tym idzie powstający stan zapalny (McGrath i wsp., 2008).

3.2. Hipotezy schizofrenii

3.2.1. Hipoteza dopaminowa

Ze względu na różnorodność objawów i ich nawracanie, przebieg schizofrenii u pacjentów jest wieloraki. W leczeniu klinicznym istnieje klasyfikacja schizofrenii ze względu na przewagę w występowaniu konkretnych objawów i wyróżnia się: schizofrenię paranoidalną, gdzie w przewadze występują objawy pozytywne, hebefreniczną, w trakcie której pojawia się dezintegracja funkcji poznawczych, katatoniczną z zaburzeniami psychomotorycznymi, a także schizofrenię prostą i rezydualną, w których dominują objawy negatywne bez epizodów psychozy, bądź z jednym epizodem zapoczątkowującym chorobę. Lekarze opisują także schizofrenię niezróżnicowaną, w której objawy występują naprzemiennie i bez wyraźnej dominacji którejś grupy syndromów oraz schizofrenię lekooporną, w której pacjenci nie reagują na właściwe leczenie (Mishara i wsp., 2004). Naukowcy wysnuli kilka najbardziej rozpowszechnionych hipotez co jest przyczyną konkretnych objawów schizofrenii. Hipoteza dopaminowa, to teoria skupiająca się na układzie dopaminowym i jego nieprawidłowym funkcjonowaniem, co prowadzi do konkretnych zaburzeń. Objawy wytwórcze są tłumaczone nadaktywnością układu dopaminowego w szlaku mezolimbicznym, a zaburzenia funkcji poznawczych i negatywne syndromy obniżeniem aktywności szlaku dopaminowego w strukturach mezokortykalnych (Lau i wsp., 2013).

3.2.2. Hipoteza glutaminianowa

Druga teoria tłumacząca występowanie charakterystycznych objawów jako nieprawidłowe neuroprzekaźnictwo glutaminergiczne nazwana jest hipotezą glutaminową. Założenia tej hipotezy oscylują wokół niedostatecznego funkcjonowania receptorów NMDA w ośrodkowym układzie nerwowym, a szczególnie ważną rolę w patomechanizmie zgodnym z tą hipotezą mają szlaki: korowo-wzgórzowy, wzgórzowo-korowy, korowo-pniowy, korowo-korowy i korowo-prążkowy (Uno i Coyle, 2019).

3.2.3. Hipoteza neurodegeneracyjna i neurorozwojowa

Dwie kolejne hipotezy to: hipoteza neurodegeneracyjna, która zakłada, że przyczyną występowania objawów jest neurodegeneracja w ośrodkowym układzie nerwowym, spowodowana czynnikami endogennymi, takimi jak apoptoza oraz zewnętrznymi np. urazami głowy oraz hipoteza neurorozwojowa. W ostatnich latach naukowcy skupiają się bardziej na tej hipotezie ponieważ odkryto, że istotne dla rozwinięcia się schizofrenii są zmiany biochemiczne zachodzące w mózgu na etapie jego rozwoju, to jest podczas trwania życia prenatalnego lub na wczesnym etapie życia postnatalnego. Czynnikami zaburzającymi prawidłowy rozwój mózgu na jego wczesnym etapie rozwoju mogą być: stan zapalny, nikotyna, substancje psychoaktywne, czy zaburzenia w równowadze redoks (Murray i wsp., 2017).

3.3. Glutation

Glutation jest głównym przeciwutleniaczem występującym we wszystkich komórkach ludzkiego organizmu. Ze względu na swoją budowę chemiczną jest tripeptydem i tiolem. Składa się z trzech aminokwasów: glicyny, cysteiny i kwasu glutaminowego. Jego właściwości antyoksydacyjne polegają na odtwarzaniu grup tiolowych w wewnątrzkomórkowych białkach. Najważniejszą rolę przeciwutleniacza glutationu pełni w mózgu - gdzie zapobiega zachwianiu równowagi redoks i w wątrobie - gdzie bierze udział np. w metabolizmie leków w tym popularnego paracetamolu (Tsugawa i wsp., 2019). Zmniejszenie endogennego poziomy glutationu poprzez zablokowanie jego syntezy prowadzi do zaburzeń w równowadze redoks, a tym samym do generowania toksycznych dla żywych komórek cząsteczek tzw. wolnych rodników. Znane są badania, których wyniki sugerują, że w chorobach ośrodkowego układu nerwowego, a w szczególności chorobie Alzheimera oraz Parkinsona oraz w chorobach psychicznych takich jak schizofrenia, depresja, choroba afektywna dwubiegunowa występuje znaczny deficyt poziomu glutationu (Gu i wsp., 2015; Yang i wsp., 2019).

3.4. Terapie farmakologiczne

Leczenie farmakologiczne w schizofrenii obejmuje głównie dwa etapy. Leczenie w trakcie ostrych epizodów psychozy oraz leczenie podtrzymujące, które u większości pacjentów należy stosować stale do końca życia. Obecnie leki przeciwpsychotyczne tzw. neuroleptyki dzieli się na dwie grupy: klasyczne leki oraz leki nowej generacji. W terapii schizofrenii klasyczne leki przeciwpsychotyczne hamują objawy pozytywne natomiast nie wpływają na objawy negatywne czy zubożenie funkcji poznawczych. Do tej grupy leków przykładowo należą: haloperidol, chlorpromazyna, droperidol i są to antagoniści receptora dopaminowego D_2. W aktualnej farmakoterapii odchodzi się od stosowania neuroleptyków I generacji, gdyż ich zażywanie wiąże się z obecnością pozapiramidowych objawów ubocznych takich jak sztywność mięśni czy występowaniem drżeń (Nord i Farde, 2011).

Lepszą tolerancją wśród pacjentów cieszą się neuroleptyki nowej generacji. Leki atypowe mają wyższe powinowactwo do receptora 5-HT_{2A} niż do receptora dopaminowego D_2, dzięki czemu mogą częściowo łagodzić objawy negatywne oraz nieznacznie poprawiać funkcje poznawcze. W tej grupie znajdziemy leki takie jak: rysperydon, klozapina, olanzapina oraz arypiprazol (ARI). Obecnie znacznie częściej stosowane są terapie z użyciem wyżej wymienionych leków, jednakże również występują skutki uboczne ich zażywania np. ARI zwiększa apetyt i prowadzi do nagłego wzrostu masy ciała pacjenta. Badania ostatnich lat sugerują, że w leczeniu schizofrenii powinno się zwrócić uwagę również na epizody depresyjne, które towarzyszą temu schorzeniu. Dlatego pojawiła się koncepcja nowej formy terapii, gdzie do atypowego leku przeciwpsychotycznego dołącza się lek przeciwdepresyjny. Nowa terapia ma dawać poprawę zaburzeń funkcji poznawczych, złagodzenie objawów negatywnych oraz eliminowanie epizodów depresji w przebiegu choroby. Słuszność tej teorii potwierdzają wstępne badania kliniczne, w których dodanie leku przeciwdepresyjnego – mirtazapiny (MIR), będącej antagonistą receptora adrenergicznego oraz serotoninowego do terapii rysperydonem dawało polepszenie funkcji poznawczych bardziej efektywne niżeli w zastosowaniu terapii tylko z rysperydonem. Również badania przedkliniczne pokazują, że dodanie escitalopramu (ESC), będącego lekiem z grupy selektywnych inhibitorów wychwytu zwrotnego serotoniny (SSRI) do terapii rysperydonem zwiększa efektywność leczenie i może stanowić alternatywę do klasycznego leczenia schizofrenii (Horacek i wsp., 2006).

3.5. Leki - charakterystyka

Leki przeciwpsychotyczne nowej generacji zdominowały obecne leczenie schizofrenii. Zasługą tego jest przede wszystkim brak skutków ubocznych w postaci objawów pozapiramidowych, a także dłuższy czas działania wywołany mniejszą dawką leku. Poniżej przedstawię charakterystykę leków zastosowanych w badaniach naukowych stanowiących podstawę mojej rozprawy doktorskiej.

3.5.1. Arypiprazol (ARI)

ARI znany również pod nazwą marketingową jako Abilify, a także nazwą chemiczną 7-{4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butyloxy}-3,4-dihydro-2(1H)-quinolinone. Jest pochodną chinolinonu i zaliczany jest do atypowych leków nowej generacji. Często określany jako lek III generacji, gdyż jego mechanizm działania wyróżnia się na tle neuroleptyków II generacji (Mailman i Murthy, 2010). Wykorzystywany w terapii schizofrenii, zaburzeń afektywnych dwubiegunowych, epizodów maniakalnych i innych zaburzeń na tle psychicznym (Shapiro i wsp., 2003). Opracowany został w połowie lat 90. Agencja Food and Drug Administration (FDA) zarejestrowała go jako lek przeciwpsychotyczny w 2002 roku. W Europie zarejestrowany jest jako lek dla osób dorosłych oraz dla młodzieży powyżej 13 roku życia. W Stanach Zjednoczonych przeznaczony jest zaś do leczenia schizofrenii u pacjentów w przedziale wiekowym 13-17 lat, leczenia manii u młodzieży w wieku 10-17 lat, a także do leczenia objawów związanych z autyzmem u dzieci w wieku 6-10 lat (Kirino, 2014).

ARI jest antagonistą receptora serotoninowego 5-HT_{2A} oraz agonistą receptora dopaminowego D_2 i 5-HT_{1A}. Stabilizuje aktywność dopaminową i serotoninową w obrębie: jądra półleżącego- *nucleus accumbens,* obszaru brzusznego nakrywki- *ventral teamental area* oraz kory czołowej- *frontal cortex*. Ponadto może modyfikować wewnątrzkomórkową sygnalizację poprzez receptor D_2. ARI ma wysokie powinowactwo do receptora D_2 i może zmieniać swój mechanizm działania w zależności od gęstości tego receptora na komórce. Gęstość receptorowa przyczynia się do tego, czy ARI będzie presynaptycznym agonistą czy postsynapstycznym antagonistą (Tuplin i Holahan, 2017). W badaniach na liniach ludzkich embrionalnych komórek nerki HED-D2L oraz linii komórkowej chomika chińskiego CHO-DL2 wykazano wzrost ekspresji receptora D_2 po zastosowaniu ARI, a zatem w tym badaniu wykazuje działanie agonisty (Shapiro i wsp., 2003). Natomiast w badaniach na szczurzym modelu Parkinsona, mimo iż nie jest to główny lek wykorzystywany w leczeniu tej choroby, wykazuje mechanizm działania antagonisty (Kikuchi i wsp., 1995).

W przeciwieństwie do klasycznych leków przeciwpsychotycznych, ARI łagodzi nie tylko objawy pozytywne ale także negatywne oraz poprawia funkcje poznawcze. Jest dobrze tolerowany przez pacjentów, a dzięki temu, że jego stężenie dłużej utrzymuje się na stałym poziomie we krwi jest bardziej skuteczny w swoim działaniu. Może być przyjmowany w postaci tabletek doustnie lub iniekcji o przedłużonym działaniu lub też iniekcji szybko działających w celu zastosowania doraźnego w nagłych epizodach psychozy. W terapii ARI zazwyczaj nie występują objawy pozapiramidowe, niekiedy mogą się one pojawić u pacjentów w bardzo podeszłym wieku. ARI ma jeden z bezpieczniejszych profili działania, gdyż nie powoduje zmian metabolicznych ani hiperprolaktynemii polekowej, nawet u młodych pacjentów. Główne skutki uboczne długotrwałego stosowania leku to przede wszystkim wzrost masy ciała, który jak pokazują badania jest trzykrotnie niższy niż przy zastosowaniu np. olanzapiny. Z racji swojego bezpiecznego działania może być stosowany zarówno u osób starszych jak i młodzieży (Mierzejewski, 2017).

3.5.2. Escitalopram (ESC)

ESC to wysoko selektywny lek, zaliczany to grupy związków zwanych selektywnymi inhibitorami wychwytu zwrotnego serotoniny (SSRI). Jest to związek organiczny, będący S-enancjomerem citalopramu. Enancjomer ten wykazuje większą selektywność i dwukrotnie silniejsze działanie w porównaniu do mieszaniny racemicznej (Sanchez i wsp., 2003). Główny mechanizm działania escitalopramu polega na zablokowaniu transportera serotoninowego, tak aby uniemożliwić mu transport zwrotny serotoniny z przestrzeni międzysynaptycznej do aksonu komórki nerwowej. Transporter ten posiada dwa miejsca łączenia się z lekami z grupy SSRI. Miejsce wiązania podstawowego, o wysokim powinowactwie do leków, które odpowiada za bezpośrednie zahamowanie transportu serotoniny oraz miejsce allosteryczne, które moduluje wiązanie się leku z miejscem podstawowym. ESC wiążąc się z miejscem allosterycznym na transporterze modyfikuje swoje wiązanie z miejscem pierwotnym poprzez zmniejszenie stałej dysocjacji, a co za tym idzie zwiększa się stabilność wiązania. Ma to swoje odzwierciedlenie w efektowniejszym zablokowaniu transportu zwrotnego serotoniny, przez co lek jest bardziej skuteczny w leczeniu objawów depresyjnych (Landowski i Rybakowski, 2001).

W roku 2001 został wprowadzony do leczenie przez firmę H. Lundbeck. Jest stosowany w terapii depresji, zwykle w stanach o nasilonym przebiegu, ale także w terapii stanów lękowych, fobii, czy zaburzeń obsesyjno-kompulsywnych. Jest dobrze tolerowany przez

pacjentów. Podawany drogą pokarmową jest całkowicie absorbowany i po około 3-4 godzinach osiąga najwyższe stężenie w osoczu (Roa, 2012).

3.5.3. Mirtazapina (MIR)

MIR należy do czteropierścieniowych leków przeciwdepresyjnych. Zaliczana jest także do leków o działaniu noradrenergicznym oraz serotoninergicznym tzw. związków NaSSA (ang. *Noradrenergic and specific serotonergic antideressants*). MIR jest antagonistą receptorów: serotoninowych 5-HT_2 i 5-HT_3, histaminowych H_1 oraz adrenergicznego α_2. Stosowana w zaburzeniach obsesyjnych, stanach lękowych oraz epizodów depresji głównie o przebiegu ciężkim lub średnim. Głównymi efektami ubocznymi terapii MIR jest sedacja, senność i wzrost masy ciała. Ze względu na swoje efekty uboczne może być także stosowana doraźnie jako lek nasenny lub w epizodach depresyjnych, w których u pacjentów występuje brak apetytu. MIR stosuje się raz dziennie, na noc, swoje maksymalne stężenie w surowicy osiąga po 2 godzinach. Dedykowana jest dla pacjentów dorosłych, przez których jest dobrze tolerowana (Croom i wsp., 2009).

4. Cel pracy

Przedmiotem niniejszej rozprawy doktorskiej jest hipoteza, w której skojarzona terapia lekami przeciwdepresyjnymi (MIR lub ESC) z lekiem przeciwpsychotycznym (ARI) w najniższej działającej dawce daje pozytywne efekty w odwróceniu deficytów socjalnych i kognitywnych u dorosłych szczurów rasy Sprague-Dawley z objawami przypominającymi symptomy schizofrenii. Dodatkowe założenie, które bezpośrednio wiąże się z tą hipotezą to zaburzenie równowagi redoks w mózgu szczura na wczesnym etapie życia postnatalnego skutkuje pojawieniem się zachowania charakterystycznego dla schizofrenii oraz obniżeniem na poziomie genetycznym jak i potranslacyjnym białka BDNF w korze czołowej i hipokampie szczura. Hipotezę tą sprawdzono w dwóch modelach badawczych. Wykonano także badania biochemiczne, które miały za zadanie wykazanie czy interakcja wyżej wymienionych leków działa pozytywnie na stężenie BDNF u szczurów, u których wywołano objawy schizofrenii.

5. Materiały i metody (metodyka)

5.1. Test interakcji socjalnej

Jednym z typowych objawów negatywnych wśród pacjentów chorych na schizofrenię jest wycofanie z życia społecznego i trudność w nawiązywaniu kontaktów socjalnych. Test interakcji socjalnej sprawdza zachowanie dwóch nieznanych sobie szczurów względem siebie, w momencie gdy zostają umieszczone w jednej klatce eksperymentalnej. W pierwszym dniu każdy szczur pojedynczo ma czas na habituację klatki. Następnego dnia dwa osobniki umieszcza się na 10 minut w interakcji bezpośredniej. W tym czasie mierzone są trzy parametry: czas interakcji, ilość interakcji oraz lokomotoryczność (Kamińska i Rogóż, 2015).

5.2. Test rozpoznania nowego obiektu (NOR)

Kolejnym testem wykorzystanym w artykułach zawartych w rozprawie doktorskiej jest test NOR. Test ten bada zaburzenia funkcji poznawczych w schizofrenii Jego głównym celem jest ocena pamięci, nauki oraz aktywności szczura umieszczonego w klatce eksperymentalnej, w którym znajdują się dwa identyczne pod względem wizualnym oraz zapachowym obiekty: A1 i A2. Szczur przed rozpoczęciem testu poznaje klatkę tak aby nie stanowił dla niego ciekawego, nowego środowiska. Pierwsza sesja trwa przez 5 minut. Szczur eksploruje środowisko, interesując się dowolną ilość czasu obiektami. Następnie umieszczany jest w swojej klatce domowej, gdzie czeka na drugą sesję. Po godzinie obiekt A2 jest podmieniany na

obiekt B - musi być on wyraźnie inny w kształcie i kolorze. I podobnie szczura umieszcza się na 5 minut w klatce z dwoma obiektami tj. A1 i B oraz mierzony jest czas zainteresowania się każdym z obiektów oraz lokomotoryczność. W obliczeniach zmian wykorzystuje się także stosunek zainteresowania się nowym obiektem (B) względem starego obiektu (A2) (Antunes i Biala, 2012).

5.3. Test otwartego pola

Test ten wykorzystywany jest do badania neurobiologicznych podstaw zachowań lękowych u gryzoni oraz do screeningowych badań nad nowymi lekami. Zwierzę wystawione jest na dobrze oświetloną platformę, która nie posiada żadnych elementów dodatkowych, jest płaska. Szczur eksploruje otoczenie, podczas gdy obserwator mierzy jego czas chodzenia, wychylanie się, czy wznoszenie (Kraeuter i wsp., 2019).

5.4. Mikrodializa

W jednym z modeli - symptomatycznym zastosowano także mikrodializę u szczurów swobodnie się poruszających. Sondę mikrodializacyjną umieszczono w korze czołowej. Poziom stężenia monoamin oraz ich metabolitów mierzono przy pomocy wysokosprawnej chromatografii cieczowej (ang. *high-performance liquid chromatography*- HLPC) po iniekcji zastosowanych leków (Hammarlund-Udenaes, 2017).

5.5. Test immunoenzymatyczny ELISA

ELISA to jeden z najbardziej powszechnych testów wykrywających stężenie określonych białek w homogenacie. Mechanizm działania opiera się na wykorzystaniu przeciwciał monoklonalnych lub poliklonalnych odpowiednio skoniugowanych z enzymem (Lequin, 2005).

5.6. Real-time qPCR

Reakcja łańcuchowa polimerazy w czasie rzeczywistym jest metodą ilościową bazującą na technikach fluorescencyjnych. Pozwala na ocenę ilości genu (amplifikacji) w czasie trwania reakcji (Singh i Roy-Chowdhuri, 2016).

5.7. Krótkotrwały model symptomatyczny (część I rozprawy)

Krótkotrwały model symptomatyczny polega na jednokrotnym podaniu dorosłym (trzymiesięcznym) samcom szczurów razy Sprague-Dawley związku wywołującego objawy behawioralne zbliżone do objawów typowych dla schizofrenii. Symptomy te wywołuje się

podaniem substancji MK-801. Związek ten nazywany także dizocypliną jest niekompetytywnym antagonistą receptora NMDA. Dzięki temu mechanizmowi powoduje on deficyty w zachowaniu zwierząt, typowe dla objawów schizofrenii. Powszechnie jest wykorzystywany w modelach zwierzęcych schizofrenii zarówno do sprawdzenia objawów pozytywnych poprzez wywołanie hiperaktywności, a także do oceny syndromów negatywnych mierzonych w testach behawioralnych używanych do oceny tych objawów (Vanderschuren i wsp., 1998).

Schemat modelu symptomatycznego polegał na podaniu ESC lub MIR dootrzewnowo, następnie po 30 minutach iniekcji dootrzewnej ARI w niskiej dawce samo niedziałającej. MK-801 w teście interakcji socjalnej podano 4 godziny wcześniej, a w teście NOR 30 minut przed I sesją. W celu weryfikacji mechanizmów działania leków w wybranej konfiguracji zwierzętom podano antagonistę receptora serotoninowego $5HT_{1A}$ - związek WAY 100635 oraz antagonistę receptora dopaminowego D_1 - związek SCH 23390. Szczury poddano następnie testom behawioralnym, oceniającym negatywne objawy schizofrenii (Kamińska i Rogóż, 2015).

5.8. Model neurorozwojowy (część II rozprawy)

Mając wiedzę dotyczącą deficytu glutationu a występowaniem schizofrenii opracowano model neurorozwojowy. Powszechnie stosowane w modelach badawczych szczury rasy Sprague-Dawley po raz pierwszy wykorzystano w następującym schemacie eksperymentu: w celu obniżenia stężenia glutationu szczurzym samcom w okresie wzmożonej neurogenezy tj. od 5 do 16 dnia postnatalnego, podawano chronicznie inhibitor syntezy glutationu, związek BSO (3,8 mmol/kg s.c.) oraz inhibitor wychwytu zwrotnego dopaminy, związek GBR 12909 (5 mg/kg s.c.) w wariancie łącznego podania lub podania każdego związku oddzielnie (Górny i wsp., 2020). Po upływie 69 dni, kiedy szczury osiągają dorosłość wprowadzono terapię farmakologiczną w postaci trzytygodniowych, chronicznych iniekcji leku przeciwpsychotycznego ARI (0,1; 0,3; 1 mg/kg) oddzielnie lub łącznie z lekiem przeciwdepresyjnym: ESC. Następnie szczury poddano testom behawioralnym, oceniającym objawy negatywne schizofrenii oraz zaburzenia funkcji poznawczych. Wykorzystano test interakcji socjalnej, test NOR oraz test otwartego pola. Wykonano także badania biochemiczne. Mierzono poziom ekspresji genu oraz stężenie białka dla BDNF w korze czołowej i hipokampie. BDNF jest białkiem należącym do neurotrofin, odpowiada za wzrost nerwów, ich przeżywalność oraz tworzenie nowych projekcji w obszarach mózgu odpowiedzialnych za emocje oraz funkcje poznawcze w szczególności w rozwijającym się

mózgu. Obniżenie poziomu BDNF jest charakterystyczne w chorobach ośrodkowego układu nerwowego, w tym schizofrenii i depresji (Phillips, 2017).

6. Dyskusja

Niniejszą rozprawę doktorską stanowią cztery oryginalne artykuły naukowe, w których prezentowane wyniki badań zostały przeprowadzone w dwóch zwierzęcych modelach schizofrenii. Zgodnie z danymi literaturowymi jak i doniesieniami klinicznymi obecne leczenie objawów negatywnych oraz zaburzeń funkcji poznawczych występujących w schizofrenii połączonych dodatkowo z nawracającymi epizodami depresyjnymi wymaga wprowadzenia nowego, dającego lepsze efekty terapeutyczne leczenia farmakologicznego. Słuszność koncepcji terapii niską dawką ARI wraz z niskimi dawkami leków przeciwdepresyjnych potwierdzono eksperymentalnie zarówno w pierwszej jak i drugiej części badań składających się na obecną rozprawę doktorską.

Zgodnie z hipotezą glutaminianową, schizofrenia to zaburzone funkcjonowanie receptorów NMDA, które powoduje wystąpienie epizodów psychozy. MK-801 podobnie jak ketamina lub fencyklidyna jest antagonistą receptorów NMDA. Istnieje wiele modeli schizofrenii opartych na zastosowaniu wymienionych antagonistów, są to modele zarówno krótkotrwałe jak i chroniczne, gdzie antagonistę podaje się przez kilka dni. Jednorazowe podanie fencyklidyny powoduje zwiększenie aktywności lokomotorycznej u szczurów, która utrzymuje się przez dłuższy okres. Natomiast chroniczne podawanie nie powoduje zmian w lokomotoryce zwierząt lecz ma wpływ na zmniejszenie ilości receptorów 5-HT w korze przedczołowej szczurów (Adell, 2020).

Grupa Eyjolfsoon i wsp. wybrała model badawczy, bazujący na chronicznym podawaniu MK-801 przez 6 dni samcom szczurów rasy Sprague-Dawley w dwóch dawkach 0,1 mg/kg i 0,5 mg/kg. Jak wykazały badania, chroniczne podawanie MK-801 może nasilać neurotoksyczność spowodowaną nadmiarem uwalnianego kwasu glutaminowego. Glutaminian jest neuroprzekaźnikiem pobudzającym stanowiącym ligand dla receptorów NMDA. Kiedy antagonista zwiąże się z receptorem, glutaminian gromadzi się nadmiernie w synapsie i może być toksyczny dla otaczających neuronów. Za metabolizm glutaminianu w dużej mierze odpowiadają astrocyty, których we wspomnianym modelu funkcje zostają osłabione. Ponadto niższa dawka MK-801 powodowała podwyższoną lokomotoryczność i podnosiła reakcje zwierząt w teście hamowania przedsygnałowego (PPI), ale nie dawała zmian na poziomie

biochemicznym. Wyższa dawka powodowała zmiany zarówno behawioralne jak i biochemiczne. Podania MK-801 w niższej dawce obrazują toksyczne efekty zaburzonej funkcji receptora NMDA, natomiast iniekcje w wyższej dawce, imitują objawy zbliżone do pierwszego epizodu schizofrenii u pacjentów (Eyjolfsson i wsp., 2006).

W pierwszej części badań stanowiących podstawę rozprawy bazowano na krótkotrwałym modelu symptomatycznym. Ostre podanie MK-801 4 h lub ½ h przed testami behawioralnymi powodowało wystąpienie symptomów charakterystycznych dla schizofrenii. Istotne zmiany w zachowaniu były obserwowane w testach: interakcji socjalnej oraz w NOR. Ponadto zastosowanie leku przeciwdepresyjnego w dawce 5 mg/kg jednorazowo może powodować zmniejszenie lokomotoryczności, a także wzmożone wąchanie i grooming. Zablokowanie receptorów NDMA wiąże się z upośledzeniem ich roli w neuroplastyczności w hipokampie. Zaburzona zostaje także synaptogeneza, co skutkuje upośledzeniem pamięci i funkcji kognitywnych u szczurów traktowanych MK-801 (Manahan-Vaughan i wsp., 2008). Zastosowanie MK-801 jako modelowej substancji wywołującej epizody schizofrenii wydaje się być jak najbardziej zasadne, stanowią o tym zarówno dane literaturowe, przedstawiające wyniki z chronicznych oraz jednokrotnych iniekcji, jak i własne doświadczenie eksperymentalne.

Test interakcji socjalnej trwał 10 minut, MK-801 podano 4h przed testem a następnie, 60 minut przed jego rozpoczęciem szczurom podano leki: MIR i ESC oddzielnie lub w kombinacji. ARI podawano 30 minut przed testem. W celu weryfikacji mechanizmów działania leków, 20 minut przed testem szczurom podano także związki: WAY 100635 oraz SCH 23390. Uzyskane wyniki, wskazują, że podanie ARI w dawkach 0,1 i 0,3 mg/kg odwraca efekty wywołane MK-801. Zastosowanie niedziałającej dawki ARI (0,03 mg/kg) wraz z lekami przeciwdepresyjnymi także odwraca deficyty wywołane w modelu symptomatycznym, a efekt ten znosi zablokowanie: receptorów 5-HT_{1A} przez ich antagonistę substancję WAY 100635 i częściowo receptorów D_1 blokowanych przez SCH 23390. Wyniki te sugerują, że połączenie antypsychotycznego działania ARI z działaniem leków przeciwdepresyjnych na indukowane podaniem MK-801 negatywne objawy schizofrenii u szczurów angażuje receptory serotoninowe 5-HT_{1A} oraz w mniejszym stopniu z receptorami dopaminowymi D_1.

Badania z zastosowaniem innego neuroleptyku nowej generacji - rysperydonu wraz z lekami przeciwdepresyjnymi MIR i ESC wykazały, że interakcja tych leków odwraca deficyty spowodowane ostrym podaniem MK-801 w teście interakcji socjalnej (Kamińska i Rogóż, 2015). Inne badania sugerują, że zastosowanie ESC wzmacnia przeciwpsychotyczne działanie

niskiej dawki rysperydonu. Zbadano to wykorzystując test warunkowej reakcji unikania, oceniający negatywne symptomy schizofrenii (Marcus i wsp., 2012). Również badania na myszach, donoszą o wzmocnieniu działania przeciwpsychotycznego rysperydonu lub ARI spowodowanym interakcją z lekami przeciwdepresyjnymi w testach behawioralnych oceniających wszystkie trzy grupy typowych objawów schizofrenii (Rogóż 2013; Rogóż i wsp., 2018).

Kolejnym zastosowanym testem behawioralnym był NOR. Składał się z dwóch sesji eksperymentalnych. Pierwsza sesja trwała 5 minut, druga po godzinie i po zamianie jednego z obiektów również 5 minut. Związek MK-801 został podany 30 minut przed rozpoczęciem testu, natomiast leki przeciwdepresyjne oraz ARI odpowiednio 60 lub 30 minut przed testem. Związki WAY 100635 oraz SCH 23390 zostały podane również jak w teście interakcji socjalnej w celu weryfikacji mechanizmu działania leków 20 minut przed startem testu. Uzyskane wyniki wskazują na pozytywne efekty zastosowania ARI w dawce niedziałającej w kombinacji z lekami przeciwdepresyjnymi w odwracaniu deficytów wywołanych podaniem MK-801. Efekty te były blokowane przez antagonistę receptora 5-HT_{1A} oraz receptora D_1.

W badaniach na myszach ARI odwracał deficyty wywołane fencyklidyną w teście NOR. Te pozytywne efekty znosiło zastosowanie związków WAY 100635 oraz SCH 23390 (Nagai i wsp., 2009). Inni autorzy wykorzystujący do badań samce szczurów rasy Sprague-Dawley również wywołali syndromy schizofrenii indukowane fencyklidyną. Jako terapię zastosowano ARI w interakcji ze związkiem SSR181505, który aktywuje receptory 5-HT_{1A} i D_1. Zastosowane leki odwracały deficyty zachowania w teście rozpoznawania nowego obiektu. Weryfikacja mechanizmu odpowiedzialnego za te efekty odbywała się przez podanie WAY 100635, który blokował pozytywną odpowiedź na terapię (Bruins Slot i wsp., 2005). Bazując na własnych doświadczeniach oraz na danych literaturowych można stwierdzić, że równowaga między aktywnością receptorów 5-HT_{1A} i dopaminy D_1 może mieć istotny wpływ na mechanizm wzmocnienia działania leków przeciwpsychotycznych przez leki przeciwdepresyjne w testach behawioralnych oceniających zaburzenia funkcji poznawczych, a także negatywne objawy schizofrenii.

Chcąc sprawdzić wpływ łącznego podania ARI z lekami przeciwdepresyjnymi na poziom monoamin i ich metabolitów wykonano mikrodializę u swobodnie poruszających się szczurów. Sondę mikrodializacyjną umieszczono operacyjnie w korze czołowej. Dializaty zbierano przez 180 minut, co 20 minut. W trakcie trwania eksperymentu zwierzętom podawane były leki w odpowiednich dawkach. Następnie poziomy monoamin oraz ich metabolitów

zmierzono za pomocą HPLC. Uzyskane wyniki wykazały, że zastosowanie ARI (0,3 mg/kg) z ESC (5 mg/kg) znacząco podnosi poziom noradrenaliny i serotoniny w korze czołowej szczura, natomiast istotnie obniża metabolity dopaminy, serotoniny i noradrenaliny. Łączne podanie ARI z MIR (10 mg/kg) powoduje wzrost poziomu noradrenaliny i jednocześnie obniżenie poziomu metabolitów dopaminy, serotoniny, a także noradrenaliny. Wyniki te sugerują, że wzrost zewnątrz komórkowego poziomu noradrenaliny lub serotoniny w korze czołowej wywołany łącznym podaniem ARI z lekami przeciwdepresyjnymi może mieć kluczowe znaczenie w terapii deficytu funkcji poznawczych, a także objawów negatywnych schizofrenii.

Za zwiększenie poziomu serotoniny w korze czołowej szczurów, którym podano kombinację leków: ARI + ESC może odpowiadać zablokowanie transporteru dla serotoniny (Hopwood i Stamford, 2001). Przeciwnie wyniki opisano podając szczurom wysokie dawkami ARI (3-30 mg/kg), które powodowały obniżenie poziomu serotoniny w korze przedczołowej oraz w jądrze grzbietowym szwu (Bartollozzi i wsp., 2007). Co więcej w celu zwiększenia uwalniania serotoniny postsynaptyczne α-adrenoreceptory stymulowały wzrost uwalnianej noradrenaliny przez neurony noradrenergiczne (Baraban i Aghajanian, 1980).

