mgr inż. Marta Matuszewska

Rola białek z rodziny BET w regulacji zależnych od komórek mikrogleju procesów neurodegeneracji

Rozprawa na stopień naukowy doktora
w dziedzinie nauk medycznych i nauk o zdrowiu
w dyscyplinie nauki medyczne

Promotor: dr hab. Grzegorz A. Czapski, prof. IMDiK

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„Niczego w życiu nie należy się bać, należy to tylko zrozumieć."

~ Maria Skłodowska-Curie

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"What I find fantastic is any notion that there are answers beyond the realm of science. The answers are there. You just have to know where to look."

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Wykaz skrótów

Aβ — amyloid-beta
ABF — fibrylarny amyloid-beta
AD — choroba Alzheimera (ang. *Alzheimer's disease*)
AKT — kinaza białkowa alfa serynowo-treoninowa (ang. *alpha serine/threonine-protein kinase*)
AMPK — kinaza aktywowana AMP (ang. *AMP-activated protein kinase*)
BD — bromodomena (ang. *bromodomain*)
BID — domena bogata w reszty zasadowe (ang. *basic residue-enriched domain*)
BET — białka bromodomeny i domeny pozaterminalnej (ang. *bromodomain and extraterminal domain*)
BRD2 — bromodomenowe białko 2 (ang. *bromodomain-containing protein 2*)
BRD3 — bromodomenowe białko 3 (ang. *bromodomain-containing protein 3*)
BRD4 — bromodomenowe białko 4 (ang. *bromodomain-containing protein 4*)
c-MYC — onkogen komórkowy mielocytomatozy (ang. *cellular myelocytomatosis oncogene*)
CBX4 — białko chromobox 4, pełniące rolę E3-ligazy SUMO (ang. *chromobox protein homolog 4*)
CD33 — receptor transbłonowy SIGLEC3 (ang. *sialic acid binding Ig like lectin 3*)
CD36 — klaster różnicowania 36 (ang. *cluster of differentiation 36*)
CLEC7A — receptor lektynowy typu C 7A (ang. *C-type lectin domain containing 7A*)
CK2 — kinaza serynowo-treoninowa 2 (ang. *casein kinase 2*)
CPS — miejsce fosforylacji w części C-terminalnej (ang. *C-terminal phosphorylation site*)
CRP — białko C-reaktywne (ang. *C-reactive protein*)
CSF1 — czynnik stymulujący tworzenie kolonii 1 (ang. *colony-stimulating factor 1*)
CSF1R — receptor dla CSF1 (ang. *colony-stimulating factor 1 receptor*)
DAM — mikroglej związany z procesem chorobowym (ang. *disease-associated microglia*)
DMSO — dimetylosulfotlenek (ang. *dimethyl sulfoxide*)
ELISA — immunoenzymatyczny test oznaczeń ilościowych (ang. *enzyme-linked immunosorbent assay*)
ERK — kinaza regulowana sygnałami zewnątrzkomórkowymi (ang. *extracellular signal-regulated kinase*)
(ang. *extra-terminal domain*)
FAD — rodzinna postać choroby Alzheimera (ang. *familial Alzheimer's disease*)
FBS — płodowa surowica bydlęca (ang. *fetal bovine serum*)
GFAP — kwaśne białko włókienkowe komórek glejowych (ang. *glial fibrillary acidic protein*)
GWAS — badania asocjacyjne całego genomu (ang. *genome-wide association studies*)

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GRB2 — białko adaptacyjne związane z receptorem czynnika wzrostu 2 (ang. *growth factor receptor-bound protein 2*)
HDAC — deacetylaza histonowa (ang. *histone deacetylase*)
HMW PIC — kwas poliinozynowy policytydylowy o dużej masie cząsteczkowej (ang. *high molecular weight polyriboinosinic:polyribocytidylic acid*)
HPRT — fosforybozylotransferaza hipoksantynowa (ang. *hypoxanthine phosphoribosyltransferase*)
IBA1 — jonizowana cząsteczka adapterowa wiążąca wapń 1 (ang. *ionized calcium-binding adapter molecule 1*)
IL-1β — interleukina 1 beta (ang. *interleukin 1 beta*)
IL-6 — interleukina 6 (ang. *interleukin 6*)
IL-34 — interleukina 34 (ang. *interleukin 34*)
ITGAM — integryna alfa M (ang. *integrin subunit alpha M*)
JAK2 — kinaza janusowa 2 (ang. *Janus kinase 2*)
JNK — kinaza aktywowana stresem N-końcowa c-Jun (ang. *c-Jun N-terminal kinase*)
LPS — lipopolisacharyd (ang. *lipopolysaccharide*)
MEK — kinaza kinazy aktywowanej mitogenami (ang. *mitogen-activated protein kinase kinase*)
MIA — aktywacja układu odpornościowego matki (ang. *maternal immune activation*)
MCP1 — białko chemotaktyczne monocytów-1 (ang. *chemokine (C-C motif) ligand 2*)
MIP-1β — białko zapalne makrofagów-1β (ang. *chemokine (C-C motif) ligand 4*)
miRNA — mikroRNA (ang. *micro RNA*)
MSS — skala do monitorowania i oceny nasilenia posocznicy (ang. *murine sepsis score*)
mTORC1 — mechanistyczny cel rapamycyny kompleks 1 (ang. *mechanistic target of rapamycin complex 1*)
MTT — bromek 3-(4,5-dimetylo-2-tiazolilo)-2,5-difenylotetrazoliowy
NF-κB —jądrowy czynnik transkrypcyjny kappa B (ang. *nuclear factor kappa B*)
NOR — test rozpoznania nowego obiektu (ang. *novel object recognition test*)
NPS — miejsce fosforylacji w części N-terminalnej (ang. *N-terminal phosphorylation site*)
NSAID — niesteroidowy lek przeciwzapalny (ang. *nonsteroidal anti-inflammatory drug*)
OF — test otwartego pola (ang. *open field test*)
OUN — ośrodkowy układ nerwowy
PICALM — białko adaptorowe wiążące fosfatydyloinozytol i związane z endocytozą zależną od klatryny (ang. *phosphatidylinositol binding clathrin assembly protein*)
PID — domena oddziałująca z P-TEFb (ang. *P-TEFb interacting domain*)
PIP_3 — fosfatydyloinozytol (3,4,5)-trisfosforan (ang. *phosphatidylinositol (3,4,5)-trisphosphate*)

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PI3K — fosfatydyloinozytol 3-kinaza (ang. *phosphoinositide 3-kinase*)
P-TEFb — pozytywny czynnik elongacyjny transkrypcji b (ang. *positive transcription elongation factor b*)
PTEN — fosfataza i homolog tensyny (ang. *phosphatase and tensin homolog*)
PROTAC — chimeryczne cząsteczki ukierunkowanej degradacji białka (ang. *proteolysis targeting chimeras*)
PSD95 — białko gęstości postsynaptycznej 95 (ang. *postsynaptic density protein 95*)
qPCR — ilościowa reakcja PCR w czasie rzeczywistym (ang. *quantitative polymerase chain reaction*)
RAF —białko z rodziny GTP-az (ang. *rat sarcoma*)
RAS — kinaza serynowo-treoninowa (ang. *rapidly accelerated fibrosarcoma*)
RNA — kwas rybonukleinowy (ang. *ribonucleic acid*)
ROS — reaktywne formy tlenu (ang. *reactive oxygen species*)
SAD — sporadyczna postać choroby Alzheimera (ang. *sporadic Alzheimer's disease*)
SIGLEC1 — sialoadhezyna (ang. *sialic acid binding Ig like lectin 1*)
SIRPB1A — białko regulatorowe sygnału β-1A (ang. *signal regulatory protein beta 1A*)
siRNA — małe interferujące RNA (ang. *small interfering RNA*)
SOS — czynnik wymiany nukleotydów guaninowych SOS (ang. *son of sevenless)*
SPOP — białko adaptorowe systemu ligazy ubikwityny E3 typu speckle z domeną POZ (ang. *speckle-type POZ protein*)
STAT3/5 — przekaźnik sygnału i aktywator transkrypcji 3 i 5 (ang. *signal transducer and activator of transcription 3 and 5*)
TLR3 — receptor toll-podobny 3 (ang. *toll-like receptor 3*)
TNF-α — czynnik martwicy nowotworów α (ang. *tumor necrosis factor alpha*)
TREM2 — receptor aktywujący na komórkach mieloidalnych 2 (ang. *triggering receptor expressed on myeloid cells 2*)
TSC1-TSC2 — kompleks stwardnienia guzowatego 1 i 2 (ang. *tuberous sclerosis complex 1 and 2*)
UTR — nieulegający translacji region mRNA (ang. *untranslated region*)
Y721 — tyrozyna 721 (reszta aminokwasowa w CSF1R) (ang. *tyrosine 721, residue in CSF1R*)
ZYX — zyksyna (ang. *zyxin*)

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Streszczenie

Choroby neurodegeneracyjne stanowią poważne wyzwanie medyczne ze względu na rosnącą liczbę zachorowań oraz brak skutecznych metod terapii. Kluczowym elementem patomechanizmu wielu z tych chorób jest przewlekłe neurozapalenie, w którym nieprawidłowa aktywacja mikrogleju prowadzi do uszkodzenia neuronów i zaburzeń funkcji synaptycznych. Co więcej, przewlekłe neurozapalenie przyczynia się do akumulacji agregatów białek, takich jak np. zewnątrzkomórkowe agregaty amyloidu-β (Aβ) i wewnątrzkomórkowe agregaty nadmiernie fosforylowanego białka Tau w chorobie Alzheimera (AD). Próby kliniczne ograniczenia negatywnych skutków neurozapalenia w chorobach neurodegeneracyjnych z użyciem niesterydowych leków przeciwzapalnych nie dały jednoznacznie pozytywnych wyników, dlatego uzasadnione jest poszukiwanie nowych punktów uchwytu w terapii przeciwzapalnej mającej na celu ograniczenie nadmiernej aktywności mikrogleju. Jednym z bardziej obiecujących celów badań wydają się białka z rodziny BET (ang. *bromodomain and extraterminal domain*). Białka te wiążą się z acetylowanymi histonami, modulując chromatynę i regulując ekspresję licznych genów, w tym tych zaangażowanych w odpowiedź zapalną.

Pomimo dostępnych danych wskazujących na przeciwzapalne działanie inhibitorów białek BET, brak było kompleksowych informacji na temat znaczenia białek BET w komórkach mikrogleju. Dlatego celem niniejszej rozprawy doktorskiej było zbadanie roli białek BET w regulacji funkcji mikrogleju, a także w zależnych od mikrogleju procesach zapalnych z uwzględnieniem ich potencjalnego wpływu na rozwój zaburzeń neurodegeneracyjnych.

Badania przeprowadzono na trzech modelach doświadczalnych: i) *in vitro* na komórkach mikrogleju myszy BV2, utrzymywanych jako monokultura oraz ko-kultura z komórkami neuronalnymi myszy HT22, w warunkach natywnych oraz po stymulacji z użyciem lipopolisacharydu (LPS) i Aβ; ii) *in vivo* u dorosłych samców myszy C57BL/6J po ogólnoustrojowej aktywacji układu odpornościowego LPS-em (model endotoksemii); iii) *in vivo* u starych samców myszy C57BL/6J, które w fazie swojego rozwoju embrionalnego doświadczyły aktywacji matczynego układu odpornościowego (model MIA, ang. maternal immune activation). W celu zbadania, jaki udział w obserwowanych zjawiskach mają białka z rodziny BET, zastosowano metody inhibicji farmakologicznej (JQ1, OTX-015) i genetycznej (siRNA). Do analizy wykorzystano m.in. metody biologii

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molekularnej, metody immunochemiczne, mikroskopowe, cytometryczne, wykonano także testy behawioralne.

We wszystkich zastosowanych układach doświadczalnych zaobserwowano specyficzny wzrost ekspresji jedynie białka BRD4, co sugeruje jego szczególną rolę w kontroli ekspresji genów prozapalnych i kontroli aktywności mikrogleju, podczas gdy BRD2 i BRD3 modulują subtelniejsze aspekty homeostazy mikrogleju, w tym regulację genów antyoksydacyjnych i odpowiedź na stres. Farmakologiczne i genetyczne zahamowanie białek BET znacząco modyfikuje funkcjonowanie mikrogleju na wielu poziomach. Blokada BET prowadzi do obniżenia w komórkach mikrogleju BV2 ekspresji genów kodujących CD33, TREM2 i innych genów związanych z fagocytozą oraz hamuje aktywność fagocytarną. Jednocześnie inhibitory białek BET powodują w komórkach stymulowanych zmniejszenie ekspresji cytokin prozapalnych, np. IL-1β, IL-6 i TNF-α, oraz ograniczają neurotoksyczne działanie mikrogleju na komórki neuronalne HT22. Również w badaniach na mysim modelu endotoksemii obserwowano immunosupresyjne działanie JQ1, w tym obniżenie poziomu cytokin w surowicy krwi, przejściowe zahamowanie ekspresji genów prozapalnych w hipokampie oraz zmniejszenie nasilenia objawów chorobowych. Badania wykazały, że oprócz działania przeciwzapalnego, inhibitory białek BET wpływają na poziom Aβ, który jest nie tylko neuropatologicznym markerem AD, ale również kluczowym elementem patomechanizmu tej choroby. Zarówno JQ1 w modelu endotoksemii jak i OTX-015 w modelu MIA powoduje znaczące obniżenie stężenia Aβ w hipokampie, bez wpływu na fosforylację białka Tau. Dodatkowo, OTX-015 poprawia funkcjonowanie pamięci u myszy. Co istotne, redukcja poziomu Aβ po inhibicji białek BET nie wydaje się być wyłącznie konsekwencją modulacji fagocytozy mikrogleju, a raczej wskazuje na działanie dodatkowych mechanizmów molekularnych niezależnych od klasycznej odpowiedzi zapalnej.

Białka z rodziny BET – w szczególności BRD2, BRD3 i BRD4 – pełnią kluczową rolę w regulacji funkcji mikrogleju, kontroli ekspresji genów prozapalnych, aktywności fagocytarnej oraz poziomu Aβ, a w konsekwencji funkcji poznawczych. Selektywna ich modulacja stanowi obiecującą, epigenetyczną strategię terapeutyczną, zdolną do ograniczenia nadmiernej aktywności mikrogleju, redukcji patologii amyloidowej i spowolnienia postępu chorób neurodegeneracyjnych, w tym choroby Alzheimera – otwierając nowy kierunek w przeciwdziałaniu neurozapaleniu i utracie funkcji poznawczych.

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Streszczenie anglojęzyczne (Summary)

Neurodegenerative diseases represent a significant medical challenge due to the increasing incidence and the lack of effective therapeutic approaches. A key component of the pathomechanism underlying many of these disorders is chronic neuroinflammation, in which aberrant microglial activation leads to neuronal damage and synaptic dysfunction. Moreover, chronic neuroinflammation contributes to the accumulation of protein aggregates, such as extracellular amyloid-β (Aβ) deposits and intracellular aggregates of hyperphosphorylated Tau in Alzheimer's disease (AD). Clinical trials aimed at mitigating the detrimental effects of neuroinflammation in neurodegenerative disorders using non-steroidal anti-inflammatory drugs have not yielded unequivocally positive outcomes, highlighting the need to identify novel targets for anti-inflammatory therapy aimed at limiting excessive microglial activity. One of the most promising candidates in this regard appears to be the bromodomain and extraterminal domain (BET) family of proteins. These proteins bind acetylated histones, modulating chromatin structure and regulating the expression of numerous genes, including those involved in the inflammatory response.

Despite existing data suggesting anti-inflammatory effects of BET inhibitors, comprehensive information on the role of BET proteins in microglial cells has been lacking. Therefore, the primary aim of this doctoral dissertation was to investigate the role of BET proteins in the regulation of microglial function, as well as in microglia-dependent inflammatory processes, with particular consideration of their potential impact on the development of neurodegenerative disorders.

The studies were conducted using three experimental models: (i) *in vitro* on mouse BV2 microglial cells maintained as monocultures or in co-culture with mouse HT22 neuronal cells, under basal conditions and following stimulation with lipopolysaccharide (LPS) or Aβ; (ii) *in vivo* in adult male C57BL/6J mice following systemic immune activation with LPS (endotoxemia model); and (iii) *in vivo* in aged male C57BL/6J mice that had experienced maternal immune activation (MIA) during embryonic development. To assess the contribution of BET proteins to the observed phenomena, both pharmacological (JQ1, OTX-015) and genetic (siRNA) inhibition approaches were employed. Analyses included molecular biology, immunochemical, microscopy, and cytometric methods, as well as behavioural testing.

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In all experimental systems employed, a specific increase in the expression of BRD4 protein was observed, suggesting its particular role in the regulation of pro-inflammatory gene expression and the control of microglial activity, whereas BRD2 and BRD3 modulate more subtle aspects of microglial homeostasis, including the regulation of antioxidant genes and stress responses. Pharmacological and genetic inhibition of BET proteins markedly alters microglial function at multiple levels. BET blockade in BV2 microglial cells reduces the expression of genes encoding CD33, TREM2, and other phagocytosis-related genes, while simultaneously suppressing phagocytic activity. At the same time, BET inhibitors decrease the expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in stimulated cells, and mitigate the neurotoxic effects of microglia on HT22 neuronal cells. Similarly, in the murine endotoxemia model, JQ1 exhibited immunosuppressive effects, including reduced serum cytokine levels, transient inhibition of pro-inflammatory gene expression in the hippocampus, and attenuation of disease symptoms. Furthermore, our results demonstrated that, in addition to anti-inflammatory actions, BET inhibitors influence Aβ levels, which are not only a neuropathological hallmark of Alzheimer's disease but also a key element in its pathomechanism. Both JQ1 in the endotoxemia model and OTX-015 in the maternal immune activation (MIA) model significantly lowered hippocampal Aβ concentrations without affecting Tau phosphorylation. Moreover, OTX-015 improved memory performance in mice. Importantly, the reduction in Aβ levels following BET protein inhibition does not appear to result solely from modulation of microglial phagocytosis but rather indicates the involvement of additional molecular mechanisms independent of the classical inflammatory response.

BET family proteins – in particular BRD2, BRD3, and BRD4 – play a central role in the regulation of microglial function, controlling pro-inflammatory gene expression, phagocytic activity, and amyloid-β levels, with consequent effects on cognitive function. Their selective modulation represents a promising epigenetic therapeutic strategy capable of limiting excessive microglial activity, reducing amyloid pathology, and slowing the progression of neurodegenerative diseases, including Alzheimer's disease – thereby opening a novel avenue for combating neuroinflammation and cognitive decline.

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Innowacyjność rozprawy

Rozprawa ta stanowi krok w kierunku pogłębionego zrozumienia mechanizmów epigenetycznych leżących u podstaw aktywacji mikrogleju oraz patologii związanych z chorobami neurodegeneracyjnymi. Integracja różnych modeli eksperymentalnych *in vitro* i *in vivo* umożliwiła kompleksową ocenę funkcji białek z rodziny BET, a także ocenę potencjału terapeutycznego wybranych inhibitorów białek BET, JQ1 i OTX-015, w kontekście neurodegeneracji. Zastosowanie tej wielopłaszczyznowej strategii może stanowić podstawę do opracowania nowych, ukierunkowanych terapii epigenetycznych w chorobach neurodegeneracyjnych.

W niniejszej rozprawie:

1. Zastosowano integrację modeli *in vitro* (BV2 i HT22) oraz modelu zapalenia ogólnoustrojowego *in vivo* do kompleksowej oceny funkcji mikrogleju.
2. Po raz pierwszy wykazano, że jednorazowe podanie inhibitora białek BET obniża poziom Aβ w hipokampie myszy w warunkach zapalnych i niezapalnych.
3. Zidentyfikowano selektywny wpływ JQ1 na ekspresję cytokin prozapalnych i genów związanych z aktywacją mikrogleju.
4. Wykazano, że aktywacja mikrogleju wiąże się ze specyficznym wzrostem ekspresji białka Brd4, co sugeruje jego kluczową rolę w regulacji reakcji zapalnej w mózgu.
5. Stwierdzono, że hamowanie aktywności białek BET w aktywowanym mikrogleju zmniejsza jego cytotoksyczny wpływ na komórki neuronalne, wskazując na potencjalne zastosowanie terapeutyczne inhibitorów BET w ochronie neuronów w warunkach neurozapalnych.
6. Udokumentowano epigenetyczną regulację ekspresji genu CD33 – ważnego receptora mikrogleju związanego z ryzykiem AD.

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Wstęp

Mikroglej – strażnik homeostazy neuroimmunologicznej

Mikroglej stanowi wyspecjalizowaną populację rezydujących w ośrodkowym układzie nerwowym (OUN) komórek immunologicznych, morfologicznie i funkcjonalnie przypominających makrofagi [Hristovska i wsp. 2015, Le i wsp. 2021]. Pełni kluczową rolę w utrzymaniu homeostazy środowiska śródmózgowego – nie tylko poprzez działania obronne, lecz również dzięki zaawansowanym funkcjom regulacyjnym, troficznym i rozwojowym. W warunkach fizjologicznych mikroglej nieustannie monitoruje mikrośrodowisko neuronalne, błyskawicznie reagując na zmiany metaboliczne, sygnały uszkodzenia oraz zaburzenia transmisji synaptycznej [Nayak i wsp. 2014].

Już na etapie rozwoju OUN komórki mikrogleju aktywnie uczestniczą w remodelowaniu sieci neuronalnych – eliminują nadmiarowe synapsy oraz fagocytują komórki apoptotyczne, wspomagając prawidłowe formowanie obwodów nerwowych [Eyo i wsp. 2013; Paolicelli i wsp. 2011]. Zdolność do selektywnej fagocytozy zachowują również w mózgu dorosłego osobnika, gdzie współdecydują o integralności i plastyczności sieci synaptycznych [Brown i wsp. 2012; Brown i wsp. 2019; Brown i wsp. 2021; Fricker i wsp. 2012; Paolicelli i wsp. 2011; Shay i wsp. 2013].

Przeżycie, proliferacja i różnicowanie mikrogleju są ściśle zależne od dwóch ligandów: czynnika stymulującego tworzenie kolonii 1 (CSF1) oraz interleukiny-34 (IL-34), które oddziałują z receptorem CSF1R [Blasi i wsp. 1990; Ginhoux i wsp. 2010; Gómez-Nicola i wsp. 2013]. Złożoność tej sygnalizacji podkreśla, że mikroglej nie jest jedynie „komórką sprzątającą" – to aktywny i dynamiczny regulator równowagi neuroimmunologicznej (Ryc. 1.).

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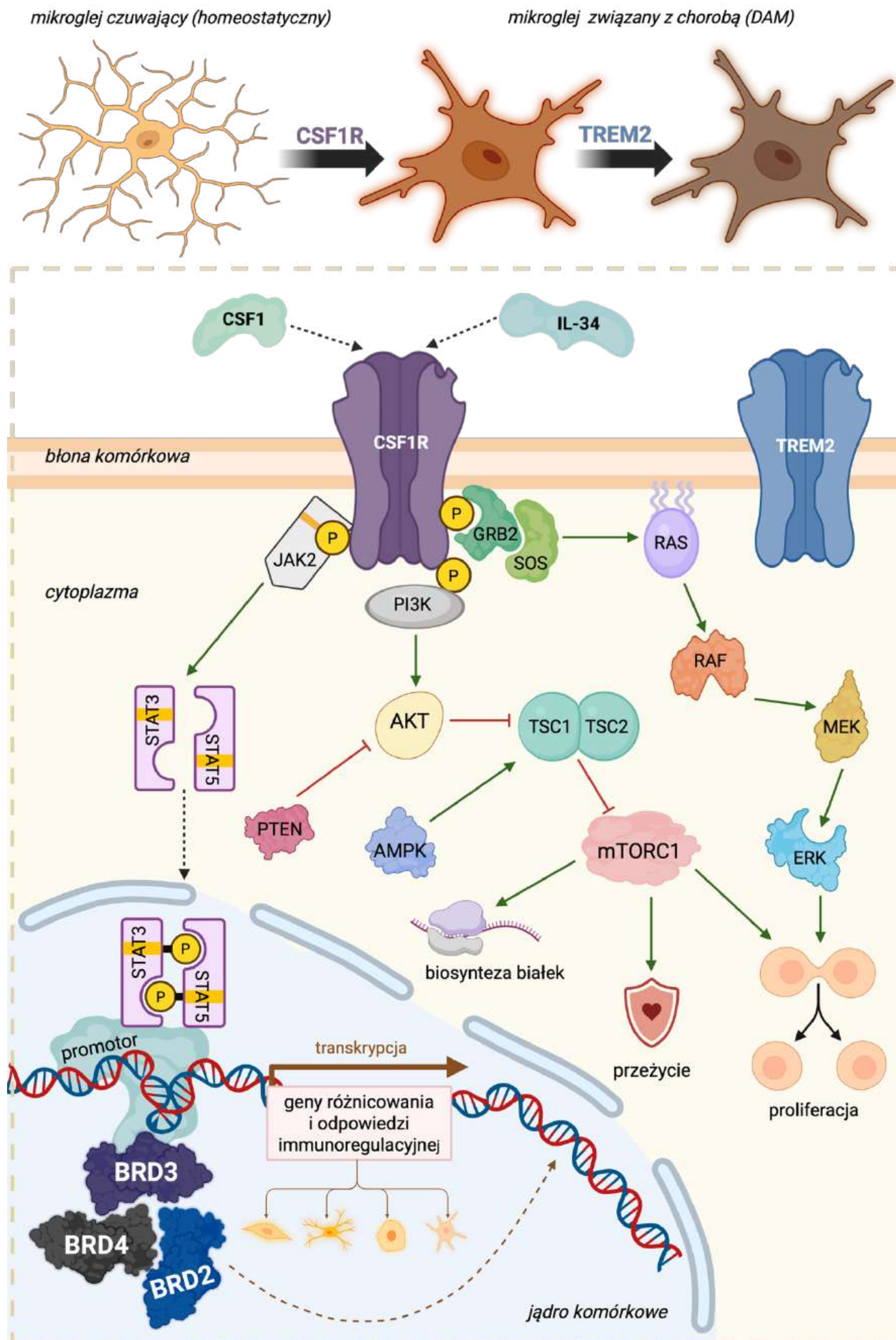

Rycina 1. Sygnalizacja CSF1/IL-34 przez receptor CSF1R reguluje przeżycie, proliferację i różnicowanie mikrogleju.

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Ligandy CSF1 i IL-34 wykazują różne profile ekspresji i uzupełniające funkcje w OUN - oba są niezbędne do rozwoju i utrzymania puli mikrogleju, przy czym IL-34 w większym stopniu wspiera populacje w istocie szarej, a CSF1 w istocie białej [Easley-Neal i wsp. 2019]. Wiązanie ligandów indukuje autofosforylację domeny cytoplazmatycznej CSF1R i aktywację kaskad sygnałowych [Stanley i wsp. 2014]. Fosforylacja reszty Y721 umożliwia rekrutację PI3K i aktywację osi PI3K/Akt, która wspiera przeżycie mikrogleju i regulację metabolizmu [Sampaio i wsp. 2011]. Następnie Akt hamuje kompleks TSC1-TSC2, co prowadzi do odblokowania aktywności mTORC1 i nasilonej biosyntezy białek, proliferacji oraz metabolicznej adaptacji komórek [Murga-Zamalloa i wsp. 2020]. Dodatkowymi negatywnymi regulatorami osi są PTEN (hamujący PI3K/AKT przez defosforylację PIP_3) oraz AMPK (aktywująca TSC1-TSC2 w warunkach stresu energetycznego). Równolegle CSF1R aktywuje szlak RAS/RAF/MEK/ERK, który wspiera cykl komórkowy i proliferację [Gómez-Nicola i wsp. 2013], oraz szlak JAK/STAT (STAT3/5), kluczowy dla różnicowania i odpowiedzi immunoregulacyjnych [Stanley i wsp. 2014]. Integracja tych osi czyni mikroglej dynamicznym regulatorem równowagi neuroimmunologicznej, a nie wyłącznie „komórką sprzątającą". W warunkach patologicznych (np. w chorobach neurodegeneracyjnych) pojawia się populacja DAM (disease-associated microglia), której ekspansja i fenotyp powiązane są z sygnalizacją CSF1R oraz innymi osiami (np. TREM2). DAM pełnią funkcje zarówno ochronne, jak i szkodliwe — nasilają fagocytozę, metabolizm lipidów i produkcję cytokin, ale także przyczyniają się do neurotoksyczności [Deczkowska i wsp. 2018; Keren-Shaul i wsp. 2017]. Białka z rodziny BET (BRD2/3/4) współdziałają z czynnikami transkrypcyjnymi w regulacji ekspresji genów mikrogleju; modulują ekspresję genów i funkcje mikrogleju, co może wpływać na fenotyp DAM, szczególnie w warunkach patologicznych [DeMars i wsp. 2018; Singh i wsp. 2020; Wang i wsp. 2018; Wang i wsp. 2021]. Rycinę opracowano przy użyciu programów Adobe Illustrator i BioRender, przeznaczonych do tworzenia ilustracji i grafik naukowych.

W warunkach patologicznych, takich jak choroby neurodegeneracyjne, obserwuje się specyficzną populację komórek mikrogleju związaną z chorobami (DAM), pojawiającą się na różnych etapach neurodegeneracji i pełniącą zarówno funkcje protekcyjne, jak i szkodliwe w progresji choroby. Komórki DAM lokalizują się przede wszystkim na granicach struktur OUN, w tym w rejonach bariery krew–mózg oraz w pobliżu ognisk uszkodzenia lub degeneracji, gdzie dochodzi do apoptozy. Takie usytuowanie nadaje im kluczowe znaczenie w modulacji odpowiedzi neuroimmunologicznej [Deczkowska i wsp. 2018]. W tej formie mikroglej intensyfikuje fagocytozę, metabolizam lipidów, produkcję cytokin prozapalnych, chemokin, reaktywnych form tlenu (ROS) oraz innych cytotoksycznych mediatorów [Block i wsp. 2005; Chartier-Harlin i wsp. 1991; Galatro i wsp. 2017]. Skutkuje to wtórnym uszkodzeniem neuronów, degradacją połączeń synaptycznych oraz zaburzeniami transmisji neuronalnej.

Neurozapalenie jako rdzeń patogenezy choroby Alzheimera

Choroba Alzheimera (AD) jest najczęstszą chorobą neurodegeneracyjną powodującą demencję u osób starszych [Ferrari i wsp. 2021; Ferreira i wsp. 2020; Korczyn i wsp. 2024; Scheltens i wsp. 2021]. W początkowych fazach choroby mikroglej reaguje na obecność agregatów amyloidu β (Aβ) w mózgu i je usuwa, preciwdziałając w ten sposób ich toksycznym skutkom [Ferrera i wsp. 2014; Fricker i wsp. 2012; Ganz i wsp. 2022;

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Griciuc i wsp. 2021; Kepp i wsp. 2023; Korczyn i wsp. 2024]. Jednak przewlekła ekspozycja mikrogleju na Aβ prowadzi do jego nadmiernej aktywacji i nasila stan zapalny – komórki tracą zdolność efektywnego usuwania toksycznych złogów, a dodatkowo zaczynają fagocytować zdrowe synapsy i neurony [Abanto i wsp. 2024; Armstrong i wsp. 2014; Baker i wsp. 2017; Ball i wsp. 1985; Brown i wsp. 2014; Butler i wsp. 2021; Cheng i wsp. 2020; Gao i wsp. 2022; Leng i wsp. 2021; Morris i wsp. 2014; Pons i wsp. 2021; Querfurth i wsp. 2010]. W efekcie zapalenie, pierwotnie stanowiące odpowiedź obronną, staje się czynnikiem przyspieszającym neurodegenerację. Aktywowane komórki glejowe uwalniają liczne cytokiny i chemokiny zapalne, takie jak TNF-α, IL-1β czy IL-6, które zaburzają homeostazę neuronalną i przyspieszają śmierć komórek nerwowych [Brown i wsp. 2019; Heneka i wsp. 2015; Ransohoff i wsp. 2016; Seok i wsp. 2013].

Modele zwierzęce oraz analizy post mortem ujawniają zwiększoną ekspresję genów związanych z odpowiedzią zapalną, a także podwyższone stężenia mediatorów zapalnych w mózgach osób z AD [Adamu i wsp. 2024; Barroeta-Espar i wsp. 2019; Salter i wsp. 2017; Sobue i wsp. 2023]. Obecność komponentów bakteryjnych (np. lipopolisacharydu – LPS) w OUN pacjentów z AD [Bu i wsp. 2015; Douros i wsp. 2021; Su i wsp. 2022], a także możliwość wywołania u zwierząt zmian neuropatologicznych charakterystycznych dla AD po ekspozycji na LPS [Kim i wsp. 2021; Wang i wsp. 2018], wskazują na związek pomiędzy infekcją, odpowiedzią zapalną a procesem neurodegeneracyjnym. Wykazano również zależność między chorobami przyzębia, które poprzez przewlekły stan zapalny lokalny mogą wywoływać konsekwencje systemowe, a ryzykiem rozwoju AD [Borsa i wsp. 2021; Heppner i wsp. 2015; Peeters i wsp. 2015].

W sporadycznej postaci choroby Alzheimera (SAD) najważniejszym czynnikiem genetycznym pozostaje allel ε4 genu *APOE*, choć badania asocjacyjne całego genomu (GWAS) ujawniły szereg dodatkowych wariantów ryzyka. Polimorfizm genów kodujących receptory mikroglejowe, takie jak *TREM2* czy *CD33*, wykazuje ścisły związek z ryzykiem choroby oraz regulacją odpowiedzi zapalnej [Barroeta-Espar i wsp. 2019; Bertram i wsp. 2008; Tortora i wsp. 2022]. Warianty te modulują zdolność mikrogleju do fagocytozy Aβ oraz kształtują profil cytokinowy, nasilając przewlekłe zapalenie [Bertram i wsp. 2019; McGeer i wsp. 2016]. Podobnie, warianty w genach związanych z układem dopełniacza, np. *CR1*, mogą prowadzić do dysregulacji mechanizmów obronnych i tworzenia neurotoksycznego środowiska [Bertram i wsp. 2019; Bertram i wsp. 2020; Wightman i wsp. 2021].

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W przypadku rodzinnej postaci choroby Alzheimera (FAD) kluczową rolę odgrywają deterministyczne mutacje w genach *APP*, *PSEN1* i *PSEN2*, które prowadzą do nadprodukcji Aβ i wczesnej aktywacji mikrogleju, niezależnie od innych genetycznech i środowiskowych czynników ryzyka. Te mutacje determinują wczesny początek choroby i przyspieszają progresję neuropatologiczną, co odróżnia FAD od SAD pod względem mechanizmów molekularnych [Dai i wsp. 2017].

Dowody epidemiologiczne wskazują, że przewlekłe choroby zapalne zwiększają ryzyko rozwoju AD. Pacjenci z reumatoidalnym zapaleniem stawów, cukrzycą typu 2 czy przewlekłymi infekcjami wykazują wyższe prawdopodobieństwo rozwoju otępienia alzheimerowskiego, przy czym badanie Framingham Heart Study wykazało, że wczesne wystąpienie cukrzycy typu 2 wiązało się z 2–3-krotnie wyższym ryzykiem demencji, a hospitalizacje z powodu ciężkich infekcji zwiększały długoterminowe ryzyko demencji niezależnie od lokalizacji infekcji [Cooper i wsp. 2023; Sipilä i wsp. 2021; Wang i wsp. 2021]. Co istotne, stosowanie długotrwałej terapii niesteroidowymi lekami przeciwzapalnymi (NSAIDs) wiązało się w części badań z obniżonym ryzykiem AD, co dodatkowo potwierdza rolę procesów zapalnych w etiologii choroby [Côté i wsp. 2012; in t' Veld i wsp. 2001; McGeer i wsp. 2007].

Na poziomie klinicznym w płynie mózgowo-rdzeniowym i osoczu pacjentów z AD obserwuje się podwyższone stężenia markerów zapalnych, takich jak IL-6, MCP-1 czy białko C-reaktywne (CRP), które korelują z tempem progresji choroby [Belkina i wsp. 2013; Meng i wsp. 2014]. Biomarkery te wskazują, że proces zapalny nie jest wyłącznie lokalny, ale odzwierciedla systemową odpowiedź organizmu.

W badaniach eksperymentalnych wykazano, że farmakologiczne lub genetyczne ograniczenie aktywacji mikrogleju zmniejsza akumulację Aβ i łagodzi deficyty poznawcze u zwierząt transgenicznych [Biscaro i wsp. 2012; Flores i wsp. 2022; Wang i wsp. 2022]. Z kolei nadmierna stymulacja receptorów TLR, odpowiedzialnych za aktywację mechanizmów wrodzonych odpowiedzi immunologicznej, prowadzi do przyspieszenia neurodegeneracji i zaburzeń synaptycznych [Hughes i wsp. 2020; Liu i wsp. 2018].

Szczególnie niepokojąca jest trwałość aktywacji mikrogleju – komórki te utrzymują stan pobudzenia nawet po ustąpieniu pierwotnego bodźca, co wskazuje na udział długofalowych mechanizmów regulacyjnych związanych z epigenetycznym

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reprogramowaniem („immune memory") mikrogleju [Martins-Ferreira i wsp. 2021]. Istotną rolę w tym zakresie przypisuje się białkom z rodziny BET – molekularnym „czytnikom" epigenetycznych modyfikacji chromatyny [Filippakopoulos i wsp. 2010].

Białka BET – architekci epigenetycznej regulacji

Rodzina białek BET (ang. *bromodomain and extra-terminal domain*) obejmuje konserwatywną grupę epigenetycznych regulatorów transkrypcji, określanych jako „czytniki" (ang. *readers*), rozpoznających specyficzne modyfikacje histonowe – zwłaszcza acetylowane reszty lizyny – i przekładających ten „epigenetyczny kod" na zmiany ekspresji genów [Eischer i wsp. 2023]. W genomie człowieka występuje ponad 60 bromodomen zgrupowanych w około 50 białkach, spośród których cztery zaliczane są do rodziny BET: BRD2, BRD3, BRD4 oraz BRDT. Trzy pierwsze są szeroko eksprymowane w komórkach somatycznych, natomiast BRDT występuje głównie w komórkach linii płciowej [Filippakopoulos i wsp. 2010; Kwon i wsp. 2021; Taniguchi i wsp. 2016]. Białka BET uczestniczą w kontroli ekspresji genów, naprawie DNA, aktywacji komórek glejowych oraz odpowiedzi na stres komórkowy. Ich działanie zależy od kontekstu biologicznego i bywa przeciwstawne, co czyni je interesującym celem terapeutycznym [Cheung i wsp. 2017; Roberts i wsp. 2017; Belkina i wsp. 2013]. Funkcjonalna aktywność białek BET jest modulowana przez post-translacyjne modyfikacje oraz sygnały stresowe i zapalne, obejmujące aktywację szlaków NF-κB, MAPK (ERK, JNK, p38) i JAK/STAT w odpowiedzi na cytokiny prozapalne (TNF-α, IL-1β, IL-6), lipopolisacharyd (LPS) czy stres oksydacyjny [Belkina i wsp. 2013; Cheung i wsp. 2017; Devaiah i wsp. 2012a; Nicodeme i wsp. 2010]. Aktywacja tych kaskad prowadzi zarówno do zmian w acetylacji histonów, jak i do bezpośrednich modyfikacji białek BET, takich jak fosforylacja BRD4 [Cheung i wsp. 2017; Devaiah i wsp. 2012a], wpływając na ich powinowactwo do chromatyny oraz zdolność rekrutowania kompleksów transkrypcyjnych. W efekcie białka BET integrują sygnały środowiskowe z mechanizmami epigenetycznymi, dynamicznie modulując ekspresję genów odpowiedzi zapalnej i stresowej w zależności od kontekstu biologicznego [Belkina i wsp. 2013; Nicodeme i wsp. 2010].