Łączne podanie ARI z MIR powodowało wzrost poziomu noradrenaliny, ale co istotne bez jednoczesnego wzrostu serotoniny. Może być to związane z aktywacją receptorów adrenergicznych. Autoreceptory adrenergiczne α_2 zlokalizowane na neuronach w korze przedczołowej mogą działać hamująco na uwalnianie noradrenaliny (Gobert i wsp., 1998). W jądrze grzbietowym szwu pobudzenie neuronów uwalniających serotoninę jest ściśle związane z toniczną aktywnością układu adrenergicznego. Zlokalizowane presynaptycznie na tych neuronach heteroreceptory-α_2 adrenergiczne są tonicznie aktywowane przez endogenną noradrenalinę. Z danych literaturowych wiadomo, że aktywacja autoreceptorów jak i heteroreceptorów adrenergicznych - α_2 powoduje spadek transmisji serotoninergicznej. Ponadto inhibitory α_2-autoreceptorów, zlokalizowanych na neuronach adrenergicznych miejsca sinawego, stymulują spadek pobudzenia tych neuronów a następnie obniżenie transmisji serotoninergicznej wynikającej z blokowania aktywności receptorów α_1 zlokalizowanych na jądrze szwu, które są niezbędne do produkcji serotoniny. Co ciekawe, podawanie ogólnoustrojowo antagonistów receptora α_2 zwiększa neurotransmisję serotoniny poprzez bezpośrednie zahamowanie α_2-heteroreceptorów zlokalizowanych na aksonach neuronów serotoninowych oraz pośrednio stymulację α_1 receptorów poprzez inhibicję α_2-autoreceptorów (Feuerstein i wsp., 1993; Mongeau i wsp., 1993; Fredman i wsp., 1984). Wiadomo także, że zablokowanie receptorów histaminowych H_1 może przyczyniać się do zwiększonego

uwalniania noradrenaliny po łącznym podaniu ARI i MIR. Stężenie noradrenaliny w dializatach pochodzących z jądra przykomorowego zwiększa się po dodaniu antagonisty receptora H_1-triprolidyny. Wskazuje to, że histamina wywiera hamujący wpływ na uwalnianie noradrenaliny w jądrze przykomorowym (Kurose i Terashima, 1999). MIR wykazuje wysokie powinowactwo do receptora histaminowego i jego zablokowanie może prowadzić do zwiększenia uwalniania noradrenaliny w korze przedczołowej szczura (Croom i wsp., 2009).

Wyżej opisane mechanizmy działania interakcji leków przeciwdepresyjnych z ARI świadczą o wzmocnieniu działania przeciwpsychotycznego i może mieć istotne znaczenie w terapii pacjentów chorych na schizofrenię.

Jak wspomniano we „Wstępie'' odpowiednia równowaga w reakcjach redoks jest niezbędna do zachowania homeostazy w ośrodkowym układzie nerwowym. Obniżenie stężenia glutationu w okresie rozwojowym prowadzi do stresu oksydacyjnego, czego następstwem są zaburzenia charakterystyczne dla schizofrenii (Boskovic i wsp., 2011). Potwierdzają to badania wykonane na szczurach ODS (ang. *Osteogenic Disorder Shionogi*). Mutanty ODS, podobnie jak ludzie, nie syntetyzują kwasu askorbinowego, który w organizmie pełni między innymi funkcję antyoksydacyjną (Castagne i wsp., 2004; Cabungcal i wsp., 2006). W celu obniżenia poziomu glutationu zastosowano substancję BSO oraz GBR 12909. BSO jest inhibitorem syntezy glutationu, działając hamująco na syntetazę gamma-glutamylocysteiny, enzymu katalizującego pierwszy etap syntezy glutationu (Shivakumar i Ravindranath, 1992; Abdelhamid i El-Kadi, 2015). GBR 12909 jest to związek zaliczany to selektywnych inhibitorów wychwytu zwrotnego dopaminy, który w połączeniu z obniżeniem syntezy glutationu na wczesnym etapie neurogenezy może powodować zmniejszenie gęstości kolców dendrytycznych na neuronach piramidowych w korze czołowej u szczura (Andersen, 1989; Do i wsp., 2004).

W drugiej części badań stanowiących podstawę niniejszej rozprawy doktorskiej opracowano model neurorozwojowy schizofrenii. Podawanie szczurom inhibitora syntezy glutationu (BSO) z inhibitorem wychwytu zwrotnego dopaminy (GBR 12909) chronicznie we wczesnym okresie postnatalnym skutkowało pojawieniem się schizofrenio-podobnych objawów oraz obniżeniem poziomu BDNF u dorosłych szczurów rasy Sprague-Dawley. W celu oceny symptomów schizofrenicznych u szczurów wykonano testy behawioralne (test integracji socjalnej, test NOR, test otwartego pola) w 4 punktach czasowych. Natomiast zmiany w

amplifikacji genu dla BDNF oceniano przy użyciu reakcji Real-Time PCR, a poziom białka BDNF badano stosując test ELISA.

Podawanie modelowych substancji w okresie neurorozwojowym prowadzi do długofalowych deficytów, utrzymujących się do późnej dorosłości. Pierwsze deficyty związane z syndromami negatywnymi zaobserwowano w grupach traktowanych BSO oraz BSO w kombinacji z GBR 12909 w interakcjach socjalnych już w okresie dojrzewania, w 42-43 dniu życia i zaburzenia te utrzymywały się aż do dorosłości (90-91 dzień). Zaburzenia w relacjach socjalnych są charakterystyczne dla osób chorujących na schizofrenię. Podobnie w teście otwartego pola tylko w grupie BSO + GBR 12909 zaobserwowano wzmożoną eksplorację otoczenia i nadpobudliwość w ruchliwości, oceniane jako równoważne z objawami pozytywnymi u pacjentów. Syndromy te również pojawiają się w okresie dojrzewania i trwają do okresu dorosłości. Jednakże u szczurów traktowanych tylko GBR 12909 nie zaobserwowano istotnych zmian w zachowaniach socjalnych ani w okresie dorosłości ani dojrzewania. Niewielkie zmiany w funkcjach poznawczych mierzone w teście NOR pojawiły się około 43 dnia, lecz nie utrzymywały się w dalszym życiu. W teście NOR szczury z grupy BSO wykazywały duże deficyty w rozpoznaniu różnic między obiektem A i B. Deficyty pamięci pojawiały się w okresie dojrzewania i utrzymywały się zarówno we wczesnej jak i późnej dorosłości. Odpowiedzialne za te zmiany może być obniżenie syntezy glutationu w okresie noworodkowym, które zaburza funkcje receptorów NDMA w dalszym życiu. Również w grupie BSO + GBR 12909 zauważalne były deficyty kognitywne utrzymujące się do dorosłości. Może być to związane z nasileniem uwalniania dopaminy wynikającej z zastosowania inhibitora syntezy glutationu w okresie postnatalnym. Zaburzenia funkcji kognitywnych w teście NOR u szczurów naśladują zaburzenia jakie obserwuje się u pacjentów schizofrenicznych (Danion i wsp., 1999; Doniger i wsp., 2001; Hecker i wsp., 2000).

W testach behawioralnych w grupie BSO + GBR 12909 zaobserwowano równocześnie z deficytami kognitywnymi zachowanie, które odwzorowywało pozytywne objawy schizofrenii. Syndromy te zauważalne były we wspomnianym wcześniej teście otwartego pola. Ponadto tylko w tej grupie (BSO + GBR 12909) podawana w dorosłości amfetamina powodowała nasilenie objawów pozytywnych poprzez wzmożoną aktywność lokomotoryczną, co cechuje pacjentów chorych na schizofrenię. Obserwowane w tym modelu (BSO + GBR 12909) zarówno objawy pozytywne jak i negatywne oraz istotnie zaburzone funkcje poznawcze dowodzą zasadności opracowania tego modelu neurorozwojowego jako zwierzęcego modelu schizofrenii.

BDNF pełni kluczową rolę w rozwoju układu nerwowego oraz synaptogenezie. Jest on także głównym modulatorem transmisji GABA-nergicznej i monoaminergicznej dlatego niezbędne było uwzględnienie go w badaniach biochemicznych. W korze czołowej obserwowano spadek zarówno na poziomie amplifikacji genu kodującego BDNF jak i ilości białka w grupie traktowanej BSO oddzielnie lub łącznie z GBR 12909. Natomiast w hipokampie obserwowano podobne zmiany tylko w grupie BSO. W przypadku łącznego podania BSO + GBR 12909 nie było istotnie statystycznych różnic na poziomie mRNA, jednakże spadek w poziomie białka był istotny w porównaniu do kontroli. Jak wspomniano wcześniej w grupie GBR 12909 nie obserwowano istotnych zaburzeń socjalnych ani kognitywnych, jednakże w korze czołowej obserwowano spadek poziomu BDNF zarówno na poziomie mRNA jak i białka. Co więcej, nie notowano takiego spadku w przypadku hipokampa, wręcz zauważalny był wzrost stężenia białka BDNF. Podniesiony poziom białka w hipokampie może mieć charakter kompensujący i może powodować normalizację funkcji poznawczych. Mechanizm polegający na efekcie kompensującym nie ma swojego zastosowania w grupach BSO i BSO + GBR 12909 w hipokampie. Sugeruje to, że zaburzona równowaga redoks, powodowana obniżeniem syntezy glutationu we wczesnym okresie postnatalnym może obniżać ekspresję genu dla BDNF, a także syntezę tego białka w dorosłości (Do i wsp., 2019; Perkins i wsp., 2020; Steullet i wsp., 2016; Lorenc-Koci, 2015). Podobnie w inny modelu schizofrenii, gdzie w okresie noworodkowym u szczurów wykonywane były lezje brzusznego hipokampa, a w dorosłości wykazywały obniżenie zarówno mRNA jak i białka BDNF w hipokampie i w korze przedczołowej (Lipska i Weinberg, 2000). Zatem różne neurorozwojowe modele schizofrenii pokazują, że nieprawidłowości w okresie postnatalnym rzutują na zaburzenia w ilości białka BDNF i redukcję ekspresji genu dla BDNF w dorosłości. Niedostateczna ilość tej neurotrofiny skutkuje zaburzeniami w dojrzewaniu neuronów i neuroplastyczności w dalszych etapach życia (Buckley i wsp., 2007; Roceri i wsp., 2004; Fumagalli i wsp., 2005).

Wyniki badań pokazują, że obniżenie poziomu glutationu wynikające z zastosowania inhibitora jego syntezy na wczesnym etapie życia postnatalnego indukuje zmiany w równowadze redoks prowadzące do zaburzenia zachowania społecznego oraz funkcji kognitywnych u szczurów w okresie dorosłości, a także zaburzenie w transmisji dopaminergicznej wywołanej inhibitorem wychwytu zwrotnego dopaminy mogą prowadzić do wystąpienia objawów pozytywnych charakterystycznych dla pacjentów schizofrenicznych. Ponadto zaburzona równowaga redoks powoduje obniżenie poziomu BDNF zarówno ekspresji

genu jak i produkcji białka w korze czołowej, czego nie zaobserwowano w hipokampie. Redukcja ilości kluczowej w rozwoju neurotrofiny może przyczyniać się do wystąpienia syndromów schizofrenii w badanym modelu neurorozwojowym.

Poszukując biochemicznych markerów zmian zapoczątkowanych chronicznym podaniem BSO i GBR 12909 w okresie poporodowym sprawdzono wpływ tych substancji na poziom glutationu, aminokwasów siarkowych (cysteina i metionina), a także na globalną metylację DNA w korze przedczołowej i hipokampie u dorosłych szczurów rasy Sprague-Dawley. Wyniki tych badań sugerują, że zmiany w poziomie glutationu i metioniny we wczesnym okresie życia postnatalnego prowadzą do zmian epigenetycznych w korze czołowej i hipokampie oraz do manifestacji deficytów społecznych i poznawczych u dorosłych szczurów (Górny i wsp., 2019). Ponadto zbadano u 16-dniowych szczurów aktywność enzymów antyoksydacyjnych (dysmutaza ponadtlenkowa, katalaza, peroksydaza glutationowa i reduktaza dwusiarczkowa glutationu) oraz poziom peroksydacji lipidów w korze czołowej i hipokampie. Wyniki analizowano w odniesieniu do stężenia glutationu i aminokwasów siarkowych. Analiza ta wykazała, że przewlekłe podawanie kombinacji BSO + GBR 12909 skutkowało znacznym obniżeniem poziomu peroksydacji lipidów w badanych strukturach mózgu, co wskazuje na osłabienie siły oksydacyjnej ich komórek i ostatecznie prowadzi do zmian w równowadze redoks, co skutkuje wystąpieniem deficytów społecznych i poznawczych w dorosłości (Górny i wsp., 2020).

W świetle powyższych danych w modelu neurorozwojowym zastosowano terapię lekiem przeciwpsychotycznym (ARI) wraz z lekiem należącym do selektywnych inhibitorów wychwytu zwrotnego serotoniny - ESC. Uzyskane wyniki zarówno w teście integracji socjalnej jak i teście NOR sugerują, że tylko w grupach traktowanych ARI w wysokiej dawce oraz ARI + ESC występowało odwrócenie deficytów socjalnych i kognitywnych spowodowanych obniżeniem poziomu glutationu. Rezultaty te wykazują zbieżność z wynikami uzyskanymi w krótkotrwałym modelu symptomatycznym. Badania *in vivo* potwierdzają, że chroniczne zastosowanie atypowego leku przeciwpsychotycznego zwiększa ekspresję mRNA dla BDNF oraz zwiększa stężenie tego białka, przeciwnie do zastosowania typowych leków przeciwpsychotycznych np. haloperidolu, której nie ma istotnego wpływu na poziom BDNF (Bai i wsp., 2003; Park i wsp., 2006; Park i wsp., 2009; Luoni i wsp., 2014).

Leki przeciwdepresyjne, do których zaliczamy selektywne inhibitory wychwytu zwrotnego serotoniny (SSRI) np. ESC wykazują pośrednie, agonistyczne działanie na receptory dla monoamin. SSRI szybko blokują transporter serotoninowy *in vitro*, natomiast w

zastosowaniu klinicznym efekty te są obserwowane dopiero po kilku tygodniach. To opóźnienie sugeruje, że molekularny mechanizm depresji, może być bardzo złożony. Odpowiedzialne za to opóźnienie mogą być wtórne zmiany w strukturze, funkcjonowaniu lub połączeniach komórek, które są niezbędne w procesie plastyczności neuronalnej (Diniz i wsp., 2021). Odkryto związek pomiędzy chronicznym stosowaniem leków przeciwdepresyjnych, a nasileniem proliferacji komórek oraz zwiększeniem stężenia BDNF u dorosłych szczurów. Badania te sugerują, że SSRI wykazują pozytywny wpływ na neurogenezę. Chroniczne zastosowanie tych leków zwiększa transmisję serotoninergiczną, a to z kolei nasila ekspresję genu dla BDNF głównie w warstwie komórek ziarnistych zakrętu zębatego hipokampa, efektu tego nie jest obserwowano podczas ostrego, jednokrotnego podawania leków (Malberg i wsp., 2000). Inne badania na gryzoniach wykazały, że do zaburzenia neurogenezy mogą przyczyniać się: wiek, stres i glikokortykosteroidy, a leki przeciwdepresyjne mogą znosić te efekty (Duman i wsp., 2001; Deltheil i wsp., 2008).

BDNF jako neurotrofina jest głównym czynnikiem biorącym udział w neuroplastyczności (Edelmann i wsp., 2014). Szereg badań wskazuję na to, że przyczynia się do neurogenezy poprzez wsparcie przeżywania komórek i tworzenia się nowych połączeń synaptycznych (Edelmann i wsp., 2014; Mattson i wsp., 2004; Park i wsp., 2012). W chorobach psychicznych takich jak depresja, w badaniach *post mortem* odkryto niski poziom BDNF w mózgu i w surowicy pacjentów. Poziom ten wracał do normy po zastosowaniu leków przeciwdepresyjnych. Potwierdzają to także badania na zwierzęcych modelach depresji (Adachi, 2014; Sen i wsp., 2008). Przypuszcza się zatem, że niski poziom BDNF stanowi podstawę mechanizmu odpowiedzialnego za pojawienie się stanów depresyjnych. Obniżony poziom BDNF powoduje słabszą i wolniejszą odpowiedź pacjentów na SSRI w kilku pierwszych tygodniach leczenia (Lee i wsp., 2020). Natomiast wraz z długością trwania leczenia, poprzez jego związek z układem serotoninergicznym, obserwuje się pozytywne efekty terapeutyczne, co może tłumaczyć dłuższą odpowiedź na SSRI w klinice, niż jest to obserwowane *in vitro* (Duclot i wsp., 2015; Duman i Monteggia, 2006; Monteggia i wsp., 2004).

Transporter wychwytu zwrotnego serotoniny (SERT), który jest blokowany przez leki z grupy SSRI, kodowany przez gen SLC6A4 prezentuje polimorfizm funkcjonalny w rejonie promotora - 5-HTTLPR. Generuje dwa warianty allelu dla SERT: krótki i długi (Diniz i wsp., 2021; Monteggia i wsp., 2004). Krótki allel powoduje obniżenie transkrypcji transportera w przeciwieństwie do długiego wariantu (Lesch i wsp., 1996) i jest to związane z większym

ryzykiem wystąpienia depresji również u pacjentów schizofrenicznych (Bleys i wsp., 2018; Caspi i wsp., 2003; Fanelli i Serretti, 2019; Homberg i wsp., 2014). Wariant krótki indukuje zmniejszoną odpowiedź na SSRI oraz może być odpowiedzialny za zmniejszony poziom BDNF w mózgu i we krwi nawet u zdrowych osób (Kroeze i wsp., 2012; Benedetti i wsp., 2017; Bhang i wsp., 2011; Molteni i wsp., 2010). Podobnie w badaniach na modelach zwierzęcych, gdzie u szczurów z całkowitym nokautem genu kodującego SERT ($SERT^{-/-}$) obserwowano znaczący spadek mRNA dla BDNF, a także spadki w poziomie białka, szczególnie w rejonach hipokampa i kory przedczołowej. Ponad to gryzonie te wykazywały zachowana lękowe i depresyjne w testach behawioralnych (Molteni i wsp., 2010; Calabrese i wsp., 2014; 201; Kalueff i wsp., 2010). Podobne zachowania jak i obniżenie poziomu mRNA dla BDNF obserwowano w modelu neurorozwojowym, stanowiącym część badań w rozprawie doktorskiej. Natomiast zniesienie tych deficytów w behawiorze oraz normalizacja poziomu ekspresji genu następowały po zastosowaniu terapii łącznych podań ARI + ESC. Sugeruje to, że stany depresyjne wywołane u szczurów zostały przez obniżenie poziomu glutationu we wczesnym okresie postnatalnym, a nie przez zmiany w allelach predysponujących do zachowań prodepresyjnych. W badaniach z 2013 na grupie pacjentów schizofrenicznych, przejawiających zachowania depresyjno-lękowe wprowadzono leczenie ESC. Obserwowano pozytywne efekty po kilku tygodniach stosowania, gdzie u pacjentów coraz rzadziej i mniejszym stopniu obserwowano objawy depresyjne, kompulsywne i lękowe. Zmniejszył się także odsetek pacjentów myślących o samobójstwie (Stryjer i wsp., 2013). Jednak zastosowanie samego ESC nie znosiło syndromów typowych dla schizofrenii. Dlatego lepszą formą terapeutyczną schizofrenii z epizodami depresji wydaje się być skojarzona terapia ARI + ESC, która nie tylko łagodzi objawy schizofreniczne, ale także podnosi poziom BDNF, kluczowego czynnika w chorobach psychicznych.

Warto nadmienić, że w trakcie publikacji jest artykuł nie będący podstawą niniejszej rozprawy doktorskiej zawierający kontynuację badań nad interakcją leków przeciwdepresyjnych wraz z ARI (Lech i wsp., 2021). Część wyników w owych badaniach zaprezentowano na 4th Central European Biomedical Congress. W modelu neurorozwojowym wykonano łączne podanie ARI z MIR, lekiem przeciwdepresyjnym mającym zupełnie różny mechanizm działania od ESC. MIR należy do grupy leków przeciwdepresyjnych NaSSA i blokuje presynaptyczne autoreceptory α_2 i heteroreceptory 5-HT_1 występujące na neuronach noradrenergicznych oraz autoreceptory 5-HT_1 i heteroreceptory α_2 obecne na neuronach serotoninowych. Tym samym nasila neurotransmisję serotoninergiczną i noradrenergiczną w

ośrodkowym układzie nerwowym (Gupta i wsp., 2016). Natomiast podobnie do zastosowania ESC, podanie niedziałającej dawki MIR łącznie z niedziałającą dawką ARI odwracało deficyty behawioralne spowodowane obniżeniem poziomu glutationu w okresie noworodkowym. Do oceny objawów schizofrenii użyto testu interakcji socjalnej oraz test NOR. W celu zbadania mechanizmu działania interakcji leków wykonano badania biochemiczne, określające poziom mRNA dla BDNF. Obniżenie poziomu BDNF było charakterystyczne dla grup potraktowanych BSO i BSO + GBR, natomiast w grupach ARI 1 mg/kg oraz ARI + MIR następowało podniesienie poziomu ekspresji genu, a szczególnie istotnie statystyczna zmiana zauważalna była w hipokampie w 92 dniu życia szczurów.

Badania kliniczne z 2011 opisane w artykule przeglądowym sugerują słuszność zastosowania MIR wraz z lekami przeciwpsychotycznymi (haloperidol, klozapina, rysperydon) w leczeniu pacjentów schizofrenicznych z przewagą objawów negatywnych. U większości pacjentów leczenie łączne MIR wraz z niską dawką leku antypsychotycznego powodowało zdecydowanie polepszenie i złagodzenie syndromów negatywnych w okresie kilku tygodni. Po 12 tygodniach leczenia również objawy pro-lękowe uległy minimalizacji. MIR wydaje się być dobrze tolerowana, działa szybko i jej pozytywne efekty były zauważalne w dowolnym czasie trwania terapii (Phan i Kreys, 2011).

Znany jest fakt, że BDNF pełni ważną rolę w patofizjologii depresji oraz ma znaczący wpływ na mechanizm leków przeciwdepresyjnych. Wyniki badań behawioralnych myszy pokazują, że lokalna iniekcja BDNF do śródmózgowia i hipokampa działa przeciwdepresyjnie w testach behawioralnych oceniających stany depresyjne (Monteggia i wsp., 2004). W 2005 roku opisano wpływ chronicznego podania MIR na poziom mRNA dla BDNF. MIR podawana samcom szczurów rasy Wistar w dawce 10 mg/kg podnosiła poziom mRNA o 26,5% w hipokampie, a w korze przedczołowej o 41,5%. Niższa dawka 5 mg/kg również dawała istotne efekty w badanych strukturach mózgu. Wyniki te sugerują, że wzmocnienie ekspresji genu dla BDNF po chronicznym (14 dni) podawaniu MIR może stanowić mechanizm działania tego leku i mieć istotne znaczenie w leczeniu klinicznym pacjentów (Rogóż i wsp., 2005). Efekty MIR są podobne do efektów jakie powoduje przyjmowanie klasycznych leków przeciwdepresyjnych np. imipraminy. Leki te wzmacniają noradrenergiczną i serotoninergiczną transmisję. Badania te dostarczają mocne dowody na to, że zwiększenie ekspresji genu dla BDNF w hipokampie i korze mózgowej jest dalszym efektem nasilonej neurotransmisji serotonicznergicznej lub noradrenergicznej i właśnie to może być odpowiedzialne za mechanizm działania MIR w efektywnej terapii depresji (McGrath i wsp., 1998; Rogoz i wsp., 2005).

7. Podsumowanie i wnioski

1. W modelu symptomatycznym schizofrenii, polegającym na podaniu pojedynczej dawki MK-801, zastosowane leki przeciwdepresyjne (ESC lub MIR) łącznie z lekiem przeciwpsychotycznym (ARI), spowodowały potęgujące działanie przeciwpsychotyczne ARI w odwracaniu deficytów socjalnych i poznawczych oraz, ze ta pozytywna interakcja angażuje receptory serotoninowe typu 5-HT_{1A} i dopaminowe D_1.
2. W modelu mikrodializy wykazano, że łączne, jednorazowe podania ESC z ARI podnosi zewnątrzkomórkowy poziom serotoniny i noradrenaliny, natomiast podanie MIR z ARI podnosi zewnątrzkomórkowy poziom noradrenaliny. Wyniki te sugerują, że otrzymane biochemiczne zmiany mogą mieć istotne znaczenie w terapii pozytywnych i kognitywnych symptomów schizofrenii.
3. W neurorozwojowym modelu schizofrenii, polegającym na deficycie glutationu indukowanym chronicznym podaniem inhibitora syntezy glutationu (BSO) oddzielnie i łącznie z inhibitorem wychwytu dopaminy (GBR 12909) we wczesnym okresie noworodkowym, wykazano ujawnienie się objawów przypominających schizofrenie już od 42 dnia życia, które utrzymywały się do końca eksperymentu tj. do 91 dnia oraz, że te deficyty były odwracane u szczurów dorosłych przez wysoką dawkę ARI, a także przez łączne podanie niedziałających dawek ARI z ESC.
4. W opisanym wyżej neurorozwojowym modelu schizofrenii, u szczurów dorosłych wykazano obniżenie ekspresji mRNA dla BDNF oraz, że ARI w wyższej dawce odwracał te zmiany, a także łączne zastosowanie niższej dawki ARI z ESC powodowało podwyższenie ekspresji mRNA dla BDNF, tylko w korze czołowej, ale nie w hipokampie.

Badania zawarte w artykułach naukowych stanowiących rozprawę doktorską potwierdziły słuszność postawionej hipotezy naukowej. Wykazano , że leki przeciwdepresyjne, o różnym profilu farmakologicznym, podawane z ARI tj. lekiem przeciwpsychotycznym w najniższej dawce mogą odwracać deficyty zachowania charakterystyczne dla schizofrenii w obu modelach badawczych. Dodatkowo interakcja ESC + ARI powodowała podniesienie ekspresji mRNA dla BDNF, kluczowej dla rozwoju mózgu neurotrofiny. Badania te sugerują, że interakcja leków przeciwdepresyjnych z ARI może stanowić skuteczną i bezpieczną terapię, a tym samym prowadzić do poprawy jakości życia pacjentów schizofrenicznych.

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9. Oświadczenia

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polegał na wykonaniu i analizie testów behawioralnych, zebraniu materiału tkankowego od zwierząt, wykonaniu analiz biochemicznych (oznaczenie białka metodą ELISA i ekspresji genów metodą qRT-PCR), a także merytorycznej analizie wyników badań w oparcie o literaturę i przygotowaniu manuskryptów

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polegał na opracowaniu koncepcji badań, pozyskaniu finansowania oraz koordynacji wykonywanych doświadczeń, a także na nadzorowaniu przygotowania manuskryptów i ich korekcie

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polegał na pomocy w statystycznym opracowaniu uzyskanych wyników doświadczalnych oraz w przygotowaniu manuskryptów do druku

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polegał na pomocy w doświadczeniach behawioralnych oraz w graficznym opracowaniu uzyskanych wyników

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polegał na wykonaniu mikrodializy oraz opracowaniu wyników z tej części badań.

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polegał na graficznym opracowaniu wyników z tej części badań.

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10. Artykuły naukowe, stanowiące podstawę rozprawy doktorskiej, w wersji oryginalnej

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Short communication

Co-treatment with antidepressants and aripiprazole reversed the MK-801-induced some negative symptoms of schizophrenia in rats

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ABSTRACT

Background: Schizophrenia is a chronic, most devastating psychiatric illness that impairs mental and social functioning. A few clinical reports have suggested that antidepressant drugs are able to augment the activity of atypical antipsychotic drugs, thus effectively improving treatment of some negative symptoms of schizophrenia.
Methods: The aim of the present study was to investigate the effect of the antidepressant escitalopram or mirtazapine and aripiprazole (an atypical antipsychotic), given separately or jointly, on the deficits induced by MK-801(a noncompetitive *N*-methyl-D-aspartate receptor antagonist) in the social interaction test in male Sprague-Dawley rats. The social interaction was measured for 10 min, starting 4 h after MK-801 (0.1 mg/kg) administration. Antidepressants and aripiprazole were given 60 and 30 min before the test, respectively. WAY 100635 (a 5-HT_{1A} antagonist) and SCH 23390 (a dopamine D_1 antagonist) were give 20 min before the tests.
Results: The present results showed that MK-801 (0.1 mg/kg)-induced deficits in the social interaction test. Aripiprazole (0.1 and 0.3 mg/kg) reversed those effects. Co-treatment with an ineffective dose of aripiprazole (0.03 mg/kg) and escitalopram (5 and 10 mg/kg) or mirtazapine (5 mg/kg) abolished the deficits evoked by MK-801, and those effects were especially blocked by a 5-HT_{1A} receptor antagonist (WAY 100635) or partly by dopamine D_1 receptor antagonist (SCH 23390).
Conclusions: The obtained results suggest that amelioration of the antipsychotic-like effect of aripiprazole by antidepressants in the MK-801-induced some negative symptoms of schizophrenia in rats may be associated with serotonin 5-HT_{1A} and to a lesser degree with dopamine D_1 receptors.

Introduction

Schizophrenia is a severe mental disorder which affecting about 1% of the world population. This disease is characterized by the impaired perception of oneself and the surrounding world as well as by distorted thinking and emotions. The complicated clinical picture of schizophrenia suggests that an extremely complex and ambiguous mechanism may underlay this disease. Furthermore, despite intensive research the search for the basis of schizophrenia has not been successful, so far, what makes it extremely difficult to develop an efficient therapy. Lack of the proper therapy imposes a serious social and economic burden [1]. Clinically, the symptoms of the disorder can be divided into three main categories: positive symptoms (delusions, hallucinations, thought disorder and incoherence), negative symptoms (lack of motivation and deficits in social function, flat affect) and cognitive deficits (impairment of attention, memory and executive functions) [2]. Furthermore, approximately 50% of schizophrenic patients, besides the typical symptoms, suffer from comorbid depression, the presence of which not only worsens rehabilitation of patients but also increases the recurrence rate of symptoms [3].

In the therapy of schizophrenia, classical antipsychotic drugs (*i.e.* antagonist of dopamine D_2 receptors) inhibit mainly the positive symptoms but do not affect the negative symptoms or the impaired cognitive processes [1–4]. In contrast to conventional antipsychotics, atypical antipsychotic drugs, partly alleviate the negative symptoms and slightly improve the impaired cognitive functions [5]. Recent studies suggest that in the treatment of schizophrenia an improvement of patients' mood should also be taken into account, and therefore the administration of antidepressant drugs (ADs) is strongly recommended [6,7]. Furthermore, a few clinical and preclinical studies demonstrated that the addition of ADs with different pharmacological profiles escitalopram (ESC) and/or mirtazapine (MIR) to the treatment with the atypical antipsychotic, such as risperidone, enhanced the efficacy

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1734-1140/

of the latter drug in alleviating negative symptoms and in the improvement of the cognitive performance, to a much greater extent than when risperidone was given alone [6–8]. The above data clearly suggest that the administration of some ADs in combination with atypical antipsychotic drugs may be of great importance for clinical practice.

Recently, aripiprazole, a novel atypical antipsychotic drug with a unique pharmacology, has become the focus of researchers' interest. This drug improves both positive and negative symptoms of psychosis without producing extrapyramidal side effects or increasing in serum prolactin [9]. Aripiprazole has high affinity for a large number of monoaminergic receptors, and acts as an antagonist of both 5-HT_{2A} and postsynaptic dopamine D_2 receptors as well as a partial agonist of 5-HT_{1A} and presynaptic dopamine D_2 receptors [10,11]. These pharmacological properties may play an important role in the therapeutic effects of this drug. Although aripiprazole has been reported to enhance cognitive function in schizophrenic patients [9,11], the mechanism of its action is unclear.

The early behavioral studies indicated that the NMDA receptor antagonists (*e.g.*, phencyclidine, MK-801 or ketamine) induced not only positive but also negative, and cognitive abnormalities in animals, which were similar to those observed in patients with psychosis [2,12].

It has been shown that sub-chronic phencyclidine treatment induced deficits in social interactions in female rats, and those effects were reversed by single dose of aripiprazole and fluoxetine (a selective inhibitor of serotonin reuptake) but not by chlordiazepoxide (an anxiolytic agent) or WAY 100635 (a 5-HT_{1A} receptor antagonist). Moreover, WAY 100635 given before aripiprazole reversed the deficits in social interactions behavior in those animals. The above results suggest that the antipsychotic-like effect of aripiprazole in phencyclidine-induced some negative symptoms of schizophrenia in rats may be associated with serotonin 5-HT_{1A} receptors [13].