Struktura białek BET obejmuje dwie tandemowe bromodomeny (BD1 i BD2), umożliwiające wiązanie się z acetylowanymi histonami H3 i H4, oraz domenę ET (extra-

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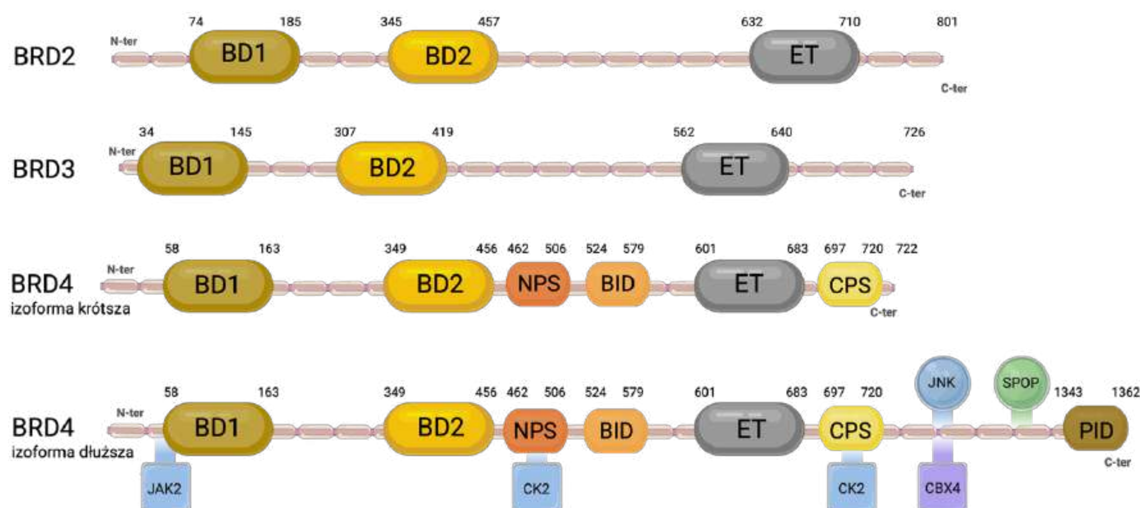
terminal), odpowiedzialną za rekrutację kofaktorów transkrypcyjnych [Badrikoohi i wsp. 2022; Banham i wsp. 2022; Yu i wsp. 2022], co schematycznie przedstwiono na Ryc. 2.



Rycina 2. Domeny strukturalne białek z rodziny BET.

Wszystkie białka BET posiadają dwie tandemowe bromodomeny N-terminalne (BD1 i BD2) oraz domenę pozaterminalną (ET). Numery oznaczają granice aminokwasowe poszczególnych domen w ludzkich białkach BET. Sekwencje aminokwasowe zostały zestawione na podstawie publicznej bazy GenPept (NCBI Reference Sequence): Brd2 – NP_005095.1; Brd3 – NP_031397.1; Brd4 – NP_490597.1. Dodatkowo oznaczono specyficzne domeny i miejsca modyfikacji białka BRD4: PID (P-TEFb interacting domain) – domena oddziałująca z P-TEFb, BID (basic residue-enriched domain) – domena bogata w reszty zasadowe; NPS (N-terminal phosphorylation site) – miejsce fosforylacji w części N-terminalnej; CPS (C-terminal phosphorylation site) – miejsce fosforylacji w części C-terminalnej. Na rycinie uwzględniono również wybrane modyfikacje potranslacyjne białka BRD4. Fosforylacja przez kinazę CK2 w rejonach NPS i CPS znosi auto-inhibicję BD2 i sprzyja zwiększeniu aktywności transkrypcyjnej [Wu i wsp. 2013; Malvezzi i wsp. 2021]. Podobny efekt wywołuje fosforylacja reszt Tyr97/98 przez JAK2, która stabilizuje białko i wzmacnia jego wiązanie z chromatyną [Liu i wsp. 2022]. Odmiennie działa fosforylacja reszt Thr1186 i Thr1212 przez JNK w części C-terminalnej, prowadząca do zmiany funkcji BRD4 i obniżenia jego aktywności [Devaiah i wsp. 2024]. Istotną rolę odgrywa także sumoilacja Lys1111 przez CBX4, która chroni BRD4 przed degradacją i zwiększa jego stabilność [Zhou i wsp. 2024], natomiast ubikwitynacja z udziałem białka SPOP kieruje białko do degradacji proteasomalnej i ogranicza jego aktywność [Dai i wsp. 2017]. Na schemacie modyfikacje prowadzące do wzrostu aktywności/stabilności oznaczono kwadratami, a te skutkujące jej spadkiem – kółkami; kolor niebieski odpowiada fosforylacji, fioletowy sumoilacji, a zielony ubikwitynacji. Rycinę opracowano przy użyciu programów Adobe Illustrator i BioRender, przeznaczonych do tworzenia ilustracji i grafik naukowych.

Oprócz samej budowy domenowej, istotne jest funkcjonalne działanie białek BET – zdolność bromodomen do rozpoznawania acetylowanych histonów i domen ET do rekrutacji kofaktorów transkrypcyjnych pozwala na selektywną modulację ekspresji genów, w tym genów prozapalnych i onkogennych. Mechanizm ten, wraz z jego farmakologiczną modulacją przez inhibitory małocząsteczkowe i związki typu PROTAC przedstawiono schematycznie na Ryc. 3.

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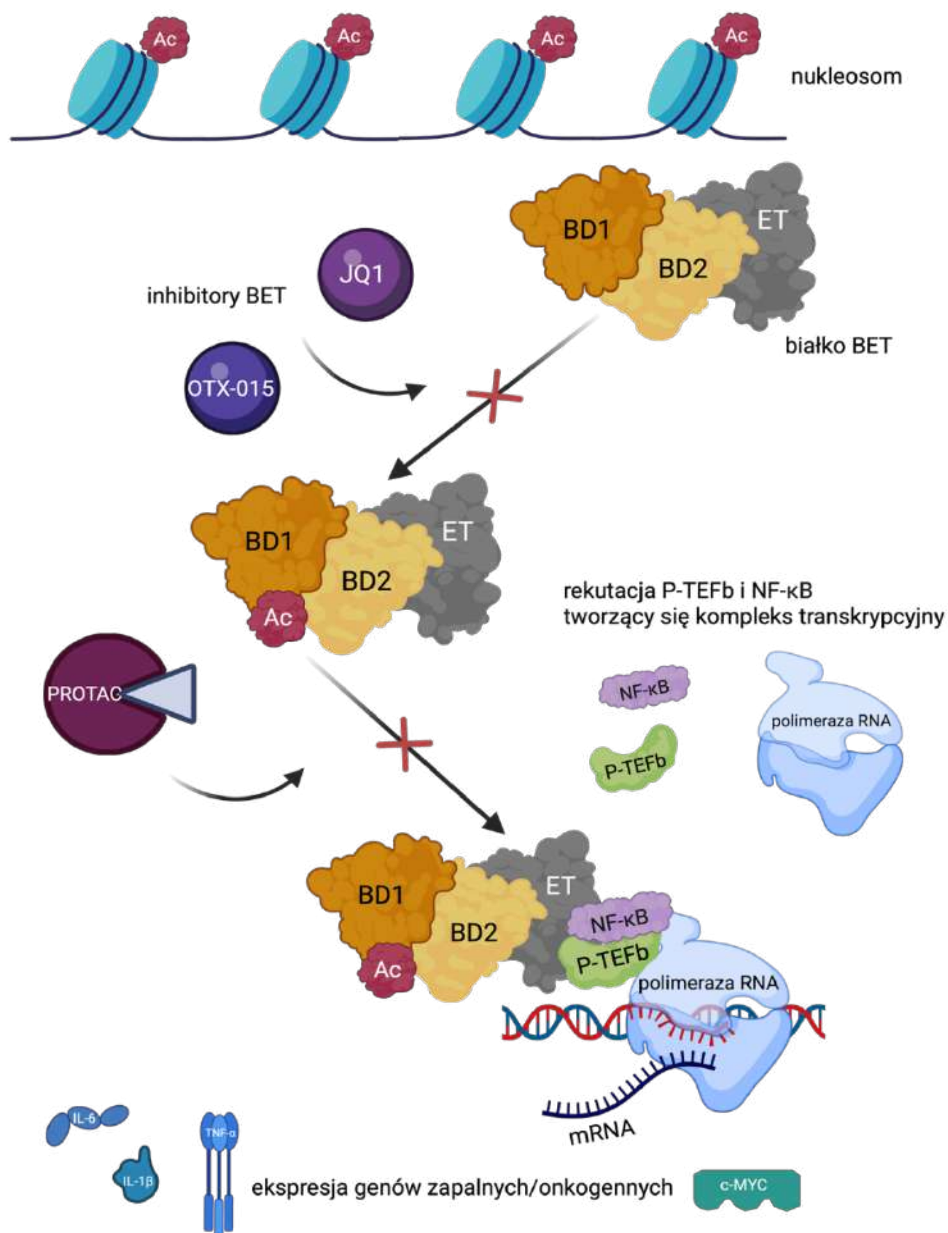

Rycina 3. Mechanizm działania białek z rodziny BET.

Bromodomeny (BD1 i BD2) białek BET rozpoznają acetylowane reszty lizyny w histonach (głównie H3 i H4) oraz w innych białkach chromatyny, co umożliwia rekrutację kompleksów transkrypcyjnych, w tym elongacyjnego kompleksu P-TEFb, czynnika NF-κB oraz mediatorów transkrypcji [Zhou i wsp. 2012; Zou i wsp. 2014]. Domeny ET uczestniczą w interakcjach z kofaktorami transkrypcyjnymi i stabilizują aktywne kompleksy na promotorach i super-enhancerach genów. BRD4, najlepiej scharakteryzowany członek rodziny BET, pełni funkcję „pamięci transkrypcyjnej", utrzymując aktywność genów zapalnych i onkogennych także po ustąpieniu bodźca indukującego [Zou i wsp. 2014]. Inhibitory małocząsteczkowe (np. JQ1, OTX-015) wiążą się konkurencyjnie z kieszeniami acetylo-lizynowymi bromodomen, blokując rozpoznawanie histonów i zakłócając interakcje z P-TEFb. Nowsze strategie terapeutyczne obejmują związki typu PROTAC, które rekrutują ligazę E3 i prowadzą do ubikwitynacji oraz degradacji proteasomalnej białek BET, co skutkuje bardziej trwałym zahamowaniem ich aktywności. Efektem obu mechanizmów jest osłabienie rekrutacji polimerazy RNA II, spadek transkrypcji genów regulowanych przez NF-κB i c-MYC oraz obniżenie ekspresji mediatorów prozapalnych (IL-1β, IL-6, TNFα) i onkogennych [Berenguer-Daizé i wsp. 2016; Bondeson i wsp. 2015; Filippakopoulos i wsp. 2010]. Rycinę opracowano przy użyciu programów Adobe Illustrator i BioRender, przeznaczonych do tworzenia ilustracji i grafik naukowych.

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Chociaż wszystkie białka z rodziny BET pełnią podobne funkcje w kontroli ekspresji genów, istnieją pomiędzy nimi różnice dotyczące np. zakresu oddziaływań z czynnikami transkrypcyjnymi oraz repertuarem kontrolowanych przez nie genów (Tab. 1.) [Wang i wsp. 2021]. BRD2 wiąże się z acetylowanym ogonem histonu H4 i uczestniczy w regulacji cyklu komórkowego oraz różnicowaniu neuronalnym – jego nadekspresja może zaburzać neurogenezę [Belkina i wsp. 2013; Cheung i wsp. 2017]. BRD3, choć strukturalnie zbliżony do BRD2, pełni funkcje zależne od typu komórki i pozostaje słabiej poznany [Deeney i wsp. 2016]. BRD4 to najlepiej scharakteryzowany członek rodziny BET i kluczowy regulator elongacji transkryptu oraz aktywacji genów zapalnych [Filippakopoulos i wsp. 2010; Eischer i wsp. 2023]. Występuje w dwóch izoformach – krótszej (BRD4-S) i dłuższej (BRD4-L), które powstają w wyniku alternatywnego splicingu mRNA [Das i wsp. 2024; Drumond-Bock i wsp. 2021; Drumond-Bock i wsp. 2023]. Izoforma długa zawiera dodatkowe domeny, takie jak domena P-TEFb, które stabilizują kompleksy transkrypcyjne i modulują aktywność enzymów modyfikujących histony, takich jak kinazy i acetylotransferazy [Zhou i wsp. 2024]. BRD4 podlega różnym modyfikacjom potranslacyjnym, w tym fosforylacji, ubikwitynacji i sumoilacji, które wpływają na jego funkcje i interakcje z innymi białkami [Liu i wsp. 2022]. Te modyfikacje umożliwiają BRD4 elastyczne dostosowanie ekspresji genów do zmieniających się warunków komórkowych, takich jak odpowiedź na stres oksydacyjny, cytokiny prozapalne czy aktywację szlaków sygnalizacyjnych, takich jak NF-κB, MAPK czy JAK/STAT [Devaiah i wsp. 2012a; Devaiah i wsp. 2020; Nicodeme i wsp. 2010]. Sekwencja 3′UTR mRNA genu BRD4 zawiera miejsca wiązania dla mikroRNA, takich jak miR-9, co sugeruje dodatkową warstwę regulacji translacyjnej. W odpowiedzi na bodźce stresowe, ekspresja miR-9 jest obniżana, co prowadzi do derepresji BRD4 i wzrostu jego aktywności transkrypcyjnej [Stratton i wsp. 2016].

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Tabela 1. Zestawienie wybranych czynników transkrypcyjnych i genów docelowych regulowanych przez białka z rodziny BET.

Czynnik transkrypcyjny	Geny regulowane przez białka z rodziny BET	Przypisane białka z rodziny BET (według literatury)
RELA (NF-κB)	*NLRP3, IL1B, IL6, TNF, IL8, IL10, IL17, CSF2, CCL2, CXCL9*	**BRD4** wiąże się z acetylowanym RelA (K310) i współdziała z nim, zwiększając ekspresję genów zależnych od NF-κB; dodatkowo integruje sygnalizację NF-κB z odpowiedzią zapalną [Huang i wsp. 2009]
JUN (AP-1)	*ESM1, SPRY2, TGIF1, FST, FGF2, EGR2, FLT1, FOXP1, NPPB, CCN2*	**BRD4** rekrutuje aktywne kompleksy AP-1 (c-Jun) do genów docelowych w określonych kontekstach (np. regulacja MMP-9, stany zapalne) [Lee i wsp. 2017; Wu i wsp. 2016]
CEBPA	*CD274*	**BRD2/BRD4** - przeglądy wskazują zmienne rekrutacje BET do genów różnicowania w zależności od zastosowanego układu doświadczalnego [Cheung i wsp. 2021]
TP53	*ULBP1, ULBP3, PVR, NECTIN2*	**BRD4** wpływa na programy stresowe i geny zależne od TP53 w wielu modelach; jednak interakcje są złożone i kontekstowe [Altendorfer i wsp. 2022]
PPARG	*INS, TNF, IL1B*	**BRD4** reguluje geny metaboliczne i odpowiedź zapalną; **BRD2/BRD4** bywają rekrutowane w regulacji genów metabolicznych [Cheung i wsp. 2021; Lee i wsp. 2017]
E2F1/3	*CCL3, IL1B, TNF*	**BRD2** jest historycznie powiązany z rekrutacją czynników E2F i regulacją genów cyklu komórkowego [Peng i wsp. 2007; Sinha i wsp. 2005]
IRF1/3/4/5/7/8	*IFNB1, IL6, IL1B, IL12A, CCL12, CXCL9, CSF3, IFIT2, MX1, CMPK2*	**BRD4** uczestniczy w regulacji odpowiedzi interferonowej i współpracuje z układami IRF/INF w infekcji i zapaleniu [Shi i wsp. 2014; Tian i wsp. 2017]
RBPJ / EGR2 / BATF	*IL4, IL5, IL13, CSF2, CSF1, ICOS, CD40LG, ICAM1, CD58*	**BRD2/BRD4** rekrutowane są do enhancerów aktywowanych w limfocytach; często **BRD4** pojawia się w SE/aktywowanych enhancerach immunologicznych [Cheung i wsp. 2021; Lee i wsp. 2017]
MYC	*CD274, IL1B, IFNB, ISG15,*	**BRD4** reguluje transkrypcję MYC i kontrolę jego programu genowego (również

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	ULBP1/3, PVR, NECTIN2, MICA	mechanistyczne dowody) [Devaiah i wsp. 2020; Kotekar i wsp. 2023]
RORC	*IL17A, IL21, CSF2, IL22, IL10*	głównie **BRD4**, częściowo **BRD2** wspierają aktywację czynnika RORC i regulację genów charakterystycznych dla limfocytów Th17 (np. IL-17A, IL-21, IL-22) [Cheung i wsp. 2021]
FOXM1	*PLK1, CCL5, CCL2, CXCL8, FOXM1*	**BRD4** reguluje geny proliferacyjne i cyklu komórkowego, powiązania z FOXM1 opisane w kontekście proliferacji nowotworowej [Altendorfer i wsp. 2022]
FOXO3	*IL6, TNF, IL1B, PTHLH*	**BRD2 /BRD4** wpływają na odpowiedzi stresowe i programy transkrypcyjne związane z FOXO [Altendorfer i wsp. 2022; Cheung i wsp. 2021]
AIRE	*ALOX12, S100A9, CD4, CELF2, KRT13*	brak jednoznacznych dowodów przypisujących wyłącznie jedno białko BET, możliwa jest rekrutacja BET do regulatorów specyficznych tkankowo [Cheung i wsp. 2021]
SP7 / RUNX2	*SLPI, RUNX2, SP7*	**BRD2/BRD4** pojawiają się przy genach związanych z różnicowaniem kostnym i regulatorami typu RUNX; dane zależne od tkanki [Antony i wsp. 2020; Lee i wsp. 2017]
STAT1/2/3/5	*IL6, CCL2, CSF2, MX1, CMPK2, NPPB, CCN2, ACOD1*	**BRD4** koordynuje aktywność enhancerów i genów odpowiedzi zapalnej w tandemie ze STAT; obserwacje w modelach zapalnych i nowotworowych [Shi i wsp. 2014; Tian i wsp. 2017]
ETS1 / CDX2 / LIN54 / FOXL1	*ESM1, SPRY2, TGIF1, FST, FGF2, EGR2, FLT1, FOXP1*	**BRD2/BRD3/BRD4** - rekrutacja do enhancerów regulowanych przez czynniki szlaku rozwojowego są raportowane, z różnymi wzorcami przyciągania przez białka z rodziny BET [Cheung i wsp. 2021; Lee i wsp. 2017]

Poszczególni członkowie rodziny BET (BRD2, BRD3, BRD4) różnią się zakresem interakcji i repertuarem kontrolowanych genów, co podkreśla ich nie w pełni nakładające się funkcje w regulacji ekspresji genów, mimo że ogólnie uczestniczą w tym procesie poprzez interakcje z szerokim spektrum czynników transkrypcyjnych [Altendorfer 2022; Borck i wsp. 2020; Cheung 2021; Kotekar 2023; Shi 2014]. Białka z rodziny BET uczestniczą zarówno w regulacji odpowiedzi immunologicznej (np. współpraca z RELA, IRF, STAT czy RORC) [Tian 2017; Wu 2016], jak i w procesach różnicowania komórkowego (np. poprzez RUNX2, GATA1 czy FOXM1) [Lee 2017; Peng 2007; Sinha 2005] czy odpowiedzi na stres (np. z udziałem TP53, FOXO3) [Kotekar 2023]. Widoczne jest także ich zróżnicowane zaangażowanie w regulację cytokin prozapalnych (IL-1B, IL-6, TNF), chemokin (CCL2, CXCL9), czynników wzrostu (FGF2, CSF2) czy regulatorów cyklu komórkowego (CCN2, PLK1) [Altendorfer 2022; Cheung 2021]. Tak szeroki, a zarazem zróżnicowany zakres funkcji wskazuje, że poszczególne białka BET nie są wymienne, lecz pełnią częściowo unikalne role zależne od kontekstu komórkowego i zestawu aktywnych czynników transkrypcyjnych [Kotekar 2023; Shi 2014].

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Białka BET jako cel terapeutyczny

Pierwsze inhibitory białek BET były rozwijane głównie w onkologii, niektóre z nich testowano w badaniach klinicznych u pacjentów z nowotworami hematologicznymi i guzami litymi [Riganti i wsp. 2018]. Przeprowadzono także badanie fazy 1 dotyczące stosowania OTX-015 u pacjentów z chłoniakiem lub szpiczakiem mnogim, a ponieważ dawka była dobrze tolerowana to stanowi punkt wyjścia do dalszych badań klinicznych [Amorim i wsp. 2016]. Wykazano także, że OTX-015 hamuje wzrost komórek białaczkowych, indukując zatrzymanie cyklu komórkowego i apoptozę. Działanie to jest związane z obniżeniem ekspresji c-MYC, co sugeruje potencjalne zastosowanie OTX-015 w leczeniu białaczek [Coudé i wsp. 2015]. Opracowano również nowe pochodne tieno[2,3-d]pirymidyny, które działają jako selektywne inhibitory BRD4 i HDAC, indukując śmierć komórkową w komórkach raka okrężnicy. Powszechnie uznaje się duży potencjał terapeutyczny tych związków w leczeniu nowotworów [Pan i wsp. 2020]. Choć ich zastosowanie w chorobach OUN nie zostało jeszcze ocenione w badaniach klinicznych, wyniki badań przedklinicznych oraz obserwacje w chorobach autoimmunologicznych, takich jak toczeń rumieniowaty układowy, w którym inhibitor BET JQ1 łagodził objawy w modelu myszy MRL/lpr [Wei i wsp. 2015], sugerują możliwość ich zastosowania w leczeniu chorób OUN.

Ważnym kierunkiem badań dotyczących roli białek BET jest analiza ich udziału w regulacji funkcji komórek układu odpornościowego. W odpowiedzi na bodźce zapalne, takie jak TNF-α, IL-1β czy LPS, białka z rodziny BET, zwłaszcza BRD4, rekrutują się do acetylowanych histonów w promotorach i enhancerach genów prozapalnych, umożliwiając ich transkrypcję. Hamowanie funkcji BET w makrofagach obniża ekspresję cytokin indukowanych przez LPS, podkreślając kluczową rolę tych białek w amplifikacji odpowiedzi zapalnej [Belkina i wsp. 2013; Nikkar i wsp. 2022]. Inhibitory białek BET, takie jak JQ1, OTX-015 czy I-BET762, skutecznie obniżają ekspresję genów kodujących mediatory zapalne: TNF-α, IL-6, IL-1β, MCP1, MIP-1β [Berenguer-Daizé i wsp. 2016; Boi i wsp. 2015; Li i wsp. 2017; Magistri i wsp. 2016; Meng i wsp. 2014; Nicodeme i wsp. 2010; Peeters i wsp. 2015].

Poza aktywnością przeciwzapalną wykazano również, że inhibitory białek BET modulują ekspresję genów związanych z plastycznością synaptyczną oraz czynnikami

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neurotroficznymi. W modelach zwierzęcych prowadzi to do poprawy pamięci i uczenia się niezależnie od poziomu Aβ [Roy i wsp., 2022; Scheeren i wsp., 2021]. Ponadto, w badaniach nad starzeniem wykazano, że hamowanie białek BET redukuje stres oksydacyjny i stabilizuje funkcje mitochondriów, co może ograniczać utratę synaps [Boi i wsp., 2015; Dombret i wsp., 2014; Yu i wsp., 2022]. W badaniach *in vivo* wykazano, że zahamowanie BET/BRD4 w modelu ostrej toksyczności Aβ u szczura zmniejsza dysfunkcję synaptyczną i poprawia wyniki w testach pamięci przestrzennej, co wskazuje na potencjał inhibitorów białek BET jako cząsteczek immunomodulujących i neuroprotekcyjnych [Nikkar i wsp., 2022].

Na podstawie dostępnych danych literaturowych można przypuszczać, że zastosowanie inhibitorów białek BET w warunkach neurozapalenia, poprzez modulację aktywności gleju i zmniejszenie ekspresji cytokin prozapalnych, może być skuteczną strategią ochrony neuronów i zachowania funkcji poznawczych [Korb i wsp., 2015; Nikkar i wsp., 2022; Yu i wsp., 2022]. Nieliczne dane dotyczące wpływu inhibitorów białek BET na funkcje komórek gleju sugerują, że i tutaj mamy do czynienia z działaniem immunosupresyjnym [Baek i wsp., 2021; Sánchez-Ventura i wsp., 2019]. Wyniki te otwierają drogę do zastosowania inhibitorów białek BET nie tylko w terapii, ale również w potencjalnej prewencji zaburzeń neurorologicznych o podłożu zapalnym.

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Cel pracy

Choroby neurodegeneracyjne, takie jak choroba Alzheimera (AD), cechuje przewlekła aktywacja mikrogleju i astrogleju w obrębie OUN [Bertram i wsp. 2020; McGeer i wsp. 2007; Serrano-Pozo i wsp. 2011]. Uważa się, że procesy te odgrywają kluczową rolę w nasilaniu neurodegeneracji i pogarszaniu funkcji poznawczych [Heneka i wsp. 2015]. Jednocześnie dotychczasowe próby kliniczne wykorzystujące klasyczne leki przeciwzapalne (np. NSAID) przynosiły ograniczone rezultaty [Breitner i wsp. 2011; Leoutsakos i wsp. 2012]. W ostatnich latach coraz większą uwagę zwraca się na epigenetyczne mechanizmy regulacji odpowiedzi zapalnej, w tym na rolę białek z rodziny BET, które jako „czytniki" acetylacji histonów i innych białek chromatyny, uczestniczą w tworzeniu kompleksów transkrypcyjnych regulujących ekspresję genów zapalnych [Belkina i wsp. 2013; Filippakopoulos i wsp. 2010; Liu i wsp. 2021; McGeer i wsp. 2007].

Cel ogólny

Celem niniejszej rozprawy doktorskiej było zbadanie roli białek z rodziny BET w zależnych od mikrogleju procesach zapalnych i ich potencjalnego wpływu na rozwój zaburzeń neurodegeneracyjnych. Główna hipoteza badawcza zakłada, że farmakologiczne lub genetyczne zahamowanie aktywności białek BET może modulować fenotyp mikrogleju, a przez to ograniczać efekty jego cytotoksycznego działania w OUN.

Cele szczegółowe

1. Zbadanie wpływu inhibicji białek BET na aktywność fagocytarną mikrogleju *in vitro.*

2. Analiza wpływu inhibicji białek BET na ekspresję genów prozapalnych i cytotoksyczne działanie komórek mikrogleju *in vitro*.

3. Zbadanie w mysim modelu, jak MIA wpływa na poziom neuropatologicznych markerów choroby Alzheimera, Aβ i fosforylowanego białka Tau, oraz aktywność gleju w hipokampie potomstwa w wieku 12 miesięcy.

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4. Ocena wpływu inhibitora białek BET (OTX-015) na pamięć oraz poziom neuropatologicznych markerów choroby Alzheimera, Aβ i fosforylowanego białka Tau w hipokampie 12-miesięcznych myszy narażonych w prenatalnej fazie rozwoju na aktywację układu odpornościowego matki (MIA).

5. Zbadanie roli białek BET w epigenetycznej kontroli ekspresji wybranych genów o potwierdzonym znaczeniu w patomechanizmie choroby Alzheimera w hipokampie w warunkach endotoksemii u myszy.

6. Ocena wływu inhibitora białek BET JQ1 na stan zapalny, zachowania chorobowe i poziom patologicznych białek Aβ i fosforylowanego białka Tau w hipokampie myszy w warunkach endotoksemii.

7. Analiza zmian ekspresji BRD2, BRD3 i BRD4 w mikrogleju *in vitro*, a także w mózgu myszy w warunkach aktywacji procesów neurozapalnych.

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Metodyka

Inhibitory białek BET jako narzędzie badawcze

Selektywne inhibitory białek BET, takie jak JQ1, I-BET762 (molibresib), OTX-015 (birabresib), CPI-0610, GSK525762A (zinabryst), PFI-1 (PF-6405761) oraz RVX-208 (apabetalon), zostały opracowane jako małocząsteczkowe ligandy zdolne do specyficznego blokowania bromodomen i uniemożliwiające ich wiązanie z acetylowanymi histonami oraz niehistonowymi białkami regulatorowymi [Belkina i wsp. 2013; Benito i wsp. 2017; Jang i wsp. 2017; Jostes i wsp. 2017; Magistri i wsp. 2016; Mertz i wsp. 2011]. W rezultacie dochodzi do zahamowania ekspresji wielu genów kontrolowanych przez białka BET, w tym genów zaangażowanych w regulację funkcji immunologicznych komórek układu odpornościowego [Dombret i wsp. 2014].

Centralnym narzędziem farmakologicznym zastosowanym w badaniach opisanych w niniejszej dysertacji był związek JQ1 (S)-4-(4-chlorofenyl)-2,3,9-trimetyl-6H-tieno[3,2-f]triazolo[4,3-a]diazepino-6-octowy kwas, ester 1,1-dimetyloetylowy – wybiórczy inhibitor białek z rodziny BET [Filippakopoulos i wsp. 2010; Lambert i wsp. 2019; Li i wsp. 2020; Li i wsp. 2022] występujący w dwóch formach enancjomerycznych: (+)-JQ1 (S) i (−)-JQ1 (R). Aktywną biologicznie formą jest (+)-JQ1, który skutecznie zmniejsza ekspresję genów docelowych białek BET, podczas gdy (−)-JQ1 nie wykazuje takiego działania. W dalszej części tekstu użyto skróconej nazwy JQ1, mając na myśli (+)-JQ1 [Wang i wsp. 2018].

Farmakokinetyka JQ1 charakteryzuje się krótkim okresem półtrwania w osoczu (około 1–2 h u myszy), wysoką biodostępnością po podaniu dootrzewnowym oraz efektywną dystrybucją do tkanki mózgowej, co pozwala na stosowanie go w badaniach procesów neurozapalnych i neurodegeneracyjnych [Belkina i wsp. 2013; Filippakopoulos i wsp. 2010]. W modelach zwierzęcych wykazano, że JQ1 szybko przenika do mózgu i skutecznie redukuje ekspresję cytokin zapalnych, takich jak *Il1b* i *Tnf* [Sánchez-Ventura i wsp. 2019]. Ograniczeniem zastosowania JQ1 jest jego nieselektywność w stosunku do poszczególnych białek BET oraz potencjalny wpływ ogólnoustrojowy, co może utrudniać rozdzielenie efektów centralnych i obwodowych [Meng i wsp. 2014]. W przyszłości wskazane byłoby zastosowanie selektywnych inhibitorów BET lub narzędzi genetycznych (np. siRNA, knockout specyficzny dla mikrogleju), które umożliwią dokładniejsze zdefiniowanie roli poszczególnych członków rodziny BET w regulacji

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stanu zapalnego w mózgu [Devaiah i wsp. 2012b; Qiu i wsp. 2022; Wang i wsp. 2018; Wang i wsp. 2023]. JQ1 jest szeroko stosowany w badaniach przedklinicznych jako narzędzie do badania funkcji białek BET w modelach nowotworów, chorób neurodegeneracyjnych i zapalnych [Jung i wsp. 2015]. Pomimo ograniczeń farmakokinetycznych, jego silne działanie biologiczne czyni go cennym narzędziem badawczym [Feng i wsp. 2023; Orihara i wsp. 2023; Shorstova i wsp. 2021; Yellapu i wsp. 2023].

OTX-015 (birabresib, MK-8628, czyli 2-[(6S)-4-(4-chlorofenyl)-2,3,9-trimetyl-6H-tieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl]-N-(4-hydroksyfenyl)acetamid) jest inhibitorem białek BET (BRD2, BRD3, BRD4), który wykazuje działanie przeciwzapalne i neuroprotekcyjne [Coudé i wsp. 2015; Liu i wsp. 2021; Servidei i wsp. 2021]. W badaniach przedklinicznych udowodniono, że OTX-015 skutecznie przenika przez barierę krew–mózg (BBB) [Berenguer-Daizé i wsp., 2016]. Badania kliniczne natomiast wykazały, że OTX-015 jest dobrze tolerowany, a jego farmakokinetyka w organizmie ludzkim jest opisana modelem jednoprzestrzennym z liniową eliminacją [Odore i wsp. 2016].

Modele badawcze

W rozprawie zastosowano trzy modele eksperymentalne umożliwiające analizę roli białek z rodziny BET: model komórkowy *in vitro,* model pobudzenia immunologicznego OUN wywołanego u dorosłych myszy obwodowym podaniem LPS (model endotoksemii) [Zhao i wsp. 2019] oraz model starzejących się myszy, które w okresie rozwoju embrionalnego narażone były na aktywację układu odpornościowego matki (model MIA, ang. *maternal immune activation*) [Knuesel i wsp. 2014].

Modele eksperymentalne *in vitro* – linie komórkowe BV2 i HT22

Badania prowadzono na liniach komórkowych: mikroglejowej BV2 (pochodzenia mysiego) oraz neuronalnej HT22 (pochodzącej z hipokampu myszy). Komórki BV2 stanowią unieśmiertelnioną mysią linię mikroglejową uzyskaną w wyniku zakażenia pierwotnych komórek mikrogleju myszy szczepu C57BL/6 retrowirusem przenoszącym onkogeny *v-raf/v-myc* [Bocchini i wsp. 1992]. Ze względu na swoje właściwości, komórki BV2 są szeroko stosowane jako substytut mikrogleju pierwotnego w badaniach

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mechanistycznych nad neurozapaleniem [Blasi i wsp. 1990; Carata i wsp. 2024; Chen i wsp. 2024; Henn i wsp. 2009]. Linia BV2 wykazuje wysoką reaktywność na LPS, co czyni ją cennym narzędziem do oceny efektów działania związków immunomodulujących [Baek i wsp. 2021; Das i wsp. 2016; Henn i wsp. 2009; Huang i wsp. 2025; Lund i wsp. 2006; Moretti i wsp. 2022].

Z kolei komórki HT22 to linia wyprowadzona z linii HT4 - unieśmiertelnionych neuronów hipokampu myszy [Morimoto i wsp. 1990]. Komórki HT22 stanowią wartościowy model i*n vitro* do badań nad mechanizmami neurodegeneracyjnymi. Wysoka wrażliwość komórek linii HT22 na reaktywne formy tlenu (ROS) umożliwia badanie procesów śmierci komórkowej oraz mechanizmów ochrony antyoksydacyjnej neuronów, co czyni je szczególnie użytecznymi w poszukiwaniu potencjalnych strategii neuroprotekcyjnych w chorobach neurodegeneracyjnych [He i wsp. 2013; Murphy i wsp. 1988]. Linia komórek HT22 jest często wykorzystywana w badaniach mechanizmów neurotoksyczności i stresu oksydacyjnego, jak również w badaniach nad neuroprotekcją [Jacques i wsp. 2023, Prasansuklab i wsp. 2023; Suh i wsp. 2007; Wang i wsp. 2025; Yang i wsp. 2024]. W prezentowanych badaniach linia ta została użyta do oceny wpływu inhibitorów białek BET na cytotoksyczną aktywność pobudzonych komórek mikrogleju BV2.

W celu wywołania aktywacji mikrogleju stosowano LPS oraz Aβ na różnych etapach agregacji (oligomery, protifibryle, fibryle) [Dubnovitsky i wsp. 2013; Hughes i wsp. 2020]. Aby zahamować białka z rodziny BET komórki inkubowano w obecności inhibitorów: JQ1, OTX-015, IBET-762, GSK1210151A, PFI-1. Aby zachować kontrolowane i porównywalne warunki eksperymentu we wszystkich grupach doświadczalnych, do grup kontrolnych dodawano analogiczne objętości rozpuszczalnika. Do wyciszania genów białek BET w komórkach BV2 zastosowano transfekcję siRNA dla *Brd2*, *Brd3* i *Brd4*, z użyciem lipofektaminy [Quezada i wsp. 2021]. W grupach kontrolnych dodawano analogiczne objętości samego rozpuszczalnika, samą lipofektaminę oraz jako kontrolę negatywną niecelowane siRNA. Interakcje mikroglej–neuron badano stosując ko-kulturę komórek BV2 i HT22 w systemie Transwell (Ryc. 4.) oraz dodając kondycjonowane medium hodowlane z komórek BV2 do komórek HT22.

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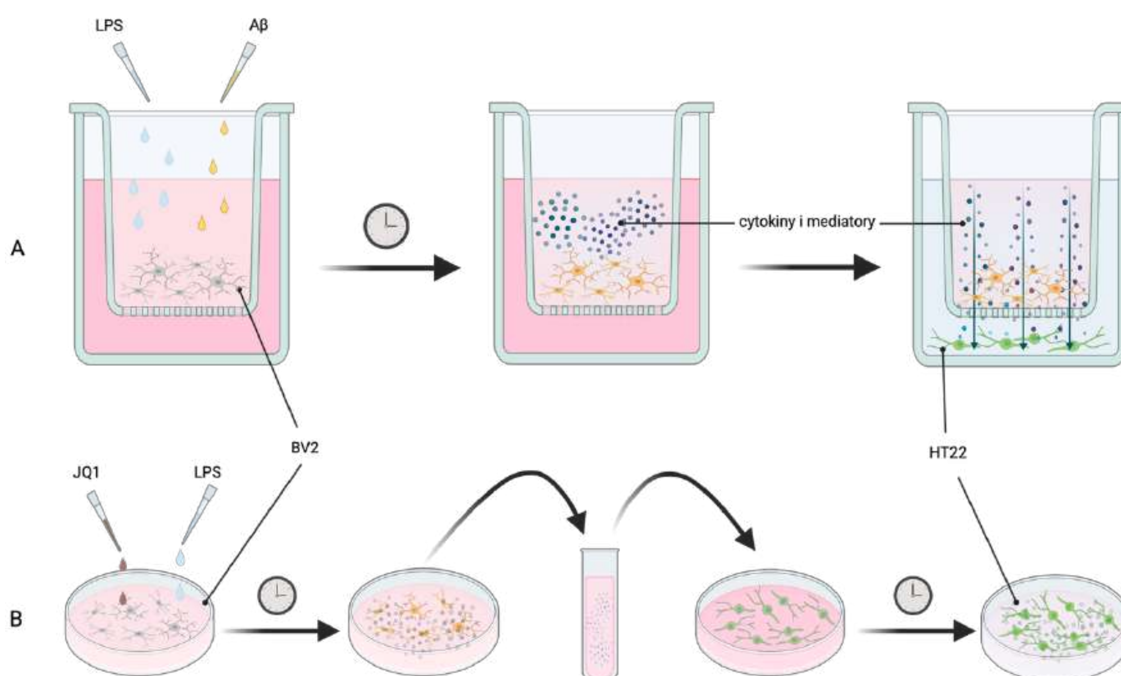

Rycina 4. Schemat analizy oddziaływania aktywnych komórek mikrogleju BV2 na komórki neuronalne HT22 *in vitro* (Publikacja II).

(A) Komórki mikrogleju BV2 hodowane są w systemie Transwell we wkładce z dnem w formie półprzepuszczalnej membrany. Po stymulacji komórek BV2 z wkładki usuwa się medium, po czym przenosi się ją do szalki, w której znajdują się komórki HT22. Dzięki półprzepuszczalnej membranie, wydzielane przez mikroglej do medium czynniki (cytokiny i inne rozpuszczalne mediatory) mogą na drodze dyfuzji oddziaływać na HT22 bez bezpośredniego kontaktu między komórkami. Taki układ eksperymentalny zapobiega przeniesieniu czynnika stymulującego do hodowli HT22. (B) Po stymulacji hodowli komórek BV2 medium kondycjonowane zawierające wydzielone cytokiny i inne rozpuszczalne mediatory przenosi się do hodowli HT22, w celu oceny wpływu wydzielanych przez mikroglej czynników na neurony. Taki układ eksperymentalny nie zapobiega przeniesieniu czynnika stymulującego do hodowli HT22. Rycinę opracowano przy użyciu programów Adobe Illustrator i BioRender, przeznaczonych do tworzenia ilustracji i grafik naukowych.