In the present study we evaluated the effect of the ADs ESC (selective inhibitor of serotonin reuptake) [14] or MIR (which enhances noradrenergic and serotonergic neurotransmission by blocking of α_2-adrenoreceptors) [15] and an atypical antipsychotic aripiprazole (given separately or jointly) in the animal test, which is used for evaluation of some negative symptom of schizophrenia, namely; the MK-801-induced deficit in social interaction test in rats. We also studied the effects of WAY 100635 (a 5-HT_{1A} antagonist) and SCH 23390 (a dopamine D_1 antagonist) on the amelioration of antipsychotic-like effect of aripiprazole by ADs on the MK-801-induced deficits in the social interaction test. The effect of co-treatment with ESC or MIR and aripiprazole on the MK-801-induced changes in the social interaction test in rats has not been studied before.

Materials and methods

Animals

The experiments were carried out on male Sprague-Dawley rats (220–250 g) derived from Charles River Laboratories, Sulzfeld (Germany). The animals were housed 4 per cage (57 cm × 35 cm × 20 cm) in a colony room kept at 21 ± 1 °C with a 40–50% humidity, on a 12-h light-dark cycle (the light on at 7 a.m.). The rats had free access to food and water before the experiments. All the experiments were conduced during the light phase, and were carried out according to the procedures approved by the Animal Care and Use Committee at the Maj Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Drugs administration

Escitalopram oxalate (ESC, Sigma-Aldrich, Saint Louis, USA) and (+)-MK-801 maleate (MK-801, Tocris Bioscience, Bristol, UK) were dissolved in a 0.9% NaCl while aripiprazole (Abcam Biochemicals, Cambridge, UK) and mirtazapine (MIR, Tocris Bioscience, Bristol, UK) were dissolved in 0.1 M tartaric acid and the solution was adjusted to pH 6–7 with 0.1 N NaOH. Antidepressants, aripiprazole, or SCH 23390 (Sigma-Aldrich, Saint Louis, USA) were given intraperitoneally (*ip*) while WAY 100635 (Tocris Bioscience, Bristol, UK) or MK-801 was injected subcutaneously (*sc*) in a volume of 2 ml/kg.

MK-801-induced deficits in social interaction in rats

The procedure for the measurement of the social interaction has been described previously by Kamińska and Rogóż [8]. The social interaction test was performed using a black PCV box (67 cm × 57 cm × 30 cm, length × width × height) divided into six symmetrical sectors. The arena was dimly illuminated with an indirect light of 18 lx. Each social interaction experiment involving two rats was carried out during the light phase of the light/dark cycle. The animals were selected from separate housing cages to make a pair for the study. The paired rats were matched for body weight within 15 g. The social interaction was measured 4 h after administration of MK-801 (0.1 mg/kg, *sc*), and 60 or 30 min after administration of MIR (2.5 and 5 mg/kg, *ip*) or ESC (2.5, 5 and 10 mg/kg, *ip*) and aripiprazole (0.03 mg/kg, *ip*), respectively. SCH 23390 (0.25 mg/kg, *ip*) and WAY 100635 (0.1 mg/kg, *sc*) were given 20 min before the test. Each pair of rats was diagonally placed in opposite corners of the box facing away from each other. The social interaction of the animals was measured over a 10-min period. The test box was wiped clean with 10% ethanol between each trial. Social interaction between two rats was expressed as the total time spent in social behavior, such as sniffing, genital investigation, chasing and fighting with each other. The number of episodes was also counted. In addition, the number of sector line crossings (ambulation) was determined as a measure of locomotor activity of those rats. Each group consisted of twelve animals (six pairs).

Statistical analysis

The data were evaluated by a one-way analysis of variance (ANOVA) followed by individual comparisons using Dunnett's test. The statistically significant differences between the studied groups were also calculated using a two-way ANOVA followed by the Tukey test (Figs. 2–5); $p < 0.05$ was considered statistically significant.

Results

A one-way analysis of variance (ANOVA) showed that in the social interaction test in rats, MK-801 given at dose of 0.1 mg/kg, *sc,* 4 h before the test evoked deficits in the parameters studied (decreased the time of interactions and the number of episodes by c.a. 62 and 49%, respectively). Moreover, aripiprazole at higher doses (0.1 and 0.3 mg/kg) reversed those effects of MK-801, although, at lower doses (0.01 and 0.03 mg/kg) it did not change those deficits [$F(5,30) = 46.3$; $p < 0.001$ and $F(5,30) = 59.35$; $p < 0.001$, respectively] (Fig. 1A, B). The locomotor activity of those rats was not altered in any of the treatment groups with MK-801 or MK-801 with aripiprazole [$F(5,38) = 1.68$; *ns*] (Fig. 1C).

In addition, our data showed that combined treatment with an ineffective dose of aripiprazole (0.03 mg/kg) and ESC (2.5, 5 and 10 mg/kg) dose-dependently abolished deficits induced by MK-801 (0.1 mg/kg), increased the time of interactions and the number of

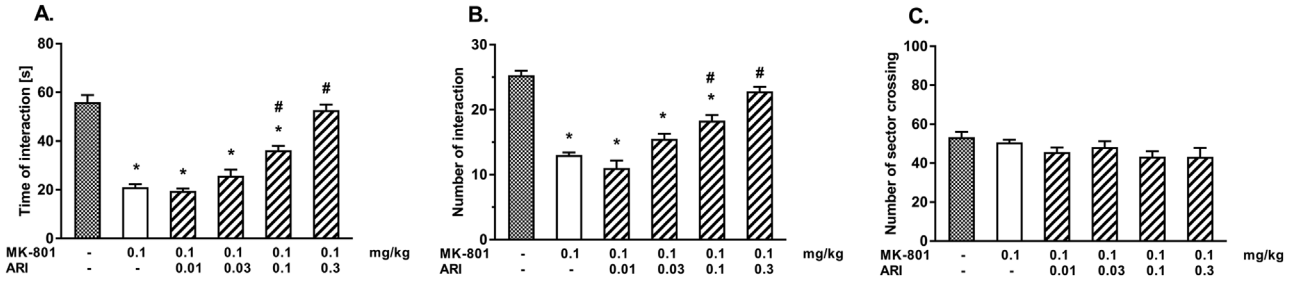

Fig. 1. The effect of aripiprazole (ARI, 0.01, 0.03, 0.1 and 0.3 mg/kg, *ip*) on the MK-801-induced deficits in the social interaction test: (**A**) the time of interaction (s), (**B**) the number of social interaction, (**C**) the locomotor activity. The social interaction was measured 4 h after the MK-801 (0.1 mg/kg, *sc*) administration. ARI was given 30 min before the test. Data are presented as the mean ± SEM of 6 pairs/group. The data were statistically evaluated by ANOVA, followed by individual comparisons using Dunnett's test. $^{*}p < 0.001$ *vs.* control group: $^{\#}p < 0.001$ *vs.* MK-801-treatment group.

episodes [$F(5,30) = 8.59$; $p < 0.001$ and [$F(5,30) = 16.26$; $p < 0.001$], respectively (Fig. 2 A, B). The locomotor activity of those rats was not altered in any of the treatment groups [$F(3,20) = 0.33$; *ns*] Fig. 2C. Two-way ANOVA demonstrated the following effects of aripiprazole 0.03 or/and ESC 2.5, 5, 10 mg/kg on the deficit induced by MK-801 (0.1 mg/kg) in the time of interactions: an effect of aripiprazole [$F(1,20) = 7.66$; $p < 0.01$], an effect of ESC 2.5 mg/kg [$F(1,20) = 8.534$; $p < 0.01$] and an interaction between ESC 2.5 mg/kg and aripiprazole [$F(1,20) = 4.634$; $p < 0.05$], or an interaction between ESC 5 mg/kg and aripiprazole $F(1,20) = 16.936$; $p < 0.001$], and an interaction between ESC 10 mg/kg and aripiprazole [$F(1,20 = 5.318$; $p < 0.05$]. Moreover, regarding the effects of aripiprazole 0.03 or/and ESC 2.5, 5, 10 mg/kg on the number of interactions of those rats, statistical analysis showed: no effect of aripiprazole [$F(1,20) = 0.068$; *ns*], no effect of ESC 2.5 mg/kg [$F(1,20) = 1.088$; *ns*] and an interaction between ESC 2.5 mg/kg and aripiprazole [$F(1,20) = 4.345$; $p < 0.05$] or an interaction between ESC 5 mg/kg and aripiprazole [$F(1,20) = 15.703$; $p < 0.001$], and an interaction between ESC 10 mg/kg and aripiprazole [$F(1,20) = 14.391$; $p < 0.05$].

The following experiment showed that combined treatment with an ineffective dose of aripiprazole (0.03 mg/kg) and MIR (5 mg/kg but not **a** lower dose of 2.5 mg/kg) also abolished deficits induced by MK-801 (0.1 mg/kg), namely, it increased in the time of interactions and the number of episodes [$F(4.25) = 17.60$; $p < 0.001$ and [$F(4.25) = 14.87$; $p < 0.001$, respectively] (Fig. 3A, B). The locomotor activity of those rats was decreased only in the group treated with MK-801 together with MIR (2.5 mg/kg) and aripiprazole [$F(4,25) = 12.99$; $p < 0.001$ (Fig. 3C). Two-way ANOVA demonstrated the following effects of aripiprazole or/and MIR 2.5 mg/kg on the deficit induced by MK-801 in the time of interactions: an effect of aripiprazole [$F(1,20) = 4.094$; $p = 0.06$], no effect of MIR 2.5 mg/kg [$F(1,20) = 1.357$; *ns*], and no interaction between MIR 2.5 mg/kg and aripiprazole [$F(1,20) = 1.357$; *ns*]. Moreover, regarding the effects of aripiprazole or/and MIR 2.5 mg/kg on the deficit induced by MK-801 in the number of interactions of those groups, the analysis revealed: no effect of aripiprazole [$F(1,20) = 0.252$; *ns*], an effect of MIR [$F(1,20) = 5.102$; $p < 0.05$], and no interaction between MIR and aripiprazole [$F(1,20) = 0.772$; *ns*]. In addition, two-way ANOVA demonstrated also the following effects of aripiprazole of/and MIR 5 mg/kg on the deficit induced by MK-801 in the time of interactions: an effect of aripiprazole [$F(1,20) = 14.824$; $p < 0.001$], an effect of MIR [$F(1,20) = 12.316$; $p < 0.002$], and an interaction between MIR and aripiprazole [$F(1,20) = 8.388$; $p < 0.01$]. Moreover, regarding the effects of aripiprazole or/and MIR on the number of interactions of those groups, the analysis indicated: an effect of aripiprazole [$F(1,20) = 4.085$; $p = 0.056$], an effect of MIR [$F(1,20) = 11.347$; $p < 0.01$], and an interaction between MIR and aripiprazole [$F(1,20) = 5.702$; $p < 0.05$].

On the other hand, we also showed that WAY 100635 (a 5-HT_{1A} antagonist) at a dose of 0.1 mg/kg did not change the deficits evoked by MK-801 (0.1 mg/kg) but it reversed the positive effect of interaction of combined administration of aripiprazole (0.03 mg/kg) and ESC (5 mg/kg) or MIR (5 mg/kg) on the deficits evoked by MK-801 in the social interaction test. For groups treated with MK-801and aripiprazole plus ESC and WAY 100635, the time of interaction and number of episodes, statistical analysis showed: [$F(4,25) = 70.78$; $p < 0.001$ and [$F(4.25) = 36.26$; $p < 0.001$], respectively (Fig. 4A, B). In the case of groups treatment with MK-801and MIR plus aripiprazole and WAY 100635, the time of interaction and number of episodes, statistical analysis showed [$F(4,25) = 64.13$; $p < 0.001$ and [$F(4.25) = 53.39$; $p < 0.001$], respectively (Fig. 4A, B). The locomotor activity of the above rats was not altered in any of the group treatment, in particular, for groups treated with MK-801 and ESC or MIR plus aripiprazole and WAY 100655 statistical analysis showed $F(4,25) = 0.46$; *ns* and $F(4,25) = 0.38$; *ns*, respectively (Fig. 4C).

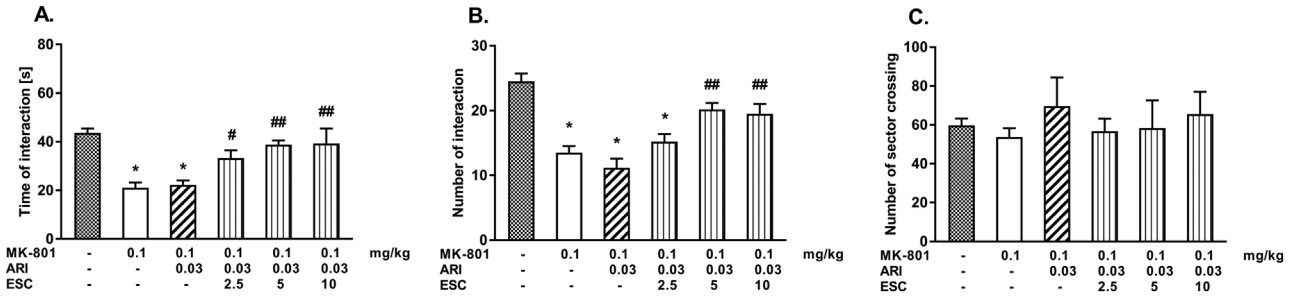

Fig. 2. The effect of escitalopram (ESC, 2.5, 5 and 10 mg/kg, *ip*) given alone or in combination with aripiprazole (ARI, 0.03 mg/kg, *ip*) on the MK-801-induced deficits in the social interaction test: (**A**) the time of interaction (s), (**B**) the number of social interaction, (**C**) the locomotor activity.The social interaction was measured 4 h after the MK-801 (0.1 mg/kg, *sc*) administration. ESC was given 60 and ARI 30 min before the test The results are shown as the mean ± SEM. Each group consisted of 6 pairs/group. The data were statistically evaluated using a two-way ANOVA following by the Tukey test. $^{*}p < 0.001$ *vs.* vehicle-treatment group; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ *vs.* MK-801-treatement group.

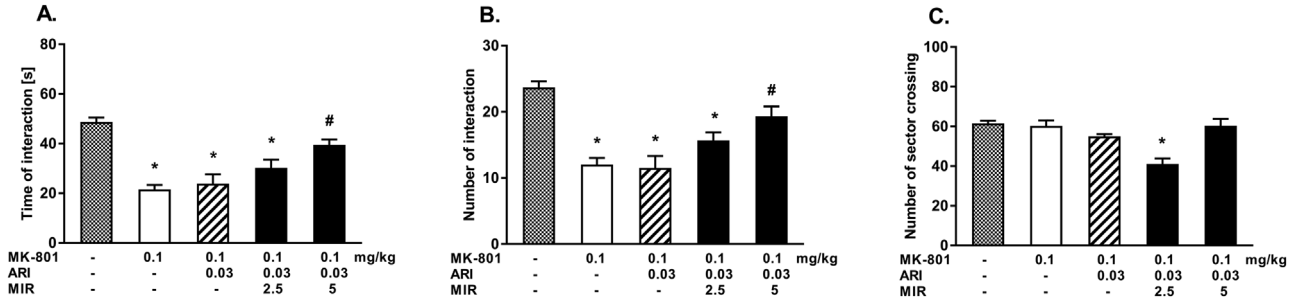

Fig. 3. The effect of mirtazapine (MIR, 2.5 and 5 mg/kg, *ip*) given alone or in combination with aripiprazole (ARI, 0.03 mg/kg, *ip*) on the MK-801-induced deficits in the social interaction test: (**A**) the time of interaction (s), (**B**) the number of social interaction, (**C**) the locomotor activity. The social interaction was measured 4 h after the MK-801 (0.1 mg/kg, *sc*) administration. MIR was given 60 and ARI 30 min before the test. The results are shown as the mean ± SEM. Each group consisted of 6 pairs/group. The data were statistically evaluated using a two-way ANOVA following by the Tukey test. $^{*}p < 0.001$ *vs.* vehicle-treatment group: $^{\#}p < 0.001$ *vs.* MK-801-treatment group.

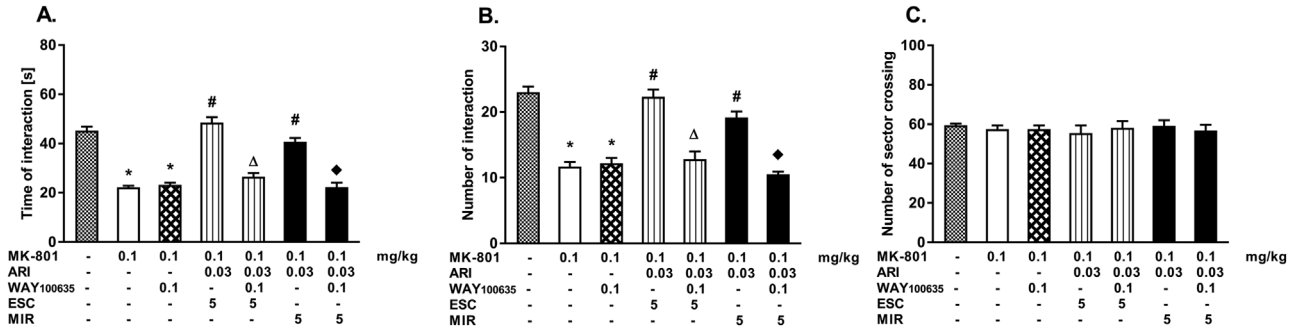

Fig. 4. The effect of WAY 100635 (0.1 mg/kg, *sc*) on the ameliorate antipsychotic-like effect of aripiprazole (ARI, 0.03 mg/kg, *ip*) by escitalopram (ESC, 5 mg/kg, *ip*) or mirtazapine (MIR, 5 mg/kg, *ip*) in the MK-801-induced deficits in the social interaction test: (**A**) the time of interaction (s), (**B**) the number of social interaction, (**C**) the locomotor activity. The social interaction was measured 4 h after the MK-801 (0.1 mg/kg, *sc*) administration. ESC or MIR were given 60, ARI 30 min and WAY 100635 20 min before the test. The results are shown as the mean ± SEM. Each group consisted of 6 pairs/group. The data were statistically evaluated using a two-way ANOVA following by the Tukey test. $^{*}p < 0.001$ *vs.* vehicle-treatment group; $^{\#}p < 0.001$ *vs.* MK-801 group; $\Delta p < 0.001$ *vs.* MK-801 + ESC + ARI and $^{\blacklozenge}$ $p < 0.001$ *vs.* MK-801 + MIR + ARI group.

Two-way ANOVA demonstrated the following effects of aripiprazole 0.03, ESC 5 or/and WAY 100635 (0.1 mg/kg) on the deficit induced by MK-801 (0.1 mg/kg) in the time of interactions: an effect of WAY 100635 [F(1.20) = 51.147; $p < 0.0001$], an effect of aripiprazole plus ESC [$F(1,20) = 102.075$; $p < 0.00001$], and an interaction between WAY 100635 and aripiprazole plus ESC [$F(1.20) = 61.353$; $p < 0.0001$]. Moreover, regarding the effects of aripiprazole, ESC or/and WAY 100635 on the number of interactions in those groups, the analysis revealed: an effect of WAY 100635 [$F(1,20) = 21.254$; $p < 0.0001$], an effect of WAY 100635 and aripiprazole, plus ESC [$F(1,20) = 33.703$; $p < 0.00001$], and an interaction between WAY 100635 and aripiprazole plus ESC F(1.20) = 26.239; $p < 0.00001$].

Two-way ANOVA demonstrated the following effects in groups treated with MIR 5 mg/kg and aripiprazole 0.03 mg/kg and WAY 100635 (0.1 mg/kg) on the deficit induced by MK-801 (0.1 mg/kg) in the time of interactions: an effect of WAY 100635 [$F(1,20) = 51.147$; $p < 0.0001$], an effect of aripiprazole plus MIR [$F(1,20) = 102.075$; $p < 0.00001$], and an interaction between WAY 100635 and aripiprazole plus MIR $F(1.20) = 61.353$; $p < 0.0001$]. Moreover, regarding the effects of MIR and aripiprazole and WAY 100635 on the number of interactions in those groups, the analysis revealed: an effect of WAY 100635 [$F(1,20) = 21.254$; $p < 0.0001$], an effect of WAY 100635 and aripiprazole plus MIR [$F(1,20) = 33.703$; $p < 0.00001$], and an interaction between WAY 100635 and aripiprazole plus MIR $F(1.20) = 26.239$; $p < 0.00001$].

In the next experiment we showed that SCH 23390 (a dopamine D_1 antagonist) at a dose of 0.25 mg/kg abolished the antipsychotic-like effect of combined treatment with MK-801 (0.1 mg/kg) plus aripiprazole (0.03 mg/kg) and ESC (5 mg/kg) or MIR (5 mg/kg) on the deficits induced by MK-801in the social interaction test. In the case of groups treated together with MK-801 and aripiprazole plus ESC and SCH 23390; the time of interactions and number of episodes, the statistical analysis indicated [$F(4,25) = 101.54$; $p < 0.001$ and $F(4.25) = 40.84$; $p < 0.001$, respectively] (Fig. 5A, B). For groups treated with MK-801 and MIR plus aripiprazole and SCH 23390, the time of interactions and number of episodes, the analysis revealed [$F(4,25) = 96.29$; $p < 0.001$ and [$F(4.25) = 60.18$; $p < 0.001$, respectively] (Fig. 5A, B). The locomotor activity of the above rats was decreased in the group treated with MK-801plus SCH 23390 by ca. 21% or in the group treated with MK-801 and ESC (5 mg/kg) or MIR (5 mg/kg) plus SCH 23390 by ca. 24 and 34% [$F(4,25) = 10.83$; $p < 0.001$ and $F(4,25) = 35.38$; $p < 0.001$], respectively (Fig. 5C). SCH 23390 used at a lower dose (0.1 mg/kg) did not change the antipsychotic-like effect of combined treatment with MK-801 (0.1 mg/kg) plus aripiprazole (0.03 mg/kg) and ESC or MIR (5 mg/kg) on the deficits induced by MK-801in this test (data no showed).

Two-way ANOVA demonstrated the following effects of aripiprazole 0.03, ESC 5 or/and SCH 23390 (0.25 mg/kg) on the deficit induced by MK-801 (0.1 mg/kg) in the time of interactions: an effect of SCH 23390 [$F(1.20) = 90.318$; $p < 0.0000$], an effect of aripiprazole plus ESC [$F(1,20) = 112.069$; $p < 0.0000$], and an interaction between SCH 23390 and aripiprazole plus ESC [$F(1.20) = 45.384$; $p < 0.0000$]. Moreover, with respect to the effect of aripiprazole, ESC or/and SCH 23390 on the number of interactions in those group, the analysis showed: an effect of SCH 23390 [$F(1,20) = 37.809$; $p < 0.0000$], an effect of aripiprazole plus ESC [$F(1,20) = 51.883$; $p < 0.0000$], and an interaction between SCH 23390 and aripiprazole plus ESC $F(1.20) = 22.500$; $p < 0.0001$].

Two-way ANOVA demonstrated the following effects in groups treated with MIR and aripiprazole plus SCH 23390: on the deficit

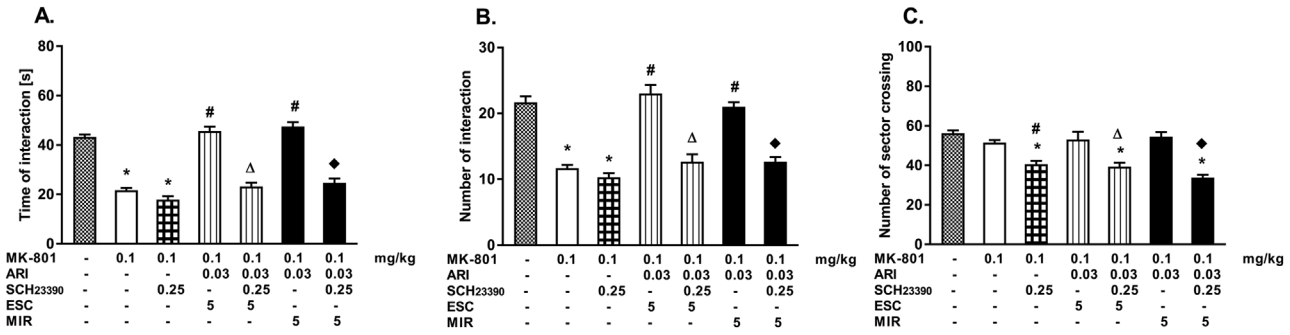

Fig. 5. The effect of SCH 23390 (0.25 mg/kg, *ip*) on the ameliorate antipsychotic-like effect of aripiprazole (ARI, 0.03 mg/kg, *ip*) by escitalopram (ESC, 5 mg/kg, *ip*) or mirtazapine (MIR, 5 mg/kg, *ip*) in the MK-801-induced deficits in the social interaction test: (**A**) the time of interaction (s), (**B**) the number of social interaction, (**C**) the locomotor activity. The social interaction was measured 4 h after the MK-801 (0.1 mg/kg, *sc*) administration. ESC or MIR were given 60, ARI 30 min and SCH 23390 20 min before the test. The results are shown as the mean ± SEM. Each group consisted of 6 pairs/group. The data were statistically evaluated using a two-way ANOVA following by the Tukey test. $^{*}p < 0.001$ *vs.* vehicle-treatment group; $^{\#}p < 0.001$ *vs.* MK-801 group; $\Delta p < 0.001$ *vs.* MK-801 + ESC + ARI and $^{\blacklozenge}$ $p < 0.001$ *vs.* MK-801 + MIR + ARI group.

induced by MK-801 on the time of interactions: an effect of SCH 23390 [$F(1,20) = 82.368$; $p < 0.0000$], an effect of aripiprazole plus MIR [$F(1,20) = 123.604$; $p < 0.0000$], and an interaction between SCH 23390 and aripiprazole plus MIR $F(1.20) = 41.815$; $p < 0.000$]. Moreover, the effects of the above drugs combinations on the number of interactions were as follows: an effect of SCH 23390 [$F(1,20) = 56.067$; $p < 0.00000$], an effect of MIR and aripiprazole [$F(1,20) = 81.667$; $p < 0.00001$], and an interaction between SCH 23390 and aripiprazole plus MIR [$F(1.20) = 29.400$; $p < 0.0001$].

Discussion

In the present study, we demonstrated that MK-801-induced deficits in the social interaction test, and aripiprazole at higher doses reversed those effects. Moreover, co-treatment with an ineffective dose of aripiprazole and ESC or MIR abolished the deficits evoked by MK-801, and those effects were especially blocked by a 5-HT_{1A} receptor antagonist (WAY 100635) or partly by dopamine D_1 receptor antagonist (SCH 23390).

Our earlier study also indicated that co-treatment with an ineffective dose of another antipsychotic drug, risperidone (0.1 mg/kg) and ESC (5 mg/kg) or MIR (2.5 and 5 mg/kg) abolished the deficits induce by MK-801 (0.1 mg/kg) in the social interaction test in male Wistar rats [8]. In addition, other authors also showed that ESC enhanced the antipsychotic-like activity of a low dose of risperidone, in the conditional avoidance response test in rats, this test is used to evaluate some negative symptoms of schizophrenia in animals [16]. Moreover, the positive interactions between risperidone or aripiprazole and ADs were demonstrated in behavioral tests in mice which evaluated the positive and some cognitive symptoms of schizophrenia [17,18]. The above data suggest that ADs may enhance the antipsychotic-like effect of risperidone or aripiprazole in the animal tests used for evaluation of some positive, negative and cognitive symptoms of schizophrenia.

In addition, some behavioral studies indicated that aripiprazole reversed the phencyclidine-induced deficits in animal tests. For example, the cognitive deficits in a novel object recognition memory test evoked by phencyclidine in mice were blocked by aripiprazole and this effect was reversed by serotonin 5-HT_{1A} receptor antagonist (WAY 100635) or dopamine D_1 antagonist (SCH 23390) [19]. Moreover, phencyclidine-induced deficits in the novel object recognition test in male Sprague-Dawley rats were also reversed by aripiprazole and compound SSR181505 which showed dual 5-HT_{1A}/dopamine D_2 activation. The positive interactions of the two above drugs (aripiprazole and compound SSR181505) were blocked by WAY 100635. These findings suggested that the balance between activity at 5-HT_{1A} and dopamine D_2 receptors might be important in the action of antipsychotic drugs on some negative symptoms of schizophrenia [20].

The above data are in line with the previous study which showed that 5-HT_{1A} receptors stimulation in the prefrontal cortex evoked dopamine D_1 receptor activation by the mesocortical dopaminergic pathway, which was involved in the phencyclidine-induced cognitive impairment and this action was blocked by aripiprazole [19,21–23]. In addition, aripiprazole, a 5-HT_{2A} receptor antagonist increased dopamine release in the prefrontal cortex, and this effect was blocked by the 5-HT_{1A} receptor antagonist WAY 100635 [24].

Our present results suggest that amelioration of the antipsychotic-like effect of aripiprazole by ADs (ESC or MIR) on the MK-801-induced negative symptoms of schizophrenia in rats may be especially associated with serotonin 5-HT_{1A}, since WAY 100635 reversed the positive interaction of combined administration of aripiprazole and ADs in this test. Moreover, locomotor activity in those rats was not changed in any tested groups. On the other hand, SCH 23390 also blocked that amelioration of the antipsychotic-like effect of aripiprazole by ADs on the MK-801-induced negative symptoms of schizophrenia in rats. However, SCH 23390 alone and in combination of treatment groups reduced the locomotor activity in the studied rats, thus, the effect associated with dopamine D_1 receptors in this test may be less specific.

In summary, the present results suggest that amelioration of the antipsychotic-like effect of aripiprazole by ADs (ESC or MIR) on the MK-801-induced some negative symptoms of schizophrenia in rats may be associated with serotonin 5-HT_{1A} and to a lesser degree with dopamine D_1 receptors.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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Effect of combined treatment with aripiprazole and antidepressants on the MK-801-induced deficits in recognition memory in novel recognition test and on the release of monoamines in the rat frontal cortex

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ABSTRACT

According to preclinical and clinical studies, the antidepressant-induced increase in the activity of atypical antipsychotics may efficiently improve the treatment of negative and some cognitive symptoms of schizophrenia. In the present study, we aimed to evaluate the effects of the antidepressants escitalopram and mirtazapine and the atypical antipsychotic drug aripiprazole, administered separately or in combination, on the MK-801-induced deficits in the recognition memory test and on the extracellular levels of monoamines and their metabolites in the rat frontal cortex.

Based on the results of the behavioral tests, co-treatment with an ineffective dose of aripiprazole (0.1 mg/kg) and escitalopram (2.5 and 5 mg/kg) or mirtazapine (5 mg/kg) abolished the deficits evoked by MK-801 in the novel object recognition test, and those effects were blocked by the 5-HT_{1A} receptor antagonist (WAY 100,635) or the dopamine D_1 receptor antagonist (SCH 23,390). Moreover, co-treatment with aripiprazole (0.3 mg/kg) and escitalopram (5 mg/kg) significantly increased the levels of noradrenaline and serotonin, decreased the level of its metabolite, and did not alter level of dopamine, but decreased the levels of its metabolites. In addition, co-treatment with aripiprazole (0.3 mg/kg) and mirtazapine (10 mg/kg) significantly increased the level of noradrenaline, did not change the levels of dopamine and serotonin, but increased the levels of their metabolites. Based on these results, the increase in the extracellular levels of noradrenaline or serotonin in the cortex induced by co-treatment with an antidepressant and aripiprazole may be very important for the pharmacotherapy of negative and some cognitive symptoms of schizophrenia.

1. Introduction

Aripiprazole, a novel atypical antipsychotic with a high affinity for a large number of monoaminergic receptors, functions as an antagonist of both 5-HT_{2A} and postsynaptic dopamine D_2 receptors, as well as a partial agonist of 5-HT_{1A} and presynaptic dopamine D_2 receptors [1,2]. It is also a potent partial dopamine D_3 and D_4 receptor agonist [3]. These pharmacological properties may have important contributions to the therapeutic effects of this drug.

Aripiprazole alleviates both positive and negative symptoms of psychosis and partially improves the cognitive function of patients with schizophrenia without producing extrapyramidal side effects or increasing the serum prolactin level [1]. Although aripiprazole has been reported to improve the cognitive function of patients with schizophrenia [2,4], its mechanism of its action remains unclear.

Early behavioral studies revealed positive symptoms, negative symptoms and cognitive abnormalities in animals treated with NMDA receptor antagonists (e.g., phencyclidine (PCP), MK-801 or ketamine) that were similar to the symptoms observed in patients with psychosis [5,6].

Aripiprazole mitigates the PCP-induced disturbance in prepulse inhibition [7]. Moreover, repeated PCP treatments induce a recognition memory deficit in the novel object recognition test in mice. Acute treatment with aripiprazole at a dose of 1 mg/kg reverses the deficit in this test; unfortunately, this dose significantly decreases the total exploration time in the training session. Moreover, repeated treatment

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with aripiprazole (0.03 and 0.1 mg/kg) for 7 days significantly abolishes the PCP-induced deficit in recognition memory without changing the total exploration time in both training and retention sessions. However, acute and repeated treatments with haloperidol (0.3 and 3 mg/kg) do not reverse the PCP-induced cognitive impairment. The effect of aripiprazole on ameliorating the recognition memory in PCP-treated mice is blocked by dopamine D_1 and serotonin 5-HT_{1A} receptor antagonists. Based on these results, the antipsychotic-like effect of aripiprazole on the PCP-induced cognitive symptoms associated with schizophrenia in mice may be associated with dopamine D_1 and serotonin 5-HT_{1A} receptors [8].

In addition, the results of biochemical studies showed that acute injections of atypical antipsychotics, e.g., clozapine, risperidone, olanzapine, quetepine and ziprasidone, induce greater increases in extracellular dopamine (DA) and acetylcholine levels in the medial prefrontal cortex (mPFC) than in the nucleus accumbens [9–13]. The increase in DA release in the mPFC has been suggested to contribute to the ability of these atypical antipsychotics to ameliorate the cognitive dysfunction and negative symptoms of schizophrenia [10,14]. In addition, WAY 100,635, a 5-HT_{1A} receptor antagonist, partially inhibits the increase in DA release in the mPFC induced by clozapine, quetiapine and ziprasidone, i.e., the drugs that are partial 5-HT_{1A} receptor agonists. Thus, the stimulation of 5-HT_{1A} receptors might be necessary for the increase in cortical DA release induced by the atypical antipsychotic drugs [11,10–13]. In addition, the administration of low doses of aripiprazole (0.1 and 0.3 mg/kg) increases DA release in the hippocampus, and the administration of a dose of 0.3 mg/kg slightly but significantly increases DA release in the mPFC, but not in the nucleus accumbens. These increases were inhibited by WAY 100,635 (a 5-HT_{1A} receptor antagonist) [15].

During the treatment of schizophrenia, classical antipsychotic drugs (i.e., antagonists of dopamine D_2 receptors) mainly inhibit the positive symptoms, but do not affect the negative symptoms or the impaired cognitive processes [16,17]. In contrast to conventional antipsychotics, atypical antipsychotic drugs partially alleviate the negative symptoms and slightly improve the impaired cognitive functions [5,18]. According to recent clinical and preclinical studies, the addition of antidepressant drugs (ADs) with different pharmacological profiles to the treatment with the atypical antipsychotic increases the efficacy of the latter drug in alleviating negative symptoms and in improving the cognitive performance to a much greater extent than the atypical antipsychotic alone [19,20]. Based on these data, the administration of some ADs in combination with atypical antipsychotic drugs may be very important in clinical practice.

Thus, in the present study, we aimed to evaluate the effects of the AD escitalopram (ESC, selective serotonin reuptake inhibitor) [21] or mirtazapine (MIR, which enhances noradrenergic and serotonergic neurotransmission by blocking α_2-adrenoreceptors) [22] and the atypical antipsychotic aripiprazole (administered separately or in combination) on the MK-801-induced deficits in recognition memory in the novel recognition test in rats. MK-801 was administered before the first introductory session, in which animals were tested for their ability to discriminate between an old, familiar and a novel object. We also studied the effects of WAY 100,635 (a 5-HT_{1A} antagonist) and SCH 23,390 (a dopamine D_1 antagonist) on ameliorating the antipsychotic-like effect of aripiprazole combined with ADs on the MK-801-induced deficits in recognition memory in the novel recognition test to investigate the mechanism of action of the combination of aripiprazole and ADs. Moreover, we evaluated the release of monoamines in the frontal cortex (FCX) of freely moving rats using a microdialysis approach. The effects of co-treatment with ESC or MIR and aripiprazole on the MK-801-induced deficits in recognition memory in the novel recognition test and on the release of monoamines in the rat FCX have not been studied previously.

2. Materials and methods

2.1. Animals

All experiments were conducted on male Sprague-Dawley rats obtained from Charles River Laboratories, Sulzfeld (Germany). The animals were housed in standard polyacrylic cages (57 cm × 35 cm × 20 cm, 4 animals/cage). The animals had free access to standard laboratory food and tap water and were housed in a colony room at a temperature of 21 ± 1 °C with 40–50 % humidity on a 12-h light-dark cycle (the lights turned on at 7 a.m.). The experiments were conducted during the light phase.

The experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health and were approved by the Bioethics Commission as compliant with the Polish law.

2.2. Drugs and treatment

Escitalopram oxalate (ESC, Sigma-Aldrich, Saint Louis, MO, USA) and (+)-MK-801 maleate (MK-801, Tocris Bioscience, Bristol, UK) were dissolved in 0.9 % NaCl, while aripiprazole (Abcam Biochemicals, Cambridge, UK) and mirtazapine (MIR, Tocris Bioscience, Bristol, UK) were dissolved in 0.1 M tartaric acid. The pH of the solution was adjusted to 6–7 with 0.1 N NaOH. Antidepressants, aripiprazole, MK-801 and SCH 23,390 (Sigma-Aldrich, Saint Louis, MO, USA) were administered using intraperitoneal (i.p.) injections, while WAY 100,635 (Tocris Bioscience, Bristol, UK) was injected subcutaneously (s.c.) in a volume of 2 mL/kg. All doses of drugs used in the present study were selected from our earlier publication [23].

2.3. Novel object recognition test in rats

The novel object recognition (NOR) test was performed using a black PCV box (67 cm × 57 cm × 30 cm, length × width × height) divided into six symmetrical sectors. The arena was dimly illuminated with an indirect light of 18 lx. On the first day of the experiment (adaptation), rats (240 − 250 g) were placed in the box for 10 min. On the next day, the novel object recognition test was carried out 30 min after the administration of MK-801 (0.1 mg/kg, i.p.), and 60 or 30 min after the injection of ESC or MIR (2.5 and 5 mg/kg, i.p.) and aripiprazole (0.1 mg/kg, i.p.), respectively. WAY 100,635 (0.1 mg/kg, s.c.) and SCH 23,390 (0.25 mg/kg, i.p.) were injected 20 min before the T1 session. After drug administration, the animals were placed in the box (T1, introductory session) for 5 min and allowed to explore two identical objects (black tin, 5 cm wide and 14 cm high or green pyramid 5 cm wide and 14 cm high). The time the animals spent exploring each object was separately measured for each of the two objects. Then, one hour after the T1 session, the rats were again placed in the box for 5 min (T2, recognition session) and allowed to explore two different objects, one of which was the same as in previous session (old) and the other was new (black box and green pyramid). The time the animals spent exploring (sniffing, touching or climbing) the objects was separately measured for each of the two objects. The test box was wiped clean with 10 % ethanol between each trial. In addition, the number of sector line crossings (ambulation) was determined as a measure of the locomotor activity of those rats. Each group was composed of 8 rats.

2.4. Biochemical study - in vivo microdialysis

Rats (290 − 300 g) were anesthetized with ketamine hydrochloride (75 mg/kg, Biowet Puławy, Poland) and xylazine (10 mg/kg) and secured in a stereotaxic frame (Stoelting, USA). Vertical microdialysis guide cannulas (Intracerebral Guide Cannula with stylet; BAS Bioanalytical, USA) were implanted in the frontal cortex (FCX) at the following stereotaxic coordinates: A/P + 2.9, L/M + 0.8, and V/D –4.0

mm from bregma and the dura (Paxinos and Watson). Seven days after surgery, microdialysis probes were inserted into the cannulas, and the frontal cortex was perfused with artificial cerebrospinal fluid (aCSF) consisting of 140 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 mM NaH_2PO_4, and 1.7 mM Na_2HPO_4 (pH 7.4), at a flow rate of 1.5 µl/min with a microinfusion pump (Stoelting, Illinois, USA). Samples were collected from freely moving rats at 20-minute intervals after a 3 -h wash-out period. An acute dose of ESC (5 mg/kg, i.p.), MIR (10 mg/kg, i.p.) or aripiprazole (0.3 mg/kg, i.p.) was injected, and dialysis samples were collected for 180 min. In the mixed groups, ESC or MIR was administered 20 min before the aripiprazole injection. All dialysates were immediately frozen on dry ice (-70°C) until they were used in the biochemical assays.

The levels of dopamine (DA), serotonin (5-HT) and their metabolites or noradrenaline (NA) in dialysates (20 µl) were assayed using HPLC with electrochemical detection, as described below. The Dionex Ultimate 3000 chromatograph (Coulochem III, Germany) was equipped with C18 columns (Hypersil Gold; 150 mm x3 µm). The mobile phase comprised 0.05 M citrate-phosphate buffer (pH 3.5), 0.1 mM EDTA, 1 mM sodium octyl sulfonate, and 3.5 % methanol. The flow rate was maintained at 0.75 mL/min. Chromatographic data were processed using the Chromeleon Dionex computer program (Germany). The levels of DA, 5-HT, NA and their metabolites were quantified by calculating the height of the chromatograph peaks and comparing them with standards run on the day of the analysis. At the end of the experiment, frozen brains were examined histologically for correct probe placement. Each group consisted of five to six animals.

2.5. Statistical analysis

The results are presented as means ± SEM (standard errors of the means). The behavioral data were evaluated using one-way analysis of variance (ANOVA) followed by individual comparisons using Duncan's test. The data obtained from the microdialysis studies were analyzed using one-way analysis of variance (ANOVA) for repeated measures, followed by Duncan's post hoc test, if significant differences were detected. The results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Timeline of the general protocol used in the present study designed to examine the memory deficits in the novel object recognition test in rats

The antidepressants escitalopram (ESC) and mirtazapine (MIR) were administered at doses of 2.5 and 5 mg/kg, i.p. 30 min prior to the aripiprazole (ARI, 0.1 mg/kg, i.p.) injection and 60 min prior to the MK-801 (0.1 mg/kg, i.p.) injection. WAY 100,635 (0.1 mg/kg, s.c.) or SCH 23,390 (0.25 mg/kg, i.p.) were injected 10 min after MK-801 administration and 20 min before T1 (introductory session conducted for 5 min). T2 (recognition session) was performed 60 min after the T1 session and lasted for 5 min (Fig. 1).

3.2. The effect of the combined treatment with escitalopram (ESC) or mirtazapine (MIR) and aripiprazole on the performance of rats in the novel object recognition test

According to the one-way ANOVA, during T1, the introductory session in which rats received saline, MK-801 (0.1 mg/kg) and all tested drugs before the test, the animals spent a similar amount of time exploring the two identical objects (A1 and A2) (Fig. 2A), indicating a lack of preference for a certain position of the objects in the area. During T2, the recognition session (Fig. 2C), control rats spent significantly more time exploring the novel object [$F(1,14) = 35.08$; $p < 0.001$], while rats receiving 0.1 mg/kg MK-801 showed no preference for a particular object [$F(1,14) = 0.19$; N.S]. In the T1 session, the locomotor activity of rats receiving MK-801 was increased by 51.4 % [$F(1,14 = 9.20$, $p < 0.001$] (Fig. 2B), but the locomotor activity of this group was not altered in T2 (recognition session) [$F(1,14) = 0.675$; N.S] (Fig. 2D). In addition, in the T2 session, the locomotor activity of rats administered MK-801 along with 5 mg/kg ESC or 5 mg/kg ESC plus 0.1 mg/kg aripiprazole was decreased by 13.6 % and 40.2 %, respectively, compared to the MK-801 group (Fig. 2D).

According to the one-way ANOVA, the administration of a higher aripiprazole dose (0.3 mg/kg, but not 0.1 mg/kg) abolished the decrease in the memory retention of the object evoked by MK-801 (0.1 mg/kg) [$F(1,14) = 14.459$, $p < 0.01$ for the difference in the B group (time spent exploring the novel object) from the A group (time spent exploring the familiar object)] (Fig. 2C). Moreover, the administration of a lower dose of aripiprazole (0.1 mg/kg) or ESC (2.5 and 5 mg/kg) alone did not change the effect of MK-801 (0.1 mg/kg) on the object recognition memory (Fig. 2C), while the combined treatment with an ineffective dose of aripiprazole (0.1 mg/kg) and ESC (2.5 or 5 mg/kg) abolished the effect of MK-801 (0.1 mg/kg) [$F(1,14) = 10,889$, $p < 0.01$ and $F(1,14) = 26,923$; $p < 0.001$ for the difference in the B group (time spent exploring the novel object) from the A group (time spent exploring the familiar object), respectively] (Fig. 2C).

Moreover, in T2 (recognition session), the administration of MIR at doses of 2.5 and 5 mg/kg did not change the effect of MK-801 on the deficits in recognition memory, and the administration of a lower dose of aripiprazole (0.1 mg/kg) in combination with 2.5 mg/kg MIR produced no change in the effect of MK-801 [$F(1,14) = 3.486$, N.S.]. While, the administration of 0.1 mg/kg aripiprazole with a higher dose of MIR (5 mg/kg) abolished the deficits in recognition memory in the T2 session [$F(1,14) = 16.80$, $p < 0.01$ for the difference in the B group (time spent exploring the novel object) from the A group (time spent exploring the familiar object)] (Fig. 2C). In the T1 session, the locomotor activity of rats receiving MK-801 along with aripiprazole (0.1 mg/kg) and MIR (5 mg/kg) was decreased by 44.2 % compared to the control group (Fig. 2B**)**.While, in the T2 session, the locomotor activity of rats administered MK-801 with 5 mg/kg MIR or 5 mg/kg MIR plus 0.1 mg/kg aripiprazole was not altered in any tested group (Fig. 2D).

3.3. The effects of WAY 10,063 and SCH 23,390 on the efficacy of the combined treatment with escitalopram (ESC) or mirtazapine (MIR) and aripiprazole in altering the performance of rats on the novel object recognition test

One-way ANOVA did not reveal changes in the MK-801 (0.1 mg/kg)-induced recognition memory deficit in T2 (recognition session) in rats treated with WAY 100,635 (0.1 mg/kg) [$F(1,14) = 1.622$, N.S.], while it blocked the effects of aripiprazole (0.1 mg/kg) combined with ESC (5 mg/kg) or MIR (5 mg/kg) on reversing the MK-801-induced deficits in recognition memory [$F(1,14) = 0.104$, NS and $F(1,14) = 0.70$, N.S. for the difference in the B group (time spent exploring the novel object) from the A group (time spent exploring the familiar object), respectively] (Fig. 3A). Locomotor activity was reduced by 41.5 % in the T2 session only in the group receiving MK-801 with aripiprazole plus ESC (Fig. 3B).

In T2 (recognition session), the one-way ANOVA indicated that SCH 23,390 (0.25 mg/kg) reversed the deficit in recognition memory induced by MK-801 (0.1 mg/kg) [$F(1,14) = 39.092$, $p < 0.001$], and it blocked the effects of aripiprazole (0.1 mg/kg) combined with ESC (5 mg/kg) or MIR (5 mg/kg) on abolishing the deficits in recognition memory [$F(1,14) = 0.137$, N.S. and $F(1,14) = 0.038$, N.S. for the difference in the B group (time spent exploring the novel object) from the A group (time of familiar object), Fig. 3C). Locomotor activity was reduced in the T2 session by 41.5 and 33.3 % in the group receiving MK-801 along with aripiprazole plus ESC and in the group injected MK-801, aripiprazole, SCH 23,390, and MIR, respectively (Fig. 3D).

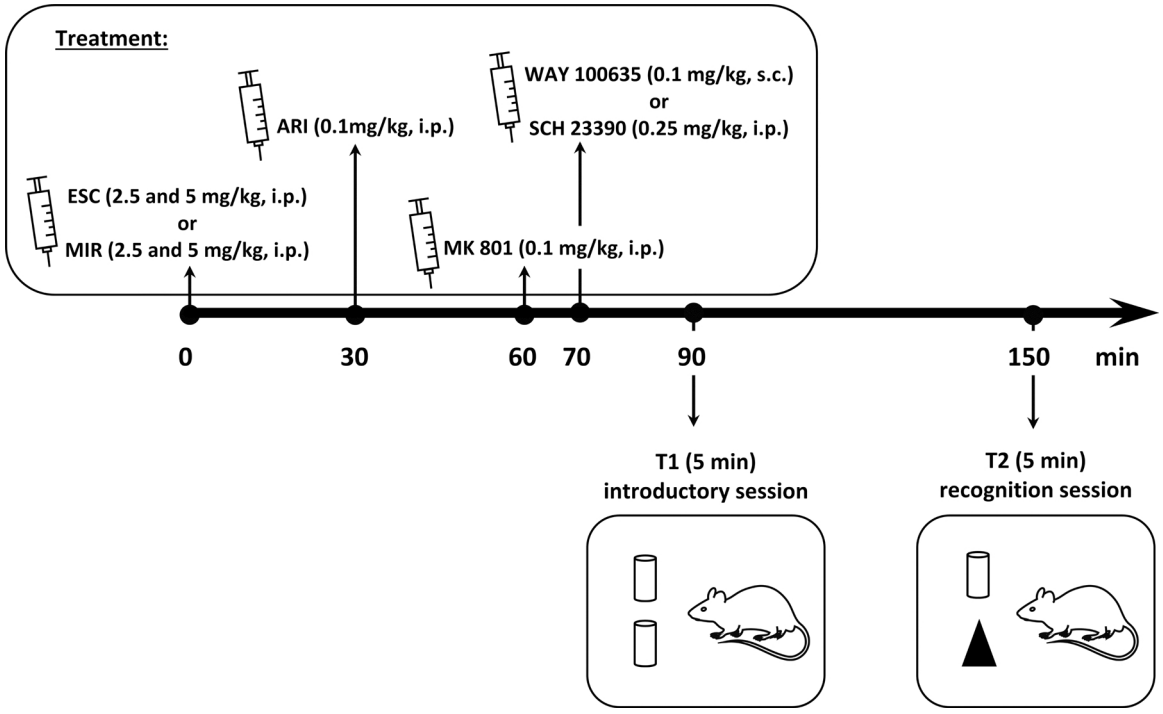

Fig. 1. Timeline of the general protocol used in the present study to examine the memory deficits in the novel object recognition test in rats. The antidepressants escitalopram (ESC) and mirtazapine (MIR) were i.p. injected at doses of 2.5 and 5 mg/kg 30 min before the aripiprazole (ARI, 0.1 mg/kg, i.p.) injection and 60 min before the MK-801 (0.1 mg/kg, i.p.) injection. WAY 100,635 (0.1 mg/kg, s.c.) or SCH 23,390 (0.25 mg/kg, i.p.) was injected 10 min after MK-801 and 20 min before T1 (introductory session lasting for 5 min). T2, the recognition session, was performed 60 min after the T1 session and lasted for 5 min.

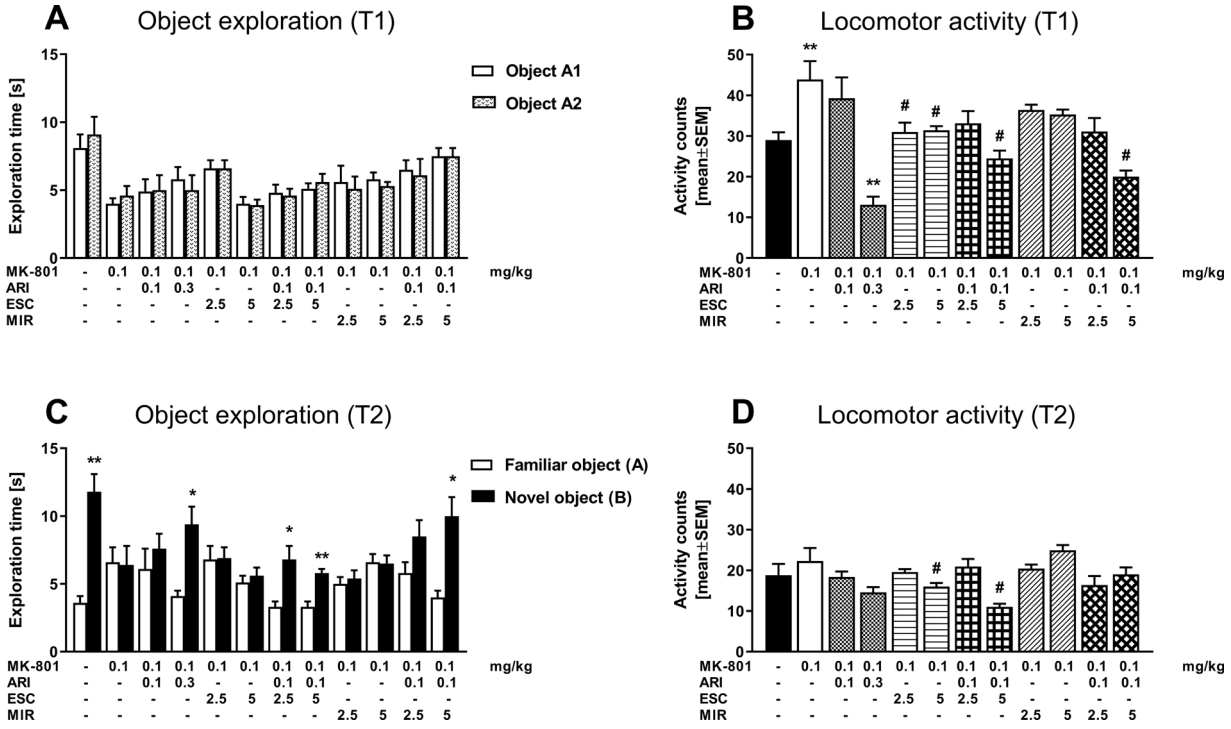

Fig. 2. Effect of the treatment with escitalopram (ESC) or mirtazapine (MIR) alone or in combination with aripiprazole (ARI) on the MK-801-induced deficits in the novel object recognition test in rats. **(A)** Effect on the exploration time (s) in T1, the introductory session. **(C)** Effect on the exploration time (s) in T2, the recognition session. **(B)** Effect on the locomotor activity in T1, the introductory session. (**D)** Effect on the locomotor activity in T2, the recognition session of the novel object recognition test, in rats. MK-801 was administered 30 min before T1, the introductory session, and recognition memory (T2 session) was tested 60 min later. ESC and MIR were administered 60 min and ARI was administered 30 min before MK-801. The results are presented as the mean ± SEM time (in seconds) spent exploring two similar objects (A1 and A2) **(A)** and both objects (A, familiar object and B, novel object) in the recognition session **(C).** Each group consisted of 8 rats. The data were statistically evaluated using ANOVA, followed by individual comparisons using Duncan's test. In **A** and **C,** (object exploration) * $p < 0.01$ and ** $p < 0.001$ for the comparison of group **B** (time spent exploring the novel object) with group **A** (time spent exploring the familiar object); in **B** and **D,** ** $p < 0.001$ compared with the vehicle-treated group.

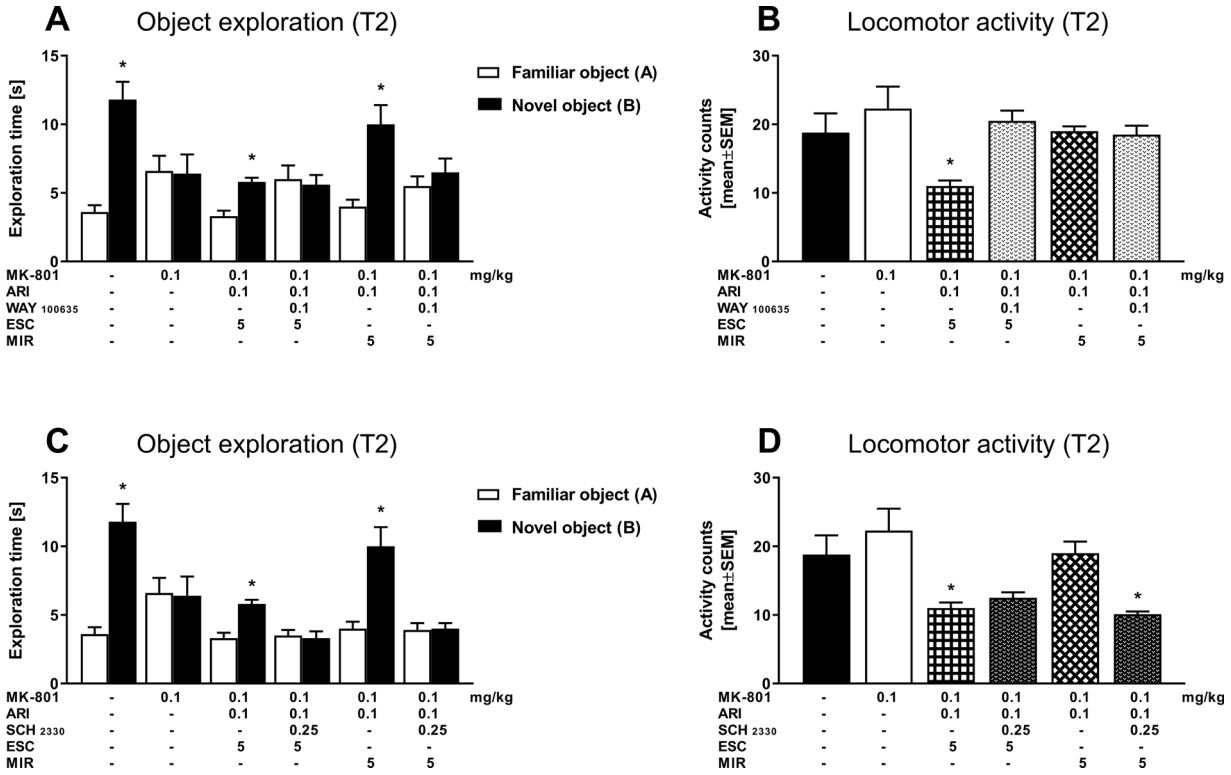

Fig. 3. Effects of WAY 100,635 and SCH 23,390 on the efficacy of the combined treatment with escitalopram (ESC) or mirtazapine (MIR) with aripiprazole (ARI) on ameliorating the MK-801-induced memory deficits in the novel object recognition test in rats in T2, the recognition session. **(A)** The effect of WAY 100,635 on the ability of the antipsychotic ARI combined with ESC or MIR to ameliorate the MK-801-induced memory deficits in the novel object recognition test in rats (as measured by the exploration time) **(B)** and on the locomotor activity. **(C)** The effect of SCH 23,390 on the ability of the antipsychotic ARI combined with ESC or MIR to ameliorate the MK-801-induced memory deficits in the novel object recognition test in rats (as measured by the exploration time) **(D)** and on the locomotor activity. ESC or MIR was administered 60 min and ARI was administered 30 min before MK-801, and WAY 100,635 or SCH 23,390 was administered 20 min before the test. The results are presented as means ± SEM. Each group consisted of 8 rats. The data were statistically evaluated using ANOVA, followed by individual comparisons using Duncan's test. * $p < 0.001$ group **B** (time spent exploring the novel object) compared with group **A (**time spent exploring the familiar object); in **B** and **D,** ** $p < 0.001$ compared with the vehicle-treated group.

3.4. The effect of the combined treatment with escitalopram (ESC) and aripiprazole on dopamine (DA) release in the rat frontal cortex (FCX)

The mean control basal extracellular concentration of DA in dialysates obtained from the FCX was approximately 4.2 ± 0.6 (pg/20 µl). One-way repeated measures ANOVA did not identify significant effects of the treatment [$F(3,18) = 0.26$, N.S.] on DA release into the extracellular space (Fig. 4A). Similarly, the effects of time [$F(12,216) = 0.72$, N.S.] and the interaction between time and treatment were not significant [$F(36,216) = 0.86$, N.S.].

3.5. The effect of the combined treatment with escitalopram (ESC) and aripiprazole on the 3,4-dihydroxyphenylacetic acid (DOPAC) concentration in the rat frontal cortex (FCX)

The statistical analysis did not detect a significant effect of the treatment [$F(3,18) = 1.92$, N.S.] on DOPAC levels in the rat FCX (Fig. 4B). However, the effects of time [$F(12,216) = 3.31$, $p < 0.01$] and the interaction between time and treatment was significant [$F(36,216) = 1.94$, $p < 0.01$]. Duncan's post hoc analysis revealed an up to 40 % decrease in the DOPAC level after treatment with the combination of ESC and aripiprazole (Fig. 4B).

3.6. The effect of the combined treatment with escitalopram (ESC) and aripiprazole on the homovanillic acid (HVA) concentration in the rat frontal cortex (FCX)

One-way repeated measures ANOVA did not reveal significant effects of the treatment [$F(3,18) = 1.93$, N.S.) on HVA concentrations in the extracellular space (Fig. 3C). However, the effects of time [$F(12,216) = 3.12$, $p < 0.01$] and the interaction between time and treatment were significant [$F(36,216) = 2.34$, $p < 0.01$]. The post hoc test revealed an up to 45 % decrease in the HVA levels after treatment with the combination of ESC and aripiprazole (Fig. 4C).

3.7. The effect of the treatment combining escitalopram (ESC) and aripiprazole on serotonin (5-HT) release in the rat frontal cortex (FCX)

The statistical analysis did not indicate a significant effect of the treatment [$F(3,18) = 2.82$, N.S.] on the 5-HT level in the rat FCX (Fig. 5A). However, the effects of time [$F(12,216) = 2.84$, $p < 0.01$] and the interaction between time and treatment were significant [$F(36,216) = 1.82$, $p < 0.01$]. Duncan's test indicated that the administration of aripiprazole alone and in combination with ESC increased the release of 5-HT by 200 % and 300 %, respectively (Fig. 5A).

3.8. The effect of the treatment combining escitalopram (ESC) and aripiprazole on the 5-hydroxyindoloamino acid (5-HIAA) concentration in the rat frontal cortex (FCX)

One-way repeated measures ANOVA revealed a significant effect of the treatment [$F(3,18) = 13.39$, $p < 0.01$] on the 5-HIAA level in the rat FCX (Fig. 5B). Additionally, the effects of time [$F(12,216) = 16.95$, $p < 0.01$] and the interaction between time and treatment were also significant [$F(36,216) = 6.24$, $p < 0.01$]. Duncan's post hoc test indicated a significant decrease in the 5-HIAA concentration by

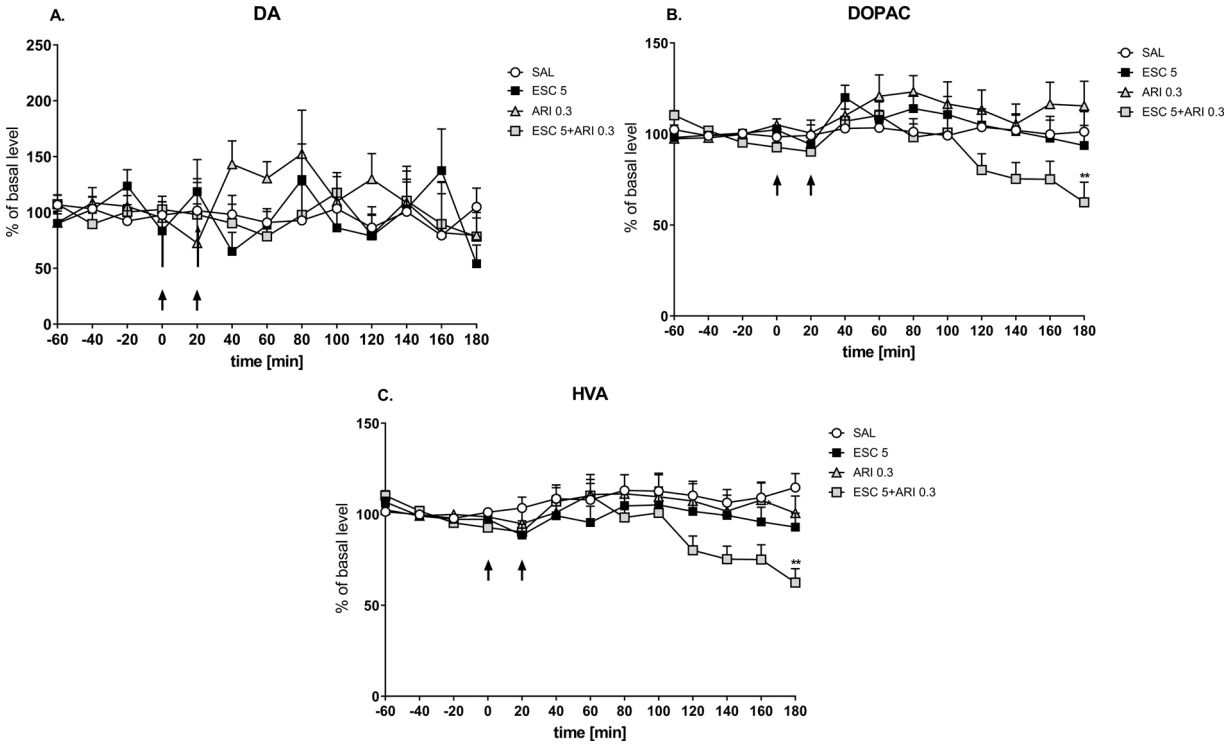

Fig. 4. Effects of the combined treatment with escitalopram (ESC) and aripiprazole (ARI) on dopamine (DA) release and the concentration of its metabolites 3,4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) in the rat frontal cortex (FCX). An acute dose of ESC (5 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, ESC was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min. The concentrations of DA **(A)**, DOPAC **(B)** and HVA **(C)** were measured. The data are reported as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

approximately 50 % after treatment with ESC alone in combination with aripiprazole compared with the basal level (Fig. 5B).