Funkcjonalne analizy komórek BV2 obejmowały fagocytozę (z użyciem fluorescencyjnych mikrosfer lub wyznakowanego fluorescencyjnie $A\beta_{1-42}$), ocenianą za pomocą mikroskopii konfokalnej [Polioudakis i wsp., 2019] oraz cytometrii przepływowej [Gu i wsp., 2014], proliferację (liczenie komórek w hodowli), cytotoksyczność (barwienie jodkiem propidyny i analiza cytometryczna), witalność (test MTT) oraz migrację (test zadrapania, ang. *scratch assay* [Liang i wsp., 2007]). Badania molekularne obejmowały analizę poziomu ekspresji genów metodą qPCR [Lloyd i wsp. 2024] oraz analizę poziomu białek za pomocą metody ELISA [Eischer i wsp. 2023].

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Ograniczeniem zastosowanych modeli eksperymentalnych *in vitro* jest ich niepełna reprezentatywność w stosunku do komórek pierwotnych, brak wpływu środowiska śródmózgowego oraz uproszczenie i skrócenie czasu interakcji międzykomórkowych [Lin i wsp. 2014; Moussaieff i wsp. 2015]. Mimo to ich zastosowanie pozwoliło na uzyskanie informacji na temat mechanizmów molekularnych działania inhibitorów białek BET [Aktories i wsp. 2022; Henn i wsp. 2009; Liu i wsp. 2009; Sarkar i wsp. 2018].

Modele eksperymentalne *in vivo*

Wszystkie eksperymenty przeprowadzono zgodnie z dyrektywą UE 2010/63 za zgodą wydaną przez II Lokalną Komisję Etyczną ds. Doświadczeń na Zwierzętach w Warszawie (numery zezwoleń: WAW2/060/2020 oraz WAW2/052/2021). Do badań wykorzystano myszy szczepu C57BL/6J hodowane w Zwierzętarni Instytutu Medycyny Doświadczalnej i Klinicznej im. M. Mossakowskiego PAN. Zwierzęta utrzymywano w standardowych warunkach laboratoryjnych (temperatura 22°C ±10%, wilgotność 55% ±10%, cykl 12/12 h światło/ciemność) i karmiono *ad libitum*. Stosowano procedury ograniczające stres i cierpienie oraz minimalizowano liczbę zwierząt [Azkona i wsp. 2023; MacArthur i wsp. 2018].

Model 1 *in vivo* – badanie wpływu inhibitora JQ1 na wzmożoną aktywność procesów neuroimmunologicznych OUN wywołaną przez obwodową iniekcję LPS (model endotoksemii)

W badaniach *in vivo* wykorzystano model systemowej aktywacji układu odpornościowego wywołanej poprzez dootrzewnowe podanie lipopolisacharydu (LPS) 3-miesięcznym samcom myszy szczepu C57BL/6J. Bakteryjny LPS jest silnym aktywatorem układu odpornościowego, w tym mikrogleju rezydującego w mózgu [Zhan i wsp. 2016; Zhao i wsp. 2017]. Jego obecność stwierdzono w mózgach osób z chorobą Alzheimera, a potencjalnym źródłem są zarówno infekcje bakteryjne, jak i mikrobiom jelitowy [Bu i wsp. 2015; Douros i wsp. 2021; Sun i wsp. 2022]. Zastosowana dawka oraz czas ekspozycji (3 h i 12 h) zostały dobrane w oparciu o wcześniejsze doświadczenia zespołu [Czapski i wsp. 2004; Czapski i wsp. 2006; Czapski i wsp. 2007; Czapski i wsp. 2013; Czapski i wsp. 2016; Czapski i wsp. 2020; Jacewicz i wsp. 2009] oraz liczne dane literaturowe [Engler-Chiurazzi i wsp. 2023; Garay i wsp. 2013; Knuesel i wsp. 2014;

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Loayza i wsp. 2023; Reisinger i wsp. 2016; Woods i wsp. 2023; Woods i wsp. 2021] potwierdzające skuteczność tego modelu w wywoływaniu odpowiedzi zapalnej w obrębie OUN.

Model ten pozwala na odtworzenie w warunkach eksperymentalnych istotnych elementów neurozapalnych, w tym wzrostu poziomu cytokin prozapalnych (IL-1β, IL-6, TNF-α), aktywacji mikrogleju, fosforylacji białka Tau oraz objawów behawioralnych, takich jak zespół zachowań chorobowych (ang. *sickness behavior*) [Birch i wsp. 2014; Cheng i wsp. 2014; Delorme i wsp. 2023; Delpech i wsp. 2016; Godbout i wsp. 2005; Kirk i wsp. 2019; Knuesel i wsp. 2014]. Zaletą modelu jest jego powtarzalność, stosunkowo niski koszt oraz zgodność z aktualnymi trendami badawczymi [Carregosa i wsp. 2024; Flores da Silva i wsp. 2024; Sheen i wsp. 2024; Wang i wsp. 2025], a ograniczeniem – brak przewlekłości procesu zapalnego oraz niepełna translacyjność do ludzkich chorób neurodegeneracyjnych [Fang i wsp. 2010; Hameete i wsp. 2021; Pierzynowska i wsp. 2019]. Dzięki dobrze poznanym podobieństwom i różnicom w ekspresji genów u ludzi i myszy, takim jak wzorce wiązania czynników transkrypcyjnych, model ten pozwala na identyfikację potencjalnych molekularnych celów terapeutycznych. W warunkach ostrej stymulacji LPS-em obejmują one receptory Toll-podobne (TLR), CD33, TREM2 oraz białka BET, których modulacja może przynieść efekt neuroprotekcyjny [Bradshaw i wsp. 2013; Butler i wsp. 2021; Crocker i wsp. 2007; Crone i wsp. 2011; Deczkowska i wsp. 2020; Harold i wsp. 2009; Jay i wsp. 2017; Liu i wsp. 2018; Morenas-Rodríguez i wsp. 2022; Partynska i wsp. 2020; Woods i wsp. 2021].

Aby zbadać rolę białek BET w odpowiedzi zapalnej, myszom podawano dootrzewnowo JQ1 rozpuszczony w DMSO i 10% 2-hydroksypropyl-β-cyklodekstrynie lub sam roztwór rozpuszczalnika. Po 30 minutach zwierzęta otrzymywały LPS bądź samą sól fizjologiczną. Ocena obejmowała monitorowanie stanu zwierząt za pomocą uproszczonej skali MSS [Shrum i wsp. 2014], pomiar poziomu 23 cytokin w surowicy metodą multipleksową oraz analizę ekspresji genów metodą qPCR, poziomu białek metodą Western blot i ELISA w hipokampie.

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Model 2 *in vivo* – badanie wpływu inhibitora OTX-015 u zwierząt 12-miesięcznych narażonych w fazie prenatalnej na aktywację układu odpornościowego matki (tzw. model MIA).

Samicom myszy C57BL/6J w 17. dniu ciąży podawano dootrzewnowo HMW PIC lub równoważną objętość roztworu soli fizjologicznej. W wieku 12 miesięcy męskie potomstwo otrzymywało przez 14 kolejnych dni inhibitor białek BET OTX-015 lub odpowiednią objętość rozpuszczalnika. Aby zminimalizować stres związany z iniekcjami lub podaniami z użyciem zgłębnika (*per os*), w eksperymencie wykorzystano procedurę dobrowolnej konsumpcji substancji wymieszanej z masłem orzechowym po uprzednim 5-dniowym treningu smakowym [Hocking i wsp. 2018, Warren i wsp., 2021].

Aktywacja układu odpornościowego matki (MIA) jest szeroko stosowana jako model eksperymentalny chorób neurorozwojowych, ponieważ pozwala na odtworzenie wpływu reakcji zapalnej w organizmie matki, w tym burzy cytokinowej, na rozwój płodu [Hameete i wsp. 2021; Knuesel i wsp. 2014; Loayza i wsp. 2023]. Indukcja MIA za pomocą PIC lub LPS aktywuje zależne od receptorów TLR mechanizmy wrodzonej odpowiedzi układu udpornościowego matki, prowadząc do przeprogramowania układu odpornościowego płodu, w tym zmian aktywności mikrogleju i zaburzeń neurozapalnych w mózgu potomstwa [Hameete i wsp. 2021; Mueller i wsp. 2019]. Skutki takich zaburzeń w fazie prenatalnej utrzymują się u potomstwa po urodzeniu [Garay i wsp. 2013; Loayza i wsp. 2023; Mueller i wsp. 2019]. Model MIA z wykorzystaniem gryzoni jest użytecznym i szeroko stosowanym narzędziem biologii eksperymentalnej, ale jak każde narzędzie posiada istotne ograniczenia. Po pierwsze, wyniki wykazują silną zależność od warunków eksperymentalnych, takich jak rodzaj zastosowanego stymulanta (PIC vs. LPS), jego dawka, czas podania w trakcie ciąży oraz szczep i płeć zwierząt potomnych, co znacząco utrudnia bezpośrednie porównywanie rezultatów między poszczególnymi badaniami [Meyer 2023]. Po drugie, reakcje immunologiczne myszy nie w pełni odzwierciedlają złożoność odpowiedzi ludzkiej, co ogranicza możliwość szybkiego i prostego przełożenia wyników do praktyki klinicznej [Masopust i wsp. 2017]. Po trzecie, model nie uwzględnia późniejszych wpływów środowiskowych ani interakcji społecznych potomstwa, które również kształtują rozwój neuronalny i zachowania. Dodatkowo, brak standaryzacji protokołów MIA może prowadzić do różnic w nasileniu neurozapalnych i behawioralnych efektów u potomstwa, co wymaga ostrożnej interpretacji wyników [Bauman i wsp. 2020; Bucknor i wsp. 2022].

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Wpływ MIA i OTX-015 na 12-miesięczne zwierzęta potomne oceniano wykonując testy behawioralne (test otwartego pola i test rozpoznawania nowego obiektu), pozwalające na ocenę aktywności eksploracyjnej i zachowań lękowych oraz pamięci rozpoznawczej. Ocena zmian molekularnych i biochemicznych w hipokampie obejmowała analizę ekspresji genów metodą qPCR oraz poziomu białek metodą Western blot i ELISA. Badania immunohistochemiczne posłużyły do oceny dystrybucji i morfologii mikrogleju i astrocytów w hipokampie [Sierra i wsp. 2010].

W badaniach *in vivo* skupiono się na analizie zmian zachodzących w hipokampie. Hipokamp jest kluczową strukturą mózgu odpowiedzialną za pamięć deklaratywną i przestrzenną, a jego uszkodzenie jest jednym z pierwszych objawów choroby Alzheimera, co czyni go istotnym obiektem badań w kontekście neurodegeneracji związanej z tą chorobą [Jaroudi i wsp. 2017; Rao i wsp., 2022; Vyas i wsp. 2020]. Struktura ta jest szczególnie podatna na skutki obwodowej stymulacji LPS-em, która indukuje w hipokampie aktywację mikrogleju i nasilenie odpowiedzi neurozapalnej [Jung i wsp. 2023; Ganesan i wsp. 2025]. W konsekwencji przewlekła lub nadmierna aktywacja mikrogleju w hipokampie może prowadzić do uszkodzenia neuronów oraz zaburzeń funkcji poznawczych, co podkreśla znaczenie tej struktury jako modelu do badań nad mechanizmami neurozapalnymi i potencjalnymi terapiami w chorobach neurodegeneracyjnych [Zhao i wsp. 2019]. Ze względu na te cechy, hipokamp stanowi istotny cel w badaniach nad mechanizmami molekularnymi i biochemicznymi związanymi z neurozapaleniem oraz potencjalnymi interwencjami terapeutycznymi w chorobach neurodegeneracyjnych.

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Podsumowanie najważniejszych wyników

1. Inhibicja białek BET zmniejsza aktywność fagocytarną mikrogleju

Badania *in vitro* z wykorzystaniem linii komórek BV2 wykazały, że białka z rodziny BET są istotnym czynnikiem regulującym aktywność i funkcje komórek mikrogleju. Farmakologiczne zahamowanie białek BET z wykorzystaniem inhibitora JQ1 selektywnie obniża ekspresję niektórych tylko genów kodujących białka zaangażowane w proces fagocytozy: *Siglec1*, *Sirpb1a*, *Cd36*, *Clec7a*, *Itgam*, *Tlr3* (Publikacja I, Ryc. 5 i 6). W konsekwencji znacząco obniża się aktywność fagocytarna mikrogleju (Publikacja I, Ryc. 2 i 3). Dzięki zastosowaniu metody wyciszania genów dla poszczególnych białek z rodziny BET ustalono, że efekt ten zależy głównie od BRD2 i BRD4, natomiast BRD3 wydaje się nie odgrywać znaczącej roli (Publikacja I, Ryc. 4e, 7).

2. Inhibicja białek BET moduluje cytotoksyczność mikrogleju wobec neuronów

Badania *in vitro* wykorzystujące medium kondycjonowane oraz ko-kulturę komórek BV2 i HT22 wykazały, że zahamowanie białek BET obniża cytotoksyczną aktywność pobudzonego mikrogleju. Stymulacja komórek BV2 LPS-em lub Aβ powodowała ich aktywację, w tym wzrost transkrypcji genów prozapalnych (*Il1b*, *IL6*, *Tnf*, *Nos2*) (Publikacja II, Ryc. 2), nasilenie fagocytozy (Publikacja II, Ryc. 4c) oraz uwalnianie do medium czynników obniżających witalność komórek HT22 (Publikacja II, Ryc. 3a, 6). Inhibitor JQ1 tłumił tę reakcję na poziomie transkrypcji (Publikacja II, Ryc. 2) i obniżał neurotoksyczne skutki aktywacji mikrogleju (Publikacja II, Ryc. 3 i 6).

3. W warunkach aktywacji prozapalnej mikrogleju zwiększa się specyficznie ekspresja jedynie BRD4

Chociaż wszystkie eksprymowane w mózgu białka BET mogą brać udział w regulacji transkrypcji, w badaniach *in vitro* i *in vivo* obserwowano wzrost poziomu mRNA i białka jedynie w przypadku BRD4 (Publikacja II, Ryc. 5, Publikacja III, Ryc. 2, Publikacja IV, Ryc. 2, Publikacja V, Ryc. 3).

4. Inhibitor JQ1 zapobiega mającemu miejsce w warunkach aktywacji mikrogleju wzrostowi ekspresji CD33

Inhibitor białek BET, JQ, powoduje w komórkach BV2 obniżenie poziomu mRNA dla genu kodującego CD33 (Publikacja I, Tab. 1, Publikacja IV, Ryc. 3a). Zjawisko to

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obserwowano również w przypadku zwiększonej ekspresji *Cd33* po stymulacji komórek BV2 czynnikami prozapalnymi (Publikacja IV, Ryc. 3a). Badania *in vivo* wykazały, że inhibicja białek BET zapobiega wzrostowi ekspresji *Cd33* wywołanemu w hipokampie w warunkach obwodowej aktywacji układu odpornościowego myszy LPS-em (Publikacja IV, Ryc. 2b).

5. Inhibitor białek BET JQ1 zmniejsza behawioralne objawy obwodowej aktywacji układu odpornościowego myszy LPS-em

W modelu endotoksemii u myszy obserwowano nasilenie zachowań chorobowych (ang. *sickness behawior*) mierzone za pomocą skali MSS oraz spadek masy ciała (Publikacja V, Ryc. 1b–d). Inhibitor JQ1 w sposób selektywny i zależny od czasu obniżał poziom niektórych cytokin w surowicy krwi oraz ekspresję niektórych genów prozapalnych w hipokampie (Publikacja V, Ryc. 2 i 4). Jednocześnie JQ1 redukował nasilenie objawów behawioralnych, nie miał jednak wpływu na wywołany LPS-em spadek masy ciała (Publikacja V, Rys. 1b-d).

6. Aktywacja układu odpornościowego matki powoduje u potomstwa utrzymujące się do 12 miesiąca życia zmiany epigenetyczne, genetyczne i biochemiczne w hipokampie

U 12-miesięcznych myszy narażonych w 17. dniu rozwoju prenatalnego na wywołaną przez iniekcję PIC aktywację układu odpornościowego matki obserwowano w hipokampie wzrost ekspresji genu *Brd4* (Publikacja III, Rys. 2A), zmiany ekspresji genów, w tym niektórych genów prozapalnych (Publikacja III, Tab. 1 i 2) oraz zwiększone stężenie peptydów Aβ (Publikacja III, Ryc. 3A-C).

7. Inhibitory białek BET obniżają poziom Aβ w hipokampie

Zastosowanie dwóch różnych inhibitorów białek BET, JQ1 i OTX-015, w dwóch różnych modelach eksperymentalnych *in vivo* skutkowało obniżeniem poziomu Aβ w hipokampie (Publikacja III, Ryc. 3; Publikacja V, Ryc. 6). W naszych badaniach udało się wykluczyć istotny udział prozapalnej aktywności mikrogleju w hipokampie jako mechanizmu odpowiadającego za obserwowane obniżenie poziomu amyloidu-β po inhibicji białek BET. Wyniki te sugerują, że wpływ BET na Aβ może zachodzić poprzez mechanizmy niezależne od klasycznej odpowiedzi zapalnej mikrogleju, co wymaga dalszych badań w celu identyfikacji potencjalnych celów molekularnych i ścieżek regulacyjnych.

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8. Zahamowanie białek BET poprawia funkcje poznawcze u myszy

U 12-miesięcznych myszy otrzymujących inhibitor białek BET OTX-015 zaobserwowano w teście pamięci rozpoznawczej poprawę w stosunku do myszy nie otrzymujących OTX-015. Efekt ten był obecny zarówno u myszy kontrolnych jak i u myszy narażonych w 17. dniu rozwoju prenatalnego na wywołaną przez iniekcję PIC aktywację układu odpornościowego matki (Publikacja III, Ryc. 9A,B). Wykazano także ujemną korelację pomiędzy poziomem Aβ a współczynnikiem dyskryminacji ID (Publikacja III, Ryc. 9C,D).

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Dyskusja

Przeprowadzone badania wykazały, że białka BET, a wśród nich zwłaszcza BRD4, są ważnymi regulatorami aktywności mikrogleju, odpowiedzi zapalnej oraz metabolizmu Aβ. Inhibicja białek BET zmniejsza fagocytozę, ogranicza cytotoksyczność mikrogleju wobec neuronów i obniża ekspresję prozapalnych cytokin, jednocześnie redukując poziom Aβ i poprawiając zdolności poznawcze związane z pamięcią. Uzyskane wyniki sugerują możliwy potencjał terapeutyczny inhibitorów białek BET w sytuacjach klinicznych związanych z neurozapaleniem i neurodegeneracją.

Rozwój choroby Alzheimera (AD) jest złożonym procesem obejmującym szereg powiązanych mechanizmów patofizjologicznych, wśród których kluczową rolę odgrywa neurozapalenie, akumulacja amyloidu-beta (Aβ) oraz patologia Tau [Adamu i wsp. 2024; Anwar i wsp. 2020; Bertram i wsp. 2008; Gylys i wsp. 2004; Lanni i wsp. 2013; Lu i wsp. 2014; Mukherjee i wsp. 2021; Spangenberg i wsp. 2019]. Dopuszczone ostatnio do leczenia pierwsze leki ukierunkowane na przyczyny choroby, przeciwamyloidowe przeciwciała monoklonalne lecanemab i donanemab, wykazują zdolność do redukcji złogów amyloidu i spowolnienia pogarszania się funkcji poznawczych u pacjentów we wczesnym stadium AD [van Dyck i wsp., 2023; Mintun i wsp., 2021]. Ich stosowanie wiąże się jednak z istotnymi ograniczeniami, w tym skutecznością głównie w łagodnych postaciach choroby oraz ryzykiem wystąpienia poważnych efektów ubocznych określanych jako ARIA (ang. *amyloid-related imaging abnormalities*), co potwierdzono zwłaszcza w przypadku donanemabu [Zimmer i wsp., 2025]. Wobec tych trudności zasadne jest poszukiwanie alternatywnych strategii terapeutycznych ukierunkowanych na mechanizmy patogenetyczne AD. Coraz więcej dowodów wskazuje, że kluczową rolę w progresji choroby odgrywa proces neurozapalny. W tym kontekście coraz większe znaczenie zyskuje rola mikrogleju jako głównego mediatora odpowiedzi immunologicznej w mózgu [Lloyd i wsp. 2024]. Mikroglej, wykazując zdolność do fagocytozy i produkcji mediatorów zapalnych, może w początkowych stadiach pełnić funkcję ochronną, jednak w warunkach przewlekłej aktywacji prowadzi do pogłębienia procesu neurodegeneracyjnego [Kong i wsp. 2020; Krstic i wsp. 2012; Munk i wsp. 2023]. Nadmierna aktywacja mikrogleju sprzyja produkcji cytokin prozapalnych, takich jak IL-1β, TNF-α czy IL-6, które nasilają akumulację Aβ, sprzyjają fosforylacji Tau i wywołują obumieranie neuronów [Anwar i wsp. 2020; Block i wsp. 2005; Bocchini i wsp. 1992; McFarland i wsp. 2024].

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W ostatnich latach szczególną uwagę zwraca się na rolę mechanizmów epigenetycznych jako kluczowego regulatora aktywności mikrogleju i genów związanych z neurozapaleniem [Lajqi i wsp. 2019; Lu i wsp. 2004; Matzuk i wsp. 2012]. Białka z rodziny BET, będące „czytnikami" acetylowanych lizyn histonowych, odgrywają istotną rolę w modulacji ekspresji genów, które są istotne dla odpowiedzi zapalnej [Belkina i wsp. 2013; Cheung i wsp. 2021]. Badania opisane w niniejszej dysertacji potwierdziły, że białka BET odgrywają centralną rolę w procesach aktywacji mikrogleju. Warto podkreślić, że poszczególne białka BET wykazują odmienne funkcje w kontekście regulacji genów mikrogleju. Na przykład BRD4 odgrywa dominującą rolę w aktywacji prozapalnych genów mikrogleju poprzez współpracę z NF-κB i innymi czynnikami transkrypcyjnymi, podczas gdy BRD2 i BRD3 mogą modulować bardziej subtelne aspekty transkrypcji genów związanych z homeostazą komórkową i odpowiedzią na stres oksydacyjny poprzez wiązanie się z acetylowanymi histonami w określonych regionach genów, regulację ekspresji genów antyoksydacyjnych oraz epigenetyczne dostrajanie podstawowej aktywności transkrypcyjnej mikrogleju [Liu i wsp. 2021; Seifritz i wsp. 2023]. Zrozumienie tych różnic jest istotne dla opracowywania selektywnych strategii terapeutycznych, ponieważ nieselektywne blokowanie wszystkich białek BET może prowadzić do efektów ubocznych związanych z zakłóceniem podstawowych procesów epigenetycznych w neuronach i komórkach glejowych [Belkina i wsp. 2013; Brown i wsp. 2014].

W publikacjach II, IV i V oraz suplemencie do publikacji I wykazano, że inhibitor białek BET, JQ1, skutecznie obniża produkcję prozapalnych cytokin, takich jak IL-1β, TNF-α i IL-6. Wyniki te są zgodne z wcześniejszymi doniesieniami, które wskazują, że białka BET odgrywają kluczową rolę w aktywacji transkrypcji genów prozapalnych w różnych typach komórek [Belkina i wsp., 2013; Cheung i wsp., 2021; Meng i wsp., 2014; Petretich i wsp., 2020; Quezada i wsp., 2021; Riganti i wsp., 2018; Wang i wsp., 2018; Wang i wsp., 2023; Yuan i wsp., 2021]. Białka BET działają jako „czytniki" epigenetycznego kodu acetylowanych lizyn, wiążąc się z chromatyną i mobilizując kompleksy transkrypcyjne do aktywacji genów prozapalnych, takich jak *Il-1β*, *Tnf*, *Il-6*, *Mcp1* [Hoffner i wsp. 2021; Huang i wsp. 2017; Liu i wsp. 2014; Nikkar i wsp. 2022]. Blokada białek BET za pomocą JQ1 prowadzi do inaktywacji genów prozapalnych, co skutkuje ograniczeniem produkcji mediatorów zapalnych i może zmniejszać aktywację mikrogleju.

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Ważnym aspektem funkcjonowania mikrogleju jest jego aktywność fagocytarna, pozwalająca na usuwanie Aβ i innych szkodliwych substancji z mózgu. Wyniki przedstawione w publikacji I pokazują, że inhibicja białek BET przez JQ1 zmniejsza aktywność fagocytarną komórek BV2, a także obniża ekspresję genów związanych z fagocytozą, w tym *Cd33*, *Trem2* czy *Itgam*. Rodzi to pytanie o możliwe skutki zahamowania białek BET w sytuacji klinicznej, ponieważ konsekwencje zahamowania fagocytozy mogą być niekorzystne. Interpretacja tego efektu zależy od kontekstu: w sytuacjach nadmiernej aktywacji prozapalnej mikrogleju, ograniczenie jego fagocytarnej aktywności przez inhibitory BET może działać ochronnie, ponieważ zmniejsza nadmierną produkcję cytokin prozapalnych i łagodzi stan zapalny; natomiast w kontekście choroby Alzheimera zahamowanie tej funkcji mikrogleju mogłoby ograniczać jego zdolność do usuwania Aβ, sprzyjając akumulacji peptydu i progresji choroby.

Taką właśnie kaskadę, która w patofizjologii choroby Alzheimera łączy zahamowanie aktywności fagocytarnej mikrogleju i akumulację Aβ, uruchamiają zaburzenia receptora CD33, będącego pod transkrypcjyjną kontrolą białek BET [Boots i wsp. 2023; Brinkman-Van der Linden i wsp. 2003; Côté i wsp. 2012; Griciuk 2020; Hollingworth i wsp. 2011; In t' Veld i wsp. 2001; Raj i wsp. 2014b]. W organizmie ludzkim, w wyniku alternatywnego splicingu genu, CD33 powstawać może w dwóch izoformach o odmiennym działaniu. Obecność długiej izoformy CD33 powoduje zahamowanie fagocytozy i akumulację Aβ w mózgu, podczas gdy obecność izoformy krótkiej, pozbawionej egzonu drugiego, ma działanie protekcyjne [Bhattacherjee i wsp. 2021]. Badania GWAS wykazały, że polimorfizm genu *CD33* jest związany z ryzykiem rozwoju AD [Bertram i wsp. 2019]. Badania eksperymentalne oraz epidemiologiczne potwierdziły, że zmiany dotyczące *CD33*, w tym wzrost jego ekspresji, także prowadzą do zahamowania fagocytarnych funkcji mikrogleju i zwiększenia poziomu Aβ w mózgu [Bhattacherjee i wsp. 2019; Podleśny-Drabiniok i wsp. 2020]. Zaobserwowany w naszych badaniach wzrost ekspresji *CD33* w hipokampie myszy po obwodowej iniekcji LPS sugeruje możliwy mechanizm łączący aktywację układu odpornościowego z patologią Alzheimerowską. Do wyjaśnienia pozostaje kwestia, czy powtarzające się w ciągu życia epizody aktywacji mikrogleju mogą poprzez zwiększenie ekspresji *CD33* sukcesywnie sprzyjać akumulacji Aβ. Krótkotrwała inhibicja białek BET po zastosowaniu JQ1 powoduje z jednej strony zahamowanie aktywności fagocytarnej mikrogleju, z drugiej zaś strony obniża ekspresję *CD33*, co powinno sprzyjać nasileniu

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funkcji fagocytarnych. Jednak nasze wyniki pokazują, że równoczesne hamowanie innych genów i szlaków niezbędnych dla funkcjonowania fagocytozy ogranicza ogólną aktywność mikrogleju. W efekcie, mimo zmniejszenia ekspresji *CD33*, sumaryczny wpływ JQ1 na fagocytozę Aβ jest hamujący, co może ograniczać praktyczny efekt terapeutyczny. Nasze wyniki podkreślają złożoność regulacji funkcji fagocytarnych mikrogleju i wskazują na potrzebę dalszych badań, które pozwolą w pełni zrozumieć mechanizmy wpływające na te procesy oraz ich potencjalne wykorzystanie w terapii chorób neurodegeneracyjnych.

Przedstawione w niniejszej dysertacji badania mają swoje ograniczenia, które zmniejszają nieco możliwość praktycznego wnioskowania na ich podstawie. Większość analiz, w tym publikacje I–V, opiera się na modelach *in vitro* oraz na zwierzętach, które nie w pełni oddają złożoność ludzkiego mózgu ani jego regulacji epigenetycznej, co utrudnia bezpośrednią translację wyników na kontekst kliniczny [Berenguer-Daizé i wsp., 2016; Ravindra i wsp., 2009]. Na przykład różnice w zakresie dostępności acetylowanych histonów i aktywności białek BET mogą się różnić w mózgu mysim i ludzkim [Adamu i wsp. 2024; Lambert i wsp. 2009; Nikkar i wsp. 2022]. Po drugie, długoterminowe skutki farmakologicznej blokady białek BET są jeszcze słabo poznane, a potencjalne skutki uboczne, takie jak immunosupresja, zahamowanie proliferacji komórek czy wpływ na funkcje neurokognitywne, jeszcze nie zostały w pełni zbadane [Bilecki i wsp. 2021; Eischer i wsp. 2023]. Również rola białek BET w modulacji innych istotnych w neurodegenracji procesów takich jak autofagia czy zaburzenia funkcji mitochondriów, nie jest jeszcze dobrze poznana [Benito i wsp. 2017; Cheung i wsp. 2021; Rahman i wsp. 2020].

Zważywszy na powyższe, uzasadnione wydaje się przeprowadzenie przyszłych badań oceniających skuteczność inhibitorów białek BET w dłuższej perspektywie. Należy także rozwinąć badania mechanistyczne, aby wyjaśnić, w jaki sposób inhibitory bałek BET wpływają na poziom Aβ w mózgu, a także jak oddziałują na funkcje innych komórek mózgu, takich jak astrocyty czy neurony [Adamu i wsp. 2024; Benito i wsp. 2017]. Kolejnym perspektywicznym kierunkiem badań jest analiza interakcji białek BET z innymi epigenetycznymi regulatorami, czy też z szlakami związanymi z metabolizmem mitochondrialnym, które odgrywają istotną rolę w neurodegeneracji [Cheung i wsp. 2021].

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Podsumowując, uzyskane wyniki wskazują, że białka z rodziny BET stanowią bardzo ważny element epigenetycznej regulacji funkcji mikrogleju. Zastosowanie inhibitorów białek BET do modulacji aktywności mikrogleju w chorobach związanych z neurozapaleniem wydaje się obiecującym podejściem terapeutycznym [Cummings i wsp. 2021; Cummings i wsp. 2024; Liu i wsp. 2019; McGeer i wsp. 2016; McGeer i wsp. 1998; Richardson i wsp. 2019]. Rozpoznanie potencjału inhibicji białek BET jako przyszłej strategii terapeutycznej ukierunkowanej na tłumienie nadmiernej aktywności mikrogleju wymaga jednak jeszcze wielu szczegółowych badań, aby wyeliminować potencjalne ryzyko, określić efekty centralne o obwodowe i zoptymalizować terapię [Passeri i wsp. 2022; Raj i wsp. 2014a]. W tym celu konieczne jest nie tylko pogłębienie badań przedklinicznych, lecz także rozwój badań klinicznych, które pozwolą na pełne zrozumienie ich roli w patogenezie i leczeniu chorób neurodegeneracyjnych.

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Wnioski

1. Inhibicja białek BET obniża ekspresję niektórych genów związanych z aktywnością fagocytarną i endocytozą w komórkach mikrogleju *in vitro*. Skutkiem tego jest zahamowanie funkcji fagocytarnej mikrogleju. Białka z rodziny BET w różnym stopniu są odpowiedzialne za to zjawisko, efekt zależy głównie od BRD2 i BRD4, co wskazuje na ich kluczową rolę w regulacji funkcji mikrogleju.

2. JQ1 hamuje ekspresję genów cytokin prozapalnych i ogranicza uwalnianie cytotoksycznych mediatorów przez aktywowane komórki mikrogleju *in vitro*. Efekt inhibicji jest zależny od czasu ekspozycji. JQ1 nie wpływa jednak na ekspresję tych genów w komórkach niestymulowanych. Wyniki te sugerują, że hamowanie aktywności białek BET może być skuteczną metodą zapobiegania skutkom nadmiernej aktywacji mikrogleju w procesach neurozapalnych.

3. U zwierząt narażonych w prenatalnej fazie rozwoju na procesy związane z aktywacją układu odpornościowego matki (MIA) znotowano długotrwałe zmiany utrzymujące się do co najmniej do 12. miesiąca życia. Obserwowano wzrost poziomu Aβ w hipokampie, podczas gdy poziom i fosforylacja białka Tau nie ulegały zmianie. Pomimo selektywnych zaburzeń trankskrypcji genów, w tym genów prozapalnych, nie obserwowano ewidentnych zmian dystrybucji i morfologii mikrogleju i astrocytów, które mogłyby wskazywać na zmianę aktywności gleju. Wyniki te sugerują istotne znaczenie czynników prenatalnych w patomechanizmie sporadycznej postaci AD, nie wskazują jednak na udział komórek gleju.

4. Inhibitor białek BET OTX-015 znacząco redukuje poziom Aβ w hipokampie myszy, ale nie ma wpływu na poziom i fosforylację białka Tau. Efekt ten nie jest związany z modulacją aktywności mikrogleju, co sugeruje istnienie alternatywnych mechanizmów, przez które mogą działać inhibitory białek BET. OTX-015 poprawia też pamięć rozpoznawczą u myszy, zarówno w grupie kontrolnej jak i w grupie MIA, co sugeruje białka BET jako warty zainteresowania cel terapeutyczny w chorobie Alzheimera.

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5. W warunkach obwodowej aktywacji układu odpornościowego za pomocą LPS-u w hipokampie myszy obserwuje się wzrost ekspresji CD33, receptora mikroglejowego odgrywającego istotną rolę w patomechanizmie AD, co potencjalnie może przyczyniać się do przyspieszenia rozowju patologii alzheimerowskiej. Ponieważ JQ1 zapobiega wzrostowi ekspresji CD33, inhibicja białek BET wydaje się być możliwą strategią przeciwdziałania tej kaskadzie. Należy jednak podkreślić, że wpływ inhibitorów BET na fagocytozę i inne funkcje mikrogleju może mieć zarówno korzystne, jak i niekorzystne konsekwencje dla homeostazy mózgu.

6. JQ1 redukuje ekspresję cytokin prozapalnych i zmniejsza nasilenie zachowań chorobowych oraz obniża poziom Aβ w hipokampie, nie wpływa jednak na fosforylację białka Tau. Zaobserwowane działanie przeciwzapalne i przeciwamyloidowe wskazuje, że inhibicja białek BET może stanowić obiecującą strategię terapeutyczną w chorobie Alzheimera.

7. Aktywacja mikrogleju *in vitro*, a także aktywacja procesów neurozapalnych w OUN wiąże się ze wzrostem ekspresji BRD4, co sugeruje, że wśród białek z rodziny BET to właśnie BRD4 odgrywa kluczową rolę w regulacji transkrypcji genów związanych z odpowiedzią neurozapalną.

Przeprowadzone badania w znacznym stopniu potwierdzają główną hipotezę badawczą, wskazując, że zahamowanie aktywności białek BET moduluje fenotyp mikrogleju i ogranicza jego cytotoksyczne działanie, a ponadto może wpływać na kluczowe procesy neuropatologiczne związane z chorobą Alzheimera. Uzyskane wyniki sugerują jednak, iż mechanizmy działania inhibitorów białek BET są złożone i nie zawsze bezpośrednio powiązane z modulacją aktywności mikrogleju, co podkreśla konieczność dalszych badań. Niezależnie od tych ograniczeń, zebrane dane jednoznacznie wskazują na białka BET – zwłaszcza BRD2 i BRD4 – jako istotnych regulatorów odpowiedzi neurozapalnej oraz perspektywiczny cel dla nowych strategii terapeutycznych w chorobach neurodegeneracyjnych.

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The Role of Bromodomain and Extraterminal (BET) Proteins in Controlling the Phagocytic Activity of Microglia In Vitro: Relevance to Alzheimer's Disease. Int. J. Mol. Sci. 2023, 24(1), 13, doi: 10.3390/ijms24010013. IF=4,9

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BIBLIOTEKA
Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk
02-106 Warszawa, ul. A. Pawińskiego 5
tel. 22 608-66-11 NIP 525-000-81-69
e-mail: library@imdik.pan.pl

KIEROWNIK BIBLIOTEKI
Katarzyna Nieszporska
mgr Katarzyna Nieszporska

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Publikacja I

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Article

The Role of Bromodomain and Extraterminal (BET) Proteins in Controlling the Phagocytic Activity of Microglia In Vitro: Relevance to Alzheimer's Disease

Marta Matuszewska [1], Magdalena Cieślik [1], Anna Wilkaniec [1], Marcin Strawski [2] and Grzegorz A. Czapski [1,*]

[1] Department of Cellular Signalling, Mossakowski Medical Research Institute, Polish Academy of Sciences, ul. Pawińskiego 5, 02-106 Warsaw, Poland
[2] Laboratory of Electrochemistry, Faculty of Chemistry, University of Warsaw, ul. Pasteura 1, 02-093 Warsaw, Poland
* Correspondence: gczapski@imdik.pan.pl

Abstract: The correct phagocytic activity of microglia is a prerequisite for maintaining homeostasis in the brain. In the analysis of mechanisms regulating microglial phagocytosis, we focused on the bromodomain and extraterminal domain (BET) proteins: Brd2, Brd3, and Brd4, the acetylation code readers that control gene expression in cooperation with transcription factors. We used pharmacological (JQ1) and genetic (siRNA) inhibition of BET proteins in murine microglial cell line BV2. Inhibition of BET proteins reduced the phagocytic activity of BV2, as determined by using a fluorescent microspheres-based assay and fluorescently labelled amyloid-beta peptides. Gene silencing experiments demonstrated that all brain-existing BET isoforms control phagocytosis in microglia. From a set of 84 phagocytosis-related genes, we have found the attenuation of the expression of 14: *Siglec1*, *Sirpb1a*, *Cd36*, *Clec7a*, *Itgam*, *Tlr3*, *Fcgr1*, *Cd14*, *Marco*, *Pld1*, *Fcgr2b*, *Anxa1*, *Tnf*, *Nod1*, upon BET inhibition. Further analysis of the mRNA level of other phagocytosis-related genes which were involved in the pathomechanism of Alzheimer's disease demonstrated that JQ1 significantly reduced the expression of *Cd33*, *Trem2*, and *Zyx*. Our results indicate the important role of BET proteins in controlling microglial phagocytosis; therefore, targeting BET may be the efficient method of modulating microglial activity.