3.9. The effect of the combined treatment with escitalopram (ESC) and aripiprazole on noradrenaline (NA) release in the rat frontal cortex (FCX)

The statistical analysis indicated a significant effect of the treatment [$F(3,17) = 88.64$, $p < 0.01$] on the NA concentration in the rat FCX (Fig. 6). Moreover, the effects of time [$F(12,204) = 37.66$, $p < 0.01$] and the interaction between time and treatment were also significant [$F(36,204) = 32.54$, $p < 0.01$]. The post hoc test revealed a significant increase in the NA concentration of approximately 300 % after treatment the combination of ESC and aripiprazole ($p < 0.01$) (Fig. 6).

3.10. The effect of the treatment combining mirtazapine (MIR) and aripiprazole on dopamine (DA) release in the rat frontal cortex (FCX)

The mean basal extracellular concentration of DA in dialysates obtained from the FCX of control rats was approximately 4.5 ± 0.5 (pg/20 µl). One-way repeated measures ANOVA did not reveal significant effects of the treatment [$F(3,18) = 1.25$, N.S.] on DA release into the extracellular space (Fig. 7A). Similarly, the effects of time [$F(12,216) = 1.21$, N.S.] and interaction between time and treatment were not significant [$F(36,216) = 1.44$, N.S.].

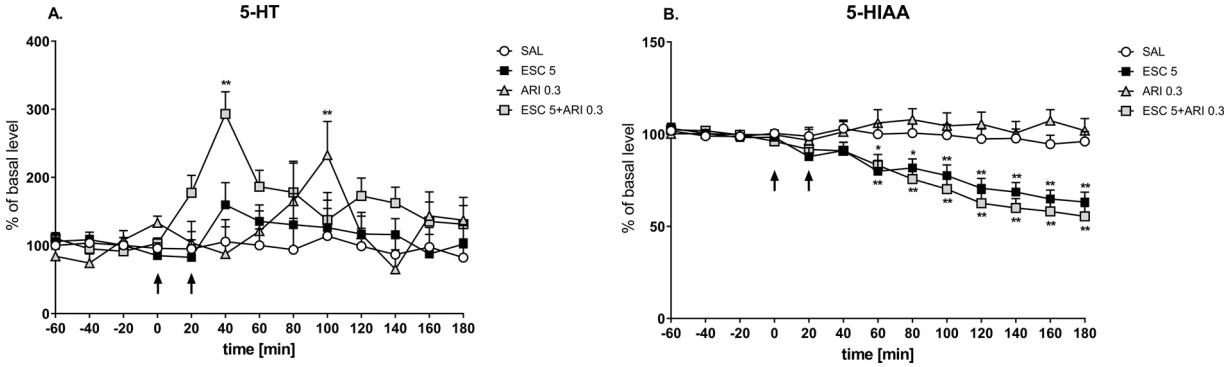

Fig. 5. Effects of the combined treatment with escitalopram (ESC) and aripiprazole (ARI) on serotonin (5-HT) release and the concentrations of its metabolite 5-hydroxyindoloamino acid (5-HIAA) in the rat frontal cortex (FCX). An acute dose of ESC (5 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, ESC was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min. The concentrations of 5-HT **(A)** and 5-HIAA **(B)** were measured. The data are presented as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

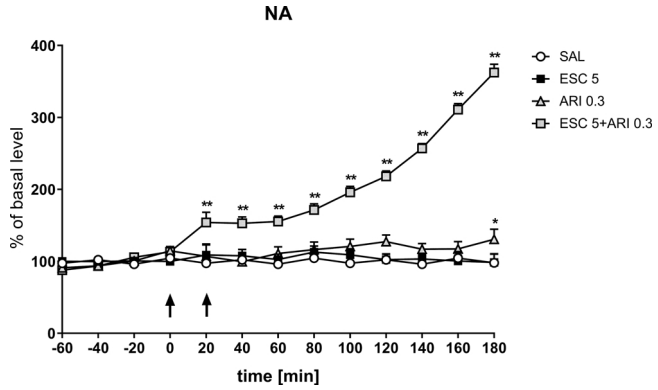

Fig. 6. Effect of the combined treatment with escitalopram (ESC) and aripiprazole (ARI) on noradrenaline (NA) release in the rat frontal cortex (FCX). An acute dose of ESC (5 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, ESC was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min, and the concentrations of NA were measured. The data are presented as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

3.11. The effect of the combined treatment with mirtazapine (MIR) and aripiprazole on the 3,4-dihydroxyphenylacetic acid (DOPAC) concentration in the rat frontal cortex (FCX)

The statistical analysis did not detect a significant effect of the treatment [$F(3,18) = 1.53$, N.S.] on the DOPAC level in the rat FCX (Fig. 7B). However, the effect of time [$F(12,216) = 4.61$, $p < 0.01$] was significant. At the same time, the effect of the interaction between time and treatment was not significant [$F(36,216) = 1.17$, N.S.]. Duncan's post hoc analysis showed that treatment with the combination of MIR and aripiprazole increased the DOPAC level by up to 60 % (Fig. 7B).

3.12. The effect of the combined treatment with mirtazapine (MIR) and aripiprazole on the homovanillic acid (HVA) concentration in the rat frontal cortex (FCX)

One-way repeated measures ANOVA revealed a significant effect of treatment [$F(3,18) = 4.32$, $p < 0.05$] on the HVA concentration in the extracellular space (Fig. 7C). In addition, the effects of time [$F(12,216) = 3.59$, $p < 0.01$] and the interaction between time and treatment were significant [$F(36,216) = 2.97$, $p < 0.01$]. Treatment with the combination MIR and aripiprazole increased the level of HVA by ca. 60 % in the post hoc test (Fig. 7C).

3.13. The effect of the combined treatment with mirtazapine (MIR) and aripiprazole on serotonin (5-HT) release in the rat frontal cortex (FCX)

The statistical analysis did not indicate a significant effect of the treatment [$F(3,18) = 1.29$, N.S.] on the 5-HT level in the rat FCX (Fig. 8A). Similarly, the effects of time [$F(12,216) = 0.81$, N.S.] and the interaction between time and treatment were not significant [$F(36,216) = 0.97$, N.S.].

3.14. The effect of the combined treatment with mirtazapine (MIR) and aripiprazole on the 5-hydroxyindoloamino acid (5-HIAA) concentration in the rat frontal cortex (FCX)

One-way repeated measures ANOVA revealed a significant effect of the treatment [$F(3,18) = 12.45$, $p < 0.01$] on the 5-HIAA level in the rat FCX (Fig. 8B). At the same time, the effect of time was not significant [$F(12,216) = 1.53$, N.S.], but the effect of the interaction

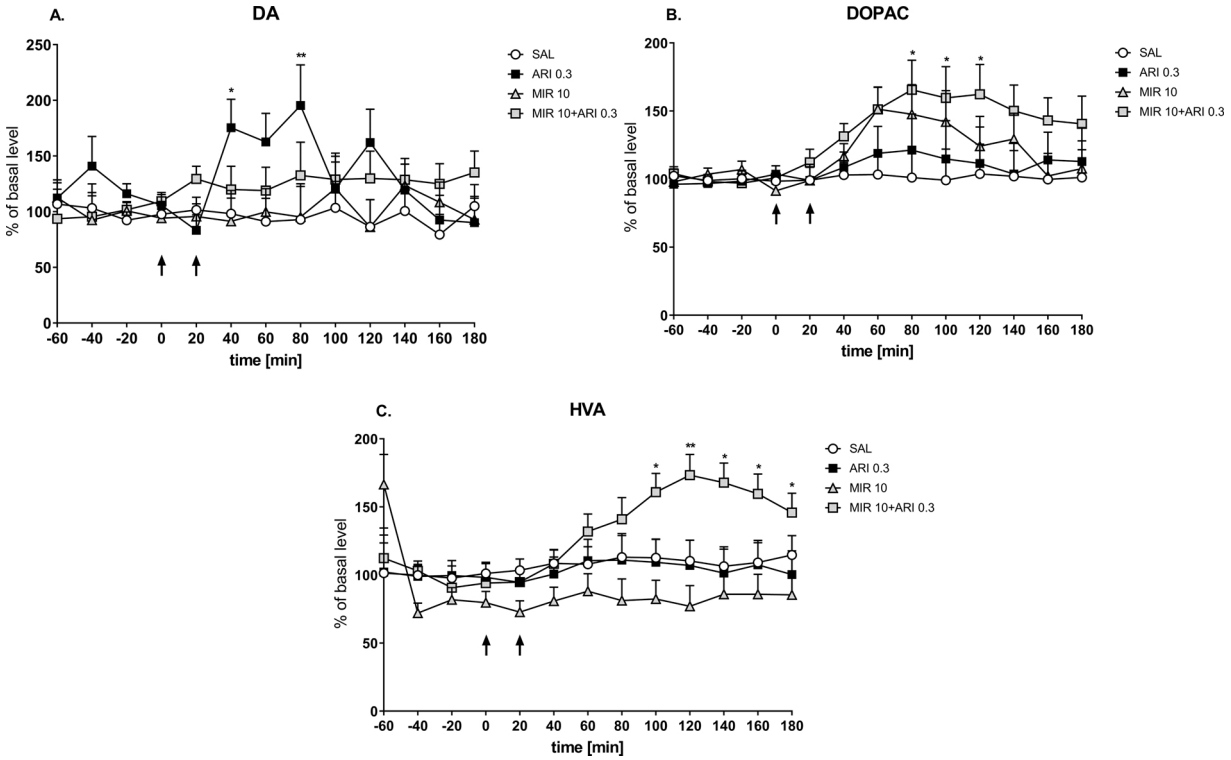

Fig. 7. Effects of the combined treatment with mirtazapine (MIR) and aripiprazole (ARI) on dopamine (DA) release and the concentrations of its metabolites 4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) in the rat frontal cortex (FCX). An acute dose of MIR (10 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, MIR was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min. The concentrations of DA **(A),** DOPAC **(B)** and HVA **(C)** were measured. The data are presented as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

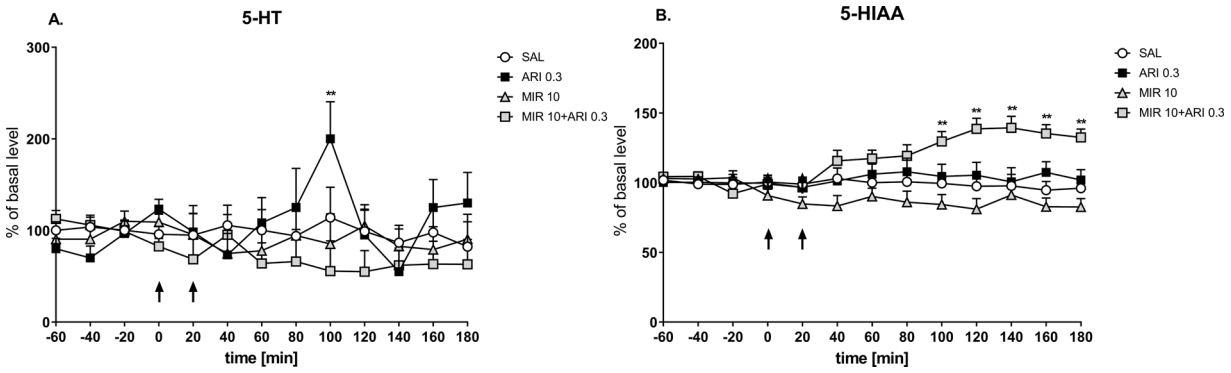

Fig. 8. Effects of the combined treatment with mirtazapine (MIR) with aripiprazole (ARI) on serotonin (5-HT) release and the concentrations of its metabolite 5-hydroxyindoloamino acid (5-HIAA) in the rat frontal cortex (FCX). An acute dose of MIR (10 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, MIR was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min. The concentrations of 5-HT **(A)** and 5-HIAA **(B)** were measured. The data are reported as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

between time and treatment was significant [$F(36,216) = 3.47$, $p < 0.01$]. Duncan's post hoc test indicated that treatment with MIR combined with aripiprazole significantly increased the concentration of 5-HIAA by ca. 40 % (Fig. 8B).

3.15. The effect of the combined treatment with mirtazapine (MIR) and aripiprazole on noradrenaline (NA) release in the rat frontal cortex (FCX)

The statistical analysis indicated a significant effect of the treatment [$F(3,14) = 6.61$, $p < 0.01$] on the NA concentration in the rat FCX (Fig. 9). Moreover, the effects of time [$F(12,168) = 4.16$, $p < 0.01$] and the interaction between time and treatment were also significant [$F(36,168) = 4.86$, $p < 0.01$]. According to the post hoc test, the combination of MIR and aripiprazole significantly increased the NA concentration by up to 650 % ($p < 0.01$) (Fig. 9).

4. Discussion

The novel object recognition test in rodents is analogous to some extent to human declarative (episodic) memory, one of a few cognitive domains that are abnormal in patients with schizophrenia [24]. In this test, the animal (rat or mouse) is tested for its ability to discriminate an old, familiar object and a novel object in two sessions (introductory and recognition sessions). An animal generally spends more time exploring a novel object in the recognition session than in examining the object presented in the introductory session. A previous study of object recognition memory indicated a decrease in memory retention when MK-801 was administered before the first introductory session [6].

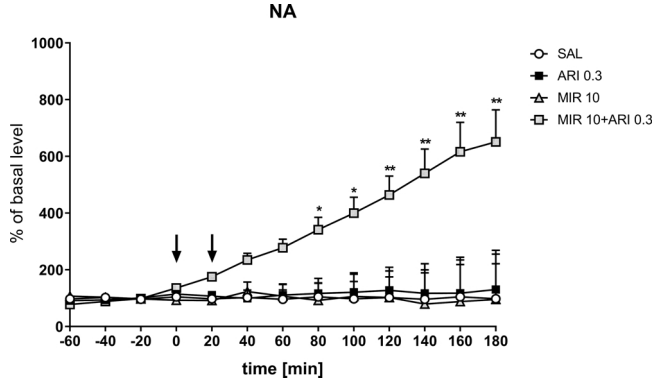

Fig. 9. Effect of the combined treatment with mirtazapine (MIR) and aripiprazole (ARI) on noradrenaline (NA) release in the rat frontal cortex (FCX). An acute dose of MIR (10 mg/kg, i.p.) or ARI (0.3 mg/kg, i.p.) was injected and dialysis samples were collected for 180 min. In the mixed groups, MIR was administered 20 min before the ARI injection. The control group was treated with saline. The dialysate was collected every 20 min, and the concentration of NA was measured. The data are presented as means ± SEM (n = 5-6). Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with the basal value (Duncan's test).

In the present behavioral study, MK-801 induced deficits in recognition memory in the novel object recognition test in rats, and the administration of high doses of aripiprazole reversed those effects. Moreover, co-treatment with an ineffective dose of aripiprazole and ESC or MIR abolished the deficits evoked by MK-801, and those effects were blocked by the 5-HT_{1A} receptor antagonist (WAY 100,635) or the dopamine D_1 receptor antagonist (SCH 23,390).

In addition, some earlier behavioral studies indicated that aripiprazole reversed the PCP-induced deficits in animal tests. The PCP-induced cognitive deficits in a novel object recognition memory test in mice were blocked by aripiprazole, and this effect was reversed by the serotonin 5-HT_{1A} receptor antagonist (WAY 100,635) or the dopamine D_1 antagonist (SCH 23,390) [8]. Moreover, PCP-evoked deficits in the novel object recognition test in male Sprague-Dawley rats were also abolished by aripiprazole and compound SSR181505, which induces 5-HT_{1A}/dopamine D_2 activation. The positive synergistic effects of aripiprazole and the compound SSR181505 were blocked by WAY 100635. Based on these findings, the balance between the activity of the 5-HT_{1A} and dopamine D_2 receptors might have an important contribution to the mechanism of action of antipsychotic drugs on some cognitive symptoms of schizophrenia [25].

The results are consistent with previous studies showing that PCP-induced cognitive dysfunction was accompanied by 5-HT_{1A} receptor stimulation in the PFC that induced dopamine D_1 receptor activation *via* the mesocortical dopaminergic pathway. This effect was blocked by aripiprazole [8,26,27].

In our previous study, the AD (ESC or MIR) ameliorated the antipsychotic-like effect of aripiprazole on the MK-801-induced deficits in the social interaction test in rats (this test is used to evaluate some behavioral deficits considered equivalent to negative symptoms of schizophrenia in animals) and may be associated with the serotonin 5-HT_{1A} receptor, since WAY 100,635 reversed the positive synergistic effects of the combined treatment with aripiprazole and ADs in this test [23].

As shown in our previous study, co-treatment with an ineffective dose of another antipsychotic drug, risperidone (0.1 mg/kg), and ESC (5 mg/kg) or MIR (2.5 and 5 mg/kg) abolished the deficits induced by MK-801 (0.1 mg/kg) in the social interaction test in male Wistar rats [28]. In addition, other authors also showed that ESC increased the antipsychotic-like activity of a low dose of risperidone in the conditional avoidance response test in rats. This test is used to evaluate some

behavioral deficits considered equivalent to negative symptoms of schizophrenia in animals [29]. The positive interactions between risperidone or aripiprazole and ADs were also observed in the behavioral tests conducted in mice, which assessed the positive symptoms and some cognitive symptoms of schizophrenia [30,31]. Based on these findings, ADs may enhance the antipsychotic-like effect of risperidone or aripiprazole on the results of animal tests used to evaluate some positive, negative and cognitive symptoms of schizophrenia.

Moreover, the present neurochemical data indicated a slight (50–60 %), but not significant increase in DA release in the PFC following the administration of aripiprazole alone, while co-treatment with ESC did not change in the DA level. However, it decreased the levels of its metabolites (DOPAC and HVA). Thus, the decreased levels of DA metabolites might indicate the inhibition of dopaminergic catabolism, which enhances neurotransmitter activity in the synapse. In addition, in this group, a significant increase in the release of 5-HT was observed simultaneously with a decrease in the level of its metabolite (5-HIIA) and an increase NA release.

Some earlier studies, using *in vivo* microdialysis indicated that aripiprazole induced a bell-shaped dose-dose-dependent DA release in the rat mPFC (maximum 50 % increase at 0.3 mg/kg, and no changes at 0.1, 1 and 3 mg/kg) [15], whereas no effects were observed between 2 and 40 mg/kg at all doses used by Jordan et al. [32] and by Assie et al. [33]. Moreover, Semba et al. [34] reported a moderate 20–25 % reduction after the administration of 10 and 40 mg/kg aripiprazole. In the mouse mPFC, a significant 80 % increase was observed after treatment with a dose of 0.3 mg/kg (but no change was observed at doses of 0.1, 1 and 3 mg/kg) [35]. However, this effect was observed 3 h after aripiprazole administration, whereas Li et al. [15] reported a short-term increase soon (80 − 90 min) after aripiprazole administration. In the present study, the s.c. injection of 0.3 mg/kg aripiprazole slightly but significantly (by 50 %) increased DA release in the rat mPFC. In addition, doses of 0.1 and 0.3 mg/kg significantly increased DA release in the hippocampus. The authors postulate that the functions of both mPFC and hippocampus may contribute to the ability of aripiprazole to alleviate negative symptoms and cognitive dysfunction in individuals with schizophrenia [15]. Moreover, 5-HT_{1A} receptor agonists were reported to increase the outflow of DA in the rat PFC [36]; thus, 5-HT_{1A} activation may be the mechanism by which aripiprazole modulates DA release. In our present study, we also observed slightly increased DA release in the mPFC (by 50–60 %, not significant) 20–60 min after aripiprazole administration. This explanations for these discrepancies have not been clearly determined, but might be attributed to the insolubility of aripiprazole in water and the different methods used to suspend or dissolve the drug in an aqueous vehicle before systemic administration.

In the present study, the administration of ESC alone or in combination with a low dose of aripiprazole (0.3 mg/kg, i.p.) increased the level of 5-HT, and its mechanism of action might be associated with the blockade of serotonin transporters [37]. In contrast, the administration of high doses of aripiprazole (3 − 30 mg/kg, i.p.) to rats reduced the 5-HT levels in the mPFC and dorsal raphe at higher doses [38]. A similar reduction in 5-HT levels in the mPFC and dorsal raphe was observed by other authors after animals were treated with higher doses (3, 10, and 30 mg/kg) [33]. Moreover, the increase in NA release from noradrenergic neurons stimulated postsynaptic α_1-adrenoreceptors on 5-HT cell bodies to increase 5-HT release [39]. Thus, in our study, the administration of a low dose of aripiprazole (0.3 mg/kg, i.p.) likely increased the level of 5-HT in the mPFC through this mechanism.

Based on the data from the present study, the administration of MIR (10 mg/kg) alone did not alter the NA level, while co-treatment with aripiprazole significantly increased the NA level but did not change the levels of DA and 5-HT. However, the levels of their metabolites (DOPAC and HVA or 5-HIIA, respectively) were altered. The increase in the levels of these metabolites increased the rates of DA and 5-HT catabolism and, consequently, decreased the activity of these neurotransmitters. Co-treatment with ESC and aripiprazole also increased NA release. As shown in our previous study, MIR (10 and 20 mg/kg) increased the extracellular levels of DA, NA and 5-HT in the cortex of male Wistar-Han rats in a dose-dependent manner [40]. According to other authors, MIR (8 and 16 mg/kg) evoked a dose-dependent increase in the extracellular DA level but did not change the level of 5-HT in the rat mPFC [41].

Treatment with a combination of MIR and aripiprazole increased the level of NA, but not 5-HT, and may have contributed to the activation of adrenergic receptors. Adrenergic α_2 autoreceptors located both on the dendrites and terminals of the fronto-cortical adrenergic pathway exert a pronounced tonic, inhibitory effect on the release of NA in the PFC [42]. Buspirone (by activating 5-HT_{1A} activation and blocking adrenergic α_2 receptors) facilitates fluoxetine-stimulated increases in the dialysate levels of NA, but not 5-HT, in the PFC [43].

In addition, the firing activity of 5-HT cells of the dorsal raphe nucleus depend on the tonically active, central adrenergic system. The α_2-heteroreceptors located on presynaptic 5-HT terminals are tonically activated by endogenous NA (4445**)**. Activation of α_2-adrenergic auto- and heteroreceptors decreases 5-HT transmission. Moreover, the stimulation of inhibitory α_2-autoreceptors located on the NA neurons in the locus coeruleus decreases firing of these neurons and subsequently decreases 5-HT transmission by reducing the activation of stimulatory α_1 receptors located on the 5-HT cell bodies in the raphe nucleus **(**46). Moreover, the systemic administration of α_2-antagonists increase 5-HT neurotransmission by directly inhibiting the α_2-heteroreceptors located on the 5-HT nerve terminals and indirectly stimulating the α_1 receptors through the inhibition of α_2-autoreceptors [37,44–47].

The blockade of histamine H_1 receptors may also contribute to the increase in NA efflux induced by the combination of aripiprazole and MIR. The NA concentration in perfusates of the paraventricular nucleus (PVN) was significantly increased by addition of the histamine H_1 receptor antagonist triprolidine to the perfusate, indicating that histamine exerted an inhibitory effect on NA release from the hypothalamic nerve terminals in the PVN [48]. Aripiprazole and MIR also show affinities for histamine H_1 receptors [3,22,49]. Thus, the blockade of these receptors by the studied drugs may be another mechanism, in addition to the actions on 5-HT_{1A} and α_2 adrenergic receptors, involved in the increase in NA release in the rat FCX.

Regarding the pharmacokinetic data, studies of human liver microsomes showed that MIR exerted minimal inhibitory effects on the cytochrome P450 isoenzymes, and thus it is not expected to induce clinically significant interactions of aripiprazole with these isoforms [50]. Moreover, the lack of a pharmacokinetic interaction was observed between MIR and the newer antipsychotics in patients with chronic schizophrenia [51,52]. In addition, aripiprazole does not exert significant effects on the pharmacokinetics of the ADs studied to date (ESC, venlafaxine, fluoxetine, paroxetine and sertraline) [53,54]. Thus, the effect of the combined administration of aripiprazole and MIR or ESC on neurotransmitter release in our study appears to be mediated by the activation or blockade of brain monoamine receptors by those drugs, but not by pharmacokinetic interactions.

In summary, the AD (ESC or MIR)-elicited amelioration of the antipsychotic-like effect of aripiprazole on the MK-801-induced cognitive symptoms of schizophrenia in rats may be mediated *via* serotonin 5-HT_{1A} and dopamine D_1 receptors. Moreover, the results of the neurochemical study suggested that the increase in extracellular levels of 5-HT or NA in the cortex evoked by the combined administration of ADs and aripiprazole may be of crucial importance to the pharmacotherapy of negative symptoms and some cognitive symptoms of schizophrenia.

Declaration of Competing Interest

Declarations of interest: none.

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Glutathione Deficiency during Early Postnatal Development Causes Schizophrenia-Like Symptoms and a Reduction in BDNF Levels in the Cortex and Hippocampus of Adult Sprague–Dawley Rats

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Abstract: Growing body of evidence points to dysregulation of redox status in the brain as an important factor in the pathogenesis of schizophrenia. The aim of our study was to evaluate the effects of L-buthionine-(*S*,*R*)-sulfoximine (BSO), a glutathione (GSH) synthesis inhibitor, and 1-[2-Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12909), a dopamine reuptake inhibitor, given alone or in combination, to Sprague–Dawley pups during early postnatal development (p5–p16), on the time course of the onset of schizophrenia-like behaviors, and on the expression of brain-derived neurotrophic factor (BDNF) mRNA and its protein in the prefrontal cortex (PFC) and hippocampus (HIP) during adulthood. BSO administered alone decreased the levels of BDNF mRNA and its protein both in the PFC and HIP. Treatment with the combination of BSO + GBR 12909 also decreased BDNF mRNA and its protein in the PFC, but in the HIP, only the level of BDNF protein was decreased. Schizophrenia-like behaviors in rats were assessed at three time points of adolescence (p30, p42–p44, p60–p62) and in early adulthood (p90–p92) using the social interaction test, novel object recognition test, and open field test. Social and cognitive deficits first appeared in the middle adolescence stage and continued to occur into adulthood, both in rats treated with BSO alone or with the BSO + GBR 12909 combination. Behavior corresponding to positive symptoms in humans occurred in the middle adolescence period, only in rats treated with BSO + GBR 12909. Only in the latter group, amphetamine exacerbated the existing positive symptoms in adulthood. Our data show that rats receiving the BSO + GBR 12909 combination in the early postnatal life reproduced virtually all symptoms observed in patients with schizophrenia and, therefore, can be considered a valuable neurodevelopmental model of this disease.

Keywords: neurodevelopmental model of schizophrenia; schizophrenia-like symptoms; levels of BDNF mRNA and its protein; effect of amphetamine

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1. Introduction

Schizophrenia is a severe chronic mental illness affecting approximately 1% of the world population [1] and characterized by three broad categories of symptoms, namely positive symptoms (delusions, hallucinations, thought disorder, and incoherence) and negative symptoms (lack of motivation and deficits in social function) as well as cognitive impairment (decline of working memory, executive function, learning, long-term memory, visual/auditory perception, and attention) [2–4]. Positive symptoms are the most striking feature of the disease, but cognitive deficits, typically present before the onset of

psychosis [5], are critical determinants of patients' quality of life and their daily functioning [6–8]. The etiology of schizophrenia still remains poorly understood, but according to the dominant hypothesis, it is increasingly recognized as a disease with an important neurodevelopmental component contributing to structural and functional changes in the brain. It is believed that the aberrant neurodevelopmental processes are induced by multiple interactions between the genetic and environmental factors [9] that occur during embryonic or early postnatal development. Detrimental effects of these interactions result in the emergence of cognitive deficits and other symptoms of schizophrenia in adolescence and/or early adulthood [3,10–13].

Although the pathomechanism of schizophrenia is not fully explored, an increasing number of studies indicate oxidative stress, the impaired redox status of cells, and epigenetic regulation disorders as key factors contributing to the pathophysiology of this disease [14–21]. Referring to the impaired redox status in schizophrenia, it has been shown that the level of its main regulator and strong antioxidant, glutathione (GSH), was clearly reduced in the cerebrospinal fluid and medial frontal cortex of drug-naïve schizophrenic patients [22] and also in the post-mortem striatum [23] and the prefrontal cortex of those previously treated with antipsychotic drugs [24]. Decreases in GSH levels were also found in erythrocytes [25–27] and in plasma [26,28,29] of antipsychotic-free schizophrenia patients as well as in those chronically medicated with these drugs. Impairment of GSH synthesis in some patients with schizophrenia has been shown to be linked with polymorphisms in genes encoding both catalytic and modifier subunits of γ-glutamate-cysteine ligase (GCL) [30–32], a key enzyme catalyzing the first stage of the two-step reaction of GSH formation. Furthermore, a significant negative correlation was found between the brain GSH levels and the severity of negative symptoms in schizophrenia patients [33].

The occurrence of schizophrenia-like symptoms [34–39], reminiscent of those observed in patients, has been described in rodents in which the GSH deficit was induced by specific compounds that reduce GSH concentration [40,41]. Behavioral consequences of brain GSH deficit, induced by chronic administration of the GCL inhibitor, L-buthionine-(*S*,*R*)-sulfoximine (BSO), in combination with a dopamine (DA) reuptake inhibitor, the compound 1-[2-Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12909), during early postnatal development (p5–p16) were studied for the first time in Osteogenic disorder Shionogi (ODS) mutant rats, which, like humans, cannot synthesize ascorbic acid [37,38]. In these studies, it was demonstrated that such combined treatment with BSO + GBR 12909 in early postnatal life caused the long-term schizophrenia-like memory deficits, assessed in the novel object recognition test (NOR) in adulthood. Correspondingly, treatment of ODS and Wistar rats during early postnatal life with BSO alone evoked impairments of some cognitive functions under conditions, when learning and discrimination tasks aimed at assessing spatial working memory performance (homing board, radial maze) were tested in the presence of controlled olfactory cues in adulthood [39].

In our recently published study by Górny et al. [42] conducted on Sprague–Dawley rats chronically treated with BSO alone or the BSO + GBR 12909 combination on postnatal days p5 to p16, cognitive deficits, as well as previously unidentified deficits in social behavior, were found in adulthood (p90–p91). However, only in rats treated with the BSO + GBR 12909 combination, the elevated values of exploratory behavior parameters (time of walking, ambulation, peeping, and rearing), which correspond to the positive symptoms in schizophrenia patients, were observed during the 3-min open field test (OFT) in adulthood (p92). The effects described above clearly indicate that inhibition of both GSH synthesis and dopamine reuptake in the early postnatal life leads to the manifestation of behaviors resembling positive symptoms of schizophrenia in adulthood, while inhibition of GSH synthesis alone at this stage of development is sufficient for the disclosure of deficits in the social behavior and cognitive functions.

The aim of the present study was to further characterize this neurodevelopmental rat model of schizophrenia and to establish at what time point after administration of BSO

alone or in combination with GBR 12909 to Sprague–Dawley pups, the first episodes of schizophrenia-like behavior appear. To address these issues, schizophrenia-like behaviors, corresponding to negative and positive symptoms as well as to cognitive deficits, were assessed using behavioral tests (social interaction test, SIT; NOR; OFT) at three time points of adolescence (p30, p42–p44, p60–p62) as well as in adulthood (p90–p92). In schizophrenia patients, it has been shown that the increased release of DA in the striatum induced by amphetamine (AMF), as determined by means of position-emission tomography, was associated with the appearance or exacerbation of positive symptoms [43–45]. Therefore, to investigate whether Sprague–Dawley rats given the BSO + GBR 12909 combination during the early postnatal life respond behaviorally in a similar way as patients with schizophrenia, we measured the AMF-stimulated locomotor activity for 30 min using actometers in adulthood, as an indicator of exacerbation of positive symptoms. In addition, to investigate whether the inhibition of GSH synthesis and dopamine reuptake in early postnatal life could affect the brain-derived neurotrophic factor (BDNF), we examined both BDNF mRNA and protein levels in the prefrontal cortex (PFC) and the hippocampus (HIP) in 93-day-old rats after completion of the last series of behavioral tests. In the brain, BDNF is an important signaling molecule responsible for neuronal growth, maturation of synapses during development, and synaptic plasticity [46–48], which is essential for learning and memory processes [49,50]. It is worth noting that administration of BSO in the early postnatal days (p5–p16) coincided with the developmental switch in the action of GABA, through $GABA_A$ receptors, from excitatory to inhibitory [51], as well as with a peak of synaptogenesis and the formation of adult neuronal networks [52]. In the rat brain, the critical period of increased excitability that takes place during the second postnatal week is within the temporal window of excitatory action of GABA [53,54]. Interestingly, a growing body of experimental evidence indicates that during this period, the excitatory action of GABA participates in the BDNF-mediated signaling and induction of synaptic plasticity in the developing hippocampus [55–58]. On the other hand, it has been demonstrated that $GABA_A$ receptor agonists inhibit BDNF expression [59–61]. In line with these facts, the formation of normal neuronal connections during early brain development depends on a precise balance between excitation and inhibition. Therefore, it is reasonable to assume that even a small impairment of this process may lead to developmental abnormalities. However, till now, it has not been explored whether inhibition of GSH synthesis and consequent impairment of the redox status of brain cells early in postnatal life [62], when GSH concentration in this tissue is the highest [63], might interfere with the excitatory action of GABA during this time, causing changes in BDNF expression in the PFC and HIP visible in adulthood. Hence, it seems that the determination of BDNF levels in the groups of rats receiving BSO may help answer this question and explain the link between the GSH deficit in early postnatal life and the occurrence of negative symptoms and memory deficits in adulthood. We hope that the obtained results bring a new quality to the search for the most adequate animal model of schizophrenia.

2. Results

2.1. The Impact of Chronic Administration of BSO and GBR 12909 during the Early Postnatal Life on the Development of Social Deficits in Adolescence and Adulthood

Social behavior was assessed by means of two parameters, i.e., the total time spent by two rats in social behavior and the number of these interactions. Figure 1A,B shows the time-dependent changes in the expression of these two parameters during adolescence and adulthood of rats that were treated chronically with BSO and GBR 12909 alone or jointly during early postnatal life (p5–p16).

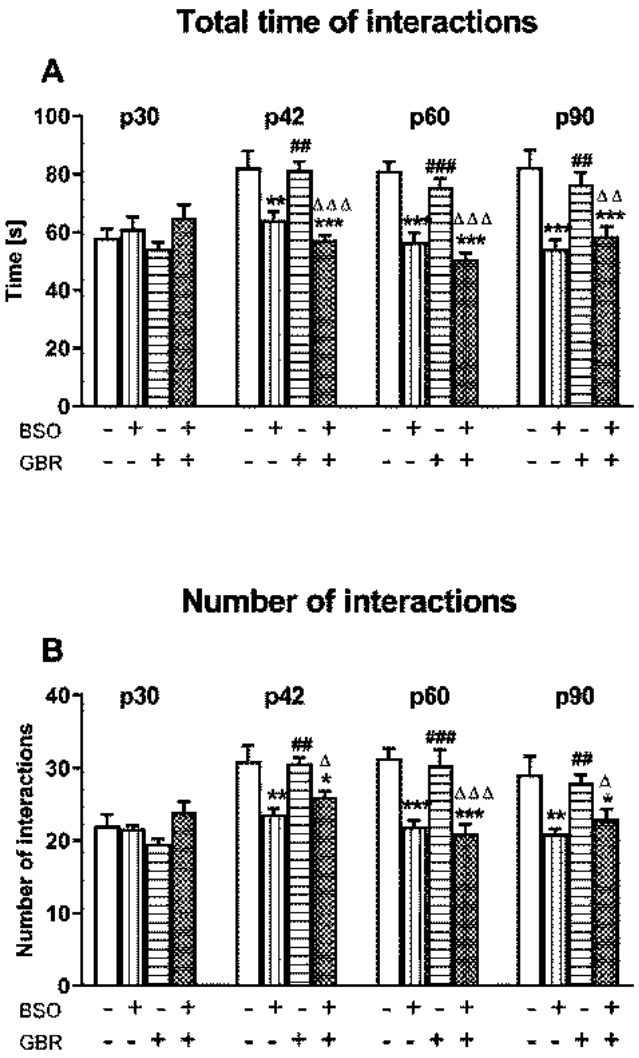

Figure 1. The effect of chronic administration of BSO and GBR 12909, alone or in combination, during postnatal days p5–p16 on the social behavior assessed as the total time spent in social interactions (**A**) and the number of interactions (**B**) in adolescent and adult Sprague–Dawley rats. Data are presented as the mean ± SEM, n = 16 (8 pairs) for each group. Statistical analysis was performed using a two-way ANOVA; symbols indicate significance of differences according to the Newman–Keuls post hoc test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control; $^{###}$ $p < 0.001$, $^{##}$ $p < 0.01$ vs. BSO- and $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta\Delta}$ $p < 0.01$, $^{\Delta}$ $p < 0.05$ vs. GBR 12909-treated groups.

A two-way ANOVA performed for the total time spent in social interactions revealed an overall treatment effect of BSO (for p42—$F_{(1,28)} = 36.327$, $p < 0.001$; for p60—$F_{(1,28)} = 70.315$, $p < 0.001$; for p92—$F_{(1,28)} = 30.258$, $p < 0.001$) but a lack of GBR 12909 treatment effect and no interaction between these two model substances in any of the studied time points (Figure 1A). Similarly, a two-way ANOVA carried out for the number of social interactions showed only a significant treatment effect of BSO (for p42—$F_{(1,28)} = 20.747$, $p < 0.001$; for p60—$F_{(1,28)} = 38.099$, $p < 0.001$; for p92—$F_{(1,28)} = 17.648$, $p < 0.001$) on this parameter at three out of four studied time points (Figure 1B).

Post hoc comparisons of the effects of model compounds on the studied parameters showed that BSO administered alone or in combination with GBR 12909 shortened the total time spent in social interactions (Figure 1A) as well as decreased the number of these interactions (Figure 1B) for the first time in rats that reached the age of 42 days. These effects were still present in late adolescence (at p60) and in adulthood (at p90). In contrast to BSO, administration of GBR 12909 alone during early postnatal life did not evoke changes in the

measured parameters of social behavior at any of the studied time points of adolescence and adulthood.

2.2. The Impact of Chronic Administration of BSO and GBR 12909 during the Early Postnatal Life on the Development of Cognitive Deficits in Adolescence and Adulthood

The NOR test serving to evaluate cognitive impairments in rodents was performed on the next day after SIT in all studied groups at each time point (Figure 2).

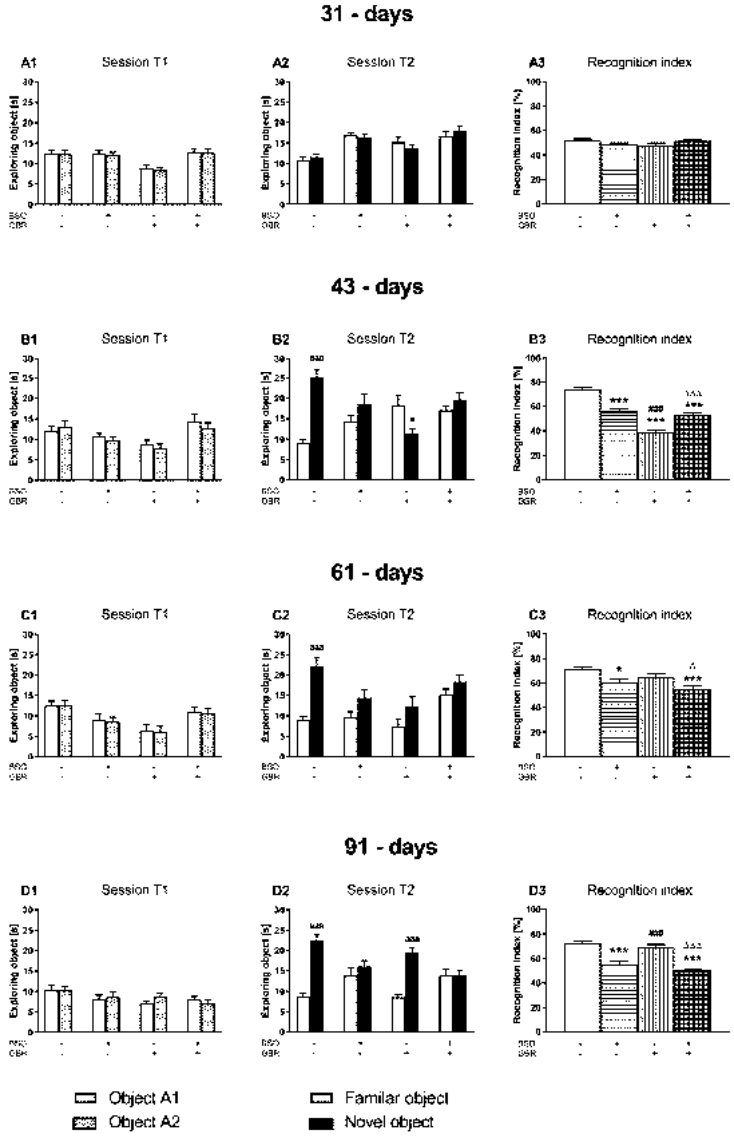

Figure 2. The effect of chronic administration of BSO and GBR 12909, alone or in combination, during postnatal days p5–p16 on cognitive functions assessed in adolescent (**A**–**C**) and adult (**D**) Sprague–Dawley rats. (**A1**–**D1**) The effects of the studied model compounds on the exploration of two identical objects in the acquisition trials (session T1). (**A2**–**D2**) The effects of the studied model compounds on the exploration of a novel and familiar object in the retention trial (Session T2). (**A3**–**D3**) The effects of the studied model compounds on the recognition index. Data are presented as the mean ± SEM, $n = 10$ for each group. Letters indicate statistically significant differences between the exploration time of a novel and familiar object in the session T2 within each studied group, according to the Student's *t*-test for independent samples, ^{aaa} $p < 0.001$, ^{a} $p < 0.05$ vs. familiar object. Statistical analysis of the recognition index was performed using a two-way ANOVA; symbols indicate significance of differences according to the Newman–Keuls post hoc test, *** $p < 0.001$, * $p < 0.05$ vs. control; $^{###}$ $p < 0.001$ vs. BSO- and $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta}$ $p < 0.05$ vs. GBR 12909-treated groups.

During the acquisition trial (session T1), all rats representing particular groups spent equal time exploring two identical objects (Figure 2A1,A2). In the retention trial (session T2) adolescent control 43- and 61-day-old, as well as adult 91-day-old control rats, but not 31-day-old animals, explored the novel object significantly longer than the familiar one. (Figure 2A2–D2). Interestingly, in 43-day-old rats that were treated with GBR 12909 alone in early postnatal life (p5–p16), exploration of the familiar object was markedly less intensive than the novel one (Figure 2B2). However, in 61-day-old rats treated with GBR 12909, an increasing tendency toward the exploration of the novel object was observed, while in 91-day-old ones, the time of exploration of the novel object was significantly longer than the familiar one, like in controls (Figure 2C2,D2).

Two-way ANOVA performed in groups of adolescent 31-day-old rats demonstrated neither the effects of the tested model compounds nor their combination on the values of the recognition indexes (Figure 2A3). However, this analysis performed for the recognition index in adolescent 42-day-old rats (Figure 2B3) revealed a significant treatment effect of GBR 12909 ($F_{(1,36)} = 109.69$, $p < 0.001$) and an interaction of BSO × GBR 12909 ($F_{(1,36)} = 79.341$, $p < 0.001$), but a lack of treatment effect of BSO alone ($F_{(1,36)} = 0.989$, NS). However, in adolescent 61-day-old rats a two-way ANOVA showed both significant treatment effect of BSO ($F_{(1,36)} = 14.797$, $p < 0.001$) and GBR 12909 ($F_{(1,36)} = 5.232$, $p < 0.05$) but no interaction between these model compounds ($F_{(1,36)} = 0.041$, NS) (Figure 2C3). Furthermore, in adult 91-day-old rats only treatment effect of BSO ($F_{(1,36)} = 71.530$, $p < 0.001$) was observed (Figure 2D3). Post hoc comparison showed that values of the recognition indexes in group of rats treated with BSO alone or in combination with GBR 12909 were significantly decreased compared to the value of this parameter in the control group in adolescent (p43, p61) and in adult rats (p91).

2.3. The Impact of Chronic Administration of BSO and GBR 12909 during the Early Postnatal Life on the Manifestation of Positive Lescence and Adulthood

On the 32nd, 44th, 62nd, and 92nd days of postnatal life, the exploratory activity in the open field test (time of walking, ambulation, peeping, and rearing) was determined in all studied groups of rats as a measure of positive symptoms (Figure 3A–C).

A two-way ANOVA performed for the time of walking revealed a significant treatment effect of BSO in middle and late adolescence and in adulthood (for p44 ($F_{(1,36)} = 4.563$, $p < 0.05$); for p62 ($F_{(1,36)} = 39.160$, $p < 0.0001$); for p92 ($F_{(1,36)} = 52.678$, $p < 0.0001$)) but not in early adolescence (for p32 ($F_{(1,36)} = 0.277$, NS). This analysis also demonstrated a significant treatment effect of GBR 12909 on this parameter, at all time points (for p32 ($F_{(1,36)} = 4.429$, $p < 0.05$); for p44 ($F_{(1,36)} = 5.003$, $p < 0.05$); for p62 ($F_{(1,36)} = 6.006$, $p < 0.05$)) except the adult 92-day-old rats ($F_{(1,36)} = 0.277$, NS). Furthermore, a significant interaction of BSO and GBR 12909 regarding the time of walking was found at all studied time points (for p32 ($F_{(1,36)} = 4.429$, $p < 0.05$); for p44 ($F_{(1,36)} = 18.904$, $p < 0.001$); for p62 ($F_{(1,36)} = 83.917$, $p < 0.001$); for p92 ($F_{(1,36)} = 67.522$, $p < 0.0001$)). Post hoc comparisons showed that the combined treatment with these model compounds resulted in the extension of walking time in the adolescent 44-, 62- and adult 92-day-old rats when compared to rats treated chronically with vehicle (control), BSO or GBR12909 alone during early postnatal life (Figure 3A). Such effect of the combined treatment was not observed in adolescent, 32-days old rats. On the other hand, in the group of rats receiving GBR 12909 alone, the time of walking was shortened at postnatal days p32, p62 and p92, but not at p44, compared to the control.

As to the second parameter, measured in OFT, i.e., the number of sector crossings, a two-way ANOVA also demonstrated a significant effect of BSO treatment in adolescent and adult rats ((for p44 ($F_{(1,36)} = 8.510$, $p < 0.01$); for p62 ($F_{(1,36)} = 7.339$, $p < 0.001$); for p92 ($F_{(1,36)} = 32.777$, $p < 0.0001$)) as well as interaction of BSO × GBR 12909 for two time points ((for p62 ($F_{(1,36)} = 28.006$, $p < 0.001$); for p92 ($F_{(1,36)} = 18.836$, $p < 0.0001$)). At none of the time points examined, a two-way ANOVA showed an impact of GBR 12909 treatment on the number of sector crossings. Post hoc analysis revealed that in rats administered BSO + GBR 12909 at postnatal days p5–p16, the number of sector crossings was significantly increased

at p44, p62 and p92 when compared to groups treated with the vehicle (control), BSO or GBR 12909 alone (Figure 3B).

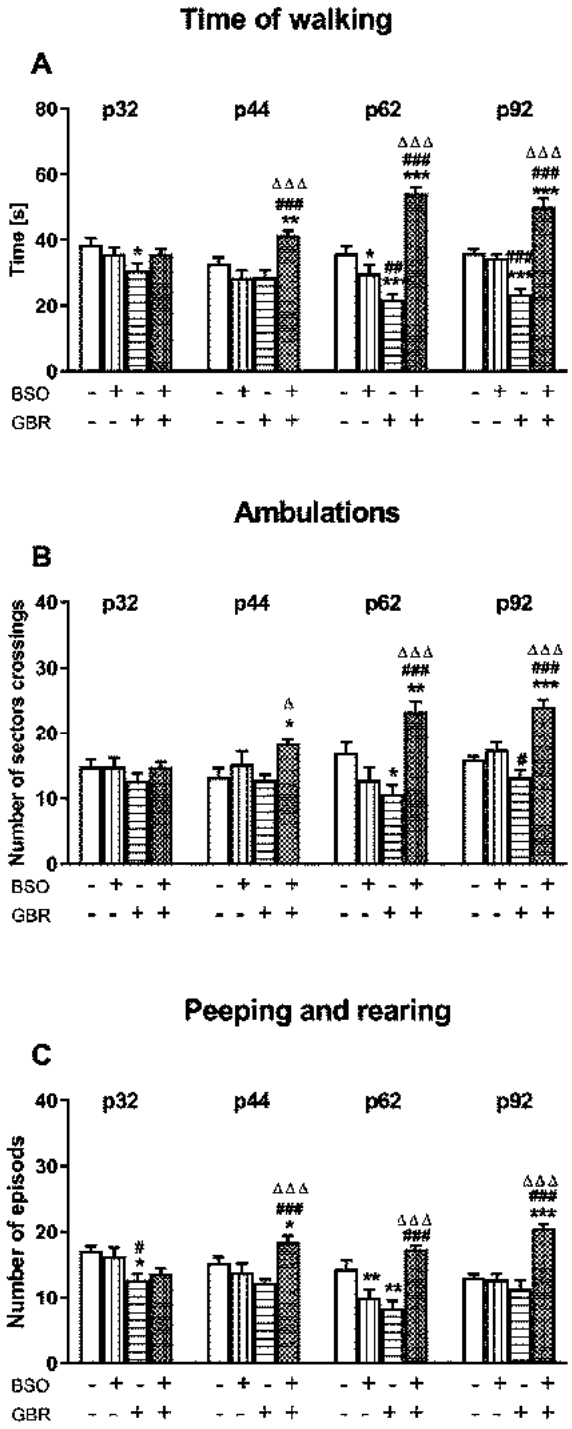

Figure 3. The effect of chronic administration of BSO and GBR 12909, alone or in combination, during postnatal days p5–p16 on positive symptoms assessed in the OFT in adolescent and adult Sprague–Dawley rats as: (**A**) the time of walking (**B**) the number of sector crossings (**C**) the number of peeping and rearing episodes. Data are presented as the mean ± SEM, $n = 10$ for each group. Statistical analysis was performed using a two-way ANOVA; symbols indicate significance of differences according to the Newman–Keuls post hoc test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control; ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ vs. BSO and $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta}$ $p < 0.05$ vs. GBR 12909-treated groups.

Regarding the third parameter measured in the OFT, i.e., the number of peeping and rearing episodes, a two-way ANOVA revealed a significant treatment effect of BSO ((for p44 ($F_{(1,36)}$ = 5.586, $p < 0.05$); for p62 ($F_{(1,36)}$ = 4.464, $p < 0.05$); for p92 ($F_{(1,36)}$ = 24.027, $p < 0.0001$)) as well as an interaction of BSO × GBR 12909 ((for p44 ($F_{(1,36)}$ = 14.004, $p < 0.001$); for p62 ($F_{(1,36)}$ = 36.760, $p < 0.001$); for p92 ($F_{(1,36)}$ = 27.376, $p < 0.0001$)). This analysis also showed a significant treatment effect of GBR 12909 for early adolescence (for p32 ($F_{(1,36)}$ = 4.429, $p < 0.05$) and for adulthood (for p92 ($F_{(1,36)}$ = 11.287, $p < 0.002$). Post hoc analysis demonstrated that at conditions of the combined administration of BSO + GBR 12909 at postnatal days p5–p16, the number of peeping and rearing episodes in adolescence (p44, p62) and adulthood (p92) was significantly higher than in groups that were treated with the vehicle (control), BSO or GBR 12909 alone during early postnatal life (Figure 3C).

2.4. The Impact of Chronic Administration of BSO and GBR 12909 during the Early Postnatal Life on the Spontaneous and AMF-Induced Locomotor Activity and Stereotypy in Adult Rats Determined Using Actometers

In order to find an association between GSH deficiency in early postnatal development (p5–p16) and hyperfunction of dopaminergic transmission and schizophrenia-like positive symptoms in adulthood, AMF at a single dose of 1 mg/kg was given to 90-day-old Sprague–Dawley adults previously treated with vehicle, BSO, GBR 12909 or BSO + GBR 12909. The effects of this DA-releasing stimulant on the total horizontal and vertical locomotor activity and on stereotypical behavior were compared with the effects of single saline injections in analogous groups of rats (Figure 4).

A two-way ANOVA performed for the total distance traveled in groups of rats receiving in adulthood a single dose of saline (Figure 4A) showed a significant treatment effect of BSO ($F_{(1,28)}$ = 11.268, $p < 0.01$), but a lack of treatment effect of GBR 12909 ($F_{(1,28)}$ = 3.360, NS) and no interaction between these two compounds ($F_{(1,28)}$ = 1.031, NS). However, the same analysis carried out for this parameter in rats receiving a single dose of AMF in adulthood, revealed significant treatment effects both of BSO ($F_{(1,28)}$ = 48.068, $p < 0.0001$) and GBR 12909 ($F_{(1,28)}$ = 20.797, $p < 0.0001$) as well as an interaction between these model compounds ($F_{(1,28)}$ = 19.459, $p < 0.0001$). Post hoc comparison of spontaneous horizontal locomotor activity, defined as the total distanced traveled, in four groups of rats treated with model compounds at postnatal days p5–p16 and receiving single doses of saline in adulthood, showed that this parameter increased significantly, only in the group receiving BSO + GBR 12909 combination when compared to the control and GBR 12909-treated group. The same comparison performed for rats receiving single doses of AMF in adulthood (Figure 4A) showed that the total distances traveled in all these groups were significantly longer than in the corresponding saline-treated rats. However, the most pronounced increase in the value of this parameter was observed in the BSO + GBR 12909 treated group receiving a single dose of AMF when compared to the same group of rats injected with saline. The latter effect means that AMF exacerbates the expression of locomotor activity, particularly in the BSO + GBR-treated group of rats.

As to the vertical locomotor activity, i.e., the total time spent on climbing, a two-way ANOVA showed only a significant treatment effect of BSO for a set of groups receiving either a single injection of saline ($F_{(1,28)}$ = 6.218, $p < 0.02$) or AMF ($F_{(1,28)}$ = 10.457, $p < 0.01$) in adulthood. Post hoc analysis of the total time spent on climbing in four groups of rats receiving a single dose of saline in adulthood showed that only in the group treated with BSO + GBR the value of this parameter was significantly higher than in the control and groups receiving only BSO or GBR 12909 (Figure 4B). A single dose of AMF increased the climbing time in all studied groups, but the strongest effect was found in the group treated with the BSO + GBR combination (Figure 4B). The latter effect clearly indicates that AMF, in addition to horizontal activity, also intensified vertical locomotor activity in the BSO + GBR-treated group.

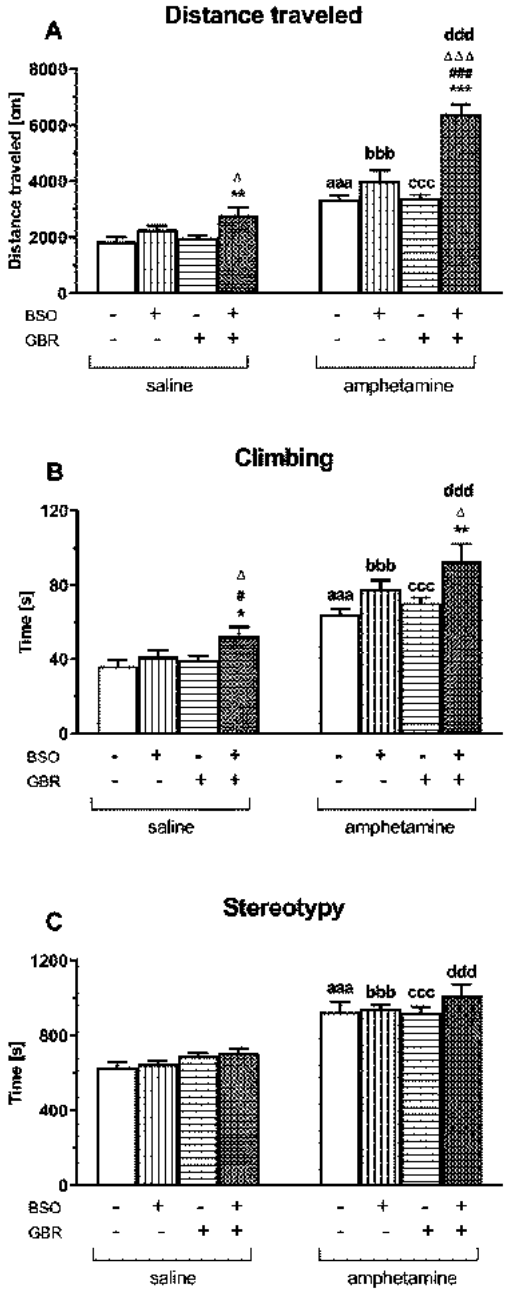

Figure 4. The effect of chronic administration of BSO and GBR 12909, alone or in combination, during postnatal days p5–p16, on the spontaneous and AMF-induced locomotor activity and stereotypy, measured in 90-day-old Sprague–Dawley rats using actometers. (**A**) Horizontal locomotor activity is presented as the total distance traveled expressed in cm, (**B**) vertical locomotor activity is shown as the total time spent climbing expressed in seconds (s), and (**C**) stereotypy as the total time devoted to stereotypical behavior expressed in seconds. These parameters were recorded during a 30–minute measurement session. Data are presented as the mean ± SEM, $n = 10$ for each group. Statistical analysis was performed using a two-way ANOVA; symbols indicate significance of differences according to the Newman–Keuls post hoc test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control; ### $p < 0.001$, # $p < 0.05$ vs. BSO-; and $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta}$ $p < 0.05$ vs. GBR 12909-treated groups. Comparisons between the corresponding groups treated with saline or AMF were performed using the Student's *t*-test for independent samples, ^{aaa} $p < 0.001$ vs. saline-treated control, ^{bbb} $p < 0.001$ vs. saline-treated BSO group; ^{ccc} $p < 0.001$ vs. saline-treated GBR 12909 group; ^{ddd} $p < 0.001$ vs. saline-treated BSO + GBR 12909 group.

A two-way ANOVA performed for the time of stereotypical behavior, expressed in seconds, in groups of rats receiving a single dose of saline in adulthood (Figure 4C), revealed only a significant treatment effect of GBR 12909 ($F_{(1,28)}$ = 6.284, $p < 0.05$), but a lack of treatment effect of BSO ($F_{(1,28)}$ = 0.575, NS) and no interaction between these two compounds ($F_{(1,28)}$ = 0.001, NS). However, a post hoc analysis did not show any differences in the values of this parameter between the studied groups (Figure 4C). As to the time of stereotypical in four groups of rats receiving a single dose of AMF in adulthood, a two-way ANOVA demonstrated neither treatment effects of BSO ($F_{(1,28)}$ = 1.622, NS) or GBR 12909 ($F_{(1,28)}$ = 0.551, NS) alone nor an interaction between these two model compounds ($F_{(1,28)}$ = 0.711, NS). However, comparisons using the Student's t-test for independent samples, regarding the time of stereotypic behavior between the corresponding controls, BSO-, GBR 12909 and BSO + GBR 12909 groups administered a single dose of saline or AMF during adulthood, showed that AMF increased the duration of stereotypy in all studied groups (Figure 4C).

2.5. The Impact of Chronic Administration of BSO and GBR 12909 during the Early Postnatal Life on BDNF mRNA and Protein Levels in the Prefrontal Cortex and Hippocampus of Adult Rats

A two-way ANOVA performed for BDNF mRNA expression in the prefrontal cortex of adult rats receiving saline, BSO, GBR 12909 or BSO + GBR 12909 during early postnatal life revealed a significant treatment effect of BSO ($F_{(1,28)}$ = 11.806, $p < 0.002$), a lack of treatment effect of GBR 12909 ($F_{(1,28)}$ = 3.5876, p = 0.07) and an interaction between these compounds ($F_{(1,28)}$ = 7.405, $p < 0.01$). The same analysis performed for hippocampal samples demonstrated only a significant interaction between BSO and GBR 12909 ($F_{(1,28)}$ = 22.516, $p < 0.0001$), but no treatment effects of these model compounds when administered alone (for BSO $F_{(1,28)}$ = 0.220, NS; for GBR 12909 $F_{(1,28)}$ = 0.387, NS) (Figure 5).

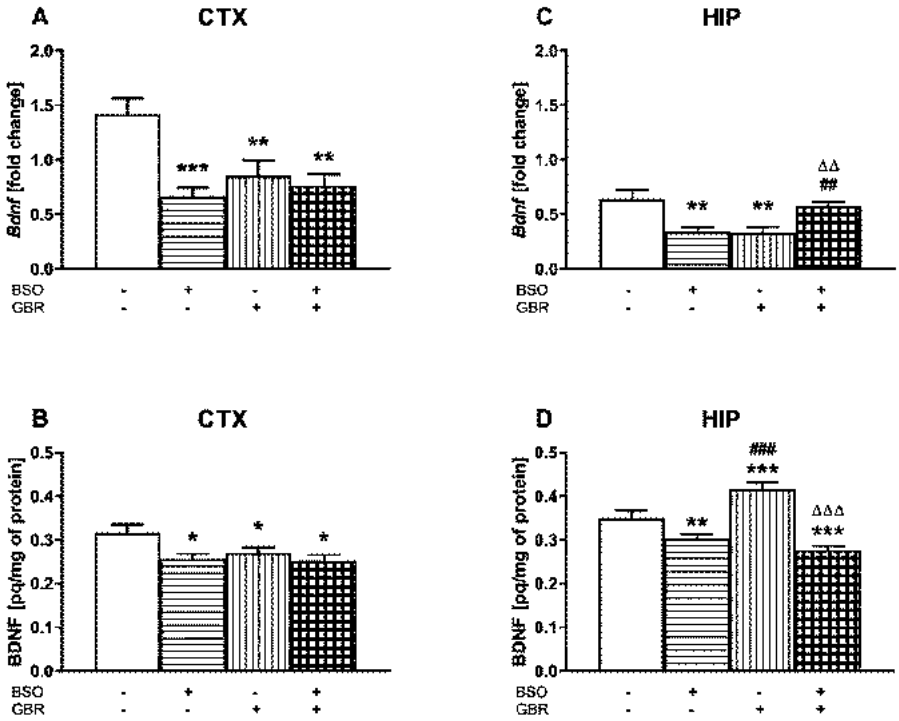

Figure 5. The effect of chronic administration of BSO and GBR 12909, alone or in combination, during postnatal days p5–p16, on BDNF mRNA and protein levels in the PFC (**A**,**C**) and HIP (**B**,**D**) of adult rats. Data are presented as the mean ± SEM, n = 7–8 for each group. Statistical analysis was performed using a two-way ANOVA; symbols indicate significance of differences according to the Newman–Keuls post hoc test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control; ### $p < 0.001$, ## $p < 0.01$, vs. BSO-; and $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta\Delta}$ $p < 0.01$ vs. GBR 12909-treated groups.

In the PFC, a post hoc comparison showed that the levels of BDNF mRNA in BSO-, GBR 12909, and BSO + GBR-treated groups were significantly lower than in the control (Figure 5A). In the HIP, BDNF mRNA levels in the groups of rats administered only BSO or GBR 12909 alone were also lower than in the control group, but after the combined treatment (BSO + GBR 12909), the content of BDNF mRNA was significantly higher than in groups receiving model compounds separately and was almost the same as in the control (Figure 5C).

Regarding BDNF protein levels in the PFCs of adult rats chronically administered saline, BSO, GBR 12909 or BSO + GBR 12909 early in postnatal life, a two-way ANOVA showed a significant treatment effect of BSO ($F_{(1,24)} = 6.745$, $p < 0.05$) but no effect of GBR 12909 ($F_{(1,24)} = 1.876$, NS) as well as no interaction between these model compounds ($F_{(1,24)} = 2.732$, NS). The same analysis performed for the BDNF protein in the HIP revealed a significant effect of BSO treatment ($F_{(1,24)} = 60.719$, $p < 0.0001$) a lack of treatment effect of GBR 12909 ($F_{(1,24)} = 2.817$, NS) and a significant interaction between these compounds ($F_{(1,24)} = 14.716$, $p < 0.001$).

Post hoc comparisons of the studied groups demonstrated that the levels of BDNF protein in the PFC of adult rats receiving during BSO, GBR, or the combination of BSO + GBR 12909 postnatal days 5–16 were significantly reduced vs. the control (Figure 5B). The same comparisons performed in the HIP showed that BSO alone or in combination with GBR 12909 significantly reduced while GBR 12909 alone increased the levels of BDNF protein in this structure (Figure 5D).