Keywords: BET; microglia; BV2; phagocytosis; Alzheimer's disease

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1. Introduction

Microglial cells, which represent the immune system in the central nervous system (CNS), play a crucial role in maintaining tissue homeostasis, a prerequisite of a healthy brain [1]. As resident brain macrophages, they are equipped with a wide array of receptors and constantly examine the local microenvironment, searching for signals indicating disorder, like infection or cell death. Detection of any sign of imbalance triggers a respective microglial reaction. However, microglia also have important functions in a healthy brain. During proper brain development, microglia shape neuronal connections by phagocyting redundant synapses, unnecessary axons, dendrites, and entire neurons [2–8]. Self-renewal and homeostasis of microglia are maintained by several factors, like colony stimulating factor 1 (CSF1) and interleukin-34 (IL-34) [9,10]. The deregulation of those factors results in microglial dysfunction and is associated with many neurodegenerative diseases, including Alzheimer's disease (AD), a form of dementia that usually appears in the elderly, characterized by partial or complete loss of memory and cognition [11,12]. Recently, it was postulated that excessive phagocytosis by microglial cells activated by disturbed neuronal signalling has a detrimental role in the progression of AD-related pathology [13]. Activation of microglia and microglia-mediated phagocytosis must be tightly controlled in time and space, in other cases, it may be detrimental to the tissue. Thus, identifying the molecular targets for

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inhibition of aberrant microglial phagocytosis could be an efficient method of attenuating inflammation. Among many targets, bromodomain and extraterminal (BET) proteins seem to be especially interesting. In the CNS, the expression of three proteins belonging to the BET family, Brd2, Brd3, and Brd4, was observed. They act as epigenetic readers of acetylation code, controlling transcription activation and elongation by the recruitment of RNA polymerase II (Pol II) and the positive transcription elongation factor (P-TEFb) [14]. They are also coactivators of p65 (RelA), a subunit of the major inflammation-related transcription factor NF-κB. BET proteins were demonstrated to be involved in the pathomechanism of malignant diseases, immunological, neurological, and cardiovascular syndromes, as well as sepsis [15–19]. They also directly regulate inflammatory responses [20]. BET proteins are involved in the activation of innate immunity, and their inhibition has been demonstrated to attenuate pro-inflammatory processes [15]. A pan-inhibitor of BET family JQ1 was shown both in vitro and in vivo to inhibit lipopolysaccharide (LPS)-stimulated expression of cytokines, including IL-1β, IL-6, IL-10, IL-18, tumor necrosis factor α (TNF-α), C–C motif chemokine ligand 2 (CCL2), CCL3, CCL4, and monocyte chemoattractant protein-1 (MCP-1) [21,22]. Accordingly, the inhibition of BET proteins prevents LPS-induced expression of proinflammatory cytokines in mouse bone marrow-derived macrophages [22]. Therefore, the BET family may become an important target in the treatment of inflammation-related diseases [23]. It is very important that we gain a deeper understanding of the overlapping and distinct functions of BET proteins in physiology and disease, as they may contribute to the activation of transcription of several genes, including those which regulate phagocytosis [24]. Therefore, the aim of our study was to analyse the role of BET proteins in regulating phagocytosis in microglial cells. The results demonstrated that Brd2, Brd3, and Brd4 control the transcription of phagocytosis-related genes, and BETs' inhibition may be the efficient method of modulating microglial phagocytosis.

2. Results

In our study, we used a murine BV2 cell line to determine the role of BET proteins in controlling the phagocytic function of microglia. To inhibit the activity of BET proteins, we used the pharmacological inhibition by JQ1 or a genetic (siRNA) approach. First, we examined the effect of different concentrations of BET inhibitor JQ1 on BV2 cells' viability to establish the optimal non-toxic concentration (Figure 1). By using an MTT assay, we observed that treatment with JQ1 for 24 h with concentrations below 0.75 μM had no impact on cell viability (Figure 1a). Similar results were obtained by using a propidium iodide (PI)-staining assay, where JQ1 at concentrations higher than 0.5 μM displayed toxic properties (Figure 1b). Therefore, LPS-stimulated cells were pre-treated with JQ1 at a non-toxic range (50–500 nM) to determine the effective inhibitory concentration. As shown in Figure 1c, all tested concentrations of JQ1 efficiently reduced LPS-evoked interleukin 1β expression. On the basis of these data, 50 nM concentration of the JQ1 was chosen for further studies. At this concentration, 24 h incubation with JQ1 did not impact BV2 cells' migration (Figure 1d), proliferation (Figure 1e), and morphology (Figure 1f). Additionally, we did not observe any change in cell morphology after 2 h of treatment with LPS (Figure 1f)—perhaps this time period is too short to observe morphological changes. We also verified the potency of other BET inhibitors, and the obtained results are presented in Supplementary Figure S1.

To analyse the role of BET proteins in controlling the phagocytic function of BV2 cells, we used the fluorescent microspheres (FMS)-based assay. Confocal microscopy analysis demonstrated that, in control conditions, BV2 cells efficiently ingested FMS, but 24 h pre-treatment with JQ1 appeared to decrease the amount of engulfed FMS (Figure 2a–c). For quantitative assessment, we used the established method based on the flow cytometry analysis (Figure 2d,e), and to verify the accuracy of this method, we used phagocytosis inhibitor cytochalasin D (CCHD; 2 μM) as a positive control. We observed that CCHD significantly reduced the number of microglial cells with the ingested FMS. We also established the level of the non-specific binding of FMS to cellular membranes by incubating the cells at 4 °C, the conditions in which microglial cells phagocytosis is completely inhibited [25]. As

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demonstrated in Figure 2d,e, a very small fraction of BV2 cells was labelled with FMS at 4 °C; therefore, we recognized the non-specific binding of FMS to cells as negligible. Our flow-cytometric analysis demonstrated that inhibition of BET proteins with 50 nM JQ1 significantly reduced the fraction of FMS-positive BV2 cells, indicating that BET proteins contribute to the regulation of microglial phagocytosis.

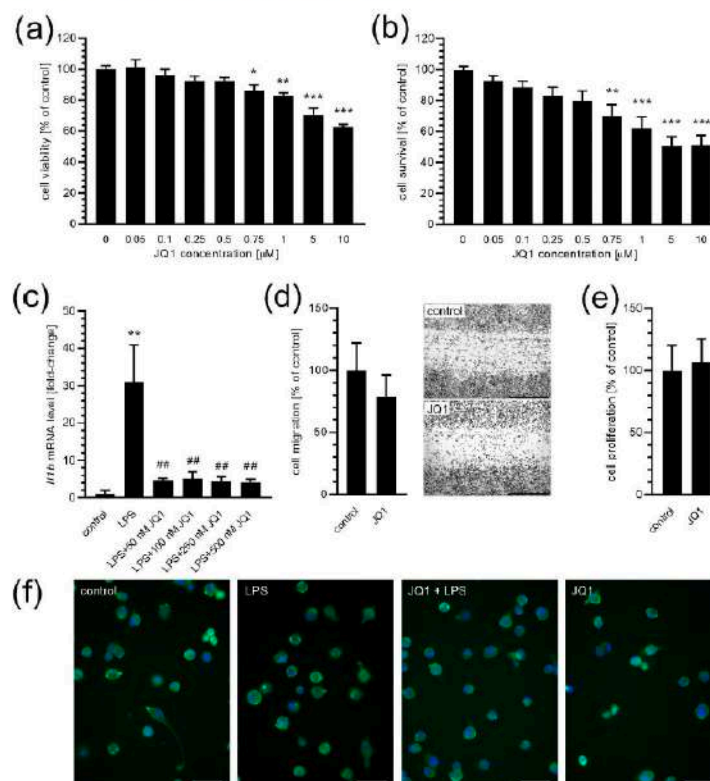

Figure 1. The effect of BET inhibitor JQ1 on microglial BV2 cells. (a) BV2 cells were incubated for 24 h in the presence of JQ1, and then an MTT assay was performed to analyse cell viability (n = 3–5). (b) BV2 cells were incubated for 24 h in the presence of JQ1, and then PI staining assay was performed to analyse cell survival (n = 7). (c) BV2 cells were incubated for 2 h in the presence of LPS (100 ng/mL) and JQ1; then, the mRNA level for *Il1b* gene was determined by using qPCR (n = 3–4). (d) BV2 cells were incubated for 24 h in the presence of 50 nM JQ1, and then a scratch assay was performed to analyse cell migration (n = 6). Typical images are presented. The scale bar: 1 mm. (e) BV2 cells were incubated for 24 h in the presence of 50 nM JQ1; then, the cell number was counted to analyse cells' proliferation (n = 6–7). (f) The morphology of BV2 cells in control conditions and in the presence of JQ1 (50 nM, 24 h) and LPS (100 ng/mL, 2 h). Cells were stained with Hoechst 33342 and Cell Mask Green Actin Tracker. The scale bar: 50 μm. Means ± SEM are presented. *, **, *** $p < 0.05$, 0.01, and 0.001, respectively, compared to the control. ## $p < 0.01$, compared to the LPS-treated group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test (a–c) or the Student t-test (d,e).

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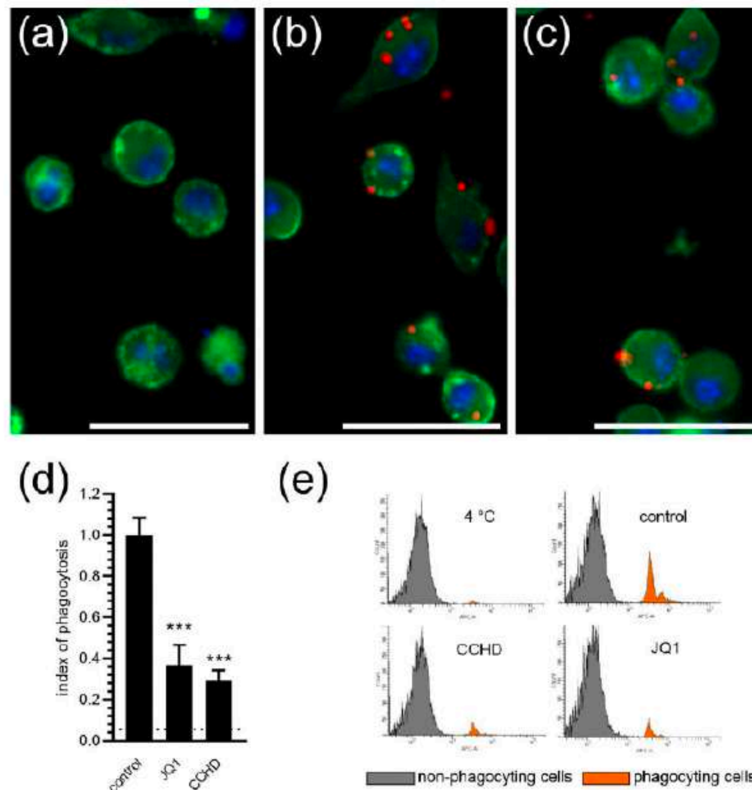

Figure 2. The effect of the BET inhibitor on phagocytosis of fluorescent microspheres by microglia. BV2 cells were incubated for 2 h without FMS (**a**), with FMS in the absence of JQ1 (**b**) or after 24 h of preincubation with 50 nM JQ1 (**c**). Cells are stained with green cytoplasmic dye and blue nuclear dye. Fluorescent microspheres are orange. The scale bar: 50 µm. (**d**) Flow cytometric analysis of the effect of BET inhibitor and CCHD on FMS' phagocytosis by microglia. The level of non-specific labelling is shown with a dotted line. (**e**) Typical flow-cytometric histograms of non-phagocytic and phagocyting BV2 cells. Means ± SEM are presented. $n = 11$–13; *** $p < 0.001$, compared to the control. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test.

Microglial phagocytosis was also assessed in the BV2 cells pre-treated with 50 nM JQ1 for 24 h and subsequently exposed to fluorescently labelled $A\beta_{1-42}$ for 2 h. During 2 h of incubation with Aβ, we did not observe changes in the morphology of the cells. The Aβ species were efficiently phagocytosed by BV2 cells during 2 h of incubation, as visualized using confocal microscopy (Figure 3a–c, green arrows) and quantified using flow cytometry (Figure 3d). Preincubation with JQ1 significantly reduced the amount of Aβ ingested by microglial cells (Figure 3c,d), indicating the important role of BET proteins in controlling Aβ phagocytosis by microglia. As visualized using atomic force microscopy (AFM), 2 h of incubation of $A\beta_{1-42}$ in a cell culture medium at 37 °C resulted in the formation of a mixture of low-molecular-weight prefibrillar oligomers (marked with a blue rectangle, for example) and larger protofibril forms (marked with red arrows) (Figure 3e; the high-resolution image is presented in Supplementary Figure S2).

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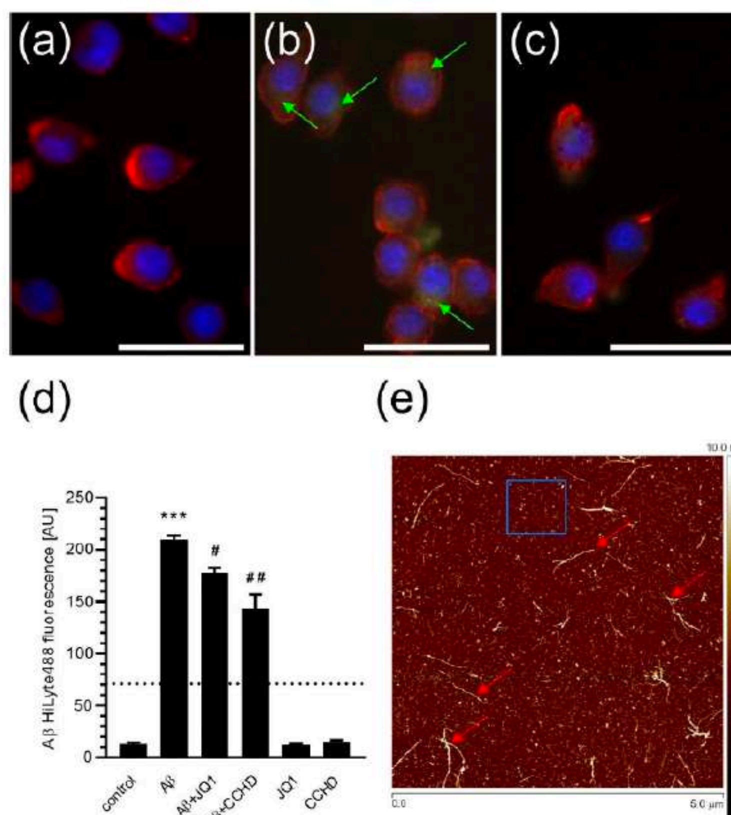

Figure 3. The effect of BET inhibitor JQ1 on Aβ phagocytosis by microglia. BV2 cells were preincubated with 50 nM JQ1 for 24 h or with 2 µM CCHD for 30 min, and then fluorescently labelled $A\beta_{1-42}$ (Aβ HiLyte488; green on the pictures) was added. After 2 h incubation, cells were stained with red cytoplasmic dye and blue nuclear dye. **(a)** Control, **(b)** cells incubated with Aβ, **(c)** cells preincubated with JQ1 and incubated with Aβ. The scale bar: 50 µm. Green arrows point to intracellular Aβ HiLyte488 staining. **(d)** Flow-cytometric analysis of the effect of the BET inhibitor and CCHD on Aβ phagocytosis by microglia. The dotted line shows the level of unspecific labelling with Aβ (at 4 °C). **(e)** The typical AFM picture of Aβ after 2 h incubation at 37 °C. Example oligomers were marked with a blue rectangle; red arrows indicate representative protofibrils. Means ± SEM are presented. $n = 6$–8; *** $p < 0.001$, compared to control, #, ## $p < 0.05$ and $p < 0.01$, compared to Aβ group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test.

To analyse which isoform of BET proteins is involved in controlling the phagocytic function of microglia, the gene silencing method was used. As demonstrated in Figure 4a–c, siRNA evoked an efficient and specific decrease in mRNA levels of all three brain-expressed isoforms of BET, *Brd2*, *Brd3*, and *Brd4*, and this siRNA treatment did not affect BV2 cells' viability (Figure 4d). The silencing of each isoform evoked a moderate inhibitory effect on microglial phagocyting activity, with the prevalent effect observed for *Brd2* and *Brd4* (Figure 4e).

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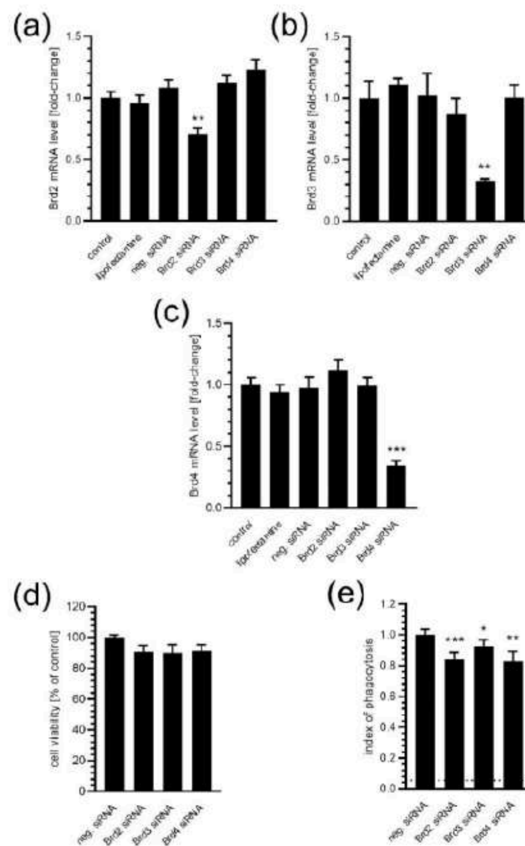

Figure 4. The effect of BET silencing on fluorescent microspheres' (FMSs) phagocytosis by microglia. **(a–c)** mRNA levels for *Brd2, Brd3, Brd4* genes 24 h after gene silencing. **(d)** The effect of *Brd2, Brd3,* and *Brd4* gene silencing on viability BV2 cells (MTT assay). **(e)** The BV2 cells were subjected to gene silencing. After 24 h, FMS were added for 2 h. Cells were stained with blue nuclear dye and flow cytometric analysis was performed. The level of non-specific labelling is shown with a dotted line. Means ± SEM are presented. $n = 8$ (a–c), $n = 4$ (d), $n = 12$ (e); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the negative siRNA-treated group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test.

Because the mechanism of BET inhibition-related changes in phagocytic activity of microglia is likely evoked by alterations in gene expression, we performed the real-time PCR screening experiment to identify the phagocytosis-associated genes that were affected by JQ1 treatment. We used a gene expression array that included a panel of 84 target and 12 control genes. Among those, the expression of 75 was detected in BV2 cells. For detailed information about this analysis, please see Supplementary Table S1. In this experiment, we pre-treated BV2 cells with 50 nM JQ1 for 24 h and subsequently induced phagocytosis by 2 h exposition to FMS, and observed that the mRNA level of 14 genes was significantly reduced (fold change (>2, $p < 0.05$) in phagocyting JQ1-pretreated BV2 cells (Figure 5). No gene expression was elevated by JQ1. The mRNA level of six genes (*Siglec1, Sirpb1a, Cd36, Clec7a, Itgam, Tlr3*) was found to be the most severely decreased; therefore, we have selected those for further detailed analysis.

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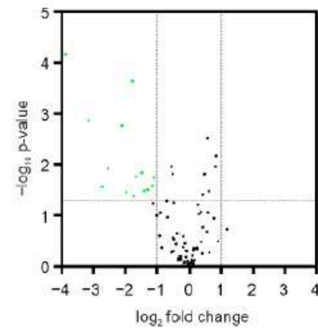

Figure 5. The effect of BET inhibitor JQ1 on the expression of phagocytosis-related genes in phagocyting microglia. BV2 cells were incubated in the absence or presence of 50 nM JQ1 for 24 h, and then FMS were added for 2 h and mRNA levels were determined using a gene expression array. The genes, the expression of which was significantly (FC > 2, $p < 0.05$) altered by JQ1, were marked on the volcano plot with green color. $n = 4$. Statistical analysis was performed by using the Student *t*-test.

Subsequently, the impact of JQ1 on *Siglec1*, *Sirpb1a*, *Cd36*, *Clec7a*, *Itgam*, and *Tlr3* expression after Aβ treatment of BV2 cells was investigated. As shown in Figure 6, in our experimental conditions, Aβ did not significantly affect the mRNA level of investigated genes, although a weak upward trend in the *Tlr3* expression was observed. BET inhibition efficiently down-regulated the mRNA level of all tested genes in control conditions and/or after stimulation with Aβ.

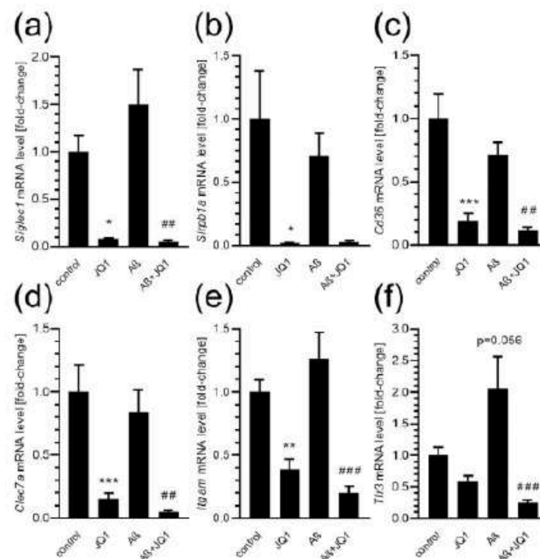

Figure 6. The effect of the BET inhibitor on microglial expression of phagocytosis-related genes. BV2 cells were incubated in the absence or presence of JQ1 for 24 h, and then Aβ was added for 2 h and mRNA levels for *Siglec1* (**a**), *Sirpb1a* (**b**), *Cd36* (**c**), *Clec7a* (**d**), *Itgam* (**e**), and *Tlr3* (**f**) were measured using the qPCR method. Means ± SEM are presented. $n = 6$–8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control, ##, ### $p < 0.01$ and $p < 0.001$, compared to Aβ group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test.

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To reveal which BET isoform is involved in the observed phenomena, we performed silencing of particular isoforms and analysed the expression of selected phagocytosis-related genes. As shown in Figure 7, the silencing of *Brd2* decreased mRNA levels of *Clec7* and *Itgam*, and the silencing of *Brd4* evoked a decline in *Siglec1* and *Sirpb1a* expression. Remarkably, silencing of *Brd3* seemed to not affect the expression of selected genes, except for *Itgam*, which was significantly elevated upon *Brd3* down-regulation.

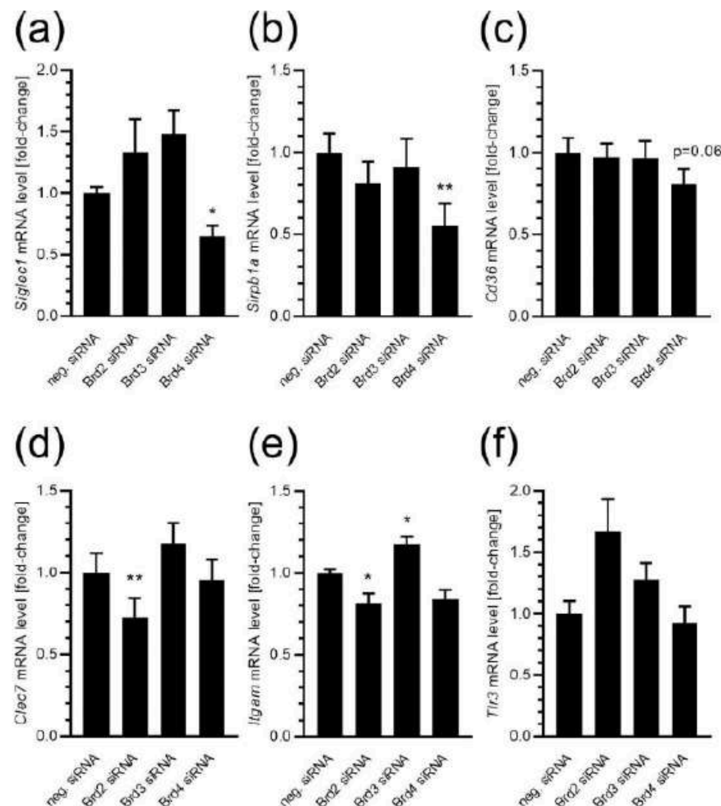

Figure 7. The effect of BET silencing on the microglial expression of phagocytosis-related genes in BV2 cells. The mRNA levels for *Siglec1* (**a**), *Sirpb1a* (**b**), *Cd36* (**c**), *Clec7a* (**d**), *Itgam* (**e**), and *Tlr3* (**f**) were determined 24 h after gene silencing by using the qPCR method. Means ± SEM are presented. $n = 8$; * $p < 0.05$, ** $p < 0.01$, compared to the negative siRNA-treated group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post hoc test.

Finally, we examined the effect of BET inhibitor JQ1 on mRNA levels of phagocytosis-related genes which were previously demonstrated to be involved in the pathomechanism of Alzheimer's disease: *Abca7*, *Bin1*, *Cd2ap*, *Cd33*, *Clu*, *Cr1l* (the murine ortholog of human *CR1*), *Picalm*, *Rab10*, *Rin3*, *Scara3*, *Trem2*, and *Zyx*. As shown in Table 1, 24 h of incubation in the presence of BET inhibitor JQ1 significantly decreased the expression of *Cd33*, *Trem2*, and Zyx. The levels of mRNA for *Abca7*, *Bin1*, *Cd2ap*, *Clu*, *Cr1l*, *Picalm*, *Rab10*, *Rin3*, and *Scara3* were not affected by JQ1.

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Table 1. The effect of the BET inhibitor on microglial expression of phagocytosis-related AD-involved genes.

	mRNA Level [Fold-Change]		
Gene	Control	JQ1	
Abca7	1.00 ± 0.09	1.13 ± 0.08	
Apoe	1.00 ± 0.30	1.38 ± 0.33	
Bin1	1.00 ± 0.11	1.08 ± 0.10	
Cd2ap	1.00 ± 0.08	1.24 ± 0.11	
Cd33	1.00 ± 0.17	0.17 ± 0.04	***
Clu	1.00 ± 0.10	1.23 ± 0.14	
Cr1l	1.00 ± 0.11	1.04 ± 0.11	
Picalm	1.00 ± 0.05	1.01 ± 0.02	
Rab10	1.00 ± 0.06	1.23 ± 0.09	
Rin3	1.00 ± 0.09	1.27 ± 0.13	
Scara3	1.00 ± 0.11	0.87 ± 0.11	
Trem2	1.00 ± 0.11	0.45 ± 0.06	***
Zyx	1.00 ± 0.11	0.61 ± 0.05	**

BV2 cells were incubated in the absence or presence of JQ1 for 24 h. Then, mRNA levels were measured using the qPCR method. Means ± SEM are presented. $n = 6–8$; ** $p < 0.01$, *** $p < 0.001$, compared to the control. Statistical analysis was performed by using the Student t-test.

3. Discussion

Microglial phagocytosis plays an important role in the development, health, and disease of the CNS. During development, microglia phagocytose excessive synapses, dendrites, axons, and neurons. To maintain a healthy brain, microglia phagocytose invading pathogens, dying or dead cells, cellular debris, or protein aggregates. Microglia may also contribute to neurodegeneration by excessive phagocytosis of live synapses or even stressed-but-viable neurons [2,7,8,26]. In our study, we showed for the first time that BET proteins control the phagocytic activity of microglia. We used immortalized murine microglial BV2 cell line, which is frequently used as a substitute for primary microglia due to similar antigen patterns, and phagocytic and cytotoxic activity [27,28]. However, this might state the major limitation of this study, since some differences between human and murine microglia, including changes in gene expression, were observed [29]. Transcriptomic analysis demonstrated that genes expressed by human and murine microglia were similar, but a limited overlap between humans and mice was found in microglial genes regulated during aging [30]. However, in our opinion, despite considerable differences between human and rodent physiology, molecular processes are similar, and using murine cell lines may provide a good introduction to the investigations on human-derived cells. To investigate the role of BET proteins in the processes of phagocytosis regulation, we used either gene silencing of specific isoforms or pharmacological inhibition. Although many proteins (over forty) contain a bromodomain in their structure, the proteins of the BET family (Brd2, Brd3, Brd4, and BrdT) have a unique domain architecture: they contain two conserved amino-terminal bromodomains that recognize acetylated lysine residues and a divergent carboxy-terminal recruitment domain. Because BrdT is not expressed in the brain, we focused our study on Brd2, Brd3, and Brd4. JQ1, a pan-inhibitor of the BET family, was used to inhibit all BET proteins [31]. JQ1 binds directly to the Kac (acetylated lysine) binding site in both bromodomains of BET proteins, but no significant binding was detected for bromodomains in proteins outside the BET family, suggesting that JQ1 is highly selective inhibitor of Brd2, Brd3, and Brd4 [31]. Therefore, to investigate the role of specific BET isoforms, we had to perform additional experiments using the gene silencing technique. Previous studies demonstrated the significant contribution of BET in regulating the expression of many genes related to immune system function; however, the role of those proteins in regulating phagocytosis has never been tested. In this study, we observed significant changes in the expression of phagocytosis-associated genes and a reduction in phagocytosis in BV2 cells after pharmacological or genetic inhibition of BETs. Our results

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showed that the pan-inhibitor of BET proteins, JQ1, at a low 50 nM concentration, which was demonstrated to be effective and non-toxic, significantly decreased phagocytosis in microglial cells. The effect of the BET inhibitor was comparable to the one observed for the inhibitor of phagocytosis, CCHD. Moreover, the application of the low concentration of the inhibitor enabled avoidance of its possible off-target action, which might appear at higher concentrations.

The previous studies analysed other aspects of BET-controlled phagocytosis in dendritic cells. Riganti et al. demonstrated that the effect of inhibitors of BET proteins, JQ1 and OTX-015, may be dependent on phagocyted cell type [32]. Authors demonstrated that after prolonged (6 days) incubation in the presence of 250 nM JQ1, the malignant pleural mesothelioma (MPM) cells, but not normal non-transformed mesothelial cells (HMC) displayed elevated expression of the "eat-me" signals, calreticulin and ERp57. Consistently, JQ1 pre-treatment had no effect on dendritic cell-mediated phagocytosis of HMC, but increased phagocytosis of MPM cells [32]. Another study focusing on the effect of JQ1 on macrophage-mediated phagocytosis of melanoma cells demonstrated an attenuation of signal regulatory protein α (SIRP-α) expression and an increase in phagocytosis [33,34]. SIRP-α is a receptor that interacts with a transmembrane protein CD47 expressed in phagocytosed cells and is known as the "do not eat me" signal. Therefore, in the above studies, inhibition of BET could affect gene expression in either phagocytosing (dendritic cells, macrophages) or phagocytosed cells (MPM, HMC, melanoma), and thus might alter the interplay between them. In our experimental model, we studied microglial cells that phagocytose fluorescent microspheres or Aβ. Therefore, inhibition of BET proteins could only affect the cellular machinery in the phagocytosing cells. This could explain why the effect of JQ1 on the phagocytosis of cells and non-cellular material (in our study) is different. An additional difference between that and our study, which might impact the result of JQ1 action, is the concentration of JQ1: 1–2.5 μM versus 50 nM, respectively. Additionally, in the study of Benham and co-workers, the high 5 μM concentration of another BET inhibitor, I-BET, was demonstrated to significantly up-regulate the expression of genes involved in phagocytic processes, thus elevating phagocytic activity of murine bone marrow-derived macrophages [35]. In our study, we demonstrated that attenuation of BET proteins decreased the expression of many genes related to the phagocytic pathway in BV2 cells. Consistently, phagocytic activity reduced by JQ1 treatment was confirmed by using the gene silencing of particular *Brd* isoforms.

The mechanism of BET protein activity is predominantly connected with the regulation of gene transcription; thus, the inhibition of those proteins is likely associated with changes in the expression of genes related to phagocytic processes. Our array analysis of mRNA levels of 84 phagocytosis-related genes identified 14 JQ1-sensitive genes that were significantly down-regulated. However, we assumed the changes in the gene expression that were higher than two-fold to be significant. We also observed that the mRNA level of some genes was significantly ($p < 0.05$) up- or down-regulated, but those changes were less than two-fold; thus, they might play a minor role in JQ1-induced phagocytosis attenuation. Interestingly, we have observed that various BET isoforms regulated the expression of phagocytosis-related genes in a different manner: while the Brd2 and Brd4 seemed to be involved in the stimulation of the above-mentioned genes, the Brd3 rather inhibited their expression. This is in agreement with the previous studies, suggesting overlapping but distinct roles for individual BET proteins in metabolic regulation [36,37]. Because of the diverse specificity of JQ1 against various BET isoforms [38–41], it can be expected that different JQ1 concentrations may change the expression pattern of various phagocytosis-related genes in a different way. It was previously demonstrated that various BET isoforms function jointly, but may also play separate roles in gene transcription. For example, in Th17 cells, over 90% of Brd4-associated genes were also connected with Brd2, but over 70% of Brd2 target genes did not overlap with target genes of Brd4 [24]. It is also possible that some effects of BET inhibitors could be evoked by BET's function that is not directly attributed to their role as acetylation code readers. Interestingly, BET family

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proteins have been identified as atypical kinases, which might affect gene transcription also by alternative mechanisms [42]. For example, Brd4 may affect gene transcription by phosphorylation of transcription machinery [43]. Brd4 possesses intrinsic kinase activity and may directly phosphorylate RNA polymerase II, TATA-box binding protein associated factor 7 (TAF7), and positive transcription elongation factor b/cyclin-dependent kinase 9 (PTEFb/CDK9) [44,45]. Moreover, Brd4 may also phosphorylate transcription factor MYC (master regulator of cell cycle entry and proliferative metabolism) at Thr58, leading to its ubiquitination and degradation [46].

The role of microglia in AD pathology is complex and not fully understood. The early studies stimulated the conception of the "autotoxic loop" [47]. According to this theory, activation of microglia by primary disease-associated factors leads to the release of several neurotoxic cytokines and other compounds which accelerate neurodegeneration and evoke the release and accumulation of cellular debris that, in consequence, may reinforce microglial activation. This concept of the detrimental role of "pro-inflammatory" activation of microglia was supported by epidemiologic studies which demonstrated that long-lasting use of non-steroid anti-inflammatory drugs reduces the risk of developing AD [48,49]. However, several studies which used genetic manipulation to modulate inflammatory pathways in animal models of AD demonstrated high variability in the results, indicating the complex role of microglia and neuroinflammation in the pathomechanism of AD [50]. The most important role of microglia in AD seems to be related to its beneficial phagocytic activity towards Aβ and to the release of detrimental pro-inflammatory cytokines; therefore, promoting microglial phagocytosis together with mitigating excessive inflammatory response was proposed to be a promising therapeutic strategy [51].

Recent studies highlighted the possibility that microglia may contribute to the spreading of Aβ pathology in AD. For example, it was demonstrated that microglia might directly contribute to amyloid plaque formation. Microglial depletion, which was evoked in 5×FAD mice by a selective brain-penetrant CSF1R inhibitor (PLX5622), prevented amyloid plaque formation in the brain parenchyma [52]. It was also recently suggested that microglia build plaques rather than remove them [53]. Authors demonstrated in a transgenic model of amyloidosis, mice APP/PS1, that impaired microglial phagocytosis results in the development of fewer dense-core plaques. The intriguing hypothesis of confinement mechanism was proposed: microglia might limit the dissemination of toxic Aβ oligomers in the brain by taking them up, compacting them in the acidic environment of the lysosomes, and finally releasing that less toxic material, and therefore contributing to building dense-core plaques.

Recent genome-wide association studies (GWAS) confirmed the previous concept that microglial phagocytosis may play a significant role in the pathomechanism of AD. These GWAS studies demonstrated that polymorphism of several phagocytosis-related genes, for example, *TREM2*, *PICALM*, *BIN1*, *CD2AP*, and *ABCA7*, increases the risk of developing AD [54–56]. Our analysis of established genetic risk factors of AD and other phagocytosis-related genes involved in the pathomechanism of AD demonstrated that among tested genes, JQ1 significantly reduced the expression of *Cd33*, *Trem2*, and *Zyx*. CD33, a member of the SIGLEC (sialic acid-binding immunoglobulin-type lectins) family, is a phagocytic receptor that was implicated in the pathomechanism of AD as a modifier of Aβ pathology. The increased CD33 (mRNA and protein) levels in the AD brains were observed, and CD33 promoted Aβ deposition and plaque formation in vivo [57]. In circulating monocytes of patients bearing the rs3865444(C) risk allele, alternative splicing of exon 2 leads to higher cell surface expression of CD33 [58]. This, in turn, increased microglial activation, enhanced accumulation of Aβ, and evoked deterioration of cognitive functions [59]. The presence of the minor rs3865444(T) protective allele was associated with reduced CD33 protein levels and also with reduced levels of insoluble Aβ in the AD brain [57]. Additionally, the inactivation of CD33 in transgenic mice $APP_{Swe}/PS1_{\Delta E9}$ reduced brain levels of insoluble Aβ42 as well as mitigated Aβ plaque pathology [57]. The mechanism of CD33-related microglial Aβ uptake was dependent on interaction with sialic acid [57]. Collectively, the data suggest that CD33, as a regulator of microglial clearance of Aβ, may be a relevant target

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for the treatment and prevention of AD. Our data thus indicate that BET protein modulation might be an indirect way by which the activity of CD33 might be down-regulated, thus slowing down the AD progression. Moreover, polymorphism of *CD33* was suggested to impact the pathomechanism of multiple sclerosis [60] and Parkinson's disease [61].

ZYX (zyxin; previously EPHA1) is a LIM domain adaptor protein that is involved in many cellular processes, including signal transduction and modulating gene expression, but also interaction of the cell with the extracellular matrix, and organisation and function of the cytoskeleton, including endocytosis [62]. It was suggested that zyxin is associated with actin filaments and participates in actin remodelling and active engulfment [63,64]. Moreover, zyxin has the pathological relevance in carcinogenesis as a regulator of the homeodomain-interacting protein kinase 2 (HIPK2)-p53 signalling axis in response to DNA damage [65–67]. In AD, the expression of zyxin is reduced, and the role of zyxin is also not limited to regulating endocytosis [68]. In vitro studies in neuronal SH-SY5Y cells demonstrated that zyxin was attenuated by intracellular Aβ peptides, leading in consequence to the deregulation of the HIPK2-p53 pathway [69]. Moreover, zyxin was proposed to be a suitable neuron-derived exosomal protein marker in serum, whose expression drops before a clinical diagnosis of AD [68]. Considering that zyxin is down-regulated in AD, attenuation of *Zyx* expression by inhibiting BET might not have beneficial effects, but this question requires further research.

TREM2 (triggering receptor expressed on myeloid cells 2), a member of the immunoglobulin superfamily, is a pattern recognition receptor highly expressed in microglia. TREM2 recognizes a wide array of ligands, including bacterial compounds (LPS), DNA, phospholipids, glycolipids, lipoproteins, apolipoproteins, and also Aβ. In the brain, TREM2 is selectively expressed in microglia, and its stimulation activates transcriptional changes, phagocytosis, chemotaxis, and other processes related to the activation of the innate immune response [70]. TREM2 plays an important role in the pathomechanism of AD; some loss-of-function mutations of the *TREM2* gene showed a high association with AD prevalence. For example, TREM2 gene variant rs75932628(T) increased by two to three times the risk for AD in European and North American populations [71]. In the brains of AD patients, TREM2 interacts with components of senile plaques, including Aβ, lipo-, and apolipoproteins [70]; and the elevation of soluble TREM2 in cerebrospinal fluid (CSF) negatively correlated with plaque growth, cortical shrinkage, and cognitive decline [72]. Experimental TREM2 deletion gives conflicting results, showing a decrease or increase in amyloid pathology, depending on the model employed, age, and brain region [73]. It seems that the outcome of TREM2 function may be protective in the early stages of AD and detrimental in the late phase of the disease [63,73]. Activation of TREM2 signalling in transgenic AD mice models using humanized monoclonal IgG1 agonistic antibody AL002 normalized behavior [74], and this antibody successfully underwent phase I clinical trials (NCT03635047) and is currently in phase II (NCT04592874), which involves participants with early AD. Considering that the role of TREM2 changes during the development of AD, being positive in the early stages and negative in the late stages, down-regulation of *Trem2* expression by the inhibition of BET family proteins might have a beneficial effect only in the late stage of AD. However, confirmation of this phenomenon requires further research.

In summation, our study demonstrated that BET proteins play an important role in controlling microglial function and BET's inhibition may significantly affect microglial phagocytosis, and thus may potentially open up new avenues for developing novel therapeutic strategies. This is especially interesting since some BET inhibitors are now undergoing clinical trials as therapeutics for several disorders, including cancer. Therefore, understanding the effect of BET inhibitors on microglial phagocytosis should be critically important for ongoing and future therapeutic strategies for AD.

4. Materials and Methods

4.1. Chemicals

Reagents for reverse transcription (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor) and quantitative PCR (Taqman Assays, TaqMan OpenArray Mouse

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Phagocytosis Panel, and TaqMan Fast Advanced Master Mix), red fluorescent microspheres (FMS), 2.0 μm, Hoechst 33342, CellMask Orange Actin Tracking Stain, CellMask Green Actin Tracking Stain, RPMI were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Opti-MEM was from Gibco, negative siRNA control was from Ambion, Lipofectamine was from Thermo Fisher Scientific, Inc., $A\beta_{1-42}$ and $A\beta_{1-42}$ HiLyte 488 were from AnaSpec, Inc. (Fremont, CA, USA). JQ1, GSK12101517, IBET-762, OTX-015, PFI-1, Cytochalasin D were obtained from Sigma-Aldrich (St. Louis, MO, USA). Heat-inactivated foetal bovine serum (FBS), Accutase solution, penicillin, streptomycin, L-glutamine, 3-(4,5-dimethyl-2-tiazolilo)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide, TRI-reagent, DNase I, dithiothreitol (DTT), anhydrous dimethyl sulfoxide (DMSO), lipopolysaccharide from *Escherichia coli* O55:B5 (toxicity 3,000,000 U/mg), and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Cell Culture

Murine microglial BV2 cells were purchased from Elabscience Biotechnology Inc. (Houston, TX, USA) [75]. The cells were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin in 5% CO_2 atmosphere at 37 °C. The passages below 20 were used for experiments. The cells were regularly tested to exclude mycoplasma contamination.

JQ1, other BET inhibitors, and Cytochalasin D (CCHD) were dissolved in DMSO at 10 mM stock solution and then were diluted with a culture medium and added to cells at a specified concentration. The final DMSO concentration was 0.1%. Lipopolysaccharide (LPS) was dissolved in saline. In all experiments, the respective vehicle was added to corresponding groups accordingly.

4.3. Silencing of Bet Proteins Expression

Gene silencing protocol was adopted from our previous study [76]. Shortly, BV2 cells were seeded on a six-well plate at a density of $3.8 \times 10^4/cm^2$ in 2.5 mL of RPMI medium supplemented with 10% FBS and 2 mM L-glutamine. Immediately, the 10 min preincubated mixture of 500 μL Opti-MEM with 2.5 μL lipofectamine and 20 μL siRNA for Brd2, Brd3, Brd4, or the negative control was added dropwise. Then, the cells were cultured in standard conditions for 24 h.

4.4. Determination of Cell Survival (MTT Reduction Assay) and Cytotoxicity (PI Uptake Assay)

Cellular viability was evaluated by the reduction of 3-(4,5-dimethyl-2-tiazolilo)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. After treatment with investigated compounds, MTT (0.25 mg/mL) was added and cells were incubated at 37 °C for the next 2 h. The medium was removed, the cells dissolved in DMSO, and the absorbance of formazan was measured at 595 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.).

Cytotoxicity was evaluated by using propidium iodide (PI) staining. After treatment with investigated compounds, PI (8 μM) was added to a culture medium, and cells were incubated in the dark at room temperature for 20 min. Then, cells were collected and subjected to flow-cytometric analysis on a FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA).

4.5. Determination of Microglial Proliferation

For analysis of cell proliferation, equal numbers of cells were seeded on a 6-well plate. After 24 h, 50 nM JQ1 or a respective vehicle were added and incubation was continued for the next 24 h. Then, cells were detached and counted by using Cell Counter (Bio-Rad, Hercules, CA, USA).

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4.6. Analysis of Microglial Migration (Scratch Assay)

Microglial migration was analysed with the scratch assay [77]. Murine microglial BV2 cells were seeded at a density of $3.8 \times 10^4/cm^2$. After 24 h, a single layer of cells was scraped in a straight line to create a "scratch" with the tip of the pipette. The floating cells were removed by washing twice with warm PBS, and then RPMI, with reduced FBS concentration (2%), to attenuate proliferation, was added and cells were incubated in standard conditions in the presence of 50 nM JQ1 for 24 h. Then, pictures were taken under the microscope and cells in the "scratch area" were counted by a blinded operator using ImageJ software (ver. 1.52p; National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/) [78].

4.7. Determination of Phagocytic Activity of Microglia (Fluorescent Microspheres Assay)

Microglial phagocytosis was analysed by flow cytometry and confocal microscopy [25,79,80]. For flow cytometry, BV2 cells were seeded to a 12-well plate at a density of 3.2×10^4 cells/cm^2. After 24 h, cells were incubated in the presence of a tested compound in standard conditions, and then FMS (0.5×10^6/mL) were added and incubation was continued for the next 2 h. Then, cells were washed three times with PBS to remove the non-phagocyted FMS and incubated in Accutase solution supplemented with and Hoechst 33342 (2 μg/mL) for 15 min in the dark at 37 °C. Cells were collected, and the presence of FMS in living microglial cells was detected by flow cytometry using BD FACS Canto II and DIVA 6 software (BD Biosciences, San Jose, CA, USA). The Hoechst-positive BV2 cell population was gated and the number of FMS-labelled cells was measured. Cytochalasin D (CCHD; 2 μM) was used as an inhibitor of phagocytosis. To evaluate the level of non-specific binding of FMS to cells, the incubation was performed at 4 °C. The index of phagocytosis was calculated as follows: index = % of FMS-labelled cells in analysed group/% of FMS-labelled cells in the control.

For microscopic confocal analysis, BV2 cells were seeded on Cellview Cell Culture Slides (Greiner) in a medium with reduced FBS concentration (2%) to attenuate proliferation. After 24 h, 50 nM JQ1 was added for the next 24 h. Then, Hoechst 33342 (2 μg/mL) and Cell Mask Green Actin Tracker Stain (×1) were added, and cells were incubated in standard conditions. After 30 min, the fluorescent microspheres were added and incubation was continued for 2 h. Then, cells were analysed under a confocal microscope.

4.8. Analysis of the Aβ Uptake by Microglial Cells Using Fluorescent Aβ HiLyte488

Aβ uptake was analysed by flow cytometry and confocal microscopy, as described previously [80]. Directly before analysis, HFIP-pretreated $A\beta_{1-42}$ HiLyte 488 was dissolved (5 mM) in anhydrous DMSO and further diluted in a cell culture medium to 100 μM concentration. After 30 s vortexing, Aβ preparations were directly used for cell treatment.

For flow cytometry, BV2 cells were seeded to a 12-well plate at a density of 3.2×10^4 cells/cm^2 for 24 h. Then, incubation was continued in the presence of JQ1 (50 nM) for 24 h or with Cytochalasin D (CCHD; 2 μM) for 30 min. Aβ HiLyte488 (1 μM) was added for the next 2 h. Then, Hoechst 33342 (2 μg/mL) and Cell Mask Orange Actin Tracker Stain (×1) were added, and cells were incubated in standard conditions for 30 min. The presence of Aβ in living microglial cells was detected by flow cytometry using BD FACS Canto II and DIVA 6 software (BD Biosciences). The Hoechst-positive BV2 cell population was gated and the mean fluorescence intensity in cells within the gate was measured. To determine the level of non-specific binding of Aβ HiLyte488 with the cell surface, the additional group was incubated at 4 °C.

For microscopic confocal analysis, BV2 cells were seeded on Cellview Cell Culture Slides (Greiner) in a medium with reduced FBS concentration (2%) to attenuate proliferation. After 24 h, 50 nM JQ1 was added for the next 24 h. Then, Hoechst 33342 (2 μg/mL) and Cell Mask Orange Actin Tracker Stain (×1) were added, and cells were incubated in standard conditions. After 30 min, $A\beta_{1-42}$ HiLyte 488 (1 μM) was added and incubation was continued for 2 h. Then, cells were analysed under the confocal microscope.

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4.9. Analysis of Gene Expression

RNA was isolated by using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. The concentration and quality of RNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Digestion of potential DNA contamination was performed by using DNase I, according to the manufacturer's protocol (Sigma-Aldrich). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.).

4.9.1. Gene Expression Array Plates

The level of mRNA for phagocytosis-related genes was analysed using TaqMan OpenArray Mouse Phagocytosis Panel (Catalog number: 4471126) according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). An amount of 40 ng of cDNA was used per each well. Data were analysed and calculated with Expression Software version 1.3 (Thermo Fisher Scientific, Inc.) using global normalization and the relative levels of mRNA were calculated using the ΔΔCt method.

4.9.2. TaqMan Assays

The level of mRNA for selected genes was analysed using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Inc.): *Actb* (Mm04394036_g1), *Gusb* (Mm01197698_m1), *Hprt* (Mm00446968_m1), *Il1b* (Mm00446190_m1), *Abca7* (Mm00497010_m1), *Apoe* (Mm01 307193_g1), *Bin1* (Mm00437457_m1), *Cd2ap* (Mm00815310_s1), *Cd33* (Mm00491152_m1), *Cd36* (Mm01135198_m1), *Clec7a* (Mm01183349_m1), *Clu* (Mm01197002_m1), *Cr1* (Mm00 785297_s1), *Itgam* (Mm00434455_m1), *Picalm* (Mm00525455_m1), *Rab10* (Mm00489481_m1), *Rin3* (Mn00617220_m1), *Scara3* (Mm00553769_m1), *Siglec1* (Mm00488332_m1), *Sirpb1* (Mm0 2525668_u1), *Tlr3* (Mm01207404_m1), *Trem2* (Mm04209424_g1), *Zyx* (Mm00496120_m1).

Quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR System using TaqMan Fast Advanced Master Mix according to the manufacturer's instructions. The relative levels of mRNA were calculated using the ΔΔCt method with *Gusb*, as a reference gene. To increase the validity and reproducibility of qPCR analysis in experiments with JQ1, the ΔΔCt calculation was extended by replacing the Ct of a single reference gene with an averaged Ct-value from three reference genes (*Actb*, *Gusb*, *Hprt*) [81].

4.10. Atomic Force Microscopy

AFM analysis was performed as described previously [82]. Shortly, amyloid samples were prepared by applying a drop of 10 μL medium on freshly cleaved mica, V1 grade (NanoAndMore GmbH, Germany). After incubation for 10 min, the sample was rinsed with deionised water (Merck Millipore Inc., Burlington, MA, USA) and dried under a gentle stream of argon. A Multimode 8 Nanoscope atomic force microscope (AFM, Bruker, Billerica, MA, USA) was used to image the surfaces of the mica substrate and the deposited amyloid structures.

4.11. Statistical Analysis

The results were expressed as mean values ± SEM. The statistical analysis of the data was performed by using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA, USA). The data distribution was analysed by using a normality test. The outliers were detected with the ROUT method. Data, depending on experimental design, were analysed using a Student *t*-test or a one-way analysis of variance (ANOVA) with Bonferroni post hoc test with correction for multiple comparisons; p values < 0.05 were considered significant. The n number refers to independent experiments.

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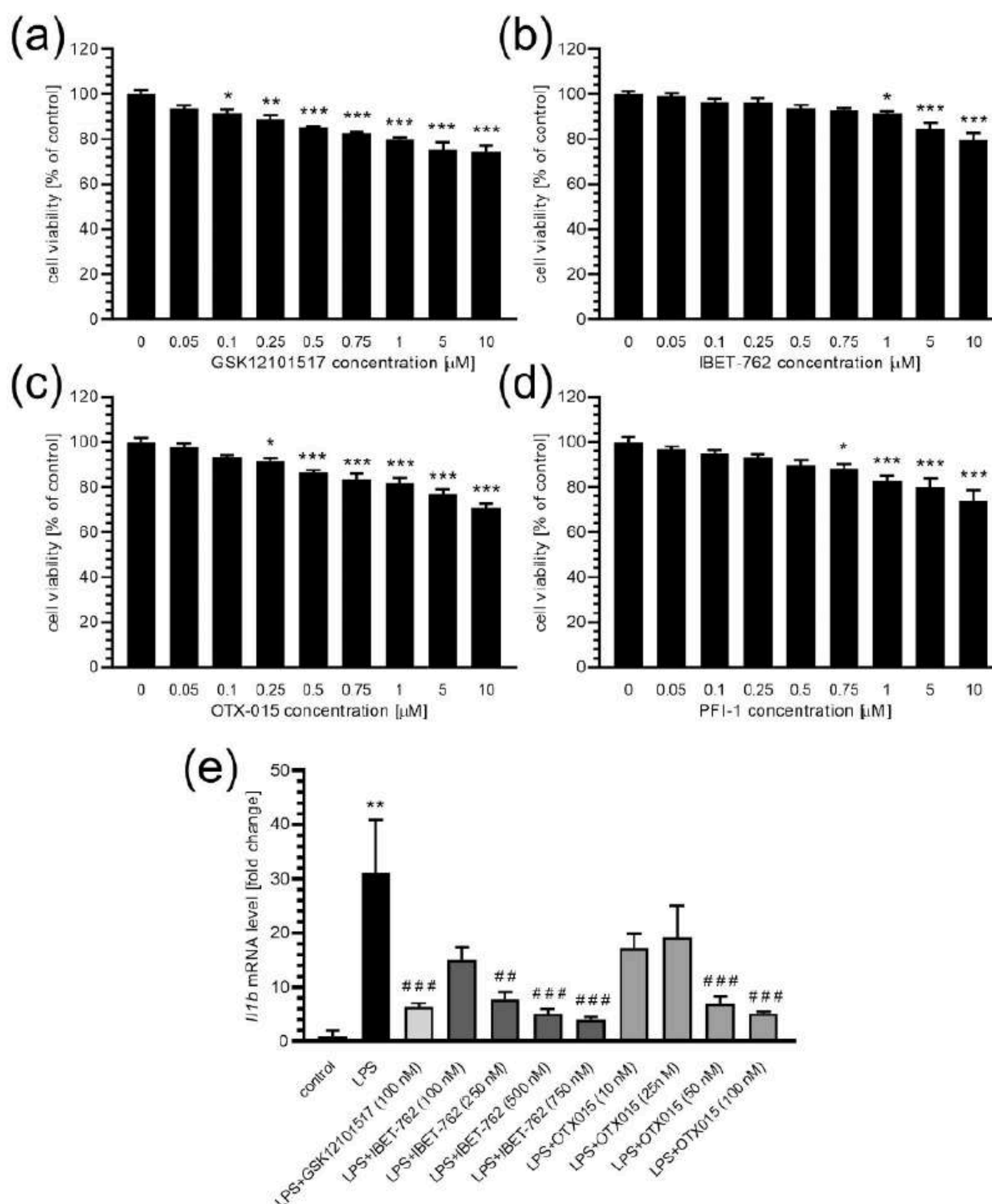

Supplementary Figure S1. The effect of BET inhibitors on microglial BV2 cells. (**a–d**) BV2 cells were incubated for 24 h in the presence of tested compounds, then MTT assay was performed to analyse cell viability (n=4). (**e**) BV2 cells were incubated for 2 h in the presence of LPS (100 ng/ml) and tested compound, then mRNA level for *Il1b* gene was determined by using qPCR (n=3-4). Means ± SEM were presented. *,**,*** $p<0.05$, 0.01, and 0.001, respectively, compared to the control. ##, ### $p<0.01$, and $p<0.001$, respectively, compared to LPS-treated group. Statistical analysis was performed by using a one-way ANOVA followed by the Bonferroni post-hoc test.

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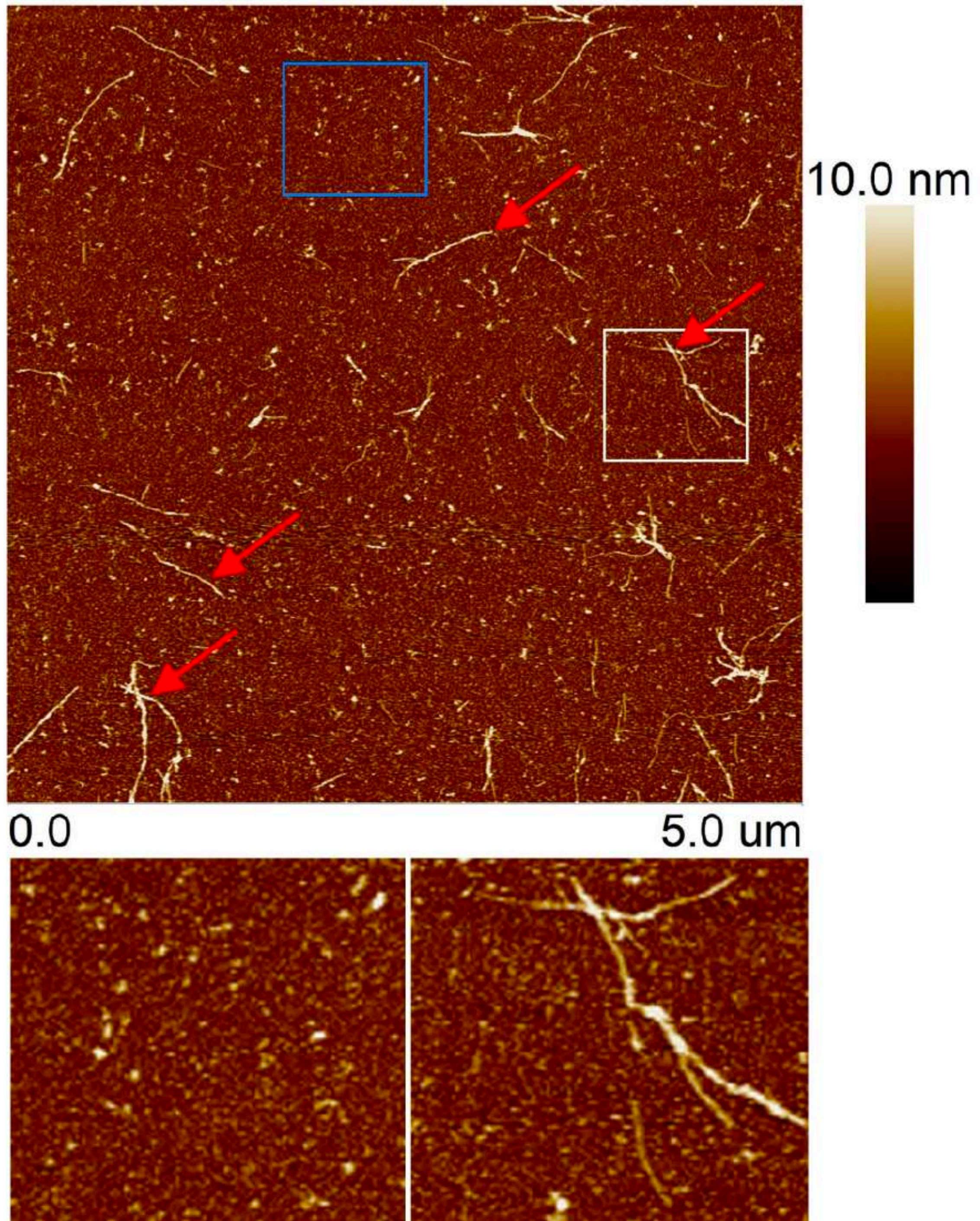

Supplementary Figure S2. High resolution AFM picture of Aβ after 2 h incubation at 37 °C. On the upper panel example oligomers were marked with a blue rectangle, red arrows indicate representative protofibrils. Lower panel shows marked areas in higher magnification.

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Supplementary Table S1. The results of gene expression array analysis.

TaqMan Array Mouse Phagocytosis 96-well plate, fast, RPU62UA

Normalization method Global Normalization

Fold Change Boundary 2.0

P-Value Boundary 0.05

Statistical Test T-test

N=4

* control

	Assay ID	Gene Symbol	Gene Name	Fold Change	P-Value	Result
*	Hs99999901_s1	18s rRNA	eukaryotic 18S rRNA	1.321	0.56012934	Insignificant
*	Mm00607939_s1	Actb	actin, beta	0.995	0.9702059	Insignificant
	Mm00456425_m1	Adipoq	adiponectin, C1Q and collagen domain containing	1.861	0.31970453	Insignificant
	Mm01134790_g1	Ager	advanced glycosylation end product-specific receptor	1.084	0.8682234	Insignificant
	Mm00440225_m1	Anxa1	annexin A1	0.411	0.031275354	Down-regulated
	Mm00437221_m1	Axl	AXL receptor tyrosine kinase	0.465	-	N.A.
*	Mm00437762_m1	B2m	beta-2 microglobulin	0.728	0.29210293	Insignificant
	Mm00437838_m1	C3	complement component 3	1.533	0.5392111	Insignificant
	Mm00482936_m1	Calr	calreticulin	0.960	0.9062711	Insignificant
	Mm00438094_g1	Cd14	CD14 antigen	0.301	0.041669343	Down-regulated
	Mm01135198_m1	Cd36	CD36 antigen	0.152	0.027002657	Down-regulated
	Mm01277163_m1	Cd44	CD44 antigen	0.501	0.10117423	Insignificant
	Mm00495011_m1	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	0.975	0.886271	Insignificant
	Mm01257219_m1	Ceacam3	carcinoembryonic antigen-related cell adhesion molecule 3	-	-	N.A.
	Mm01183349_m1	Clec7a	C-type lectin domain family 7, member a	0.172	0.012091957	Down-regulated
	Mm01179940_mH	Clic4	chloride intracellular channel 4 (mitochondrial)	0.823	0.6299917	Insignificant
	Mm01169510_m1	Cnn2	calponin 2	-	-	N.A.
	Mm01236242_m1	Colec12	collectin sub-family member 12	0.827	0.72318345	Insignificant
	Mm00467065_m1	Crk	v-crk sarcoma virus CT10 oncogene homolog (avian)	1.071	0.8084952	Insignificant
	Mm00432680_g1	Crp	C-reactive protein, pentraxin-related	-	-	N.A.
	Mm00432686_m1	Csf1	colony stimulating factor 1 (macrophage)	0.537	0.08823264	Insignificant
	Mm01290062_m1	Csf2	colony stimulating factor 2 (granulocyte-macrophage)	-	-	N.A.
	Mm00432751_m1	Csk	c-src tyrosine kinase	1.042	0.7218633	Insignificant

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	Mm00512037_m1	Cyp2s1	cytochrome P450, family 2, subfamily s, polypeptide 1	0.039	-	N.A.
	Mm01269874_m1	Dock1	dedicator of cytokinesis 1	0.985	0.9177907	Insignificant
	Mm00473720_m1	Dock2	dedicator of cyto-kinesis 2	1.021	0.8729215	Insignificant
	Mm00519109_m1	Elmo1	engulfment and cell motility 1	0.630	0.109474644	Insignificant
	Mm01204974_m1	Fas	Fas cell surface death receptor	0.886	0.85421735	Insignificant
	Mm02343757_m1	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	0.777	0.22789556	Insignificant
	Mm00438874_m1	Fcgr1	Fc receptor, IgG, high affinity I	0.257	0.034961548	Down-regulated
	Mm00438875_m1	Fcgr2b	Fc receptor, IgG, low affinity IIb	0.376	0.03289869	Down-regulated
	Mm00438882_m1	Fcgr3	Fc receptor, IgG, low affinity III	0.457	0.05749123	Insignificant
	Mm00433373_m1	Fyn	FYN proto-oncogene, Src family tyrosine kinase	0.708	0.0152459815	Flat
*	Mm99999915_g1	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	1.384	0.015334317	Flat
	Mm00518428_m1	Gulp1	GULP, engulfment adaptor PTB domain containing 1	-	-	N.A.
*	Mm00446953_m1	Gusb	glucuronidase, beta	1.042	0.8080083	Insignificant
*	Mm00660262_g1	Hmbs	hydroxymethylbilane synthase	2.248	0.1852197	Insignificant
*	Mm00446968_m1	Hprt	hypoxanthine guanine phosphoribosyl transferase	1.528	0.033382244	Flat
	Mm01168134_m1	Ifng	interferon gamma	-	-	N.A.
	Mm00516117_m1	Il1rl1	interleukin 1 receptor-like 1	0.722	0.5056077	Insignificant
*	Mm01255158_m1	Ipo8	importin 8	1.020	0.84822565	Insignificant
	Mm01182566_m1	Iqsec1	IQ motif and Sec7 domain 1	0.712	0.27312392	Insignificant
	Mm00434455_m1	Itgam	integrin alpha M	0.234	0.0017175004	Down-regulated
	Mm00434486_m1	Itgav	integrin alpha V	1.161	0.43396673	Insignificant
	Mm00434513_m1	Itgb2	integrin beta 2	0.926	0.6455412	Insignificant
	Mm01217488_m1	Lyn	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	1.191	0.060764432	Insignificant
	Mm01301009_m1	Mapk14	mitogen-activated protein kinase 14	1.486	0.087163374	Insignificant
	Mm00440265_m1	Marco	macrophage receptor with collagenous structure	0.320	0.017310344	Down-regulated
	Mm00487623_m1	Mbl2	mannose-binding lectin (protein C) 2	-	-	N.A.
	Mm00460328_m1	Mcoln3	mucolipin 3	0.685	0.528743	Insignificant
	Mm00434920_m1	Mertk	c-mer proto-oncogene tyrosine kinase	1.752	0.010825158	Flat
	Mm00500549_m1	Mfge8	milk fat globule-EGF factor 8 protein	0.868	0.4174912	Insignificant
	Mm03938638_s1	Mif	macrophage migration inhibitory factor	0.530	0.25248522	Insignificant
	Mm00447889_m1	Msn	moesin	0.871	0.2576329	Insignificant
	Mm00440338_m1	Myd88	myeloid differentiation primary response gene 88	1.108	0.7475546	Insignificant
	Mm00805062_m1	Nod1	nucleotide-binding oligomerization domain containing 1	0.467	0.018206742	Down-regulated
	Mm01242584_m1	Pecam1	platelet/endothelial cell adhesion molecule 1	0.906	0.82243997	Insignificant
*	Mm00435617_m1	Pgk1	phosphoglycerate kinase 1	1.025	0.8835571	Insignificant

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	Mm00659576_m1	Pik3cb	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	0.685	0.010959459	Flat
	Mm00476829_m1	Pip5k1a	phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha	0.967	0.8899483	Insignificant
	Mm00447040_m1	Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)	0.730	0.056154333	Insignificant
	Mm00448162_m1	Pla2g5	phospholipase A2, group V	0.805	0.69393283	Insignificant
	Mm01289339_m1	Pld1	phospholipase D1	0.360	0.014450495	Down-regulated
	Mm00447066_m1	Pld2	phospholipase D2	0.958	0.78521127	Insignificant
	Mm00440894_m1	Prkce	protein kinase C, epsilon	1.090	0.48608992	Insignificant
	Mm01343426_m1	Pros1	protein S (alpha)	0.801	0.74985003	Insignificant
	Mm00477208_m1	Pten	phosphatase and tensin homolog	1.450	0.20915312	Insignificant
	Mm00727887_s1	Rab5a	RAB5A, member RAS oncogene family	0.924	0.49013537	Insignificant
	Mm01183732_g1	Rab7	RAB7, member RAS oncogene family	0.904	0.35396162	Insignificant
	Mm01201653_mH	Rac1	RAS-related C3 botulinum substrate 1	1.280	0.085625395	Insignificant
	Mm00485472_m1	Rac2	RAS-related C3 botulinum substrate 2	1.338	0.03895043	Flat
	Mm00450933_m1	Rala	v-ral simian leukemia viral oncogene homolog A (ras related)	1.035	0.8661943	Insignificant
	Mm00469677_m1	Ralb	v-ral simian leukemia viral oncogene homolog B (ras related)	1.339	0.17033182	Insignificant
	Mm00522941_m1	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	-	-	N.A.
	Mm00834507_g1	Rhoa	ras homolog gene family, member A	1.066	0.6128333	Insignificant
*	Mm00782638_s1	Rplp2	ribosomal protein, large P2	1.775	0.006717769	Flat
	Mm00450234_m1	Scarb1	scavenger receptor class B, member 1	1.463	0.0030187257	Flat
	Mm00435860_m1	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	0.763	0.364854	Insignificant
	Mm00486060_m1	Sftpd	surfactant associated protein D	1.061	0.8901841	Insignificant
	Mm00488332_m1	Siglec1	sialic acid binding Ig-like lectin 1, sialoadhesin	0.068	0.000068767535	Down-regulated
	Mm02525668_u1	Sirpb1a	signal-regulatory protein beta 1A	0.112	0.001369061	Down-regulated
	Mm00454684_m1	Stab2	stabilin 2	-	-	N.A.
	Mm00452814_m1	Stx18	syntaxin 18	0.943	0.5024671	Insignificant
	Mm01333032_m1	Syk	spleen tyrosine kinase	1.216	0.09423082	Insignificant
*	Mm00446973_m1	Tbp	TATA box binding protein	1.284	0.32984367	Insignificant
*	Mm00441941_m1	Tfrc	transferrin receptor	1.705	0.1138525	Insignificant
	Mm00436987_m1	Tgm2	transglutaminase 2, C polypeptide	0.556	0.4245617	Insignificant
	Mm00844508_s1	Ticam1	toll-like receptor adaptor molecule 1	1.168	0.45567507	Insignificant
	Mm01207404_m1	Tlr3	toll-like receptor 3	0.293	0.00022732517	Down-regulated
	Mm00446193_m1	Tlr9	toll-like receptor 9	0.622	0.05131324	Insignificant
	Mm00443258_m1	Tnf	tumor necrosis factor	0.448	0.025960745	Down-regulated
	Mm00441906_m1	Tnfsf11	tumor necrosis factor (ligand) superfamily, member 11	-	-	N.A.
	Mm00807071_m1	Vamp7	vesicle-associated membrane protein 7	1.085	0.91970754	Insignificant
	Mm01232047_m1	Vav1	vav 1 oncogene	1.100	0.44238567	Insignificant
	Mm00494167_m1	Was	Wiskott-Aldrich syndrome homolog (human)	1.065	0.76816595	Insignificant
	Mm00437347_m1	Wnt5a	wingless-type MMTV integration site family, member 5A	-	-	N.A.

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Publikacja II

Matuszewska, M.; Wilkaniec, A.; Cieślik, M.; Strawski, M.; Czapski, G.A.

The Inhibition of Bromodomain and Extraterminal Domain (BET) Proteins Protects Against Microglia-Mediated Neuronal Loss In Vitro. Biomolecules 2025, 15(4), 528, https://doi.org/10.3390/biom15040528

Article

The Inhibition of Bromodomain and Extraterminal Domain (BET) Proteins Protects Against Microglia-Mediated Neuronal Loss In Vitro

Marta Matuszewska [1], Anna Wilkaniec [1], Magdalena Cieślik [1], Marcin Strawski [2] and Grzegorz A. Czapski [1,*]

[1] Mossakowski Medical Research Institute, Polish Academy of Sciences, Department of Cellular Signalling, ul. Pawińskiego 5, 02-106 Warsaw, Poland; mmatuszewska@imdik.pan.pl (M.M.); awilkaniec@imdik.pan.pl (A.W.); mcieslik@imdik.pan.pl (M.C.)
[2] University of Warsaw, Faculty of Chemistry, ul. Pasteura 1, 02-093 Warsaw, Poland; marcin@chem.uw.edu.pl
* Correspondence: gczapski@imdik.pan.pl

Abstract: Neuroinflammation is a key feature of all neurodegenerative disorders, including Alzheimer's disease, and is tightly regulated by epigenetic mechanisms. Among them, bromodomain and extraterminal domain (BET) proteins play a crucial role by recognizing acetylated histones and acting as transcriptional co-regulators to modulate gene expression. This study investigates the potential of inhibiting BET proteins in preventing microglia-mediated neuronal damage in vitro. Murine BV2 microglial cells were exposed to lipopolysaccharide (LPS) or amyloid-β (Aβ) to induce an inflammatory response, and the subsequent effects on murine HT22 neuronal cells were examined. Among the BET proteins tested, only Brd4 was significantly upregulated in BV2 cells upon pro-inflammatory stimulation. JQ1, a potent pan-inhibitor of BET proteins, suppressed LPS-induced upregulation of pro-inflammatory cytokine mRNA levels, including *Il1b*, *Il6*, and *Tnf*, in BV2 microglia. Pre-treatment with JQ1 attenuated the cytotoxicity of LPS-activated BV2 cells toward neurons. Additionally, conditioned media from Aβ fibril-stimulated BV2 cells induced neuronal cell death, which was partially prevented by pre-treatment with JQ1. Co-culture assays further demonstrated the beneficial effect of BET inhibition. Our findings suggest that targeting BET proteins may offer a neuroprotective strategy by modulating microglial activation, potentially providing therapeutic benefits in neurodegenerative diseases.

Keywords: neuroinflammation; bromodomain and extraterminal domain proteins; neuroprotection; Alzheimer's disease; microglia

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1. Introduction

The immune system identifies and eliminates pathogens, as well as recognizes and neutralizes abnormal self-molecules, thereby contributing to the maintenance of homeostasis within the organism. However, when dysregulated, prolonged, or excessive, immune responses can contribute to the development of pathological conditions. This is particularly evident in the central nervous system (CNS), where neuroinflammation is a central feature of a wide range of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis [1–3]. In these disorders, chronic neuroinflammation exacerbates neuronal damage and contributes to disease progression [4–6]. This process is primarily driven by activated microglia, the brain's resident immune cells, which are essential for maintaining homeostasis and responding to injury or disease. In

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their surveillance state, often inaccurately referred to as the "resting state", microglial cells monitor the CNS environment and maintain neuronal health by clearing cellular debris and secreting neurotrophic factors. However, microglia become activated in response to pathological stimuli, such as amyloid-β (Aβ) peptides in AD or α-synuclein in PD [7,8]. This activation releases pro-inflammatory cytokines, reactive oxygen species (ROS), and neurotoxic molecules, impairing neuronal function and promoting neurodegeneration [9,10]. Therefore, the modulation of microglial activation presents a potential therapeutic target for preventing or delaying the progression of neurodegenerative diseases.

Bromodomain and extraterminal domain (BET) proteins, including Brd2, Brd3, and Brd4, have emerged as key regulators of transcriptional processes [11]. These proteins function as "readers" of the histone acetylation code, recognizing acetylated lysine residues on histones and other proteins, which promotes the recruitment of transcriptional complexes and modulates gene expression [12]. BET proteins play a crucial role in various cellular processes, including inflammation, cell cycle regulation, and differentiation. Studies have shown that BET inhibitors, such as JQ1, can attenuate inflammatory signaling pathways in macrophages by blocking the transcription of pro-inflammatory genes, including TNF-α, IL-6, and IL-1β [13]. However, while the role of BET proteins in macrophage-mediated inflammation is well-established, their involvement in microglial activation and neuroinflammation is still less defined.

Given the growing evidence linking neuroinflammation to the development of neurodegenerative diseases, this study investigates the therapeutic potential of inhibiting BET proteins as a neuroprotective strategy. In this in vitro model, we specifically investigate whether inhibition of BET proteins can prevent microglia-dependent neuronal cell loss. Using the murine BV2 microglial cell line and HT22 neuronal cells, we assess the impact of BET inhibition on microglial activation and the subsequent effects on neuronal viability. This work aims to provide further insights into the role of BET proteins in neuroinflammation and their potential as therapeutic targets in neurodegenerative diseases.

2. Materials and Methods

2.1. Reagents and Materials

(S)-(+)-tert-butyl 2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo [4,3-a][1,4]diazepin-6-yl)acetate (JQ1), L-glutamine, penicillin, streptomycin, 3-(4,5-dimethyl-2-tiazolilo)-2,5-diphenyl-2H-tetrazolium bromide (MTT), DNase I, anhydrous DMSO, lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (toxicity 3,000,000 U/mg), hexafluoroisopropanol (HFIP), bovine serum albumin (BSA), and accutase solution were from Merck Millipore Inc. (Burlington, MA, USA). RPMI-1640 medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Nunc Polycarbonate Cell Culture Inserts in Multi-Well Plates, pore size 0.4 μm, TRI reagent, High-Capacity cDNA Reverse Transcription Kit (including RNase Inhibitor), TaqMan Gene Expression Assays and TaqMan Fast Advanced Master Mix, red fluorescent microspheres (FMS; 2.0 μm), and BCA Protein Assay Kits were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Ham's F12 Medium, w/o L-glutamine and phenol red, was from PAN-Biotech GmbH (Aidenbach, Germany). Amyloid-beta (Aβ1-42) was from AnaSpec, Inc. (Fremont, CA, USA). The enzyme-linked immunosorbent assay (ELISA) kits for quantitatively detecting mouse Brd2, Brd3, and Brd4 proteins were from Abbexa Ltd. (Cambridge, UK). The Mycoplasma Detection Kit was from InvivoGen Corp. (San Diego, CA, USA). Mycoplasma-Off disinfecting solution was from Minerva Biolabs GmbH (Berlin, Germany). The Mini Dialysis Kit (1 kDa cut-off) was from Cytiva (Buckinghamshire, UK). All other reagents were obtained from Merck Millipore Inc.

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2.2. Reagents and Treatments

Bacterial LPS was dissolved in PBS and added to a cell culture medium to induce the pro-inflammatory activation of BV2 microglial cells. The cells were treated with LPS at a concentration of 100 ng/mL for the specified durations [14,15]. HFIP-pretreated Aβ1–42 was initially dissolved in anhydrous DMSO at a concentration of 5 mM, followed by dilution in F12 medium to a final concentration of 100 μM. The solution was vortexed for 30 s and then incubated under different conditions to promote the formation of distinct aggregated species: (1) for oligomer formation, the solution was incubated at 4 °C for 24 h; (2) for protofibrils, it was incubated at 37 °C for 6 h, and (3) for fibrils, incubation was continued at 37 °C for 60 h [16,17]. After incubation, the samples were subjected to dialysis in F12 for 3 h using a Mini Dialysis Kit to remove any residual solvents or small molecules [16]. BET inhibitor JQ1 was initially dissolved in DMSO to create a 10 mM stock solution, which was subsequently diluted in the culture medium to achieve a 50 μM solution for application to the cells [12]. The final concentration in the cell culture medium (50 nM) was selected based on our previous studies [18]. Equal volumes of vehicles were added to each experimental group to ensure comparable conditions.

2.3. Cell Culture Experiments

Murine microglial BV2 cells [19] (Elabscience Biotechnology Inc., Houston, TX, USA) and murine neuronal HT22 cells [20] (Merck Millipore Inc., Burlington, MA, USA) were cultured in the same conditions in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The cells were usually passaged every 2 to 3 days, and the passages up to 20 were utilized for the experiments. To exclude mycoplasma contamination, the cells were regularly tested using a Mycoplasma Detection Kit (InvivoGen Corp., San Diego, CA, USA).

A 6 h stimulation period was chosen for the BV2 cells. Our preliminary experiments and published data demonstrate that the expression of most inflammation-related immediate-early genes in microglia increases rapidly and reaches its peak levels within 6 h of stimulation [21–23]. Therefore, because BET proteins regulate gene transcription, our study focused on acute processes.

The BV2 cells were pretreated with JQ1 (50 nM) and/or LPS (100 ng/mL) for 6 h for conditioned medium experiments. Subsequently, the conditioned medium was collected and transferred to HT22 neuronal cells. The conditioned medium was mixed with the existing culture medium at a 1:1 ratio [14]. Incubation was continued for 24 h.