3. Discussion

In the present study, a comprehensive analysis of the time-dependent onset of schizophrenic-like symptoms in Sprague–Dawley rats in which both GSH synthesis and dopamine (DA) reuptake were inhibited by BSO and GDR 12909, respectively, during the early postnatal life, was performed based on behavioral tests (SIT, NOR, OFT). The long-term effects of these model substances administered alone or in combination clearly showed that the first deficits in social behavior and cognitive functions appeared in mid-puberty (p42–p43) and were still present in adulthood (p90–p91) both in the group of rats receiving BSO alone or a combination of BSO + GBR 12909. However, only rats treated with BSO + GBR 12909 showed the increased exploratory behavior assessed in the OFT as the time of walking, the number of sector crossings (ambulation) as well as the number of peeping and rearing episodes that are considered to be equivalent to positive symptoms in patients with schizophrenia. They appeared for the first time on the postnatal day p44 and continued to manifest into adulthood (p92). Unlike the groups treated with BSO alone or with the BSO + GBR 12909 combination, rats receiving GBR 12909 alone in early postnatal life showed no deficits in social behavior, as measured by the total interaction time and the number of interactions, both during adolescence and early adulthood. Regarding cognitive functions in rats treated with GBR 12909, a significant transient decrease in the value of a recognition index, which is a measure of these functions, was only observed on p43, followed by recovery of the recognition index value to the control group level on days p61 and p91.

In order to introduce the principles of cognitive function assessment based on the NOR test, it should be reminded that this test is based on the spontaneous tendency of rats to investigate objects and to favor novel objects versus familiar ones. In our study, rats receiving BSO alone early in postnatal life failed to discriminate between familiar and novel objects at all time points studied. Since basal exploratory activity, as assessed by the OFT, was preserved in this group of rats at virtually all time points, significant decreases in the value of recognition index during adolescence (p43, p61) and early adulthood (p92) can be attributed to cognitive impairment.

In contrast to the BSO-treated group, rats administered GBR 12909 alone early in the postnatal life examined the novel object much less intensively than the familiar one only in the middle adolescence (p43). However, as the basal exploratory activity measured in

the OFT remained at the level of the control group, therefore, a significant decrease in the recognition index value at p43 can also be attributed to cognitive impairment. Furthermore, during subsequent adolescence and adulthood, the interest of the GBR 12909-treated rats in the investigation of the novel object gradually increased. This increased interest in studying the novel object compared to the old one was the greatest on p91 and occurred despite the significant reduction in rat exploratory activity observed in the OFT. The latter behavioral data under discussion indicate that a transient cognitive decline on p43, induced by disturbances only in the dopaminergic transmission during early postnatal life, may be gradually compensated in late adolescence (p61), eventually reaching cognitive normalization in early adulthood (p91).

In contrast, rats receiving the BSO + GBR12909 combination in early postnatal life, like those receiving BSO alone, were unable to distinguish between familiar and novel objects in the NOR test performed in adolescence and adulthood. However, the exploratory activity measured in the OFT as the time of walking was significantly increased in rats receiving the BSO + GBR combination compared to the control group and those receiving BSO- or GBR 12909 alone. Thus, the presence of cognitive impairment, assessed in the group of rats receiving the BSO + GBR 12909 combination based on the recognition index in adolescence and early adulthood, may be a consequence of the increased dopaminergic transmission that occurred under conditions of inhibited GSH synthesis in the early postnatal life. The mechanism and the underlying neuronal basis of the BSO + GBR 12909-mediated cognitive impairment remains to be elucidated. In conclusion, the observed disturbances in the NOR test in rats treated with BSO alone or BSO + GBR combination are in line with the decreased object recognition capacity of schizophrenic patients as compared to healthy control subjects [64–66].

Simultaneously with the occurrence of cognitive disturbances, in the group of rats treated with BSO + GBR 12909, behaviors corresponding to positive symptoms observed in patients with schizophrenia were also found. The presence of positive symptoms of schizophrenia in this group of rats was confirmed in the previously described OFT, and in an additional experiment carried out on 91-day-old adult rats, in which the horizontal (distance travel) and vertical (climbing) locomotor activities were measured using actometers as equivalents of these symptoms. These motor activity parameters in the BSO + GBR 12909-treated rats were significantly enhanced compared to groups treated with saline, BSO, or GBR 12909 alone, respectively, in the early postnatal life. However, the most important effect was that only in the group of rats receiving the BSO + GBR 12909 combination, AMF given in adulthood exacerbated the positive symptoms compared to the corresponding group of rats receiving saline instead of AMF. This AMF-mediated exacerbation of already elevated motor parameters in rats treated with the BSO + GBR 12909 combination clearly indicates that in this neurodevelopmental rat model of schizophrenia, like in schizophrenia patients [43–45], AMF may worsen the existing positive symptoms. These behavioral data also show that Sprague–Dawley rats treated with the combination BSO + GBR 12909 at early postnatal days (p5–p16) reproduce virtually all the symptoms seen in schizophrenia patients and can, therefore, be considered a valuable neurodevelopmental model of schizophrenia for studying the efficacy of antipsychotic drugs.

Since BDNF has an established role in neuronal development and synaptogenesis [50,67] and is an important modulator of monoaminergic and GABA-ergic neurotransmitter systems [68], in groups of rats chronically treated with BSO and GBR 12909 alone or in combination in the early postnatal period, we decided to test the long-term effects of this treatment on BDNF mRNA and its protein levels in the PFC and HIP in adulthood. Our results show that in the PFC, administration of BSO alone or the combination of BSO + GBR 12909 resulted in decreases in both BDNF mRNA and protein levels in adulthood. In the HIP, in the group of rats receiving only BSO, just as in the PFC, the decrease in the level of BDNF mRNA was accompanied by a decrease in its protein content. However, in the HIP of BSO + GBR-treated rats, despite no changes in the level of BDNF mRNA, a significant decrease in BDNF protein level was observed both when compared to the control and

GBR-12909-treated groups. Interestingly, in the group of rats receiving GBR 12909 alone in early postnatal life, in which no social and cognitive deficits were revealed in adulthood, the levels of BDNF mRNA and its protein in the PFC were significantly reduced, while in the HIP despite a significant decrease in BDNF mRNA a relatively large increase in BDNF protein was observed compared to the control group and that treated with BSO alone. The latter results show that GBR 12909 administered alone in the early postnatal period modulates BDNF protein expression in adulthood in an inverse manner, reducing its level in the PFC and simultaneously increasing it in the HIP. In addition, these results suggest that in the group receiving GBR 12909 alone, the increase in the BDNF protein level in the HIP may be of compensatory nature, ultimately leading to the normalization of cognitive functions. However, such a compensatory effect at the BDNF protein level did not occur in the rat HIP when BSO and GBR 12909 were administered in combination. Our data suggest that redox imbalance [14,62,69,70], as a result of repeated treatment with a GSH synthesis inhibitor (BSO) administered alone or in combination with a DA reuptake inhibitor (GBR 12909) during early postnatal development, may be an important factor reducing the expression of BDNF protein in the PFC and HIP in adulthood. In addition, in another model of schizophrenia [71] in rats with a neonatal ibotenic lesion of the ventral HIP, a reduction in the level of BDNF mRNA in the PFC and HIP was demonstrated [72]. In general, in other animal studies, it was shown that some early life events could produce long-lasting effects on processes to which neurotrophins contribute, thereby affecting neuronal maturation and plasticity in later periods of life [68,73,74].

According to the dopamine hypothesis of schizophrenia, it is postulated that the hypofunction of the cortical and prefrontal dopamine systems contributes to negative symptoms and cognitive deficits and that the subcortical and limbic dopamine system hyperactivity causes positive symptoms of schizophrenia [75–77]. In our study, it is difficult to explain the appearance of social and cognitive deficits in adolescence and early adulthood in the group of rats receiving BSO alone during the early postnatal life, referring only to the above-mentioned dopamine hypothesis of schizophrenia. These data show that their occurrence in the BSO-treated group may be related to disturbances in other neurotransmitter systems rather than the dopamine system [78]. Since the inhibition of GSH synthesis may impact the function of NMDA receptors [14,62,69], it seems that impairment of the excitation-inhibition balance during the early postnatal life plays a decisive role in the occurrence of social and cognitive deficits in the BSO-treated rats later in life. Our neurodevelopmental rat model of schizophrenia induced by chronic BSO treatment in the early postnatal days seems to be consistent with the NMDA receptor insufficiency model of schizophrenia postulated previously by Carlsson [79]. To validate the rat model of schizophrenia presented here in screening drugs that can be used in the therapy, we recently showed that chronic per os administration of the antioxidant *N*-acetylcysteine at a dose of 30 mg/kg to adult rats treated with BSO resulted in the reversal of social and cognitive deficits assessed by the SIT and NOR tests (data in preparation). In this model, antipsychotic drug aripiprazole administered chronically (0.3 and 1 mg/kg i.p.) was also effective in reversing social and cognitive deficits in adulthood [80]. Although in the BSO-treated rats it is difficult to determine the role of dopaminergic transmission in the development of social and cognitive deficits in adulthood, in the group of rats receiving the BSO + GBR 12909 combination in early postnatal life, the contribution of in the dopaminergic transmission in shaping them should be seriously considered especially because the changes in rats' behavior corresponding to positive symptoms in patients with schizophrenia were observed only in the latter group.

Furthermore, only in this group of rats, AMF exacerbated the existing positive symptoms. The dopamine hypothesis of schizophrenia has been linked to hippocampal hyperactivity. Dopamine released in the hippocampus improves the performance of some specific cognitive tasks at higher concentrations but has detrimental effects at levels above the optimal range [81,82]. The effect induced by AMF in the BSO + GBR 12909-treated group is consistent with the above-presented data.

4. Conclusions

Our data show that redox status disturbances caused by chronic treatment with BSO alone during early postnatal life lead to social and cognitive deficits that appeared in the middle adolescence stage and continued to occur into adulthood, as well as to the decreases in BDNF mRNA and its protein levels in the PFC and HIP in adulthood. When redox status disturbances were accompanied by disruption of dopaminergic transmission induced by GBR 12909 treatment during early postnatal life, in addition to social and cognitive deficits, rats were shown to develop behaviors corresponding to the positive symptoms in humans. In the latter case, the decreases in BDNF mRNA and its protein levels were observed only in the PFC, but in the HIP, only the level of BDNF protein was decreased. These results suggest a causal relationship between BDNF deficiency and the occurrence of schizophrenia-like symptoms in this neurodevelopmental rat model of schizophrenia.

5. Materials and Methods

The experiments were carried out in compliance with the Act on Experiments on Animals of 21 January, 2005 reapproved on 15 January, 2015 (published in Journal of Laws no 23/2015 item 266, Poland), and according to the Directive of the European Parliament and of the Council of Europe 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. The studies received also an approval of the Local Ethics Committee at the Institute of Pharmacology, Polish Academy of Sciences (permission no 3/2018 of January 2018). All efforts were made to minimize the number and suffering of animals used.

5.1. Animals and Treatment

To create the neurodevelopmental model of schizophrenia, pregnant Sprague–Dawley females at embryonic day 16 were delivered to our laboratory by the Charles River Company (Sulzfeld, Germany). They were kept in individual cages under standard laboratory conditions, at room temperature (22 °C) under an artificial light/dark cycle (12/12 h), with free access to standard laboratory food and tap water. On the day of parturition, the sex of pups was determined, and only males were left with their mother to be used in the further experimental procedure. Between the postnatal days p5 and p16, male Sprague–Dawley pups were administered the selective inhibitor of GCL, compound L-buthionine-(*S*,*R*)-sulfoximine (BSO, 3.8 mmol/kg s.c., once daily), and the dopamine reuptake inhibitor 1-[2-Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12909,5 mg/kg s.c., every second day), alone or in combination. At conditions of the combined treatment, BSO administration preceded an injection of GBR 12909. Control rats, instead of BSO or GBR 12909, received a vehicle once daily. Rats were weighed daily, and the injected volumes of the studied model compounds were adjusted accordingly to the actual body weight. On postnatal day p23, rats were weaned and housed in groups of four to five until p92. Behavioral tests assessing the expression of schizophrenia-like changes, corresponding to negative (social interaction test; SIT) and positive (open field test; OFT) symptoms, and to cognitive deficits (new object recognition test; NOR), were carried out successively in all groups of rats, in the early (p30), middle (p42–p44) and late adolescence (p60–p62) as well as in adulthood (p90–p92). In additional groups of rats receiving vehicle (control), BSO-, GBR 12909- and BSO + GBR 12909 at postnatal days (p5–p16), spontaneous and amphetamine-induced locomotor activity (horizontal and vertical) and stereotypic behavioral patterns were measured with actometers in adulthood (on the 90th day). D-amphetamine (AMF) was administered at a single dose of 1 mg/kg, s.c. and after 30 min, the measurement was initiated and lasted 30 min.

5.2. Social Interaction Test

The social interaction test (SIT) was performed using a black PCV box (67 × 57 × 30 cm, length × width × height). The arena was dimly illuminated with an indirect light of 18 Lux [83]. Each social interaction experiment involving two rats was carried out during the

light phase of the light/dark cycle. The rats were selected from separate housing cages to make a pair for the study. The paired rats were matched for body weight within 15 g. Each pair of rats was diagonally placed in opposite corners of the box facing away from each other. The behavior of the animals was measured over a 10-min period. The test box was wiped clean between each trial. Social interaction between two rats was expressed as the total time spent in social behavior, such as sniffing, genital investigation, chasing, and fighting with each other. The number of episodes was counted as a separate paradigm. The SIT was performed 4 times: on days p30, p42, p60, and p90. Each group was composed of 16 rats (8 pairs).

5.3. Novel Object Recognition Test

The novel object recognition (NOR) test was performed using a black PCV box (67 × 57 × 30 cm, length × width × height). The arena was dimly illuminated with an indirect light of 18 Lux. On the first day of the experiment (adaptation), rats were placed in the box for 10 min. On the next day, the animals were placed in the box for 5 min (T1) with two identical objects (white tin 5 cm wide and 14 cm high or green pyramid 5 cm wide and 14 cm high). The time of object exploration was measured for each of the two objects separately. Then, one hour after T1, the rats again were placed on the box for 5 min (T2), with two different objects: one from the previous session (old) and the other new (white box and green pyramid). The time of object exploration was measured for each of the two objects separately (sniffing, touching, or climbing). NOR test was performed 4 times: on days p31, p43, p61, and p91. Each group was composed of 10 rats.

5.4. Open Field Test

Exploratory activity was assessed in the elevated open field test (OFT). A black circular platform without walls having 1 m in diameter was divided into six symmetrical sectors and was elevated 50 cm above the floor. The laboratory room was dark, and only the center of the open field was illuminated with a 75 W bulb placed 75 cm above the platform. At the beginning of the test, the animal was placed gently in the center of the platform and was allowed to explore. The exploratory activity, ambulation, peeping, and rearing in the open field, i.e., respectively, the time of walking, the number of sector crossings, and the number of episodes of peeping under the edge of the arena and rearing were assessed for 3 min. The OFT was performed 4 times: on days p32, p44, p63, and p93. Each group consisted of 10 rats.

5.5. Locomotor Activity Assessed in Actometers

Spontaneous and AMF-induced locomotor activities of adult 90-day-old Sprague–Dawley rats were recorded individually for each animal using the Opto-Varimex cages (Columbus Instruments, Columbus, OH, USA) linked online to a compatible IBM-PC. Each cage (43 × 44 × 25 cm) was surrounded with a 15 × 15 array of photocell beams located 3 cm from the floor surface as previously. Interruptions of the photocell beams were used to measure horizontal locomotor activity, defined as a distance traveled and expressed in cm, while the time of vertical locomotor activity (climbing) or the time of stereotypy was expressed in seconds. These three parameters were measured for 30 min, starting 30 min after AMF (1 mg/kg, s.c.) administration.

5.6. BDNF Expression Analysis

Freshly isolated rat frontal cortex and hippocampus tissues were stored at −80 °C prior to the next analysis. Total RNA was isolated using commercially available Bead-Beat Total RNA Mini Kit (A&A Biotechnology, Gdansk, PL) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically by using NanoDrop (ND/1000 UV/Vis, Thermo Fisher NanoDrop, Waltham, MA, USA). After dissolving in water, RNA (1 µg) was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription kit with RNAse inhibitor and random hexamers (MultiScribe™,

Applied Biosystems, Life Technologies, Carlsbad, CA, USA). cDNA was synthesized by using thermal cycler T100TM Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in RT-life program conditions: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, and indefinitely at 4 °C.

The BDNF mRNA level was determined by real-time PCR using predesigned TaqMan Gene Expression Assays (Applied Biosystems, Thermo Fisher Scientific, Milton Keynes, UK). Assay IDs for the genes examined were as follows: BDNF (Rn01484925_m1) and for reference's gene HPRT1 (Rn01527840_m1). Amplification was carried out in a total volume of 10 µL (FCx). The mixture containing: 1 × FastStart Universal Probe Master (Rox) mix (Roche, Germany), 900 nM TaqMan forward and reverse primers, and 250 nM of hydrolysis probe labeled with the fluorescent reporter dye FAM at the 5′-end and a quenching dye at the 3′-end and RNAse free water. We used 50 ng of cDNA for the PCR template, real-time PCR was conducted using a thermal cycler Quant Studio 3 (Thermo Fisher Scientific, Waltham, MA, USA), and thermal cycling conditions were: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Samples were run in duplicate.

5.7. ELISA Assay

Freshly isolated rat frontal cortex and hippocampus tissues were stored at −80 °C prior to the analysis. First, the tissues were rinsed in DPBS (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) to remove excess blood, and then they were homogenized in DPBS using Tissue Lyser II (Qiagen Inc., Valencia, CA, USA). The protein measurements of all samples were performed using a BCA Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) in pursuance of the manufacturers' instructions. The protein contents were assessed using a Tecan Infinite 200 Pro spectrophotometer (Tecan, Mannedorf, Germany). Samples containing hippocampal and cortical supernatants were analyzed by enzyme-linked immunoassay (ELISA) using commercially available kits: Rat Brain-Derived Neurotrophic Factor ELISA Kit, cat.no. E0476Ra (Bioassay Technology Laboratory, Shanghai, China) according to the manufacturers' instructions. Briefly, 50 µL of standards and 40 µL of samples, respectively, were dispensed into 96-well coated plates. Next, 10 µL of anti-BDNF antibodies were added into wells containing samples, and 50 µL streptavidin-HRP was added to each well except for blank, and the samples were incubated for 60 min at 37 °C. After washing and the next steps as recommended by the manufacturer, the absorbance was determined using a Tecan Infinite 200 Pro spectrophotometer (Tecan, Mannedorf, Germany) set to 450 nm.

5.8. Statistics

The statistical analysis of the obtained behavioral data was performed using a two-way ANOVA followed (if significant) by the Newman–Keuls test for post hoc comparisons. In addition, for a comparison of two groups, the Student's *t*-test for independent samples was used.

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Data Availability Statement: Data supporting reported results are available on request from the corresponding author.

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ARTICLE

Impact of repeated co-treatment with escitalopram and aripiprazole on the schizophrenia-like behaviors and BDNF mRNA expression in the adult Sprague–Dawley rats exposed to glutathione deficit during early postnatal development of the brain

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Abstract

Background Preclinical and clinical studies have indicated that impaired endogenous synthesis of glutathione during early postnatal development plays a significant role in the pathophysiology of schizophrenia. Moreover, some studies have suggested that antidepressants are able to increase the activity of atypical antipsychotics which may efficiently improve the treatment of negative and cognitive symptoms of schizophrenia.

Methods In the present study, we investigated the influence of repeated co-treatment with escitalopram and aripiprazole on the schizophrenia-like behavior and BDNF mRNA expression in adult rats exposed to glutathione deficit during early postnatal development. Male pups between the postnatal days p5–p16 were treated with the inhibitor of glutathione synthesis, BSO (L-buthionine-(S,R)-sulfoximine) and the dopamine uptake inhibitor, GBR 12,909 alone or in combination. Escitalopram and aripiprazole were given repeatedly for 21 days before the tests. On p90–92 rats were evaluated in the behavioral and biochemical tests.

Results BSO given alone and together with GBR 12,909 induced deficits in the studied behavioral tests and decreased the expression of BDNF mRNA. Repeated aripiprazole administration at a higher dose reversed these behavioral deficits. Co-treatment with aripiprazole and an ineffective dose of escitalopram also abolished the behavioral deficits in the studied tests.

Conclusion The obtained data indicated that the inhibition of glutathione synthesis in early postnatal development induced long-term deficits corresponding to schizophrenia-like behavior and decreased the BDNF mRNA expression in adult rats, and these behavioral deficits were reversed by repeated treatment with a higher dose of aripiprazole and also by co-treatment with aripiprazole and ineffective dose of escitalopram.

Keyword Schizophrenia and glutathione deficit · Aripiprazole · Escitalopram · Social interaction test · Novel object recognition test · BDNF mRNA expression

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Abbreviations

Ads	Antidepressant drugs
ARI	Aripiprazole
BDNF	Brain-derived neurotrophic factor
BSO	L-butionine-(*S,R*)-sulfoximine
ESC	Escitalopram
GBR 12,909	1-[2-[*Bis*-4(Fluorophenyl)methoxy]ethyl]-4–3-(3-phenylpropyl) piperazine
5-HT	Serotonin
GABA	Gamma aminobutyric acid
NMDA	*N*-Methyl-D-aspartate

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Introduction

Schizophrenia is a chronic devastating psychiatric illness affecting about 0.5–1% of the world population [1, 2]. It develops progressively, remaining often undetected during childhood and adolescence, with the first episodes of psychosis that appear at early adulthood. The symptoms of the disorder can be divided into three main categories: positive symptoms, negative symptoms, and cognitive deficits [3]. Depression is a very important comorbidity occurring in approximately 50% of schizophrenic patients [4].

Schizophrenia is associated with neurodevelopmental, structural, and functional brain alterations, pathogenesis of which remains poorly understood. The prevailing hypothesis for the etiology of this disease assumes that both structural and functional abnormalities could be a consequence of multiple interactions between genetic and environmental factors during development [5] that set off a cascade of events extending into adulthood [6]. Although symptoms of schizophrenia are well characterized, a clear mechanism underlying the pathogenesis of this disease still remains unknown. However, oxidative stress as a consequence of the aberrant redox control has become an attractive hypothesis for explanation, at least partially, the pathophysiology of schizophrenia [7, 8].

Several studies have shown that the level of glutathione, the major antioxidant and redox regulator, is decreased in the cerebrospinal fluid and medial frontal cortex of drug-naïve schizophrenic patients [9] as well as in the post-mortem striatum [10] and prefrontal cortex of those treated earlier with antipsychotic drugs. In the periphery, significantly lower levels of glutathione were found in erythrocytes [11, 12] and plasma [10] in antipsychotic-free and chronically medicated schizophrenic patients in comparison to healthy control subjects.

The effects of glutathione deficit in the brain during development were studied in the animal models in adulthood [13–16]. Those studies demonstrated that chronic combined treatment of Osteogenic Disorder Shionogi (ODS) mutant rats, which, like humans, cannot synthesize ascorbic acid, with L-butionine-(*S,R*)-sulfoximine (BSO) and GBR12909 during early postnatal life induced schizophrenia-like memory deficits assessed in the novel object recognition test during adulthood. However, the effects of these compounds have not been studied in Sprague–Dawley rats, yet. The deficit in brain glutathione combined with DA reuptake inhibition during development caused a decrease in the number of dendritic spines of pyramidal neurons in the prefrontal cortex [17]. Therefore, morphological changes found in in vitro and in vivo studies could be related to morphological alteration reported earlier to occur in the prefrontal cortex of schizophrenic patients.

Glutathione, as the most abundant thiol antioxidant, plays a key role in the control of the redox state of cells and, thus in the regulation of various signaling pathways and gene expression. Hence, its deficiency can alter the functions of redox-sensitive receptor proteins and ion channels, such as NMDA and GABAA receptors, and calcium-activated potassium channels, which are involved in neurotransmission and synaptic plasticity. Changes in these redox-sensitive proteins may be associated with disturbances in the dopaminergic, glutamatergic, and GABA-ergic neurotransmitter systems known to be dysfunctional in schizophrenia [16, 18]. All these data seem to indicate that model substances, such as BSO and GBR 12,909, which, respectively, affect cell redox status and dopaminergic transmission, and may be useful for inducing the neurodevelopment rat model of schizophrenia.

Conventional, typical antipsychotics (i.e., antagonists of dopamine D_2 receptors) commonly used in the treatment of schizophrenia mainly alleviate the positive symptoms [19, 20]. Contrary to typical antipsychotic drugs, atypical drugs partially alleviate the negative symptoms and slightly improve cognitive deficits [21, 22].

The pharmacology of aripiprazole is unique among atypical antipsychotics. It has a relatively high affinity for some monoaminergic receptors and acts as an antagonist of 5-HT_{2A} receptors as well as postsynaptic DA D_2 receptors, and as a partial agonist of 5-HT_{1A} and presynaptic DA D_2 receptors [23–27]. This drug partially relieves both positive and negative symptoms of schizophrenia.

Moreover, in several previous clinical and preclinical studies, it has been shown that the addition of antidepressants (ADs) to the therapy with atypical antipsychotics significantly increases their effectiveness in alleviating negative symptoms and improving cognitive tasks compared with the treatment with atypical antipsychotics alone [28–30]. The above data suggest that a new form of treatment of schizophrenia, combining ADs with atypical antipsychotics, may be of great importance in clinical practice. Furthermore, some earlier studies indicated a low level of the serum BDNF level in schizophrenic patients compared to control subjects [31, 32].

In light of the above data, the aim of our study was to evaluate the influence of repeated treatment with aripiprazole (an atypical antipsychotic drug) and AD escitalopram (ESC, a selective serotonin reuptake inhibitor) [33] given alone or in combination on the schizophrenia-like behavior and BDNF mRNA expression in adult rats exposed to glutathione deficit during early postnatal development.

Materials and methods

Animals and treatment

Pregnant Sprague–Dawley females at embryonic day 16 delivered by Charles River Company (Sulzfeld, Germany) were kept in individual cages under standard laboratory conditions; at room temperature of 21 ± 1 °C with 40–50% humidity on a 12-h light–dark cycle (the lights turned on at 7 a.m.), with free access to standard laboratory chow and tape water. One day after birth, the sex of pups was determined, and only males were left with their mothers to be used in further experimental procedure. Between the postnatal days p5 and p16, male Sprague–Dawley pups were treated with the selective inhibitor of glutathione synthesis, BSO (3.8 mmol/kg, *sc*, daily), and the inhibitor of dopamine reuptake GBR 12,909 (5 mg/kg, *sc*, every second day), alone or in combination. Control pups were given vehicle. On postnatal day, p23 rats were weaned and housed in groups of four until p92. ESC was given 30 min before aripiprazole repeatedly, once daily for 21 days before the tests. The last dose of the studied drugs was given 24 h before the test. Behavioral tests (social interaction and novel object recognition) evaluating expression of schizophrenia-like symptoms were carried out in adulthood (at p90-91 days of age). The tissue (hippocampus and prefrontal cortex) for biochemical assays was dissected on p92.

Drugs and treatment

1-[2-[*Bis*-4(fluorophenyl)methoxy]ethyl]-4–3-(3-phenylpropyl)piperazine hydrochloride (GBR 12,909, Abcam Biochemicals, Cambridge, UK), L-butionine-(*S,R*)-sulfoximine (BSO, Sigma-Aldrich, Saint Louis, MO, USA), and escitalopram oxalate (ESC, Sigma-Aldrich, Saint Louis, MO, USA) were dissolved in 0.9% NaCl, while aripiprazole (Abcam Biochemicals, Cambridge, UK) was dissolved in 0.1 M tartaric acid. The pH of the solution was adjusted to 6–7 with 0.1 N NaOH. ESC (5 mg/kg, *ip*) and aripiprazole (0.1 and 0.3 mg/kg, *ip*) were administered once daily for 21 days using intraperitoneal (*ip*) injections in a volume of 2 ml/kg. All doses of drugs used in the present study were selected based on our earlier publications [34, 35].

Compliance with ethical standards

All the experiments were performed in according with the EU directive 2010/63/EU and with the approval of the procedures by the Animal Care and Use Committee at the Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków (permission on no 3/2018 of 11 January 2018). All efforts were made to minimize the number and suffering of animals used.

Behavioral studies

Social interaction test

The social interaction test procedure was described previously by Górny et al. [35]. The behavior of the animals was measured over a 10-min period. The last dose of the studied drugs: ESC (5 mg/kg, *ip*) and aripiprazole (0.1 and 0.3 mg/kg, *ip*) was given 24 h before the test. Social interaction between two rats was expressed as the total time spent in social behavior, such as sniffing, genital investigation, chasing, and fighting with each other. The number of episodes was also counted. The social interaction test was performed on day p90. Each group consisted of 12 animals (six pairs).

Novel object recognition test

The procedure for the novel object recognition test was described previously by Górny et al. [35]. The novel object recognition test was performed 24 h after the last dose of drugs: ESC (5 mg/kg, *ip*) and aripiprazole (0.1 and 0.3 mg/kg, *ip*) administration. Next, the animals were placed in a box (T1 session) with two identical objects for 5 min. The time of object exploration was measured for each of the two objects separately. Then, 1 h after T1 session, the rats were placed again in the box (T2 recognition session) with two different objects: one the same as in the previous session (old) and another new for 5 min. The time of object exploration was measured for each of the two objects separately (sniffing, touching, or climbing). The novel object recognition test was performed on day p91. Each group consisted of eight to twelve rats.

BDNF mRNA expression analysis (real-time PCR) The tissue (hippocampus and prefrontal cortex) for biochemical assays was dissected on p92. Freshly isolated rat tissues were stored at − 80 °C prior to the analysis. Total RNA was isolated using commercially available Bead-Beat Total RNA Mini Kit (A&A Biotechnology, PL) according to the manufacturer's instructions. After dissolving in water, RNA (1 µg) was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription kit with RNAse inhibitor and random hexamers (MultiScribe™, Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The BDNF mRNA level was determined by Real-Time PCR using predesigned TaqMan Gene Expression Assays (Applied Biosystems, UK). Assay IDs for the genes examined were as follows:

BDNF (Rn01484925 m1) and for reference gene HPRT1 (Rn01527840 m1). Amplification was carried out in a total volume of 10 µl (FCx). The mixture containing: 1 × Fast-Start Universal Probe Master (Rox) mix (Roche, Germany), 900 nM TaqMan forward and reverse primers, and 250 nM of hydrolysis probe labeled with the fluorescent reporter dye FAM at the 5′-end and a quenching dye at the 3′-end and RNAse free water. We used 50 ng of cDNA for the PCR template, Real-Time PCR was conducted using thermal cycler Quant Studio 3 (Thermo Fisher Scientific, Waltham, MA, USA), and thermal cycling conditions were as follows: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles at 9 °C for 15 s and at 60 °C for 1 min. Each group consisted of eight rats.

Statistical analysis

Statistical analysis of the obtained results was performed with the use the Statistica 64 v.13. Before the analysis, the data for each studied group were checked for normality using the Shapiro–Wilk test. The Levene's test was used to check homogeneity of variance. Since all behavioral and biochemical data met both criteria, a one-way analysis of variance (ANOVA) for planed comparisons (the so-called contrast analysis) was applied. The recognition index was calculated for each rat [(time spent exploring the novel object—time spent exploring the familiar object)/(total time spent exploring both objects during the recognition session), and was expressed in percentages. The results are presented as the means ± SEM (standard errors of the means); they were considered statistically significant when $p < 0.05$ (Fig. 1).

Results

Figure 1 presents the timeline of the general protocol used in the present experiments.