For co-culture experiments, we used polycarbonate cell culture inserts in multi-well plates, as previously described [24]. Briefly, BV2 and HT22 cells were seeded and grown separately: BV2 in inserts and HT22 in multi-well plates. After 24 h, the BV2 cells were pretreated with JQ1 (50 nM) and/or after 30 min with Aβ (5 μM) for 6 h. Subsequently, the culture medium was aspirated from the inserts. The inserts containing BV2 cells were then transferred to wells containing HT22 cells and submerged in the resident culture medium. Incubation was continued for 24 h.

2.4. MTT Assay

Cell viability was assessed based on the reduction of MTT to formazan [18]. Following treatment with the test compounds, MTT (0.25 mg/mL) was added to the culture medium, and the cells were incubated at 37 °C for an additional 2 h. The medium was gently removed, and the cells were dissolved in DMSO. The absorbance of the formazan formed was measured at 595 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

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2.5. Gene Expression Analysis

RNA was extracted using a TRI reagent following the manufacturer's instructions. The RNA concentration and quality were assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). DNase I treatment was performed according to the manufacturer's guidelines to eliminate potential DNA contamination. For reverse transcription, 1 µg of RNA was used as input for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. Relative mRNA expression levels were determined using the ΔΔCt method, and the results were expressed as relative quantities (RQs) [25]. The levels of mRNA for selected genes were quantified using TaqMan Gene Expression Assays, including *Arg1* (Mm00475988_m1), *Brd2* (Mm01271171_g1), *Brd3* (Mm01326697_m1), *Brd4* (Mm01350417_m1), *Il1b* (Mm00434228_m1), *Il6* (Mm00446190_m1), *Nos2* (Mm00440502_m1), *Tnf* (Mm00443258_m1), and *Gusb* (Mm01197698_m1) [18]. *Gusb* was used as a reference gene. Quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using TaqMan Fast Advanced Master Mix, following the manufacturer's guidelines.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits were used strictly in accordance with the manufacturer's protocols. Briefly, the cells were washed three times with PBS and then homogenized in ice-cold PBS (pH 7.2) using a syringe. Then, the samples were sonicated (40% pulse, 40% power) for 30 s using a Model 150 V/T Ultrasonic Homogenizer (Biologics Inc., Manassas, VA, USA). The sonication was a necessary step [26] to increase the solubility of the BET proteins, which, as chromatin-associated proteins, tend to form insoluble complexes. Then, the homogenates were centrifuged at 10,000× *g* for 5 min, and the supernatant was collected. The protein concentration was accurately determined using a bicinchoninic acid (BCA) assay, with bovine serum albumin (BSA) as the standard. The prepared samples were used immediately to prevent protein degradation or denaturation. After accomplishing all the incubation steps, optical density was measured at 450 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.). The concentration of the tested compound was calculated using a standard curve and normalized to the total protein level. Each sample was analyzed in duplicate.

2.7. Phagocytosis Assay

The phagocytic activity of BV2 cells was assessed using flow cytometry. FluoSpheres Carboxylate 2.0 µm fluorescent microspheres (FMSs) were pre-coated with either 3% bovine serum albumin (BSA) for 15 min at 37 °C or 5 µM Aβ for 2 h at 37 °C. The BV2 cells were incubated with 50 µL/mL of FMS for 2 h in standard cell culture conditions. The non-phagocytosed FMSs were removed by washing the cells three times with PBS. The cells were detached using accutase solution and immediately analyzed by flow cytometry (FACSCanto II, BD Biosciences, San Jose, CA, USA) using FACSDiva Software 6.0 (BD Biosciences). The phagocytic index was determined as the percentage of BV2 cells that internalized the FMSs.

2.8. Atomic Force Microscopy (AFM)

AFM analysis was performed according to a previously described protocol [22]. Briefly, amyloid samples were prepared by placing a 10 µL drop of the medium onto freshly cleaved mica (V1 grade, NanoAndMore GmbH, Wetzlar, Germany). After a 10 min incubation, each sample was rinsed with deionized water (Merck Millipore Inc., Burlington, MA, USA) and dried using a gentle stream of argon. Imaging of the mica surface and the deposited amyloid structures was performed using PeakForce Tapping® mode on a Multimode 8

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Nanoscope atomic force microscope (AFM, Bruker, Billerica, MA, USA). High-resolution probes SNL-10 (Bruker, Billerica, MA, USA) with a spring constant 0.12 Nm^{-1} were used.

2.9. Statistics

Statistical analysis was conducted using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA, USA). Comparisons were made using either Student's *t*-test or one-way analysis of variance (ANOVA) with Bonferroni's post hoc test for multiple comparisons. Normality was assessed using the Shapiro–Wilk test. The term "n" denotes the number of independent in vitro experiments.

3. Results

Considering the crucial role of neuroinflammation in the development of neurodegenerative diseases and the involvement of BET proteins in regulating inflammatory processes, we aimed to elucidate the potential neuroprotective effects of inhibiting BET proteins by examining their influence on microglial activity.

First, we analyzed the impact of LPS on mRNA and protein levels of brain-resident BET family members in BV2 microglial cells. Under control conditions, Brd2, Brd3, and Brd4 showed comparable abundance, measured at 0.46, 0.44, and 0.51 pg/µg of protein, respectively. Our findings demonstrate that Brd4 is the only BET protein significantly upregulated in BV2 microglial cells stimulated with bacterial LPS at 100 ng/mL (Figure 1). This suggests a specific role for Brd4 in microglial activation during neuroinflammation, which is consistent with previous reports implicating BET proteins in regulating inflammatory responses [13].

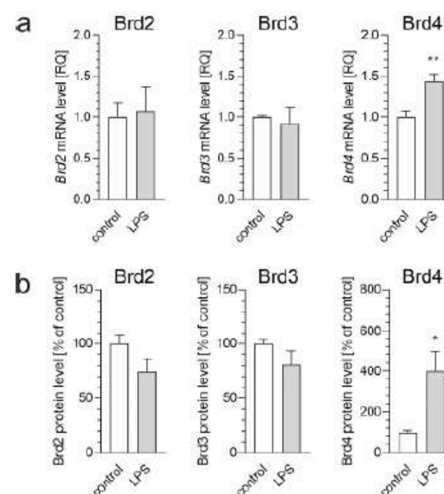

Figure 1. The effect of LPS on BET protein expression in BV2 microglial cells. BV2 cells were treated with LPS (100 ng/mL) for 6 h. (**a**) mRNA levels of *Brd2*, *Brd3*, and *Brd4* were analyzed using qPCR. (**b**) Protein levels of BET proteins were measured using ELISA assays. Data are presented as mean ± SEM; n = 4 (**a**) and 3–4 (**b**); * $p < 0.05$ and ** $p < 0.01$, compared to the control (Student's *t*-test).

Treatment with JQ1, a highly specific and potent pan-inhibitor of BET proteins, attenuated the LPS-induced transcription of key pro-inflammatory cytokines, including *Il1b*, *Il6*, and *Tnf*, in the BV2 cells. This indicates that BET inhibition effectively dampens mi-

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croglial activation, although it does not affect cytokine expression under control conditions (Figure 2).

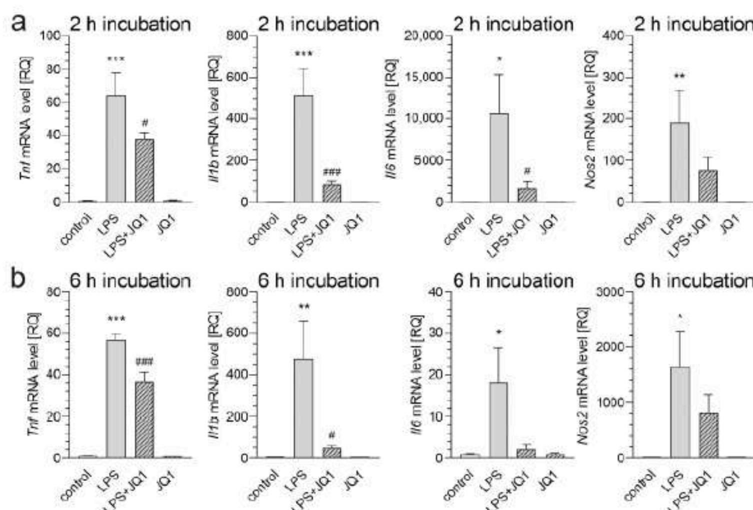

Figure 2. The effect of LPS and/or JQ1 on the mRNA levels of inflammation-related genes in BV2 cells. BV2 cells were treated with JQ1 (50 nM), and after 30 min, LPS (100 ng/mL) was added. Incubation was continued for 2 or 6 h. (**a**) mRNA levels of *Tnf*, *Il1b*, *Il6*, and *Nos2* after 2 h. (**b**) mRNA levels of *Tnf*, *Il1b*, *Il6*, and *Nos2* after 6 h. Data are presented as mean ± SEM; n = 6–9 (**a**) and 6–8 (**b**); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the control; # $p < 0.05$ and ### $p < 0.001$, compared to the LPS-treated group (ANOVA with Bonferroni post hoc test).

Activated microglial cells produce and release various mediators that may affect neighboring cells. Therefore, we used conditioned media from the LPS-stimulated BV2 cells to study the impact of microglial secretome on neuronal HT22 cells. As shown in Figure 3a, conditioned medium from the control (resting) and JQ1-treated BV2 cells did not affect HT22 cell viability. In contrast, exposure to medium from the LPS-treated BV2 cells induced cell death in the HT22 cells, indicating the neurotoxic effects of inflammation-driven microglial secretions. However, the addition of JQ1 to the BV2 culture prevented LPS-induced microglial activation and subsequent neuronal cell death, significantly preserving the viability of the HT22 cells. These findings underscore the dichotomous role of microglial-derived factors and highlight the therapeutic potential of BET inhibition in counteracting neuroinflammation-mediated toxicity.

To confirm that the cytotoxic effect of the conditioned medium on the HT22 cells was due to microglia-secreted compounds and not residual LPS, we assessed the potential toxicity of LPS at a concentration of 100 ng/mL. As shown in Figure 3b, LPS at this concentration did not affect the viability of the HT22 cells.

While microglial activation is commonly triggered by pro-inflammatory stimuli, such as LPS, it is also a central feature of neurodegenerative diseases associated with Aβ pathology. Therefore, in the following steps, we analyzed whether inhibiting BET proteins could protect neurons against the neurotoxic activity of microglia stimulated with Aβ. First, we examined how Aβ influences microglial activation (Figure 4) using two key indicators: phagocytosis and the expression of pro-inflammatory genes. Among the different forms of Aβ (oligomers, protofibrils, and fibrils), only Aβ fibrils (ABFs) effectively activated the microglial BV2 cells. The dose–response experiments showed that BV2 cell activation became detectable at an Aβ concentration of 5 μM, which was subsequently utilized in the

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following experiments. The exposure of the BV2 cells to ABFs at this concentration induced a significant upregulation of *Tnf* mRNA, yet this increase was less pronounced than the effect caused by LPS (compare Figure 2b).

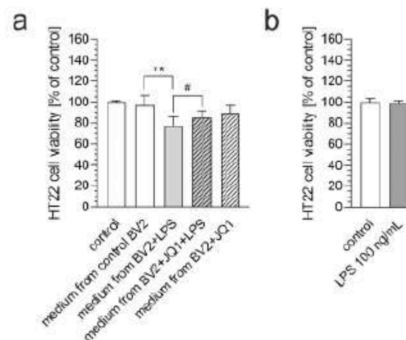

Figure 3. The effect of conditioned medium from LPS- and/or JQ1-treated BV2 cells. (**a**) BV2 cells were treated with JQ1 (50 nM), and then, after 30 min, LPS (100 ng/mL) was added, and incubation was continued for 6 h. The conditioned medium was then transferred to murine neuronal HT22 cells, which were incubated for 24 h. Cell viability was assessed using an MTT assay. (**b**) HT22 cells were treated with LPS (100 ng/mL) for 24 h, and cell viability was evaluated by an MTT assay. Data are presented as mean ± SEM; n = 8 (**a**) and 6 (**b**). ** $p < 0.01$, # $p < 0.05$, compared to the indicated group (ANOVA with Bonferroni post hoc test).

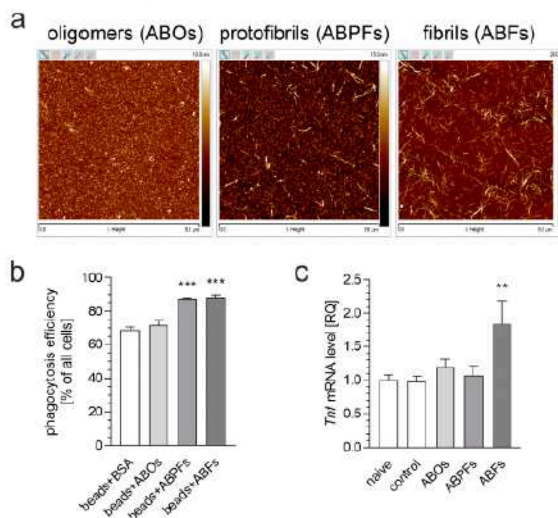

Figure 4. The effect of Aβ on BV2 cell activation. (**a**) Atomic force microscopy analysis of various Aβ preparations: oligomers (ABOs), protofibrils (ABPFs), and fibrils (ABFs). (**b**) The impact of 5 μM Aβ (ABOs, ABPFs, ABFs) on the phagocytic activity of BV2 cells. (**c**) The effect of 5 μM Aβ (ABOs, ABPFs, ABFs) on the mRNA level of the *Tnf* gene in BV2 cells after 6 h of incubation. Data are presented as mean ± SEM; n = 4 (**b**) and 6 (**c**). ** $p < 0.01$ and *** $p < 0.001$, compared to the respective control (ANOVA with Bonferroni post hoc test).

The ABFs treatment also led to the selective increase in Brd4 protein levels in the BV2 cells, with no significant alterations observed for Brd2 or Brd3 (Figure 5). This selective upregulation identifies Brd4 as a key mediator of microglial responses to amyloid pathology,

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underscoring its potential as a promising therapeutic target for neuroinflammation and Alzheimer's disease (AD).

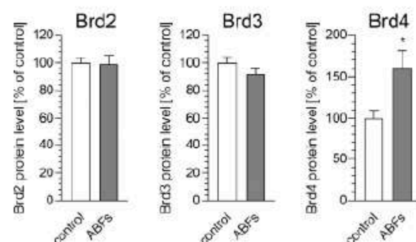

Figure 5. The effect of Aβ fibrils (ABFs) on BET protein levels in microglial BV2 cells. BV2 cells were incubated with ABFs (5 μM) for 6 h, and the levels of BET proteins were measured using ELISA assays. Data are presented as mean ± SEM; n = 3–4; * $p < 0.05$, compared to the control (Student's *t*-test).

Conditioned media from the Aβ-stimulated BV2 cells caused a reduction in HT22 neuronal cell viability, highlighting the neurotoxic potential of microglia in response to fibrils (Figure 6a). However, this effect was observed only when the fresh medium was used. Storing the conditioned medium at −80 °C for a few days abolished its neurotoxic potential. Transwell inserts were utilized to establish a co-culture system. These inserts enable the physical separation of two cell types within a single well while facilitating media exchange between compartments. This design allows for the diffusion of soluble factors, such as cytokines, growth factors, and other molecules, from one compartment to the other, allowing for the investigation of their effects on cells without requiring direct cell-to-cell contact. Pre-treatment with JQ1 preserved the viability of the neuronal cells treated with a conditioned medium, but this effect was less pronounced in the co-culture studies (Figure 6a,b). This result suggests that BET proteins play a crucial role in mediating microglia-induced neurotoxicity, and their inhibition can mitigate the damaging effects of neuroinflammation on neurons. These findings support the idea that targeting BET proteins could be a promising neuroprotective approach for preventing or slowing neurodegenerative diseases linked to chronic neuroinflammation.

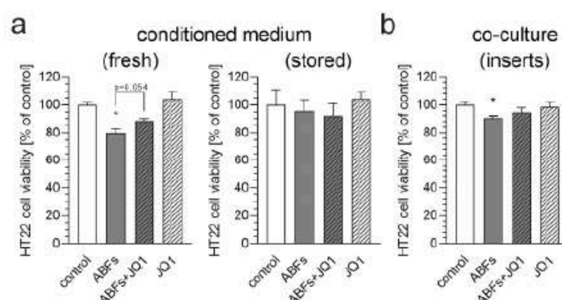

Figure 6. The effect of mediators released by ABFs-stimulated BV2 cells and/or JQ1 on the viability of HT22 cells. (**a**) BV2 cells were treated with JQ1 (50 nM) and/or after 30 min with ABFs (5 μM), and incubation was continued for 6 h. The conditioned medium was then transferred to murine neuronal HT22 cells, and after 24 h of incubation, cell viability was assessed using an MTT assay. (**b**) BV2 cells were treated with JQ1 (50 nM). After 30 min, ABFs (5 μM) were added, and incubation was continued. After 6 h, the medium was aspirated, and inserts with stimulated BV2 cells were transferred to HT22 cells. Incubation was then continued for an additional 24 h. Cell viability was assessed using an MTT assay. Data are presented as mean ± SEM; n = 4–5 (**a**), n = 8 (**b**); * $p < 0.05$, compared to the control group (ANOVA with Bonferroni post hoc test).

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4. Discussion

Increasing evidence shows that the pathogenesis of AD is related to neuronal dysfunction and immune system dysregulation. Misfolded proteins activate microglial and astrocytic pattern recognition receptors, initiating an inflammatory response that accelerates disease progression. Genome-wide studies have associated risk genes with the regulation of external factors, such as systemic inflammation, which further exacerbates these processes [2,5,27,28]. Thus, modulating immune mechanisms and associated risk factors may offer promising therapeutic strategies for AD.

This study explored the role of BET family proteins, particularly Brd4, in regulating microglial activation and neuroinflammation, emphasizing the therapeutic potential of JQ1, a potent BET inhibitor. Our results indicate that Brd4 is a key regulator of microglial responses under pro-inflammatory conditions and that the inhibition of BET proteins can mitigate both the inflammatory response and neurotoxic effects in neurodegeneration models, including those induced by LPS and Aβ. These findings highlight the potential of BET inhibition as a novel therapeutic approach for neuro-inflammatory diseases, such as AD.

Our study demonstrated a significant upregulation of Brd4 in BV2 microglial cells after pro-inflammatory stimulation, both at the mRNA and protein levels. This finding is consistent with the existing literature, which suggests that Brd4 plays a central role in the transcriptional regulation of pro-inflammatory genes in glial cells, including microglia. Specifically, Brd4 is known to facilitate the transcription of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, by interacting with acetylated histones and coactivators, thus contributing to the inflammatory cascade [11,29]. Therefore, our data further support previous findings suggesting that Brd4 may be one of the primary proteins involved in regulating microglial activation under inflammatory conditions and possibly a specific therapeutic target in neuroinflammation-related disorders. Notably, the specific upregulation of Brd4 protein levels in the brain was also observed in our in vivo studies on LPS-induced systemic inflammation in mice (Matuszewska et al., submitted for publication). The significant increase observed after only 6 h of incubation may seem surprising. However, it is essential to note that both LPS and Aβ activate innate immune response mechanisms. This evolutionarily conserved process is optimized for rapid reactions to stimuli. The proteome's highly dynamic response to LPS is well-documented, highlighting the potential for rapid and significant protein level changes even 60 min after stimulation [30]. Furthermore, considering Brd4's role in inflammatory signaling, a rapid increase in its expression is both plausible and necessary if it plays a pivotal role in regulating genes involved in inflammation. Notably, other studies have documented the exclusive upregulation of Brd4 protein under stress conditions. For instance, murine heart tissue exhibited increased Brd4 mRNA and protein levels 12 h after intraperitoneal LPS administration [31]. Additionally, our previous in vivo study demonstrated an exclusive increase in Brd4 mRNA in the hippocampi of mice after systemic inflammation induced by LPS [32]. Data indicate that the elevated level of Brd4 persists for at least 24 h [33]. The discrepancy between the slightly increased mRNA level and the significantly increased Brd4 protein level in our experiment can be explained by differences in mRNA and protein stability. Consequently, even a moderate increase in mRNA levels can result in substantial protein accumulation. We hypothesize that this phenomenon is relevant to our study.

Additionally, our data showed that Aβ-induced microglial activation led to an increase in Brd4 protein expression, highlighting the crucial role of Brd4 in mediating the Aβ-related neuro-inflammatory process. This further supports the idea that Aβ influences the expression of this BET protein, thereby exacerbating the microglial response. This observation is consistent with previous studies demonstrating that Aβ triggers microglial activation

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through multiple mechanisms, including the modulation of chromatin remodeling factors, such as Brd4, which play a key role in the regulation of inflammatory gene expression [11].

Consistent with previous studies [29,34,35], our research demonstrated that BET inhibition significantly suppresses microglial activation and cytokine release. Additionally, our data showed that BET inhibition not only reduces inflammation but also promotes neuronal cell survival in pro-inflammatory environments. Thus, we extended the previous findings by demonstrating that BET proteins are key mediators of the toxic effects induced by microglia on neighboring neuronal cells. This observation might be particularly relevant for conditions associated with chronic neuroinflammation, such as AD, where microglial activation contributes to pathogenesis and neuronal death.

We also investigated the effects of Aβ, a key player in the pathomechanism of AD, on microglial activation. Our results showed that Aβ fibrils, but not Aβ oligomers or protofibrils, effectively induced microglial activation, as evidenced by enhanced phagocytosis and the elevated expression of pro-inflammatory cytokines, such as TNF. This finding is consistent with previous studies, which demonstrate that ABFs are the most potent activator of microglia, while soluble oligomers are less efficient at triggering inflammatory responses [36–39]. Some research indicates that ABFs induce microglial phagocytosis, while ABOs attenuate it [40]. Conversely, other studies report that ABOs elicit a stronger microglial inflammatory response than ABFs [41]. These disparate findings suggest that (i) ABOs and ABFs differentially activate microglia and (ii) the conformation of Aβ and experimental conditions must be rigorously controlled.

A key finding of this study was that pre-treatment with JQ1 conferred neuroprotection of HT22 neuronal cells exposed to Aβ-activated microglia. Conditioned medium from Aβ-stimulated BV2 cells significantly decreased the viability of HT22 cells. This neurotoxic effect was attenuated by pre-treatment with JQ1, indicating that BET inhibition can mitigate microglia-mediated neurotoxicity. This result is consistent with studies showing that BET inhibitors confer neuroprotection by attenuating microglial inflammatory responses following spinal cord injury in rats [29]. However, the consequences of the global inhibition of BET proteins require further investigation. A previous study on human neuronal-like cells demonstrated that pharmacological degradation and inhibition of BET proteins significantly increased Aβ levels [42]. On the other hand, other studies have shown that JQ1 and other BET inhibitors attenuate Aβ-induced neuroinflammation, preserve neuronal viability, and reduce neuronal loss in models of AD [35,43,44]. Moreover, several studies have demonstrated the beneficial effects of BET inhibitors on cognitive function. Prolonged administration of JQ1 improved cognition deficits in rat models of AD [45,46]. Additionally, JQ1 enhanced brain plasticity across various mouse models, including wild-type and APP-expressing mice, and effectively rescued hippocampal-dependent cognitive deficits observed in C9BAC mice, an animal model of frontotemporal dementia [47,48]. Despite its positive mitigating effects on inflammation and Tau phosphorylation, JQ1 failed to improve learning and memory deficits in 7-month-old 3×Tg-AD mice [44]. In contrast, another study demonstrated that prolonged administration of JQ1 had a negative impact on memory function in mice [49]. Moreover, administering JQ1 to young rats resulted in cognitive impairment in adulthood [50]. Finally, a human randomized controlled trial demonstrated that another BET inhibitor, Apabetalone (RVX-208), improved cognitive performance in patients aged 70 or older with a baseline MoCA score of 21 or less [51]. The neuroprotective effects of JQ1 in our study further support the notion that targeting Brd4 and other BET proteins can mitigate the neurotoxic effects of activated microglia in neurodegenerative diseases [47].

An intriguing finding was that fresh conditioned medium from stimulated microglia decreased neuronal cell viability, whereas medium stored at −80 °C for approximately a

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week did not exhibit this effect. Given that the secretome of stimulated BV2 cells comprises nearly 5000 proteins [52], speculation on this issue without supporting proteomic data is unreliable. Moreover, high-resolution quantitative proteomics analysis revealed fundamental differences in protein expression between human and murine microglia, as well as across various culture conditions [53]. Therefore, our results suggest that further studies on the human microglial cell system are necessary to resolve this puzzle. Besides proteins, lipid mediators, exosomes, RNAs, and ROS may be considered. Among the mediators released by activated microglia, ROS appear particularly vulnerable to long storage conditions due to their inherently unstable nature. ROS can still undergo reactions, albeit at a slower rate, even at −80 °C. However, cytokines also exhibit significant variability in stability during storage at −80 °C [54]. Notably, TNF-α, which has been demonstrated to be neurotoxic to HT22 cells [55], has been shown to degrade even under these storage conditions [54]. Exosomes are also vulnerable to −80 °C storage and freeze–thaw cycles [56], as prolonged storage under these conditions can alter their concentration, purity, and particle size.

Despite the inherent physiological differences between humans and mice, and the resulting limitations of using murine cells in this study, the significant homology (approximately 40%) between their genomes [57] suggests that the findings remain relevant. However, even with comparable gene control systems, RNA and protein expression can differ between mice and humans [53,58,59]. Further research in human cell systems is necessary to confirm our observations.

In summary, our study demonstrates that Brd4 plays a pivotal role in the inflammatory activation of microglia in response to LPS and AβFs. BET inhibition can effectively reduce both microglial inflammatory responses and neurotoxic effects. Given that chronic microglial activation is a central feature of many neurodegenerative diseases, our results provide strong evidence for the potential of BET inhibitors as a novel therapeutic approach for treating conditions characterized by neuroinflammation. Further preclinical and clinical studies are necessary to evaluate the efficacy of BET inhibitors in these diseases and to determine their therapeutic potential in human patients.

5. Conclusions

In conclusion, our study demonstrates that inhibition of the BET proteins attenuates microglial activation and neuronal damage in vitro, providing a potential therapeutic avenue for mitigating neuro-inflammatory degeneration in neurodegenerative diseases, such as Alzheimer's disease.

Author Contributions: Conceptualization, M.M., M.C., A.W. and G.A.C.; methodology, M.M., A.W., M.C., M.S. and G.A.C.; validation, G.A.C.; formal analysis, M.M. and G.A.C.; investigation, M.M., A.W., M.C., M.S. and G.A.C.; resources, G.A.C.; writing—original draft preparation, M.M. and G.A.C.; writing—review and editing, M.M., A.W., M.C., M.S. and G.A.C.; supervision, G.A.C.; project administration, G.A.C.; funding acquisition, G.A.C. All authors have read and agreed to the published version of the manuscript.

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

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Abbreviations

The following abbreviations are used in this manuscript:

Aβ	amyloid-β
ABOs	amyloid-β oligomers
ABFs	amyloid-β fibrils
ABPFs	amyloid-β protofibrils
AD	Alzheimer's disease
AFM	atomic force microscopy
BET	bromodomain and extraterminal domain
CNS	central nervous system
DMSO	dimethyl sulfoxide
FMS	fluorescent microspheres
HFIP	hexafluoroisopropanol
LPS	lipopolysaccharide
PD	Parkinson's disease
RQ	relative quantity

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EDITED BY
Muddanna Sakkattu Rao,
Kuwait University, Kuwait

REVIEWED BY
Easwaran Ramamurthy,
Carnegie Mellon University, United States
Pravin Hivare,
The University of Utah, United States
Moorthi Ponnusamy,
University of South Florida, United States

*CORRESPONDENCE
Grzegorz A. Czapski
gczapski@imdik.pan.pl

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Inhibition of BET proteins modulates amyloid-beta accumulation and cognitive performance in middle-aged mice prenatally exposed to maternal immune activation

Marta Matuszewska[1], Anna Wilkaniec[1], Magdalena Gąssowska-Dobrowolska[1], Magdalena Cieślik[1], Gabriela Olech-Kochańczyk[1], Ewelina Pałasz[1], Elżbieta Gawinek[1], Marcin Strawski[2] and Grzegorz A. Czapski[1]*

[1]Department of Cellular Signalling, Mossakowski Medical Research Institute Polish Academy of Sciences, Warsaw, Poland, [2]Faculty of Chemistry, University of Warsaw, Warsaw, Poland

Introduction: Given the complex etiological basis of Alzheimer's disease (AD), it is reasonable to hypothesize that neuronal dysfunction and death result from the interplay of numerous factors, both genetic and environmental. Accumulating evidence implicates the immune system and inflammation as key components of the pathomechanism of AD. In the present study, we analyzed the effect of maternal immune activation (MIA) on AD-related pathological changes in middle-aged 12-month-old offspring mice. Additionally, we investigated whether the inhibition of bromodomain and extraterminal domain (BET) proteins, which are readers of the histone acetylation code, could influence these changes.

Methods: In our study, we administered a viral mimetic, polyinosinic-polycytidylic acid (PIC), on gestation day 17 to induce MIA in wild-type C57BL/6J mice. The BET protein inhibitor, OTX-015 (Birabresib), was administered orally to 12-month-old male offspring for 14 days. Subsequently, behavioral, genetic, and immunochemical analyses were conducted.

Results: Our results demonstrated several MIA-evoked molecular alterations in the brains of middle-aged offspring. We observed an increase in *App* gene expression (qPCR) and amyloid-β (Aβ) levels (ELISA), while the levels and phosphorylation of Tau protein remained unchanged (WB). The mRNA levels of selected microglial markers were also elevated in the MIA group. Treatment with OTX-015 improved memory, as observed in the novel object recognition test, and reduced Aβ levels, but did not alter the expression of inflammatory genes or amyloidogenesis-related genes.

Discussion: Our findings suggest that inhibition of BET proteins may effectively attenuate neuropathological alterations in the aged brain.

KEYWORDS
prenatal exposure delayed effects, inflammation, beta amyloid, bromodomain containing proteins, hippocampus

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1 Introduction

Alzheimer's disease (AD) is a heterogeneous disorder characterized by a multifaceted pathomechanism and an extended prodromal phase. The complex interplay between genetic, epigenetic, and environmental factors, encompassing both risk and protective elements, contributes to variability in disease dynamics, clinical manifestations, amyloid-β (Aβ) conformation, and Tau protein distribution (Ferreira et al., 2020; Ferrari and Sorbi, 2021). Consequently, a singular and simplistic explanation of the pathomechanism of AD is unlikely to exist. Multiple hypotheses have been advanced to explain the underlying mechanisms of AD, including dysfunction in cholinergic and glutamatergic neurotransmission, the role of infections and inflammatory processes, amyloid and/or Tau propagation and accumulation, lymphatic system involvement, neurovascular changes, calcium dyshomeostasis, metal ion imbalance, and the mitochondrial dysfunction (Liu et al., 2019; Yokoyama et al., 2022).

Among these, the role of inflammatory processes has gained particular attention due to mounting evidence from epidemiological, genetic, and experimental studies. The immune system-related release of mediators of inflammation with potentially neurotoxic properties was proposed as a detrimental factor over two decades ago (McGeer and McGeer, 1998). Activation of glial cells around plaques and tangles occurs linearly with progression of the AD (Serrano-Pozo et al., 2011). Furthermore, genome-wide association studies (GWAS) have identified many genetic risk factors associated with AD, the majority of which are linked to immune system functionality (Bertram and Tanzi, 2019). Also, epidemiological studies confirmed that prolonged treatment using non-steroidal anti-inflammatory drugs (NSAIDs) reduced the risk of developing AD (t'Veld et al., 2001; Côté et al., 2012). However, the clinical trials involving NSAIDs demonstrated the complexity of the pathomechanism of AD, because the effects of NSAIDs changed during the disease, being protective or noxious (Breitner et al., 2011; Leoutsakos et al., 2012). Finally, population-based studies demonstrated that regular viral or bacterial infections may accelerate the progression of the disease and enhance the risk of developing AD (Bu et al., 2015; Douros et al., 2021; Sun J. et al., 2022). For example, an association was found between periodontal bacteria and faster cognitive decline in AD (Borsa et al., 2021).

This focus on immune dysregulation aligns with emerging discussions about shared mechanisms between neurodevelopmental disorders (NDDs) and neurodegenerative diseases like AD (Boots et al., 2023; Wiegersma et al., 2023; Siguier et al., 2024). It is suggested that factors contributing to the pathomechanisms of NDDs may elevate the risk of developing dementia through mechanisms related to a lessened cognitive reserve, genetic factors, or physiopathological overlaps (Siguier et al., 2024). Maternal infections affect the functional architecture of the brain and are associated with neurodevelopmental and neuropsychiatric problems, including autism spectrum disorders, schizophrenia, or ADHD in the progeny (Kim et al., 2024). Inflammatory signaling in the mother's body during pregnancy can disturb well-orchestrated and vulnerable processes of the central nervous system's development, including cells' proliferation, differentiation, migration, synaptic formation and pruning, and the establishment of neuronal circuits (Knuesel et al., 2014; Cieślik et al., 2020a; Cieślik et al., 2020b). Data suggest that the underlying cause of neurodevelopmental disturbances is nonspecific and not directly dependent on the pathogen itself (Woods et al., 2023). Instead, it is associated with the processes activated during the response of the maternal immune system to infection.

The identification of shared pathways in NDDs and AD prompted us to investigate whether maternal immune activation (MIA), a known risk factor for NDDs, could induce AD-like alterations in the brains of aging offspring. To solve this puzzle, we used a viral mimetic, polyinosinic-polycytidylic acid (PIC), to induce MIA in pregnant wild-type mice. Then, we analyzed 12-month-old male offspring. Since MIA is believed to evoke long-lasting epigenetic alterations in offspring (Woods et al., 2021; Kleeman et al., 2022), we investigated the potential involvement of bromodomain and extraterminal domain (BET) proteins. BET proteins, the readers of chromatin acetylation code, collaborate with transcription factors in governing the expression of genes (Liu et al., 2021). Data directly linking long-lasting MIA-induced epigenetic changes specifically to BET protein-regulated genes are limited. However, alterations in the global histone acetylation profile and changes in histone deacetylase levels in the hippocampus have been observed in 8–12-week-old offspring mice prenatally exposed to PIC-induced MIA (Reisinger et al., 2016). Additionally, PIC-induced MIA in rats has been shown to evoke changes in the global histone acetylation profile in the prefrontal cortex of 60-day-old offspring, including increased H3 and H4 histone acetylation at the promoter region of *Rela*, a gene regulated by BET proteins (Wang et al., 2018; Su et al., 2022). Therefore, in our study, OTX-015, an inhibitor of BET proteins, was administered orally (voluntary intake) to 12-month-old offspring males for 14 days. Our results demonstrated that inhibiting BET proteins attenuated MIA- induced elevation of brain Aβ level and improved memory.

2 Materials and methods

2.1 Materials

High molecular weight polyinosinic-polycytidylic acid (HMW PIC) was from InvivoGen (San Diego, CA, United States). (6S)-4-(4-chlorophenyl)-N-(4-hydroxyphenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-6-acetamide (OTX-015) was from Biorbyt Ltd. (Cambridge, UK). BCA Protein Assay Kit, TRI-reagent, reagents for reverse transcription (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor), reagents for quantitative PCR (Taqman Assays and TaqMan Fast Advanced Master Mix), ELISA kits for mouse $A\beta_{1-40}$ and $A\beta_{1-42}$ were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, United States). Kits for quantitative detection of mouse bromodomain-containing proteins 2, 3, and 4 (BRD2, BRD3, and BRD4) using the enzyme-linked immunosorbent assay ELISA Kit were obtained from Abbexa Ltd. (Cambridge, UK). Clarity Western ECL Substrate was purchased from Bio-Rad Laboratories (Hercules, CA, United States). Peanut butter (smooth, 100% peanuts) was from Sante sp. z o.o. (Warsaw, Poland). Protease inhibitors cocktail Complete was purchased from Roche Diagnostics (Mannheim, Germany). DNase I, dithiothreitol (DTT), anhydrous dimethyl sulfoxide (DMSO), and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, United States). Primary antibodies: rabbit anti-GAPDH Ab, mouse anti-Tau Ab, and rabbit anti-pTau(Ser199/202) Ab were from Sigma-Aldrich, mouse anti-pTau(Ser396) Ab, rabbit anti-pTau(Ser404) Ab,

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rabbit anti-pTau(Ser416) Ab, and rabbit anti-Iba1 Ab (for WB) were from Cell Signalling Technology (Danvers, MA, United States), goat anti-GFAP Ab (for WB), goat anti-Iba1 Ab (for IHC), and rabbit anti-GFAP Ab (for IHC) were from Abcam (Cambridge, UK). Secondary HRP-conjugated antibodies: anti-mouse IgG (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), anti-rabbit IgG (Sigma-Aldrich), and anti-goat IgG (Santa Cruz Biotechnology, Dallas, TX, United States). Fluorochrome-conjugated secondary antibodies: Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 donkey anti-goat IgG were from Thermo Fisher Scientific, Inc. Vectashild Vibrance Antifade Mounting Medium with DAPI was from Vector Laboratories, Inc. (Newark, CA, United States).

2.2 Animals

The experiments were carried out on C57BL/6J mice, supplied by the Animal House of Mossakowski Medical Research Institute, Polish Academy of Sciences (Warsaw, Poland), which operates breeding of small rodents with the specific-pathogen-free (SPF) standard. The animals were maintained under controlled temperature (22°C ± 10%) and humidity (55% ± 10%) conditions on a 12 h light/dark cycle. All experiments carried out on animals were approved by the Local Ethics Committee for Animal Experiments in Warsaw (reference number WAW2/052/2021) and carried out following the ARRIVE guidelines and the EU Directive 2010/63/EU regarding animal experiments. Every effort was made to minimize the number of animals used and to reduce the risk of animals' pain and distress. For the whole experiment, we used 16 pregnant females, resulting in 48 male offspring. The use of male offspring mitigated the confounding effects of the estrous cycle in females on the experimental data.

2.3 Experimental design—maternal immune activation

The mice gestations were realized by housing an adult male and a female overnight. The following day, female mice were separated, and the pregnant ones were identified and randomly assigned to the experimental group. At gestation day 17 (GD17), MIA was evoked in 8 pregnant females by intraperitoneal (i.p.) administration of HMW PIC (20 mg/kg b.w.) (Krstic et al., 2012; Mueller et al., 2019). Eight pregnant females from the non-MIA group received i.p. administration of an analogous volume of vehicle (sterile 0.9% NaCl). All dams were allowed to give birth and nurture offspring under normal conditions. Dams from MIA group gave birth to 27 male offspring, and dams from non-MIA group gave birth to 21 male offspring. On postnatal day (PND) 22 to 23, male pups were separated and housed in groups of 3 or 4 in open polycarbonate cages in an enriched environment. For the current project, all 21 offspring males from the MIA group, and randomly selected 21 males from non-MIA group were utilized. The remaining offspring were allocated to other projects.

2.3.1 Characterization of PIC—atomic force microscopy

Multimode 8 Nanoscope atomic force microscope (AFM, Bruker, Unites States) was used to image the surfaces on V1 grade mica substrate (NanoAndMore GmbH, Germany). Silicon cantilevers, HQ: NSC19/No Al type with a spring constant of ca. 0.5 Nm^{-1} (Mikromasch, Bulgaria) were applied for imaging in PeakForce Tapping™ microscopy mode. The image presented in this work is height type images. The examination of surfaces for artefacts by AFM, and the reproducibility, was performed in the common way, i.e., by changing the AFM cantilever and moving the sample in the X or Y direction, or by varying the scanning angle and scan rate. Polyinosinic-polycytidylic acid samples were prepared by applying a drop of 10 μL medium, which was previously diluted in 10 mM $MgCl_2$ solution in a 1:100 v/v ratio, on a freshly cleaved mica. Divalent magnesium is commonly used as a linking agent between negatively charged mica surfaces and DNA/RNA chains (Lyubchenko, 2011). After incubation for 10 min, the sample was rinsed with deionised water (GENIE U 12 TOC + TR, RephiLe Bioscience Ltd.) and dried under a gentle stream of argon.

2.3.2 Characterization of PIC—agarose gel electrophoresis

Electrophoresis was performed on a 1% agarose gel containing ethidium bromide to assess the quality and size of HMW-PIC. Low molecular weight (LMW) PIC was also loaded on the gel for comparison. A 100–1,000 bp DNA ladder was used as a size marker. After electrophoresis, the gel was visualized under UV light to detect the nucleic acids.

2.3.3 Drug administration

To minimize stress, pain, and morbidity, oral self-administration of the drug by voluntary intake by 12-month-old animals was performed, using 100% natural smooth peanut butter (PB) as a vehicle (Hocking et al., 2018; Warren et al., 2021). The drug/peanut butter was given individually to each mouse on a polystyrene Petri dish (diameter 30 mm) in a separate cage. Drug self-administration of each mouse was performed in the same treatment cage throughout the experiment, and each mouse was in its own separate cage. The treatment cages were of the same type as home cages. To familiarize mice with the taste of PB, all mice were trained by giving 50 μL of PB per mouse five times during one week before the experiment. The OTX-015 (or vehicle in respective groups) was given at the end of the lights-off phase of the light–dark cycle. After consuming the drug/PB, every mouse was transferred directly to the home cage. Typically, 15–30 min was enough to consume the whole mixture. Self-administration of OTX-015/PB was performed daily at a dose of 100 mg/kg b.w. for 14 days (Figure 1A). Animals were randomly allocated to experimental groups: 10 in control group, 11 in MIA group, 10 in MIA + OTX-015 group, and 11 in OTX-015 group.

2.4 Behavioral analysis

Behavioral analysis was performed on all 42 12-month-old animals. After 7 days of self-administration of the drug, behavioral analysis was started as described previously (Czapski et al., 2021). The open field (OF) test may measure general locomotor activity, novel environment exploration, and anxiety-related behavior during 5 min. Animals were individually placed in the corner of the open field chamber (grey box, 55 cm × 55 cm × 30 cm), and the total distance and exploration of the central zone and the border zone were analyzed. The number of episodes of grooming, rearing, climbing, and

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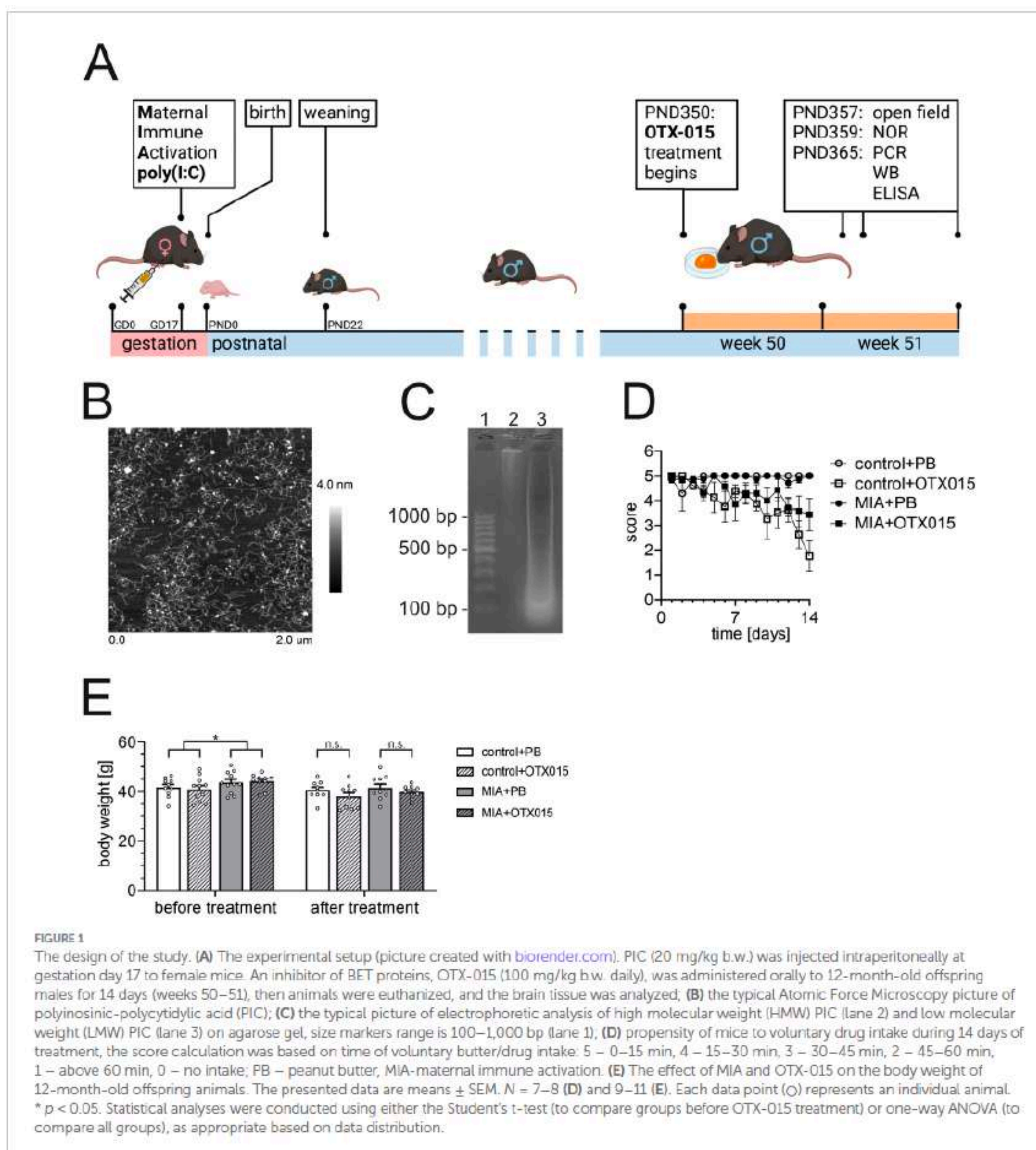

FIGURE 1
The design of the study. **(A)** The experimental setup (picture created with biorender.com). PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were euthanized, and the brain tissue was analyzed; **(B)** the typical Atomic Force Microscopy picture of polyinosinic-polycytidylic acid (PIC); **(C)** the typical picture of electrophoretic analysis of high molecular weight (HMW) PIC (lane 2) and low molecular weight (LMW) PIC (lane 3) on agarose gel, size markers range is 100–1,000 bp (lane 1); **(D)** propensity of mice to voluntary drug intake during 14 days of treatment, the score calculation was based on time of voluntary butter/drug intake: 5 – 0–15 min, 4 – 15–30 min, 3 – 30–45 min, 2 – 45–60 min, 1 – above 60 min, 0 – no intake; PB – peanut butter, MIA-maternal immune activation. **(E)** The effect of MIA and OTX-015 on the body weight of 12-month-old offspring animals. The presented data are means ± SEM. N = 7–8 **(D)** and 9–11 **(E)**. Each data point (○) represents an individual animal. * $p < 0.05$. Statistical analyses were conducted using either the Student's t-test (to compare groups before OTX-015 treatment) or one-way ANOVA (to compare all groups), as appropriate based on data distribution.

defecation was counted by the blinded operator. After two days of rest, a novel object recognition (NOR) test was performed. This test exploits the natural tendency of rodents to explore novel objects to test non-spatial memory. One day before testing, mice were submitted to a habituation session; they were allowed to freely explore the test chamber (dark grey box, 30 cm × 20 cm × 30 cm) for 5 min. The experimental session consisted of two trials. In the first trial (T1), two identical objects (O1) were placed in the chamber. During the second trial (T2), one object, O1, was replaced with the alternative object, O2. For testing the objects, we used a set of plastic bricks and a cell culture bottle. The objects presented during sessions were free of olfactory traits, and their positions in the chamber were randomized to eliminate the spatial bias in the task. At the beginning of each trial, mice were placed at the center of the box, with their heads oriented in the opposite direction to the object. The duration of T1 and T2 was 5 min. T2 started 120 min after T1. The basic measurement was the total time spent by mice exploring objects during T1 and T2 trials. Exploration of an object was defined as follows: directing the nose at a distance of 2 cm to the object and/or touching it with the nose. Climbing time was excluded from the analysis. The index of discrimination (ID) was calculated for each animal in the T2 trial and expressed as a ratio: time spent exploring the novel object/added time

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of exploring novel and known objects. All behavioral tests were performed in the morning, from 8 a.m. to noon.

2.5 Sample collection

After 14 days of voluntary drug administration, animals were deeply anesthetized (with ketamine/xylazine for immunocytochemistry or isoflurane for other analyses) and sacrificed. Brain samples for biochemical analysis (30 animals) were collected, cooled in ice-cold PBS, and the whole hippocampus was immediately dissected with the chilled scalpel on the cold dissection tray. Dissected tissue was immediately snap frozen, left and right side, separately. From each animal, the left and right sides were used for RNA and protein extraction in a random manner. For IHC staining (12 animals), mice were perfused with 4% paraformaldehyde before decapitation and brain removal. All samples were frozen and stored at −80°C upon analysis.

2.6 Western immunoblotting

The immunoreactivity of proteins was analyzed as described previously (Gąssowska-Dobrowolska et al., 2021). Tissue samples were homogenized in RIPA buffer, and protein concentration was determined using the BCA method, with bovine serum albumin as a standard. Samples were mixed with Laemmli buffer and heated at 95°C for 5 min. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane in standard conditions and then used for immunochemical analysis, followed by chemiluminescent detection using Clarity Western ECL Substrate. Densitometric analysis was performed using TotalLab4 software (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level for data normalization.

2.7 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits were used strictly according to the manufacturer's protocols. Prepared samples were used fresh to prevent protein degradation and denaturation. Each sample was analyzed in duplicate.

Shortly, for analysis of the level of $A\beta_{1-40}$ and $A\beta_{1-42}$, brain tissue was homogenized in a cold buffer (5 M guanidine-HCl, 50 mM Tris, pH 8.0). Then, samples were left on a laboratory rocker at room temperature for 4 h. The sample was then diluted tenfold with cold PBS with a protease inhibitor cocktail and centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was transferred to clean microcentrifuge tubes for further analysis according to the manufacturer's protocol.

For the analysis of the level of BET proteins, brain tissue was homogenized in ice-cold PBS (pH = 7.2) using a syringe, and then, due to the tendency of chromatin-associated BET proteins to form insoluble complexes, a sonication was performed to increase their solubility (Lambert et al., 2009). This was performed on the samples for 30 s (40% pulse, 40% power) using a Model 150 V/T Ultrasonic Homogenizer from Biologics Inc. (Manassas, VA, United States). Then, the homogenates were centrifuged at 10,000 × g for 5 min, and the supernatant was collected. After all incubation steps were completed, optical density was measured at 450 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.). The concentration of the tested compound was calculated using a standard curve and normalized to the total protein level in sample. Protein concentration was quantified using the BCA assay with bovine serum albumin (BSA) as the standard.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated by using a TRI-reagent according to the manufacturer's protocol. The concentration and quality of RNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Digestion of potential DNA contamination was performed by using DNase I, according to the manufacturer's protocol (Sigma-Aldrich). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Quantitative PCR was performed on an ABI 7500 Real-Time PCR System using TaqMan Fast Advanced Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). To increase the validity and reproducibility of qPCR analysis, the ΔΔCt calculation was extended by replacing the Ct of a single reference gene with an averaged Ct-value from three reference genes (*Gusb, Hprt, Rn18s*) (Riedel et al., 2014). The level of mRNA for selected genes was analyzed using commercially available TaqMan Gene Expression Assays: *Abca1* (Mm00442646_m1), *Adam10* (Mm00545742_m1), *Aph1b* (Mm00781167_m1), *App* (Mm01344172_m1), *Arg1* (Mm00475988_m1), *Bace1* (Mm00478664_m1), *Brd2* (Mm01271171_g1), *Brd3* (Mm01326697_m1), *Brd4* (Mm01350417_m1), *Gusb* (Mm01197698_m1), *Hprt* (Mm00446968_m1), *Il1b* (Mm00434228_m1), *Il6* (Mm00446190_m1), *Mme* (Mm01285049_m1), *Ncstn* (Mm00452010_m1), *Nos2* (Mm00440502_m1), *Psen1* (Mm00501184_m1), *Psen2* (Mm00448405_m1), *Rn18s* (Mm03928990_m1), *Tnf* (Mm00443258_m1).

2.9 Immunofluorescence staining

Mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg b.w. and 10 mg/kg b.w., respectively) and perfused through the ascending aorta initially with 0.9% NaCl in 0.1 M PBS, pH 7.4, and then with 4% paraformaldehyde. Brains were removed and post-fixed for 3 h at 4°C in the same fixative solution. Following post-fixation, brains were cryoprotected overnight in 20% sucrose solution in 0.1 M PBS, frozen on dry ice, and stored at −80°C. Coronal sections (40 μm thickness) were washed 3 times with 0.1 M PBS + 0.3% Triton X-100 for 5 min and incubated in a blocking solution (5% normal donkey serum (NDS) in 0.1 M PBS + 0.3% Triton X-100) for 1 h at room temperature (RT). The incubation with primary antibodies was performed in 1% BSA, 0.3% Triton X-100, and 0.1 M PBS for 1 h at RT and overnight at 4°C. The next day, the sections were washed with 0.1 M PBS (3 × 5 min), incubated in the dark with fluorescently labeled secondary antibodies in 1% BSA, 0.3% Triton X-100, and 0.1 M PBS for 1 h at RT, and washed with 0.1 M PBS (3 × 5 min). The sections were then mounted

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onto glass slides, air dried, and coverslipped with Antifade Mounting Medium with DAPI. Negative controls were performed using the same procedure, omitting the primary antibodies. Immunofluorescence studies were conducted in the Laboratory of Advanced Microscopy Techniques MMRI PAS using a confocal laser-scanning microscope, Zeiss LSM 780/ELYRA PS.1. (Carl Zeiss Meditec AG, Jena, Germany) platform equipped with the ZEN 2012 software, lasers (488 or 561 nm), and 405 nm diode lamp. Images were captured using a Zeiss PLN-Apo 40× /0.95 DIC III objective and further magnified by a 2 × digital zoom, resulting in an effective magnification of 80×. Z-stack acquisitions were performed from set first to set last planes, typically consisting of approximately 20 slices. Each optical slice had a thickness of 1.2 µm and was acquired at 1 µm intervals. For Sholl analysis, Z-stack images were converted from XYZ to XY dimensions using maximum intensity projection (MIP). Images were optimized for color, brightness, and contrast for best clarity. Multiple-channel images were overlaid using ZEN light software.

The morphometric analysis of microglia in the CA1 field of the hippocampus was performed according to the method described previously (Babiec et al., 2023). For every tested animal, up to nine microglial cells were randomly selected from the pyramidal cell layer, up to nine cells from the stratum radiatum, and up to nine cells from the lacunosum-moleculare, and their values were averaged to give three independent values. The extensions of individual cells were traced using the NeuronJ plugin for ImageJ Fiji software (Schindelin et al., 2012). The surface area of the cell body (soma), the number of primary and secondary extensions, and the total length of processes belonging to each cell were analyzed. The branches of individual cells were determined using the Sholl method (Sholl, 1953). Each cell was analyzed by selecting the center of its soma. Then, the number of intersections at circles of increasing diameter from the center was counted using the SNT plugin for ImageJ Fiji (Arshadi et al., 2021).

The semiquantitative analysis of microglia and astroglia density within the CA1, CA2/3 and DG region of the hippocampus was performed by manually counting Iba1- or GFAP-positive cells in six randomly selected fields (200 × 200 µm) per region per animal. Fields were selected with three from the left and three from the right hemisphere. The procedure was performed by a blinded operator and followed the method described previously (Babiec et al., 2023).

2.10 Statistical analysis

The statistical analysis of data was performed by using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA, United States). The distribution of data was analyzed using the Shapiro–Wilk test. The results were expressed as mean values ± SEM. Data were analyzed using either a Student's t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons correction for data with Gaussian distribution, or Mann–Whitney test or Kruskal-Wallis test with Dunn post hoc test for multiple comparisons correction for data with non-Gaussian distribution. p-values < 0.05 were considered significant. The N number refers to independent samples (biological replicates). To reduce the risk of litter effects, animals from at least 3 litters in each experimental group (random selection) were tested.

3 Results

In our study, we used a mouse model of MIA induced by intraperitoneal injection of PIC at gestation day 17 (GD17) (Figure 1). The murine MIA model based on PIC administration is well-characterized (Hameete et al., 2021). Therefore, to be consistent with the 3Rs principle (replacement, reduction, and refinement), we decided not to confirm activation of the immune system in pregnant mice. MIA did not affect gestation outcome in mice; the number of animals in the MIA group was unchanged, as compared to the non-MIA group (data not shown). Also, MIA did not influence the average time of voluntary intake of peanut butter (PB) or OTX-015. However, the average time of voluntary intake of OTX-015 was longer than that of PB (data not shown). For detailed analysis, we implemented a score-based approach, which demonstrated that the propensity of animals to voluntary intake of PB did not change during the 14-day-long treatment. Still, the propensity of animals to voluntary intake of OTX-015 decreased during the treatment (Figure 1D). We also observed that directly before the OTX-015 treatment, the body weight of animals in the MIA group was ca. 6% higher than that of the control group (Figure 1E). During the 14-day-long treatment with PB or OTX-015, the body weight did not increase (Figure 1E).

The effect of MIA on the level of BET proteins in the hippocampus of 12-month-old animals was analyzed using qPCR and ELISA methods. In our study, we focused on the hippocampus, a crucial brain region for memory and learning. The hippocampus is also one of the first brain regions to exhibit pathological alterations in AD (Ball et al., 1985; Jaroudi et al., 2017). Furthermore, it is a structure highly sensitive to early life stress and inflammatory insults (Dantzer et al., 2008; Czapski et al., 2010; Delpech et al., 2016). Our data showed that MIA significantly increased the mRNA level for the *Brd4* gene, but mRNA levels of *Brd2* and *Brd3* were not changed (Figure 2A). However, BET protein levels, measured by ELISA assays, did not show a significant change in the hippocampus of MIA-exposed animals (Figure 2B). Under control conditions, Brd2, Brd3, and Brd4 showed comparable abundance, measured at 1.04 ± 0.07, 1.65 ± 0.28, and 0.26 ± 0.02 pg/µg of protein, respectively.

To assess the impact of PIC-induced MIA on amyloid-related processes in middle-aged mice, we quantified $A\beta_{1-40}$ and $A\beta_{1-42}$ levels using commercial ELISA assays. As presented in Figure 3A, $A\beta_{1-40}$ showed a significant tendency to increase ($p = 0.054$) in the hippocampus. The levels of $A\beta_{1-42}$ were not increased (Figure 3B). However, the total level of Aβ, calculated as a sum of $A\beta_{1-40}$ and $A\beta_{1-42}$, was significantly increased in the MIA group (Figure 3C). Also, the mRNA level of the *App* gene was increased in the hippocampus in the MIA group (Figure 3D). Fourteen days of treatment with OTX-015 significantly reduced Aβ levels in both the MIA-exposed group and in animals not subjected to prenatal inflammatory stress. However, OTX-015 did not impact the *App* gene expression in the hippocampus.

Increased Tau phosphorylation in the brain is a typical feature of AD, but alterations of phospho-Tau levels in the hippocampus were also observed during LPS-evoked systemic inflammation in mice (Czapski et al., 2016) and in adolescent rats after LPS-evoked MIA (Cieślik et al., 2020a). Therefore, in the next step, we performed Western blot analysis of the level and phosphorylation of Tau protein in our experimental conditions. The results demonstrated that MIA did not affect the total levels of Tau protein or phosphorylation

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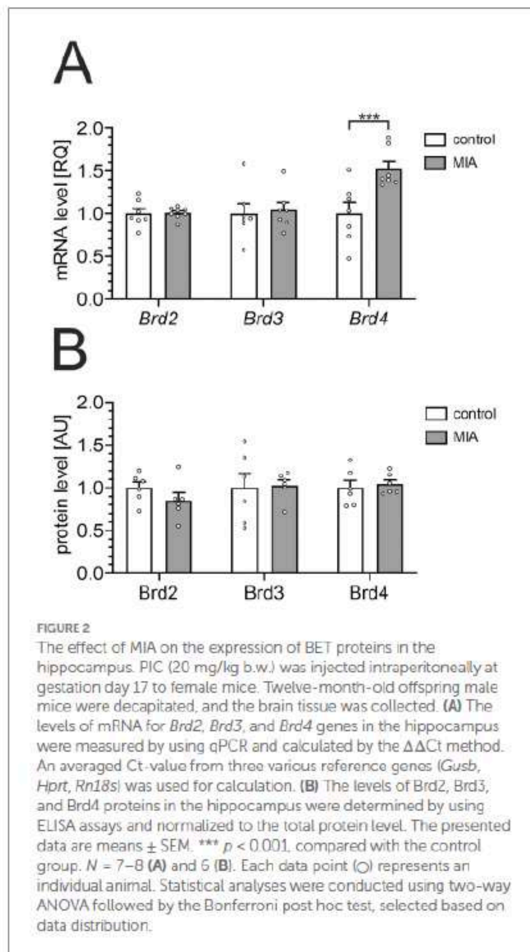

FIGURE 2
The effect of MIA on the expression of BET proteins in the hippocampus. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. Twelve-month-old offspring male mice were decapitated, and the brain tissue was collected. **(A)** The levels of mRNA for *Brd2*, *Brd3*, and *Brd4* genes in the hippocampus were measured by using qPCR and calculated by the ΔΔCt method. An averaged Ct-value from three various reference genes (*Gusb*, *Hprt*, *Rn18s*) was used for calculation. **(B)** The levels of Brd2, Brd3, and Brd4 proteins in the hippocampus were determined by using ELISA assays and normalized to the total protein level. The presented data are means ± SEM. *** $p < 0.001$, compared with the control group. $N = 7–8$ **(A)** and 6 **(B)**. Each data point (○) represents an individual animal. Statistical analyses were conducted using two-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

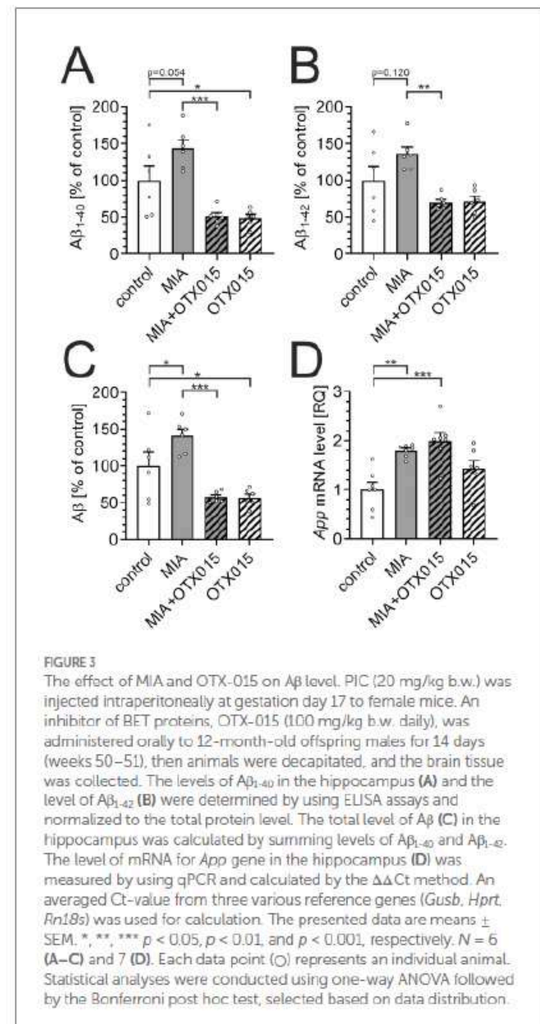

FIGURE 3
The effect of MIA and OTX-015 on Aβ level. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were decapitated, and the brain tissue was collected. The levels of $A\beta_{1-40}$ in the hippocampus **(A)** and the level of $A\beta_{1-42}$ **(B)** were determined by using ELISA assays and normalized to the total protein level. The total level of Aβ **(C)** in the hippocampus was calculated by summing levels of $A\beta_{1-40}$ and $A\beta_{1-42}$. The level of mRNA for *App* gene in the hippocampus **(D)** was measured by using qPCR and calculated by the ΔΔCt method. An averaged Ct-value from three various reference genes (*Gusb*, *Hprt*, *Rn18s*) was used for calculation. The presented data are means ± SEM. *, **, *** $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. $N = 6$ **(A–C)** and 7 **(D)**. Each data point (○) represents an individual animal. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

(Figure 4). Treatment with OTX-015 also did not significantly impact the Tau protein in the investigated groups.

Because BET proteins are epigenetic regulators of gene expression, we analyzed the possible effect of OTX-015 on the levels of mRNA for several proteins related to amyloid-β metabolism in the hippocampus. We tested selected genes for secretases (alpha-secretase *Adam10*; beta-secretase *Bace1*; gamma-secretase *Psen1*, *Psen2*, *Aph1b*, *Ncstn*), and for enzymes responsible for degradation (*Mme*) and clearing (*Abca1*) of Aβ. As presented in Table 1, MIA evoked an increase in the level of *Bace1*, *Aph1b*, and *Mme* in the hippocampi of 12-month-old progeny. OTX-015 did not prevent MIA-induced changes; on the contrary, it exacerbated the increase in *Bace1* expression in MIA animals. Additionally, OTX-015 upregulated *Bace1* expression in animals that were not exposed to prenatal stress.

An alternative mechanism that may contribute to alterations in Aβ levels in the brain is the activity of microglial cells. Therefore, we performed an immunofluorescence analysis of Iba1-positive microglial cells in the hippocampal regions: CA1, CA2/3, and DG. As demonstrated in Figures 5A,B, neither MIA nor OTX-015 had any evident impact on the morphology or density of microglia. Then, we assessed the level of microglial marker Iba1 in the hippocampus. As shown in Figures 5C,D, the immunoreactivity of Iba1 was not affected by MIA or OTX-015, confirming that the number of microglia was not changed in our experimental conditions. Also, other markers of microglial activation, like CD68 or CD206, were not changed in our experimental conditions (data not shown).

Given that a simple estimation of microglial morphology in the hippocampus may not be sensitive enough to detect subtle changes, in the next step, we conducted a detailed quantitative analysis in the next step to further investigate microglial functionality in the CA1 field. CA1 pyramidal neurons are known to be highly vulnerable to Tau accumulation, mitochondrial impairment, and early synaptic degeneration—features that are paralleled by local microglial activation in multiple models of neurodegeneration (Gylys et al., 2004; Sisková et al., 2010). This region is also more sensitive to NMDA-induced excitotoxicity than CA3 or DG, and this susceptibility is abolished in microglia-depleted preparations, indicating a microglia-dependent component of regional vulnerability (Vinet et al., 2012).

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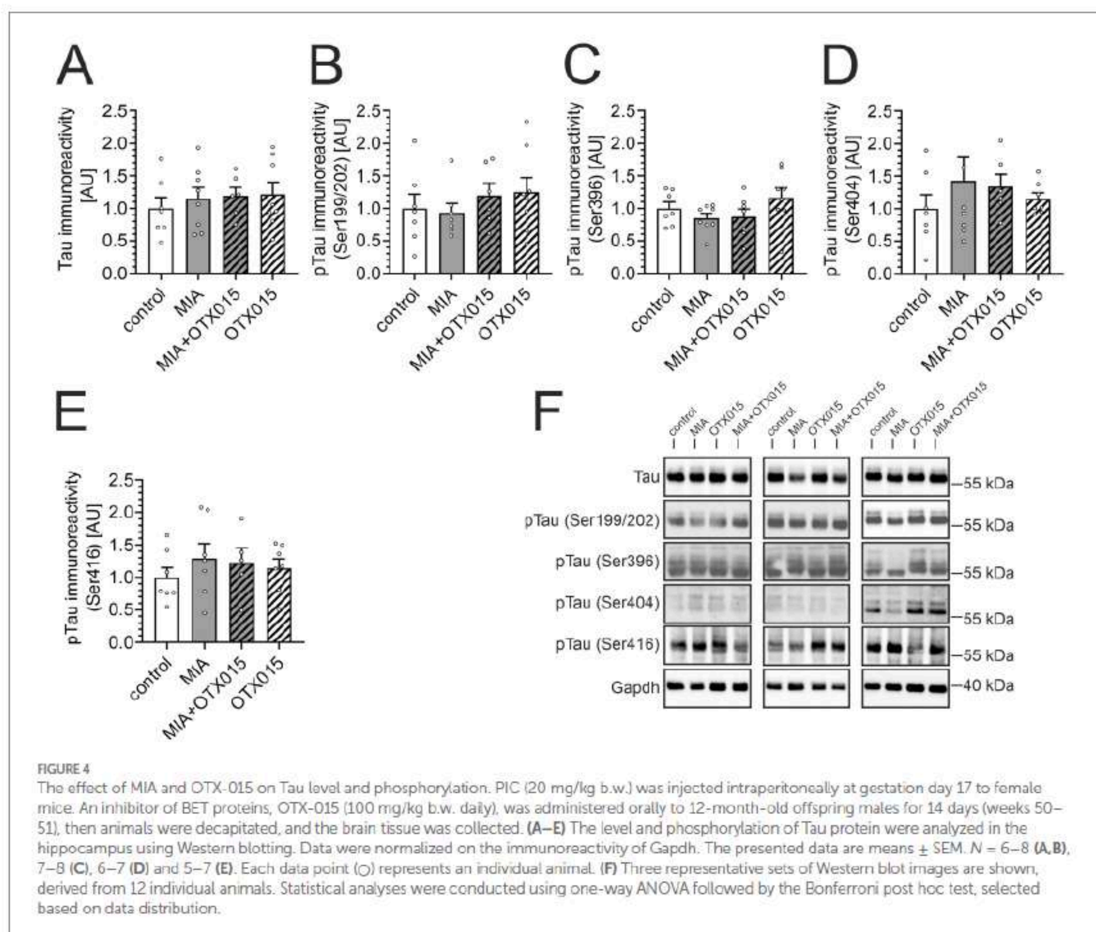

FIGURE 4
The effect of MIA and OTX-015 on Tau level and phosphorylation. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were decapitated, and the brain tissue was collected. **(A–E)** The level and phosphorylation of Tau protein were analyzed in the hippocampus using Western blotting. Data were normalized on the immunoreactivity of Gapdh. The presented data are means ± SEM. N = 6–8 **(A,B)**, 7–8 **(C)**, 6–7 **(D)** and 5–7 **(E)**. Each data point (○) represents an individual animal. **(F)** Three representative sets of Western blot images are shown, derived from 12 individual animals. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

Based on these region-specific properties, CA1 was selected as a primary site for detecting microglial responses to OTX-015 in our MIA model. The morphometric software-supported (Babiec et al., 2023) analysis of Iba1-positive cells showed no significant differences in the number of primary and secondary extensions (Figures 6C,D), the area of cell bodies (Figure 6E), or the total length of extensions (Figure 6F) in the MIA group compared to the control group. Similarly, there were no changes in the arborization of microglia (Figure 6G) in animals exposed to MIA. However, there was a slight tendency for decreased microglial arborization in MIA animals, which was not observed in MIA animals treated with OTX-015 (Figures 6D–G). Surprisingly, in the control animals, treatment with OTX-015 resulted in a decrease in microglial arborization (Figures 6C,D,F,G).

In the next step, we performed an immunofluorescence analysis of GFAP-positive astrocytes in the hippocampal regions: CA1, CA2/3, and DG. As demonstrated in Figures 7A,B, neither MIA nor OTX-015 had any evident impact on the morphology or density of astrocytes. We then assessed the level of astrocytic marker GFAP in the hippocampus. As shown in Figures 7C,D, the immunoreactivity of GFAP was not affected by MIA or OTX-015, confirming that the number of astrocytes remained unchanged under our experimental conditions.

Next, we utilized quantitative PCR, a highly sensitive technique for detecting subtle variations in mRNA levels of inflammation-related genes (Table 2). Our analysis indicated that MIA did not induce significant changes in the expression of either pro-inflammatory or anti-inflammatory genes. However, a trend toward increased expression of *Il6* and *Nos2* was observed. Notably, treatment with OTX-015 did not affect the expression of inflammation-related genes.

Finally, we measured the effect of MIA and OTX-015 on the behavior of middle-aged mice. We have investigated the exploratory activity and anxiety-related behaviors in the open-field test. As shown in Figure 8A, the total distance traveled during the test was not significantly affected in MIA-exposed or OTX-015-treated mice, indicating that neither mobility nor exploratory activity was altered under our experimental conditions. Rodents naturally avoid open areas, so changes in the frequency and duration of entries into the central zone of the open-field chamber are a measure of anxiety-related behavior. While MIA exposure did not affect central zone entries or the time spent in this area, non-MIA animals treated with

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TABLE 1 The effect of MIA and OTX-015 on the expression of amyloidogenesis-related genes in the hippocampus of 12-month-old male mice.

	Control [RQ]	MIA [RQ]	MIA+OTX015 [RQ]	OTX015 [RQ]
Adam10	1.00 ± 0.09 (7)	0.98 ± 0.12 (8)	1.14 ± 0.06 (7)	0.89 ± 0.16 (8)
Bace1	1.00 ± 0.13 (7)	1.45 ± 0.04 (7)**	1.89 ± 0.06 (6)##	1.63 ± 0.08 (6)***
Psen1	1.00 ± 0.20 (7)	0.94 ± 0.13 (8)	1.18 ± 0.11 (7)	1.04 ± 0.22 (8)
Psen2	1.00 ± 0.05 (7)	0.93 ± 0.06 (8)	1.25 ± 0.14 (7)	0.96 ± 0.07 (8)
Aph1b	1.00 ± 0.33 (7)	2.40 ± 0.25 (8)**	2.87 ± 0.17 (6)	1.27 ± 0.33 (8)
Ncstn	1.00 ± 0.22 (7)	1.61 ± 0.07 (8)	2.09 ± 0.31 (7)	1.81 ± 0.34 (8)
Mme	1.00 ± 0.34 (7)	2.71 ± 0.05 (7)***	2.49 ± 0.08 (6)	1.46 ± 0.38 (8)
Abca1	1.00 ± 0.07 (7)	1.05 ± 0.05 (8)	1.23 ± 0.04 (7)	1.19 ± 0.06 (8)

PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were decapitated, and the brain tissue was collected. The level of mRNA was measured by using qPCR and calculated by the ΔΔCt method. The presented values are mean ± SEM (*N*). **, *** $p < 0.01$, and $p < 0.001$, respectively, compared with the control group. ## $p < 0.01$, compared with the MIA group. RQ, relative quantification.

OTX-015 significantly increased time spent in the central zone (Figures 8B,C).

Additionally, behaviors such as grooming, rearing, climbing, and defecation are commonly interpreted as markers of anxiety-related responses (Figures 8D–K). In our study, MIA exposure, regardless of OTX-015 treatment, specifically reduced the frequency and duration of rearing episodes, suggesting the presence of anxiety-related behavior in MIA-exposed animals.