The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole on the schizophrenia-like behaviors in the adult Sprague–Dawley rats exposed to glutathione deficit during early postnatal brain development

The social interaction test in rats treated with BSO

The social interaction test was performed in 90-day-old rats that were chronically treated with the BSO model compound on the postnatal days p5-p16, and then in adulthood were chronically treated for 21 days with ESC (5 mg/kg) and aripiprazole (0.1, 0.3 and 1 mg/kg), alone or in combination. The analysis of the studied parameters, i.e., the time of interaction and the number of interactions, was performed using a one-way ANOVA for the planned comparisons (Fig. 2). This analysis performed for the interaction time ($F_{7,40} = 20.33$, $p < 0.001$) showed that administration of BSO in the early postnatal life significantly shortened the social interaction time assessed in adulthood, and chronic administration of 5 mg/kg ESC did not reverse this effect (Fig. 2A). Like ESC, aripiprazole at the tested doses of 0.1 and 0.3 mg/kg was ineffective in reversing the BSO-induced reduction in the social interaction time, but aripiprazole at 1 mg/kg was effective in reversing this effect. The combined treatment of an ineffective dose of ESC (5 mg/kg) with ineffective doses of aripiprazole (0.1 and 0.3 mg/kg) significantly (at

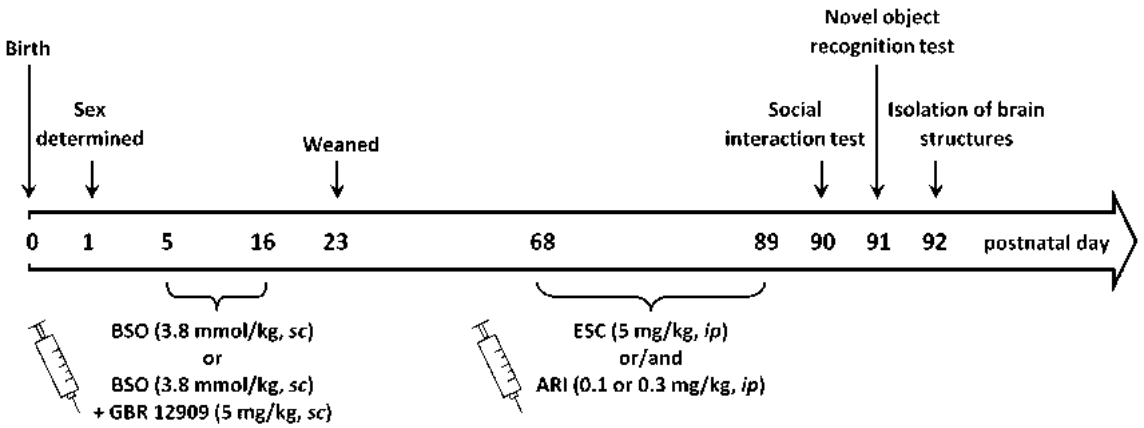


Fig. 1 Timeline of the general protocol used in the present experiments. One day after birth, the sex of pups was determined, and only males were left with their mothers to be used in further experimental procedure. Between the postnatal days p5 and p16 male Sprague–Dawley pups were treated with the selective inhibitor of glutathione synthesis, BSO (3.8 mmol/kg, *sc*, daily), and the inhibitor of dopamine reuptake GBR 12,909 (5 mg/kg, *sc*, every second day), alone or in combination. Control pups were given saline. On postnatal day p23 rats were weaned and housed in groups of four until p92. ESC was given 30 min before aripiprazole repeatedly, once daily for 21 days before the tests. The last dose of the studied drugs was given 24 h before the test. Behavioral tests (social interaction and novel object recognition) were carried out in adulthood (at p90–91 days of age). The tissue (hippocampus and prefrontal cortex) for biochemical assays was dissected on p92

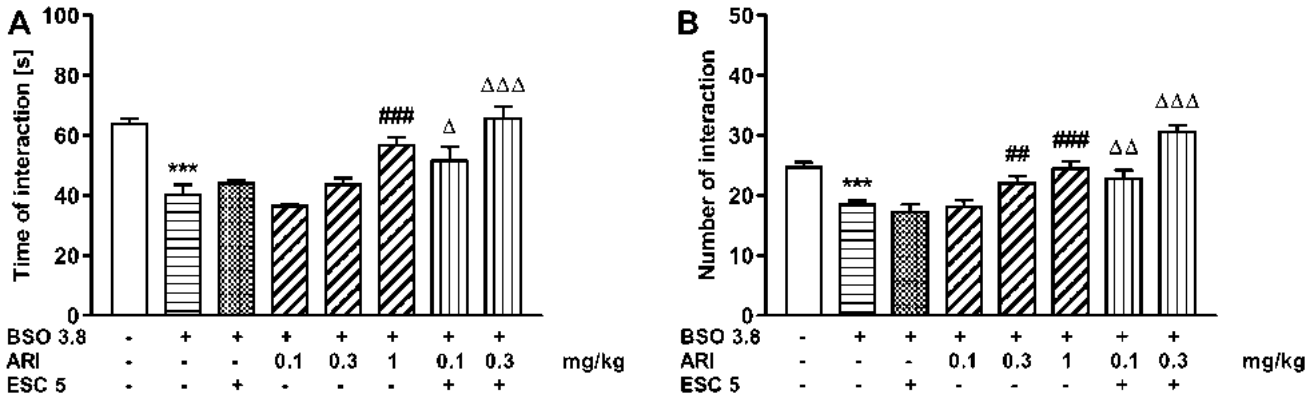


Fig. 2 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the deficits in the social interaction test performance in adult Sprague–Dawley rats induced by BSO (3.8 mmol/kg, *sc*) causing glutathione depletion during early postnatal brain development. The social interaction test performance in rats was assessed for 10 min in 90 days old rats by means of two parameters: the total time spent in social interaction (**A**) and the number of these interactions (**B**). ESC (5 mg/kg, *ip*) was given 30 min before administration of ARI (0.1 or 0.3 mg/kg, *ip*) once daily for 21 days. The results are shown as the mean ± SEM. Each group consisted of six pairs per group (12 rats). The statistically significant differences between the studied groups were calculated using a one-way ANOVA for planed comparisons. $^{***}p<0.001$ *vs.* vehicle-treatment group; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *vs.* BSO-treatment group; $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}p<0.001$ *vs.* BSO + ESC-treatment group

the levels of $p<0.05$ and $p<0.001$, respectively) prolonged the time of social interaction compared to the BSO group receiving chronically ESC alone.

Similarly, a one-way ANOVA for planned comparisons ($F_{7,40}=33.83$, $p<0.001$) for the number of social interactions showed that BSO administered early in the postnatal life had a long-lasting effect, significantly reducing their number in adulthood (Fig. 2B). Chronic treatment with ESC (5 mg/kg) or aripiprazole at a dose of 0.1 mg/kg did not change the BSO-induced reduction in the number of social interactions between the two rats, but aripiprazole at doses of 0.3 and 1 mg/kg significantly increased their number. The combined treatment with an ineffective dose of ESC (5 mg/kg) and an ineffective dose of aripiprazole (0.1 mg/kg) significantly increased the number of social interactions between two rats compared to the BSO group treated with ESC alone (Fig. 2B). However, the effect of combined therapy on the number of social interactions was much greater when an ineffective dose of ESC (5 mg/kg) was administered with an effective dose of aripiprazole (0.3 mg/kg).

The social interaction test in rats co-treated with BSO and GBR 12909

Similarly to the groups treated with BSO, also in the groups of rats receiving the combination of BSO + GBR 12909 model compounds in the early postnatal life, the social interaction test was performed in 90-day-old rats, and the studied parameters were analyzed by a one-way ANOVA for the planned comparisons (Fig. 3). The latter analysis carried out for the total time spent in social interactions ($F_{6,35}=84.08$, $p<0.01$) showed that, like in the case of BSO, also administration of the BSO + GBR 12909 combination in the early postnatal life resulted in a significant reduction in the time of social interactions (by 51%) assessed in adulthood (Fig. 3A). Chronic treatment with ESC (5 mg/kg) or aripiprazole (0.1 and 0.3 mg/kg) alone prolonged the social interaction time compared to the BSO + GBR 12909 group, and these effects although significant were only partial. Interestingly, administration of ESC (5 mg/kg) and aripiprazole at a dose of 0.1 mg/kg did not prolong the time spent in social interactions compared to the BSO + GBR 12909 group receiving chronically ESC alone. In contrast to the above effect, administration of ESC (5 mg/kg) and aripiprazole at a dose of 0.3 mg/kg significantly prolonged the social interaction time compared to the BSO + GBR 12909 group receiving chronically ESC alone (Fig. 3B). In the latter case, the time of social interactions was even slightly higher than in the control group (Fig. 3A).

As for the second studied parameter in the social interaction test, i.e., the number of social interactions, a one-way ANOVA for planned comparisons ($F_{6,35}=37.12$, $p<0.001$) showed a significant effect of the combined administration of model substances (BSO + GBR 12909) in early postnatal life on the number of social interactions assessed in adulthood (Fig. 3B). This analysis also showed that neither ESC (5 mg/kg) nor aripiprazole at a dose of 0.1 mg/kg administered chronically reversed the BSO + GBR 1290-induced decline in the number of social interactions, and only 0.3 mg/kg aripiprazole slightly but statistically significantly increased the number of these interactions (Fig. 3B). Also combined treatment with ineffective doses of ESC (5 mg/kg) and aripiprazole (0.1 mg/kg) did not increase the number of social interactions, but combined administration of 5 mg/kg ESC

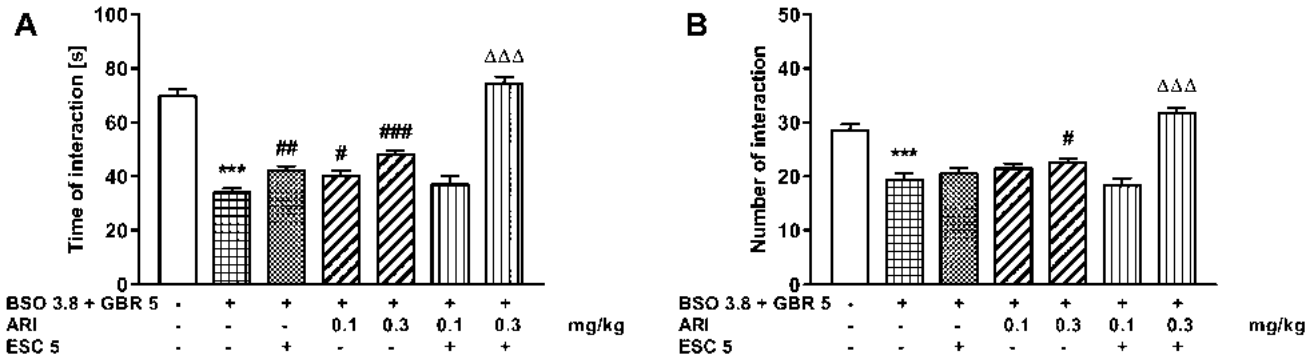


Fig. 3 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the deficits in the social interaction test performance in the adult Sprague–Dawley rats induced by BSO (3.8 mmol/kg, *sc*) given together with GBR 12,909 (5 mg/kg, *sc*) exposed to glutathione depletion during early postnatal brain development. The social interaction test performance in rats was assessed for 10 min in 91-day-old rats by means of two parameters: the total time spent in social interaction (**A**) and the number of these interactions (**B**). ESC (5 mg/kg, *sc*) was given 30 min before administration of ARI (0.1 and 0.3 mg/kg, *ip*), once daily for 21 days. The results are shown as the mean ± SEM. Each group consisted of six pairs per group (12 rats). The statistically significant differences between the studied groups were calculated using a one-way ANOVA for planed comparisons. *** $p < 0.001$ *vs.* vehicle-treatment group; $^{\#}p < 0.05$. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* BSO + GBR 12,909-treatment group; $^{\Delta\Delta\Delta}p < 0.001$ *vs.* BSO + GBR 12,909 with ESC-treatment group

with effective dose of aripiprazole (0.3 mg/kg) significantly increased their number compared to the BSO + GBR 12909 group receiving chronically ESC alone (Fig. 3B).

The novel object recognition test in rats treated with BSO

The novel object recognition test in rats treated with BSO was assessed at 91 days of age (Fig. 4). A one-way ANOVA for the planned comparisons performed for recognition indexes ($F_{7,88} = 49.70$, $p < 0.001$) calculated on the basis of the novel object recognition test showed that chronic BSO administration in the early postnatal days resulted in reduced memory retention in adult rats (Fig. 4). Chronic treatment with the higher doses of aripiprazole (0.3 and 1 mg/kg) abolished the BSO-induced memory deficits, while the lowest doses of aripiprazole (0.1 mg/kg) and ESC (5 mg/kg) were ineffective. Also, the combined administration of the ineffective dose ESC (5 mg/kg) with either the lowest dose of aripiprazole (0.1 mg/kg) or the effective dose of aripiprazole (0.3 mg/kg) significantly reversed cognitive deficits compared to the BSO group receiving chronic ESC alone (Fig. 4).

The novel object recognition test in rats co-treated with BSO and GBR 12909

The novel object recognition test in rats co-treated with BSO and GBR12909 was assessed at 91 days of age (Fig. 5). A one-way ANOVA for the planned comparisons, performed for recognition indexes ($F_{6,49} = 77.93$, $p < 0.001$) and calculated on the basis of the novel object recognition test, showed that chronic administration the BSO + GBR 12909

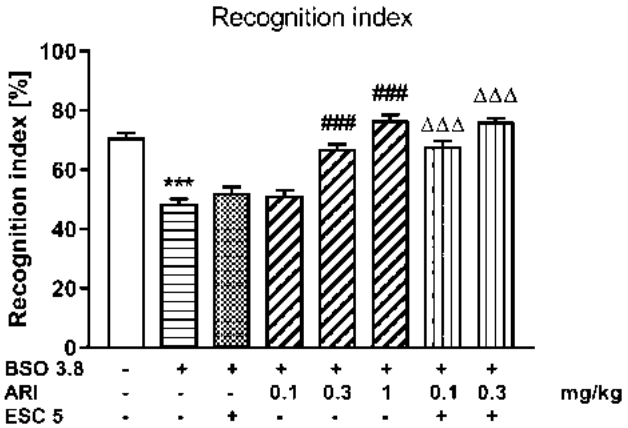


Fig. 4 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the deficits in the novel object recognition test performance in the adult 91 days old rats induced by BSO (3.8 mmol/kg, *sc*) causing glutathione depletion during early postnatal brain development. ESC (5 mg/kg, *ip*) was given 30 min before administration of ARI (0.1 and 0.3 mg/kg, *ip*), once daily for 21 days. Recognition index in the T2, recognition session was calculated for each rat [(time spent exploring the novel object—time spent exploring the familiar object)/(total time spent exploring both objects during the recognition session)], and was expressed in percentages. The results are shown as the mean ± SEM. Each group consisted of 12 rats. The statistically significant differences between the studied groups were also calculated using a one-way ANOVA for planed comparisons. *** $p < 0.001$ *vs.* vehicle-treatment group; $^{\#\#\#}p < 0.001$ *vs.* BSO-treatment group; $^{\Delta\Delta\Delta}p < 0.001$ *vs.* BSO with ESC-treatment group

combination in the early postnatal days led to disclosure of memory deficits in adulthood (Fig. 5). Chronic treatment with ESC (5 mg/kg) and a lower dose of aripiprazole (0.1 mg/kg) did not change memory deficits caused by the

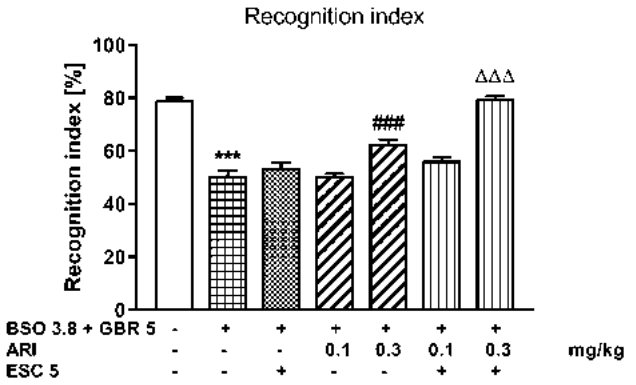


Fig. 5 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the deficits in the novel object recognition test performance in the adult 91-day-old rats induced by BSO (3.8 mmol/kg, *sc*) given together with GBR 12,909 (5 mg/kg, *sc*) exposed to glutathione depletion during early postnatal brain development. ESC (5 mg/kg, *ip*) was given 30 min before administration of ARI (0.1 and 0.3 mg/kg, *ip*), once daily for 21 days. Recognition index in the T2, recognition session was calculated for each rat [(time spent exploring the novel object—time spent exploring the familiar object)/(total time spent exploring both objects during the recognition session)], and was expressed in percentages. The results are shown as the mean ± SEM. Each group consisted of eight rats. The statistically significant differences between the studied groups were also calculated using a one-way ANOVA for planed comparisons. $^{***}p<0.001$ *vs.* vehicle-treatment group; $^{\#\#\#}p<0.001$ *vs.* BSO + GBR 12,909-treatment group; $^{\Delta\Delta\Delta}p<0.001$ *vs.* BSO + GBR 12,909 with ESC-treatment group

BSO + GBR 12909 combination, while a higher dose of aripiprazole (0.3 mg/kg) or combined chronic administration of ESC (5 mg/kg) and aripiprazole (0.3 mg/kg) reversed these memory deficits (Fig. 5).

The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole on the BDNF mRNA expression in rats treated with BSO

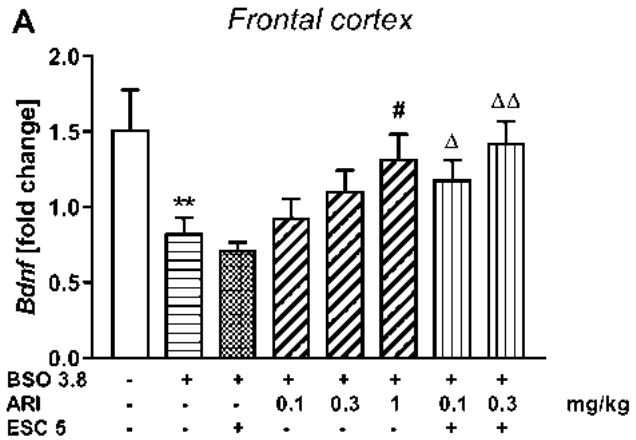
Frontal cortex

A one-way ANOVA for the planned comparisons performed for BDNF mRNA expression in the prefrontal cortex ($F_{7,56}=3.78$, $p<0.002$) showed that chronic BSO treatment in the early postnatal life significantly lowered the level of this parameter in adulthood, and chronic administration ESC (5 mg/kg) and lower doses of aripiprazole (0.1 and 0.3 mg/kg) did not reverse this effect, and only 1 mg/kg of aripiprazole was effective in reversing this change (Fig. 6A).

Hippocampus

As in the prefrontal cortex, a one-way ANOVA for the planned comparisons performed for BDNF mRNA expression in the hippocampus ($F_{7,56}=4.545$, $p<0.001$) revealed that chronic BSO treatment in the early postnatal life significantly lowered the level of this parameter in adulthood (Fig. 6B). However, in the hippocampus, unlike the prefrontal cortex, chronically administered ESC reversed this effect, while the studied doses of aripiprazole were ineffective. In addition, the combined chronic administration of ESC (5 mg/kg) and 0.1 mg/kg of aripiprazole reduced the expression of BDNF mRNA compared to the BSO group chronically treated with ESC alone (Fig. 6B).

The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole on the BDNF mRNA expression in rats treated with BSO and GBR 12909



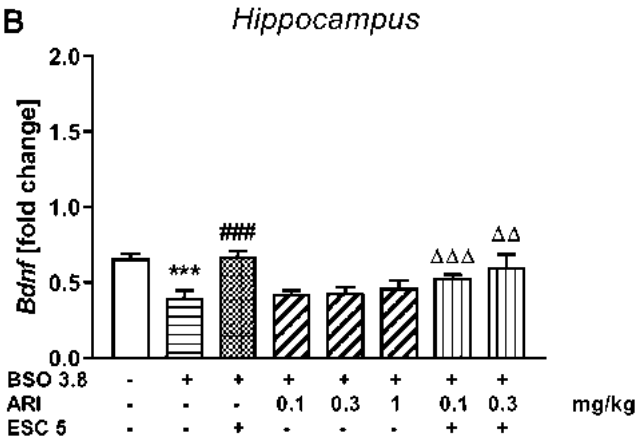


Fig. 6 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the BDNF mRNA expression in the frontal cortex (**A**) and hippocampus (**B**) in the adult 92-day-old rats in which glutathione deficit was induced by BSO administration (3.8 mmol/kg, *sc*) during early postnatal brain development. The results are shown as the mean ± SEM. Each group consisted of eight rats. The statistically significant differences between the studied groups were calculated using a one-way ANOVA for planed comparisons. $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* vehicle-treatment group; $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ *vs.* BSO-treatment group; $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}p<0.001$ *vs.* BSO with ESC-treatment group

Frontal cortex

In the groups of rats receiving the combination of BSO + GBR 12909 model compounds in the early postnatal life, similarly to the groups treated with BSO, a one-way ANOVA for the planned comparisons for BDNF mRNA expression was performed in the prefrontal cortex ($F_{6,49} = 8.653$, $p < 0.001$) in adulthood (Fig. 7A). This analysis showed that such treatment significantly decreased expression of BDNF mRNA in this brain structure (Fig. 7A). However, neither ESC (5 mg/kg) nor aripiprazole at the doses tested did not reverse the BSO + GBR 12909-induced effect (Fig. 7A). Moreover, the combined chronic administration of ESC with both the lower and the higher dose of aripiprazole significantly decreased the levels of these parameters in the studied groups compared to the long-term administration effect of escitalopram alone (Fig. 7A).

Hippocampus

A one-way ANOVA for the planned comparisons performed for BDNF mRNA expression in the hippocampus ($F_{6,49} = 8.965$, $p < 0.001$) showed that administration of the BSO + GBR 12909 combination in the early postnatal life did not induce changes in the expression of BDNF mRNA (Fig. 7B). Chronic treatment with ESC (5 mg/kg) or aripiprazole (0.1 mg/kg) alone had no effect on the expression of this parameter, while 0.3 mg/kg aripiprazole and the combined administration of ESC (5 mg/kg) + aripiprazole (0.1 mg/kg) decreased it significantly (Fig. 7B).

Discussion

Our previous study [35] showed that inhibition of glutathione synthesis by repeated treatment with BSO alone or in combination with GBR 12909 to Sprague–Dawley pups in early postnatal life (p5-p16) induced the schizophrenia-like behavior evaluated in the social interaction test and in the novel object recognition test in early adulthood (p90). The above-mentioned behavioral tests are widely used to study some negative and cognitive symptoms of schizophrenia [36, 37]. In the present study, these behavioral deficits induced by treatment with model compounds (BSO or BSO + GBR 12 909) during early postnatal developmental were reversed by repeated treatment with a higher dose of aripiprazole as well as by co-treatment with ineffective doses of aripiprazole and ESC in adulthood. These chronic effects of the studied drugs, i.e., their ability to reverse the social and cognitive deficits of the schizophrenia type in the rat neurodevelopmental model of this disease, are consistent with acute effects of these drugs, used in the same dose range and combination, in previously described model of the MK-801-induced schizophrenia-like behavior [34, 38].

The behavioral data described above clearly indicated that the potentiation of the antipsychotic effect of aripiprazole by the used ADs in the MK-801-induced rat model of schizophrenic-type negative symptoms may be related to their action both through serotonin 5-HT_{1A} and dopamine D_1 receptors [34]. In line with the latter study, it was also shown in the rat model of schizophrenic-type cognitive deficits induced by MK-801, the ADs potentiate the pro-cognitive effects of aripiprazole, and these effects may also be mediated by serotonin 5-HT_{1A} and dopamine D_1 receptors

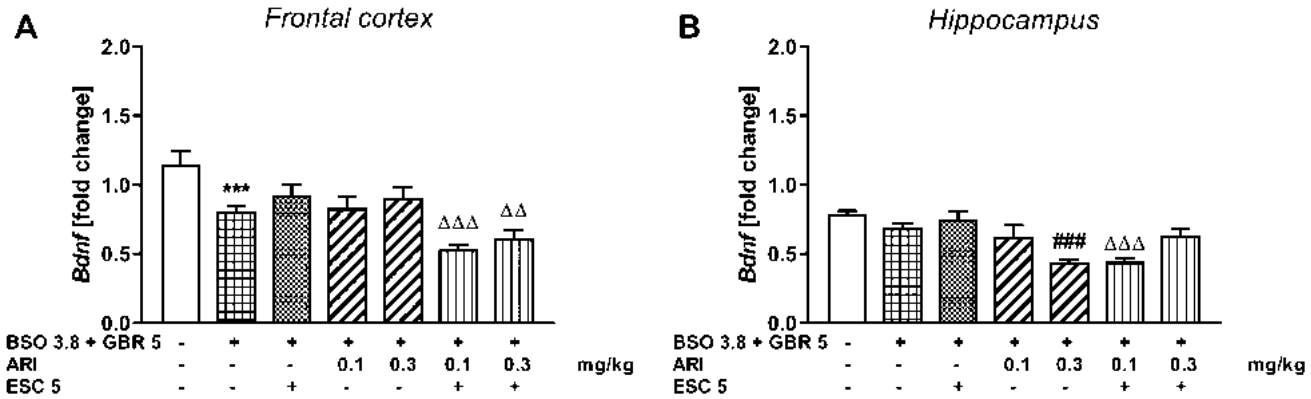


Fig. 7 The effect of repeated co-treatment with escitalopram (ESC) and aripiprazole (ARI) on the BDNF mRNA expression in the frontal cortex (**A**) and hippocampus (**B**) in the adult 92-day-old rats exposed to glutathione deficit after BSO (3.8 mmol/kg, *sc*) given together with GBR12909 (5 mg/kg, *sc*) during early postnatal brain development. The results are shown as the mean ± SEM. Each group consisted of eight rats. The statistically significant differences between the studied groups were calculated using a one-way ANOVA for planed comparisons. $^{***}p < 0.001$ *vs.* vehicle-treatment group; $^{\#\#\#}p < 0.001$ *vs.* BSO + GBR12909-treatment group; $^{\Delta\Delta}p < 0.01$ and $^{\Delta\Delta\Delta}p < 0.001$ *vs.* BSO + GBR 12,909 with ESC-treatment group

[38]. In the above-cited study, in the conditions of combined administration of aripiprazole and ADs, an increase in extracellular concentrations of 5-HT and noradrenaline in the rat cortex was also shown, which may be of great importance to alleviating the negative symptoms and improving the cognitive functions [38]. Therefore, from the above study, it is reasonable to assume that the effects of combination treatment with aripiprazole and ESC on neurotransmitter release may be related to the activation or blockade of monoaminergic receptors in some structures of the rat brain, but not to pharmacokinetic interactions of these drugs, since clinical trials conducted in healthy subjects and patients with depression showed that aripiprazole did not significantly affect the pharmacokinetics of various classes of ADs (ESC, venlafaxine, fluoxetine, paroxetine, and sertraline) [39, 40].

Pharmacological models of schizophrenia induced by MK-801 or phencyclidine are symptomatic models used for screening antipsychotic drugs, and do not reflect the pathological changes leading to their formation. Looking for the biochemical markers of changes initiated in the early postnatal period by chronic administration of BSO alone or the combination of BSO + GBR 12909, in previous studies, we checked their impact on the levels of glutathione and sulfur amino acids (cysteine, methionine) and on the global DNA methylation in the prefrontal cortex and hippocampus [35]. These data suggest that transient alterations in the content of glutathione and sulfur amino acid methionine during early postnatal life lead to changes in epigenetic status in the prefrontal cortex and hippocampus, and to manifestation of social and cognitive deficits in adult rats. To further characterize this rat model of schizophrenia, in a recently published study [41], the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, and glutathione disulfide reductase) and the levels of lipid peroxidation in the prefrontal cortex and hippocampus of 16-day-old rats from the group treated with BSO + GBR12909 were analyzed in relation to glutathione content and sulfur amino acids, methionine, and cysteine [41]. This analysis showed that chronic administration of the BSO + GBR 12909 combination resulted in a significant reduction in the level of lipid peroxidation in the examined brain structures, indicating a weakening of the oxidative power of their cells and ultimately leading to changes in redox cell signaling. As a result of the redox state disturbance in the examined brain structures in the early postnatal life, social and cognitive deficits may occur in adulthood.

The brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the brain that, in addition to promoting neuronal survival and differentiation, modulates the efficacy of synaptic transmission [32, 42]. In animal models of depression and schizophrenia, BDNF levels were found to be abnormally regulated [32]. It has been shown that the level of BDNF is markedly reduced both in the plasma and post-mortem brains of patients suffering from schizophrenia, what suggests that BDNF dysfunction plays an important role in the pathogenesis of this disease [43]. Several in vivo studies have found that atypical antipsychotics increase the levels of both BDNF mRNA and its protein, while the typical antipsychotic drug haloperidol reduces or does not affect BDNF levels [43–46].

It is known that BDNF has an important role in neuronal development, synaptogenesis and also as a modulator of monoaminergic and GABA-ergic neurotransmitter systems [47, 48]. Thus, in our early experiments, we studied the long-term effects of chronic treatment with BSO and GBR 12909 alone or in combination in the early postnatal life on BDNF mRNA and its protein levels in the prefrontal cortex and hippocampus in adulthood in rats [49]. The obtained data showed that in the prefrontal cortex, BSO given alone or in combination with GBR 12909 induced a decrease in both BDNF mRNA and protein levels in adulthood. In the hippocampus, in rats treated only with BSO, also the decrease in the level of BDNF mRNA and its protein was observed. On the other hand, in the hippocampus of BSO + GBR-treated rats, no changes in the level of BDNF mRNA were observed, while there was a significant decrease in BDNF protein level when compared to the control group. In addition, treatment with GBR 12909 alone in early postnatal life modulates BDNF protein expression in adulthood in an opposite manner, reducing it in the prefrontal cortex and increasing it in the hippocampus. The above data suggest that in rats given GBR 12909 alone, this increase in the BDNF protein level in the hippocampus may be of compensatory nature, ultimately leading to the normalization of cognitive functions. However, the observed compensatory effect at the BDNF protein level did not occur in the rat hippocampus following co-treatment BSO with GBR 12909 [49]. Moreover, our and literature data suggest that redox imbalances [7, 18, 50, 51], as a result of repeated treatment with a glutathione synthesis inhibitor administered alone or in combination with a DA reuptake inhibitor in early postnatal development may be an important factor reducing BDNF protein expression in the prefrontal cortex and hippocampus in adulthood. In another model of schizophrenia [52], a decrease in the level of BDNF mRNA in the prefrontal cortex and the hippocampus was observed [53]. Also. other animal studies have shown that some early postnatal disturbances can have long-term effects on various neurotrophin-mediated processes, thereby affecting neuronal maturation and plasticity later in life [48, 54, 55].

Moreover, our present data indicated that the decrease in BDNF mRNA expression in the frontal cortex of rats treated with BSO was abolished by a higher dose of aripiprazole, and also by co-treatment with aripiprazole and ineffective doses of ESC. In contrast, in the hippocampus of rats treated with BSO + GBR 12909 and

aripiprazole + ESC, even greater decrease in BDNF mRNA expression was observed than in rats treated with BSO and GBR12909. Our previous study showed that ESC (5 mg/kg, *ip*) administered repeatedly (once daily for 14 days) did not change in BDNF mRNA expression in the frontal cortex and hippocampus compared to the vehicle-treated group, but its higher dose (10 mg/kg, *ip*) increased this expression [56]. Moreover, it was demonstrated that repeated treatment with ESC (10 mg/kg) regulated intracellular pathway linked to neuroplasticity at both evaluated time points in an area-specific manner. For example, 7 days of treatment with ESC activated intracellular pathways linked to BDNF and increased the levels of pro-BDNF in the prefrontal cortex. On the other hand, 21 days of treatment with ESC decreased CREB/BDNF signaling, but increased p38 levels in the rat hippocampus. These data suggest that efficacy of ESC may be mediated by early and late effects on synaptic plasticity in selected brain areas [57]. In addition, it was demonstrated that aripiprazole reduced the total mRNA levels in the hippocampus (ventral and dorsal), while increasing it in the ventral hippocampus [46]. Our present experiments in which we used the whole hippocampus suggest that the decrease in the levels of the BDNF mRNA expression in rats treated with BSO + GBR 12909 and aripiprazole + ESC may be connected with this difference**.** Moreover, our present data suggest that redox imbalance after repeated treatment with a glutathione synthesis inhibitor alone or in combination with a DA reuptake inhibitor during early postnatal development may be an important factor which reduces the expression of BDNF protein in the prefrontal cortex and hippocampus in adulthood. Further studies are needed to investigate whether changes in the BDNF protein levels or mRNA expression in the prefrontal cortex and in the hippocampus of rats treated with BSO or BSO + GBR 12909 combination affect intracellular signaling pathways.

In conclusion, our present findings indicated that the inhibition of glutathione synthesis by repeated treatment with BSO alone and together with GBR 12909 in early postnatal development induced long-term deficits corresponding to schizophrenia-like behavior evaluated in the social interaction and novel object recognition tests, and decreased the expression of BDNF mRNA in the frontal cortex and hippocampus. Moreover, these behavioral deficits were reversed by repeated treatment with a higher dose of aripiprazole and also by co-treatment with ineffective doses of aripiprazole and ESC. The above data suggest that the neurodevelopment rat model of schizophrenia induced by glutathione deficit generated by repeated treatment with BSO alone and together with GBR12909 in early postnatal life may be very important for studies on the pathomechanism of schizophrenia.

Author contributions Participated in research design: ZR and ELK. Conducted experiments and collected the data: MAL, KK, ML, and ZR. Analyzed the data: ELK, KK, and ZR. Wrote or contributed to the writing of the manuscript: ZR and MAL. All authors have read and agreed to the published version of the manuscript.

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Declarations

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

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