The novel object recognition (NOR) test is a widely utilized assay to evaluate animals' memory. Animals that recall the objects presented during the initial test session prefer the novel object in the subsequent session. Therefore, the index of discrimination (ID) above 0.5 indicates that animals remember the objects, and an ID of about 0.5 shows that animals do not remember the objects. Our previous studies using the same NOR assay protocol observed that ID for young (2–3 month-old) adult male mice is above 0.6. As presented in Figure 9A, the ID for the control group is 0.56, indicating that 12-month-old animals have difficulty distinguishing between novel and familiar objects 120 min after the presentation session. This reduced ID may explain why the difference between the control and MIA groups (ID = 0.492) did not achieve statistical significance. We assume that a shorter delay between two sessions could give more conclusive results in aged animals. However, OTX-015 presented a strong tendency ($p < 0.1$) to improve cognitive function (Figure 9A). Moreover, the data were also presented as relative exploration time to display the difference between animals' interest in familiar versus novel objects (Figure 9B). It is evident that animals treated with OTX-015, regardless of MIA exposure, spent significantly more time exploring the novel object than the familiar one ($p < 0.001$), indicating that they could effectively distinguish between them and retain the memory of the familiar object. In contrast, despite some tendency in the control group, the control and

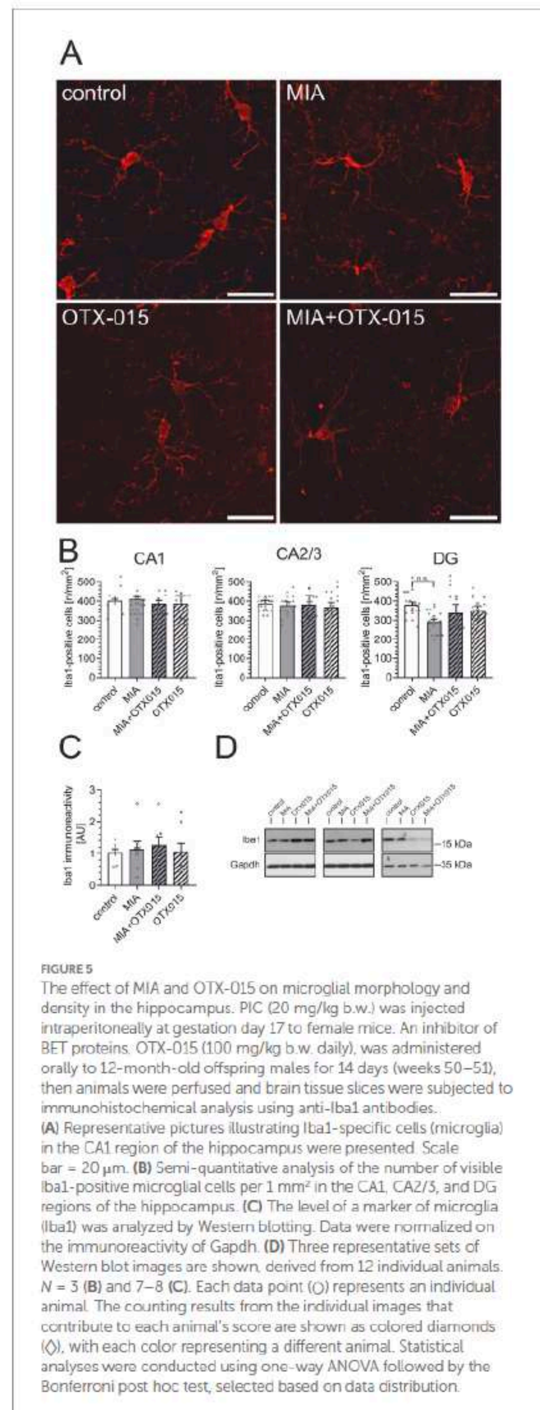

FIGURE 5
The effect of MIA and OTX-015 on microglial morphology and density in the hippocampus. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were perfused and brain tissue slices were subjected to immunohistochemical analysis using anti-Iba1 antibodies. **(A)** Representative pictures illustrating Iba1-specific cells (microglia) in the CA1 region of the hippocampus were presented. Scale bar = 20 μm. **(B)** Semi-quantitative analysis of the number of visible Iba1-positive microglial cells per 1 mm² in the CA1, CA2/3, and DG regions of the hippocampus. **(C)** The level of a marker of microglia (Iba1) was analyzed by Western blotting. Data were normalized on the immunoreactivity of Gapdh. **(D)** Three representative sets of Western blot images are shown, derived from 12 individual animals. *N* = 3 **(B)** and 7–8 **(C)**. Each data point (○) represents an individual animal. The counting results from the individual images that contribute to each animal's score are shown as colored diamonds (◊), with each color representing a different animal. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

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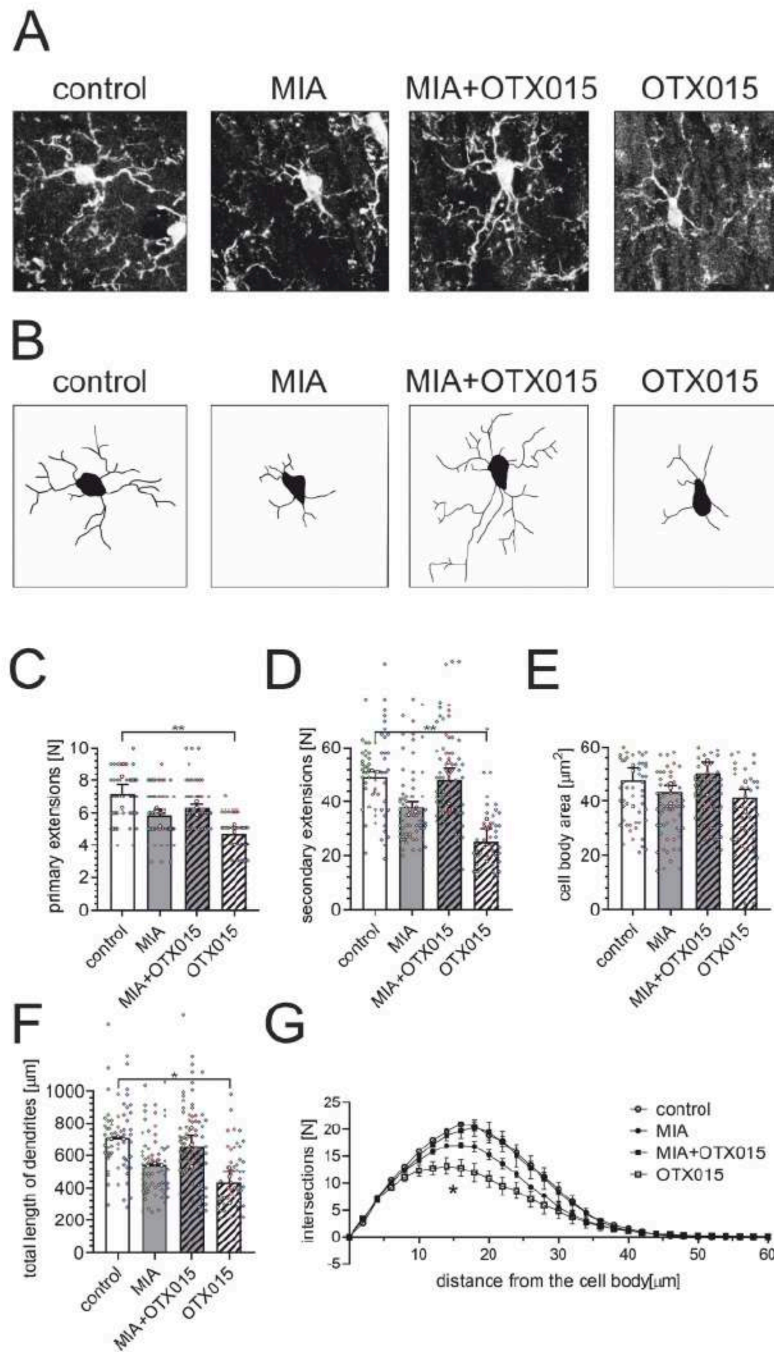

FIGURE 6
The effect of MIA and OTX-015 on glial cells in the hippocampus – a quantitative analysis. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were euthanized, and the brain tissue was analyzed. The morphometric analysis of Iba1-positive (microglial) cells in the hippocampus was performed using the NeuronJ plugin for ImageJ Fiji software. The crucial steps in the morphology analysis was converting image to an 8-bit format **(A)** and processes tracing using the NeuronJ plugin for Fiji **(B)**. Representative images were shown. The morphometric analysis included the number of primary extensions **(C)**, the number of secondary extensions **(D)**, the cell body area **(E)**, and the total

(Continued)

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FIGURE 6 (Continued)
length of dendrites (F). (G) Sholl analysis of the branching complexity of microglia in the hippocampus was performed with the SNT plugin for ImageJ Fiji. Data are presented as the mean value ± SEM. *, ** $p < 0.05$ and $p < 0.01$, respectively, compared with the control group. $N = 3$. Each data point (○) represents an individual animal. The results from the individual cells that contribute to each animal's score are shown as colored diamonds (◊), with each color representing a different animal. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

MIA groups spent similar amounts of time exploring novel and familiar objects. This suggests that these animals did not differentiate between the objects and thus failed to remember the familiar one. This result indicates the significant improvement in cognitive function in animals treated with OTX-015. Finally, we performed Pearson's correlation analysis between ID (memory function) and Aβ levels in individual animals. The results presented in Figures 9C,D show a strong negative correlation.

4 Discussion

Alzheimer's disease is characterized by the pathological accumulation of misfolded proteins in the brain, particularly Aβ and Tau. The aggregation of these proteins drives neurodegeneration, leading to neuronal loss and a consequent decline in the production of neurotransmitters essential for cognitive function. Moreover, the toxic forms of Aβ are known to activate glial cells, including microglia and astrocytes, which leads to chronic neuroinflammation. This inflammatory response contributes to synaptic loss and neuronal death, further exacerbating the cognitive decline associated with AD. Currently studied therapeutic strategies primarily target the mechanisms underlying the formation and clearance of Aβ and Tau, as well as the inflammatory response (Scheltens et al., 2021). The clinical efficacy of these treatments remains limited, underscoring the need for innovative therapeutic approaches (Passeri et al., 2022). The potential role of BET proteins in the progression of AD presents a promising avenue for exploration, although this area remains underinvestigated (Nikkar et al., 2022). In the current study, a mouse model of maternal immune activation (MIA) was utilized to investigate how prenatal immune challenges contribute to long-term alterations in Aβ levels, Tau phosphorylation, and neuroinflammation – crucial players in the pathomechanism of AD. This research model was selected based on the study by Krstic et al. (2012), which demonstrated that immune challenges during critical periods of prenatal brain development trigger neuroinflammation, promote amyloidogenesis and Tau-related pathology, resulting in learning and memory deficits in later stages of offspring life. These findings support the hypothesis that early-life immune events can increase susceptibility to neurodegenerative diseases in adulthood, warranting further research into early-life interventions. Furthermore, given emerging evidence that inhibiting BET proteins may alleviate cognitive deficits associated with AD and enhance neuroprotection, we examined the effects of BET protein modulation using OTX-015, a selective BET inhibitor (Eischer et al., 2023). The oral dose of OTX-015 was chosen based on prior literature and preclinical studies that demonstrated its efficacy and tolerability in animal models (Boi et al., 2015; Yu et al., 2022). OTX-015, similar to its analog JQ1, has been demonstrated to cross the blood–brain barrier, which makes it a valuable tool for pharmacologically targeting central nervous system pathologies (Matzuk et al., 2012; Berenguer-Daizé et al., 2016). However, it cannot be fully excluded that some peripheral effects could also contribute to its impact on the brain. The drug was administered orally to minimize animal distress, providing a more humane route of administration compared to intraperitoneal injection. After oral administration, OTX-015 penetrated the brain and demonstrated potent efficacy in a mouse glioblastoma model, offering increased survival and reduced tumor progression without significant toxicity (Berenguer-Daizé et al., 2016). This inhibitor has also been investigated in human trials for a different condition, suggesting that oral administration could be a viable therapeutic approach. These findings are consistent with previous studies, which indicate that the pharmacokinetics of the compound support oral delivery, potentially improving patient compliance and the feasibility of treatment (Dombret et al., 2014). To ensure efficient absorption and ease of administration, OTX-015 was dissolved in peanut butter, a lipophilic, palatable, and simple carrier, facilitating easy drug consumption by the mice. In compliance with ethical standards and to minimize animal suffering, we adhered to the 3Rs principle—Replacement, Reduction, and Refinement—throughout our experimental design. Specifically, the use of oral administration, a non-invasive method, reduced physical stress compared to more invasive procedures (MacArthur Clark, 2018; Azkona, 2023). Notably, we observed a significant increase in the mean duration of voluntary consumption of OTX-015 compared to peanut butter alone, which was consistent throughout the 14-day treatment period (Figure 1D). The reason for this decreasing propensity to voluntary drug intake is unknown. Information regarding the taste or smell of OTX-015 is unavailable. However, OTX-015 evoked distortion of the sense of taste in humans in a clinical phase 1 study (Amorim et al., 2016). Therefore, we cannot exclude the possibility that the attractiveness of peanut butter with OTX-015 could decrease over time. Also, this change in consumption behavior could suggest that BET inhibition may have unintended, off-target consequences that affect animals' overall well-being. Specifically, such behavioral alterations might signal underlying discomfort or an altered metabolic state, which could be indicative of toxicity or adverse physiological responses. These findings underscore the importance of further investigation into the broader physiological effects of BET inhibition, particularly in the context of prolonged exposure. Changes in voluntary consumption patterns may serve as an early indicator of potential adverse effects that extend beyond the primary therapeutic objectives, such as cognitive enhancement or amyloid reduction. Given the critical role of safety in drug development, such behavioral changes must be carefully assessed to ensure that BET inhibitors, while offering therapeutic benefits, do not pose significant risks to overall health. Further toxicological studies are essential to refine the safety profile of BET inhibitors and ensure their viability as therapeutic agents for neurodegenerative diseases, such as AD.

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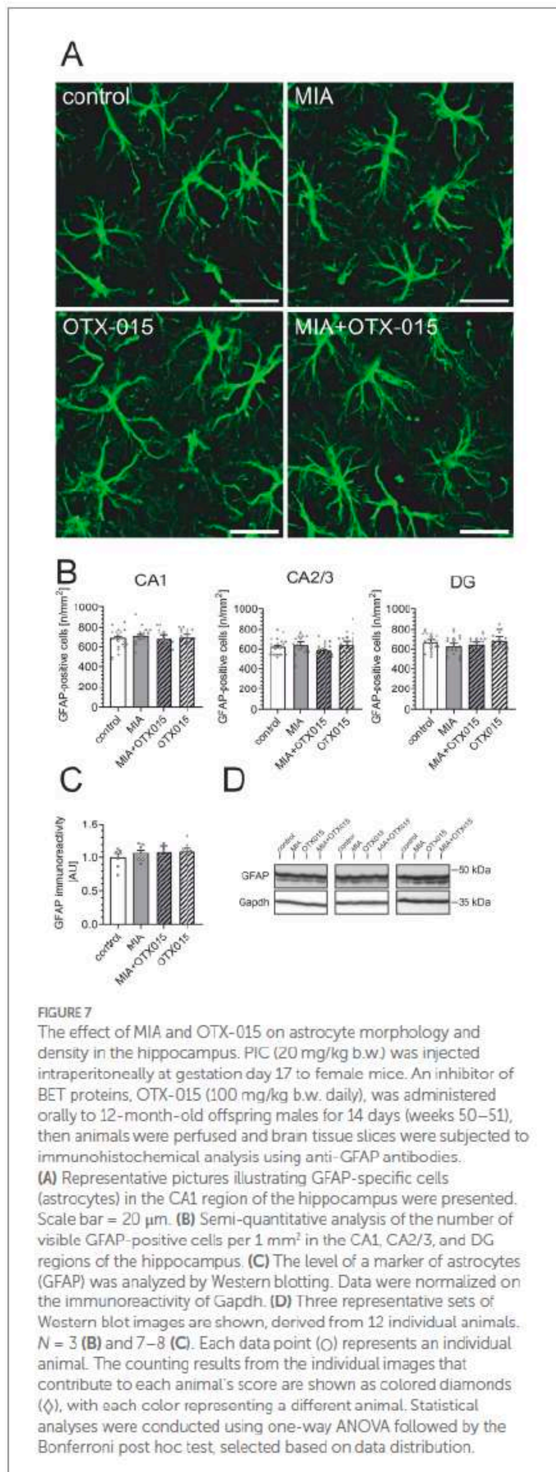

FIGURE 7
The effect of MIA and OTX-015 on astrocyte morphology and density in the hippocampus. PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were perfused and brain tissue slices were subjected to immunohistochemical analysis using anti-GFAP antibodies. **(A)** Representative pictures illustrating GFAP-specific cells (astrocytes) in the CA1 region of the hippocampus were presented. Scale bar = 20 μm. **(B)** Semi-quantitative analysis of the number of visible GFAP-positive cells per 1 mm² in the CA1, CA2/3, and DG regions of the hippocampus. **(C)** The level of a marker of astrocytes (GFAP) was analyzed by Western blotting. Data were normalized on the immunoreactivity of Gapdh. **(D)** Three representative sets of Western blot images are shown, derived from 12 individual animals. $N = 3$ **(B)** and 7–8 **(C)**. Each data point (○) represents an individual animal. The counting results from the individual images that contribute to each animal's score are shown as colored diamonds (◊), with each color representing a different animal. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

Our quantitative PCR analysis revealed a significant upregulation of *Brd4* mRNA in the hippocampus of the MIA group, while *Brd2* and *Brd3* mRNA levels remained unchanged (Figure 2). In contrast, ELISA assays failed to show a corresponding increase in Brd4 protein levels, suggesting a divergence between mRNA and protein expression. This discrepancy may reflect the inherent limitations of these methodologies, as differences in assay sensitivity and specificity are well-established. Such variations emphasize the complexity of post-transcriptional regulation and underscore the challenges in interpreting mRNA and protein expression data, particularly when utilizing distinct analytical approaches (Savino et al., 2009; Eischeid et al., 2021). Remarkably, the selective upregulation of Brd4 was also observed in our previous *in vitro* (Matuszewska et al., 2025) and *in vivo* experiments (data not shown, submitted for publication) using lipopolysaccharide to trigger an inflammatory response.

TABLE 2 The effect of MIA and OTX-015 on the expression of inflammation-related genes in the hippocampus of 12-month-old male mice.

	Control [RQ]	MIA [RQ]	MIA+OTX015 [RQ]	OTX015 [RQ]
Il1b	1.00 ± 0.16 (7)	0.83 ± 0.03 (6)	0.89 ± 0.07 (7)	0.79 ± 0.09 (8)
Il6	1.00 ± 0.19 (7)	1.53 ± 0.12 (8) &	1.71 ± 0.23 (7)	1.35 ± 0.22 (8)
Tnf	1.00 ± 0.27 (7)	1.13 ± 0.13 (8)	0.79 ± 0.15 (6)	0.78 ± 0.16 (8)
Nos2	1.00 ± 0.08 (6)	1.50 ± 0.17 (8) &	1.39 ± 0.28 (7)	0.92 ± 0.18 (8)
Argl	1.00 ± 0.08 (6)	0.98 ± 0.12 (8)	0.88 ± 0.07 (6)	0.89 ± 0.16 (8)

PIC (20 mg/kg b.w.) was injected intraperitoneally at gestation day 17 to female mice. An inhibitor of BET proteins, OTX-015 (100 mg/kg b.w. daily), was administered orally to 12-month-old offspring males for 14 days (weeks 50–51), then animals were decapitated, and the brain tissue was collected. The level of mRNA was measured by using qPCR and calculated by the ΔΔCt method. The presented values are mean ± SEM (*N*). & $p < 0.05$, compared with the control group using the Student t-test. RQ, relative quantification.

Our most interesting finding is the observation of increased Aβ levels in the hippocampi of animals prenatally exposed to inflammatory challenge (Figure 3). Moreover, BET inhibition significantly reduced Aβ levels in both control animals and those exposed to MIA. Importantly, this observation is further supported by the results of our experiments on adult mice, where we observed an attenuation of Aβ levels in the brain after using JQ1, an analog of OTX-015 (data not shown, submitted for publication). Neuroinflammation, such as that induced in the MIA model, is known to activate several transcription factors, including NF-κB, which plays a prominent role in the pathomechanism of AD (Sun E. et al., 2022a). The *App* gene is a target gene of NF-κB, therefore, neuroinflammation could upregulate Aβ levels simply by enhancing *App* expression. However, although BET protein inhibitors are known to attenuate the expression of NF-κB-controlled genes (Huang et al., 2017), in our experimental conditions, they did not impact the expression of *Il1b*, *Il6*, *Nos2*, *and Tnf*, which are NF-κB-controlled genes (Table 2). This suggests that other, NF-κB-independent, mechanisms play a primary role in the anti-amyloid effect of OTX-015. Significantly, the promoter of the gene encoding BACE1, the aspartyl protease which is directly responsible for Aβ generation, harbors a binding site for NF-κB (Rossner et al., 2006). In accordance with the above interpretation, OTX-015 did not prevent MIA-evoked increase in *Bace1* expression.

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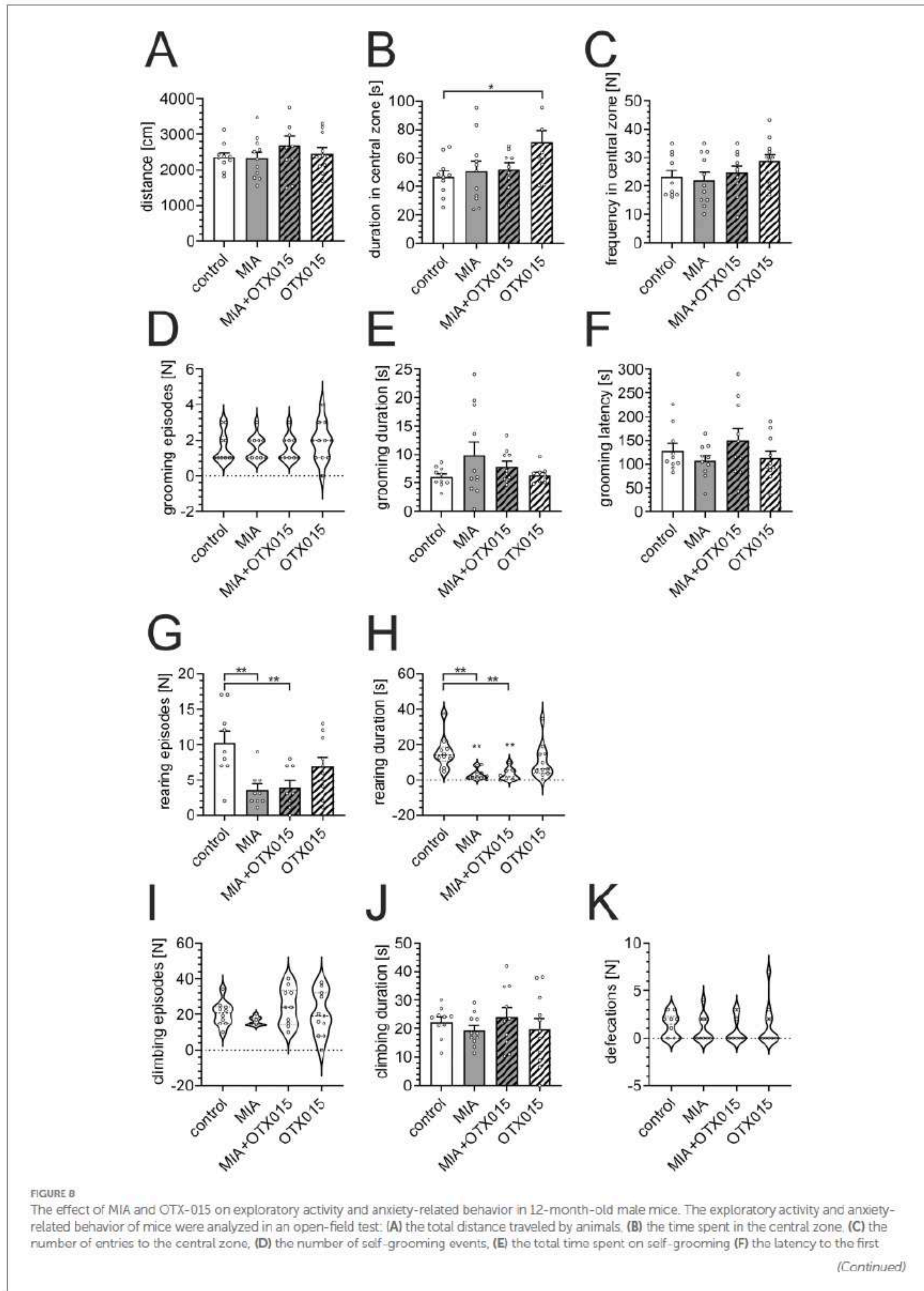

FIGURE 8
The effect of MIA and OTX-015 on exploratory activity and anxiety-related behavior in 12-month-old male mice. The exploratory activity and anxiety-related behavior of mice were analyzed in an open-field test: **(A)** the total distance traveled by animals, **(B)** the time spent in the central zone, **(C)** the number of entries to the central zone, **(D)** the number of self-grooming events, **(E)** the total time spent on self-grooming **(F)** the latency to the first

(Continued)

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FIGURE 8 (Continued)
self-grooming event, **(G)** the number of rearing events, **(H)** the duration of rearing events, **(I)** the number of climbing events, **(J)** the duration of climbing events, and **(K)** the number of defecations. Data **(A–C,E–G,J)** represent the mean values ± SEMs. Data not normally distributed **(D,H,I,K)** are presented as violin plots with all data points. *, ** $p < 0.05$ and $p < 0.01$, respectively, compared with the control group. $N = 8–11$. Each data point (○) represents an individual animal. Statistical analyses were conducted using either one-way ANOVA followed by the Bonferroni post hoc test, or the Kruskal–Wallis test followed by Dunn's post hoc multiple comparisons test, as appropriate based on data distribution.

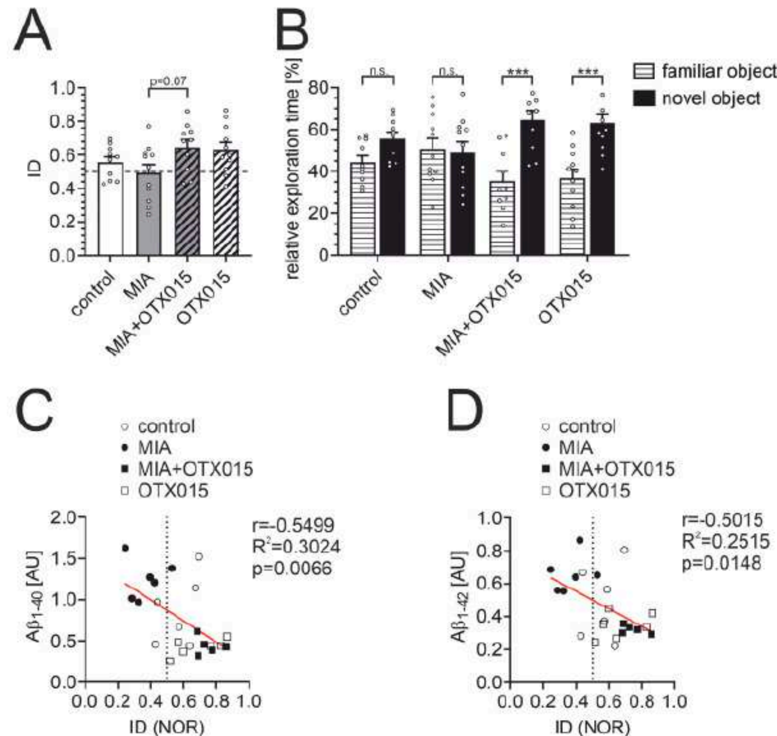

FIGURE 9
The effect of MIA and OTX-015 on memory function in 12-month-old male mice. The memory function of animals was analyzed in a novel object recognition (NOR) test. **(A)** The index of discrimination (ID) was calculated based on time spent exploring the novel object versus the familiar object, as described in the Methods section. **(B)** Relative exploration time of familiar vs. novel object in the NOR test. **(C)** The analysis of the correlation of the data from the NOR test with the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ **(D)** in corresponding animals was performed using Pearson's correlation test. Presented results are means ± SEM from $N = 10–11$ animals in each group. n.s. – not significant, ***$p < 0.001$. Each data point (○) represents an individual animal. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test, selected based on data distribution.

On the contrary, BET inhibition enhanced *Bace1* transcription, which is consistent with previous observations (Zhang et al., 2022). Further investigation into the intricate relationship between BET inhibition, neuroinflammation, and *App* regulation is needed to elucidate the specific molecular mechanisms underlying these observations. Such studies will help clarify the broader implications of BET inhibition in neurodegenerative conditions and its potential as a therapeutic strategy for diseases associated with Aβ accumulation. The possible mechanism of anti-amyloid action of OTX-015, which is worth analysis in future studies, is activating autophagy. This effect can be achieved through the modulation of signaling pathways by BET inhibitors, which indirectly promote autophagy activation (Jaeger et al., 2010; Cummings et al., 2024; Zeng et al., 2024). Prior studies have shown that BET inhibition can induce autophagy by modulating the AMPK-mTOR-ULK1 signaling cascade (Korb et al., 2015; Jang et al., 2017; Li et al., 2020). Given the role of autophagy dysregulation in AD, stimulating this cellular process has emerged as a promising therapeutic strategy for removing and clearing Aβ deposits (Friedman et al., 2015; Rahman et al., 2020). Experimental data support the efficacy of numerous autophagy activators in reducing Aβ levels (Ordóñez-Gutiérrez et al., 2018; Pierzynowska et al., 2019; Kong et al., 2020). In summary, the literature suggests that such a cascade is feasible and may present a novel therapeutic strategy for AD. However, further clinical studies are essential to confirm the efficacy and safety of this approach in humans.

In parallel, the changes in Aβ levels were correlated with cognitive functions (Figure 9). Increased levels of Aβ have been consistently correlated with cognitive dysfunction, particularly in

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neurodegenerative diseases like AD (Näslund et al., 2000; Baker et al., 2017). Studies have shown that Aβ accumulation can directly impair cognitive functions by disrupting synaptic plasticity and increasing neuroinflammation (Fang et al., 2010; Ripoli et al., 2014; Rolland et al., 2020). Furthermore, emerging evidence highlights the role of peripheral clearance mechanisms, such as lymphatic drainage, in regulating Aβ levels and, consequently, cognitive performance. The dysfunction of these pathways can exacerbate cognitive decline, suggesting that both central and peripheral processes contribute to memory deficits in AD (Cheng et al., 2020). Additionally, therapeutic interventions targeting Aβ, such as anti-Aβ monoclonal antibodies, have shown that decreases in brain Aβ levels relate to slower cognitive decline (van Dyck et al., 2023). Furthermore, the relationship between changes in Aβ levels and cognitive function is central to understanding disease progression in AD (Abanto et al., 2024). These findings align with a growing body of literature suggesting that BET protein inhibition enhances cognitive functions and memory in AD contexts. BET protein inhibitor, JQ1, improved brain plasticity in wild-type and APP-expressing mice and rescued hippocampal-dependent cognitive deficits in a murine model of frontotemporal dementia (Benito et al., 2017; Quezada et al., 2021). Also, chronic administration of JQ1 significantly improved cognition deficits in rat models of AD (Badrikoohi et al., 2022; Nikkar et al., 2022). However, in 7-month-old 3 x Tg mice, JQ1 did not ameliorate learning and memory deficits (Magistri et al., 2016). On the other hand, prolonged administration of JQ1 evoked memory deficits in mice (Korb et al., 2015). Interestingly, the administration of JQ1 to young rats induced cognitive impairment in adult rats (Bilecki et al., 2021). It is noteworthy that Apabetalone (RVX-208), a distinct BET inhibitor, positively impacted cognitive performance in a randomized controlled trial conducted on a population of patients aged 70 years and older (Cummings et al., 2021). This convergence of results across studies reinforces the hypothesis that BET modulation could serve as a viable therapeutic strategy for AD. However, it is worth noting that although Aβ accumulation is associated with an increased risk and severity of cognitive decline, it is not the sole determinant. Aβ deposits do not necessarily lead to cognitive dysfunction in all individuals, and conversely, cognitive impairment can occur in the absence of significant amyloid pathology (Armstrong, 2014; Morris et al., 2014; Kepp et al., 2023). Therefore, given the complexity and diverse clinical manifestations of AD in the population (Korczyn and Grinberg, 2024), reducing Aβ alone may not be sufficient to restore cognitive function in all patients.

Tau expression and pathological phosphorylation are of particular interest in the conditions of the prenatal immune challenge and AD. In a rat model, MIA evoked by LPS-triggered neuroinflammation and microglial activation in adolescent offspring led to disrupted normal Tau function, and reduced Tau phosphorylation at Ser199/202 and Ser396, while phosphorylation at Ser416 remained unaffected (Cieślik et al., 2020b). The Knuesel group studied Tau protein levels and phosphorylation across aging in C57BL/6 J mice after PIC-induced MIA (Krstic et al., 2012). Their observations were inconclusive, as they presented age-dependent changes in phosphorylation at threonine 205 (Thr205), which increased at 6 and 15 months but decreased at 12 months. Our results (Figure 4) demonstrated that Tau level and phosphorylation were not changed in middle-aged MIA offspring. Also, OTX-015 did not affect Tau phosphorylation.

In MIA models, prenatal immune stress results in microglial hypertrophy, as evidenced by increased cell body size and decreased arborization in neonatal and young offspring (Loayza et al., 2023). These morphological changes reflect a shift towards a more activated microglial phenotype, often associated with impaired surveillance and synaptic pruning, particularly in hippocampal regions such as the dentate gyrus and CA1 area (Delorme et al., 2023; Green and Rowe, 2024). The reduced complexity of microglial processes following MIA may disrupt normal neurogenesis and neuronal connectivity, contributing to neurodevelopmental abnormalities. Our study did not observe evidence of microglial hypertrophy in MIA-exposed middle-aged offspring (Figures 5, 6). Surprisingly, OTX-015 affected microglial morphology in MIA-not-exposed animals by inducing process retraction. This phenotype may reflect a slightly heightened inflammatory response, which could alter microglial functions. Because we did not observe any anti-inflammatory effect of OTX-015 in our conditions, we speculate that the detected effect could result from some compensatory mechanisms after prolonged OTX-015 treatment. However, further studies are necessary to elucidate the precise mechanisms through which these alterations occur and influence hippocampal function.

While our study leverages the MIA model to investigate the epigenetic ramifications of maternal immune activation on offspring, it is essential to acknowledge the limitations of our results. Our study utilizes a mouse model, which may not fully recapitulate the complexities of human disease. The MIA model, thanks to which we observed molecular changes in the offspring of a pregnant female whose immune system was activated, is well known and described (Krstic et al., 2012; Garay et al., 2013). However, as previously suggested, inflammatory responses in mouse models often exhibit limited concordance with human disease, and the underlying regulatory pathways may diverge substantially (Seok et al., 2013; Diehl and Boyle, 2018). However, other studies demonstrated significant similarities in some mechanisms of response to inflammation between humans and mice (Shay et al., 2013; Takao and Miyakawa, 2015). Also, this study utilized only male animals to mitigate the potential influence of the estrous cycle on experimental outcomes. However, given the higher prevalence of AD in females, future studies should incorporate both sexes to ensure a more comprehensive understanding of the pathomechanism of the disease. Another limitation of our study is the sample size in immunohistochemical analysis. The number of animals is sufficient to obtain reliable results. However, only repeating the tests and increasing the study group could bring us closer to testing potential drugs and planning clinical trials (Bogue et al., 2023). Other important question, we did not answer, is how stable are OTX-015 evoked changes. To date, little is known about the durability of epigenetic modulation following BET inhibition in the context of MIA. While OTX-015 has been shown to efficiently alter chromatin accessibility and transcriptional activity in various disease models, the stability of these changes following drug withdrawal remains to be elucidated. Moreover, given the systemic nature of OTX-015's effects and the complex interplay between immune activation, chromatin remodeling, and neurodevelopmental timing, long-term follow-up studies would be essential to clarify whether any MIA-induced alterations reappear or remain suppressed after treatment ends. Finally, we were unable to determine the specific molecular mechanism by which OTX-015 exhibits its anti-amyloid

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effect. Further research should explore alternative potential mechanisms.

Our results showed that orally administered BET protein inhibitor, OTX-015, reduced Aβ levels in the hippocampus and improved memory in mice. Further validation of experiments using OTX-015 for long periods and in different age frames is needed to determine the exact mechanism of the effects of BET inhibition. In conclusion, our findings support the further exploration of BET family protein inhibitors as a promising therapeutic strategy for Alzheimer's disease. Expanding the repertoire of tested inhibitors and conducting studies on human tissues will be critical steps toward validating these findings. The prospect of initiating clinical trials based on our results could pave the way for novel interventions to improve memory and cognitive function in individuals afflicted with AD. Longitudinal studies with OTX-015 across diverse age groups will also be pivotal in assessing its long-term effects on cognition and behavior in preclinical and clinical settings.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Local Ethics Committee for Animal Experiments in Warsaw, Poland. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MM: Formal analysis, Conceptualization, Methodology, Investigation, Writing – review & editing, Writing – original draft. AW: Conceptualization, Writing – review & editing, Investigation, Methodology. MG-D: Investigation, Writing – review & editing, Methodology. MC: Investigation, Conceptualization, Methodology, Writing – review & editing. GO-K: Investigation, Writing – review & editing, Methodology. EP: Investigation, Methodology, Writing – review & editing. EG: Investigation, Methodology, Writing – review & editing. MS: Writing – review & editing, Investigation, Methodology. GC: Investigation, Conceptualization, Resources, Validation, Funding acquisition, Supervision, Writing – review & editing, Formal analysis, Project administration, Methodology, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publikacja IV

Czapski, G.A.; Matuszewska, M.; Cieślik, M.; Strosznajder, J.B.

Inhibitor of bromodomain and extraterminal domain proteins decreases transcription of Cd33 in the brain of mice subjected to systemic inflammation; a promising strategy for neuroprotection. Folia Neuropathol. 2024, 62(2), 127–135, https://doi.org/10.5114/fn.2024.138140

Original paper

Inhibitor of bromodomain and extraterminal domain proteins decreases transcription of Cd33 in the brain of mice subjected to systemic inflammation; a promising strategy for neuroprotection

Grzegorz A. Czapski*, Marta Matuszewska*, Magdalena Cieślik, Joanna B. Strosznajder

Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

* *These authors contributed equally to this work.*

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Abstract

The neuroinflammation is a crucial component of virtually all neurodegenerative disorders, including Alzheimer's disease (AD). The bacterial lipopolysaccharide (LPS), a potent activator of the innate immune system, was suggested to influence or even trigger the neuropathological alterations in AD. LPS-induced neuroinflammation involves changes in transcription of several genes, thus controlling these molecular processes may be a potentially efficient strategy to attenuate the progression of AD. Since genome-wide association studies showed that the majority of AD-related genetic risk factors (AD-GRF) are connected to the immune system, our aim was to identify AD-GRF affected in the hippocampus by LPS-induced systemic inflammatory response (SIR). Moreover, we analysed the role of bromodomain and extraterminal domain (BET) proteins, the readers of the acetylation code, in controlling the transcription of selected AD-GRF in the brain during neuroinflammation.

In our study, we used a mouse model of LPS-induced SIR and mouse microglial BV2 cells. JQ1 was used as an inhibitor of BET proteins. The level of mRNA was analysed using microarrays and qPCR.

Our data demonstrated that among the established AD-GRF, only the expression of Cd33 was significantly upregulated in the hippocampus during SIR. In parallel, we observed an increase in the expression of Brd4, a BET family member. JQ1 prevented an LPS-evoked increase in Cd33 expression in the hippocampus of mice. Moreover, JQ1 reduced Cd33 expression in BV2 microglial cells stimulated with blood serum from LPS-treated mice.

Our study suggests that LPS-evoked SIR may increase Cd33 gene expression in the brain, and inhibition of BET proteins through suppression of Cd33 expression could be a promising strategy in prevention or in slowing down the progression of neuroinflammation and may potentially affect the pathomechanism of AD.

Key words: *Alzheimer's disease, systemic inflammatory response, endotoxin, bromodomain and extraterminal domain proteins, hippocampus, microglia.*

Introduction

During the last decades, a growing body of epidemiological data has indicated a substantial role of the immune system and inflammation in the pathogenesis/pathomechanism of Alzheimer's disease (AD). The association between the frequency of viral and bacterial infections and the risk of AD was presented during the last decades [10,22,54]. Several studies have demonstrated the advantageous effect of long-term use

Communicating author:

Grzegorz A. Czapski, Mossakowski Medical Research Institute, Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland, e-mail: gczapski@imdik.pan.pl

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of non-steroidal anti-inflammatory drugs (NSAIDs) on the risk of developing AD [13,33,46]. Additionally, analysis of human brains demonstrated that neuroinflammation, changes in cytokine profiles, and markers of microglia and astrocyte activation are important components of Alzheimer's pathology [1].

Recent hypotheses have highlighted the role of Gram-negative bacteria and their lipopolysaccharides (LPS) in the pathomechanism of AD [9,21,36,60]. The genome-wide association studies (GWAS) established a set of 29 genes whose polymorphism significantly affects the risk of developing AD (AD-GRF; AD-related genetic risk factors) [4]. Interestingly, many of these genes are related to the innate mechanisms of the immune system and to the function of microglia, which suggests the crucial role of neuroinflammation and microglial phagocytosis in the pathomechanism of AD [51]. LPS is a potent bacterial endotoxin that is highly resistant to degradation by mammalian enzymes, resulting in a persistent inflammatory stimulus. Intraperitoneal injection of LPS is a well-established *in vivo* model of the systemic inflammatory response (SIR) worsening brain function, cognitive functions, and memory [19,23,26,34]. However, the impact of SIR on the transcription of AD-GRF in the brain has never been studied.

The modulation of neuroinflammatory processes was demonstrated to protect the brain from LPS-induced dysfunction [17,59]. Among several anti-inflammatory strategies, inhibition of bromodomain and extraterminal domain (BET) proteins appears to be especially interesting. BET proteins are important epigenetic regulators of gene expression [12]. They are the readers of the acetylation code that in cooperation with transcription factors control the transcription [57]. Several BET inhibitors have shown anti-inflammatory properties in animal models of AD [39,43,49,50].

Therefore, in our study, we studied the impact of LPS-evoked systemic inflammation on the transcription of AD-GRF in the hippocampus of mice. Moreover, we analysed whether pharmacological inhibition of BET proteins may attenuate LPS-evoked alterations.

Material and methods

Animals and experimental design

The experiments were carried out on 3-month-old male C57Bl/6J mice supplied by the Animal House of the Mossakowski Medical Research Institute, Polish Academy of Sciences (Warsaw, Poland). The animals were maintained under standard conditions, with controlled temperature (22°C ±10%) and humidity (55% ±10%). All of the experiments conducted on the animals were approved by the II Local Ethics Committee for Animal Experimentation in Warsaw (permission WAW2/060/2020) and carried out following EU Directive 2010/63/EU on the protection of animals used for scientific purposes, and complying with the ARRIVE guidelines. All efforts were made to minimize animal suffering and reduce the animals' number. All manipulations were performed quickly and gently to reduce the animal's stress.

(S)-(+)-tert-butyl 2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetate (JQ1), a highly specific and potent inhibitor of BET proteins (Sigma-Aldrich, St. Louis, MO, USA), solution was prepared as defined previously [35]. Shortly, it was dissolved in dimethyl sulfoxide and mixed 1 : 10 with 10% 2-hydroxypropyl-β-cyclodextrin. LPS (from *E. coli* serotype O55:B5; toxicity 1.5×10^7 EU/mg; Sigma-Aldrich) was dissolved in saline. All treatments were performed in the morning. Animals were intraperitoneally (i.p.) injected with JQ1 (50 mg/kg b.w. or respective volume of the vehicle) and 30 min later with LPS (i.p.; 1 mg/kg or respective volume of the vehicle). After 3 and 12 h, animals were anesthetized by isoflurane inhalation and decapitated. The blood was collected 12 hours after the administration of LPS. Directly after the formation of the clot, samples were centrifuged at 1000 × g for 5 minutes to separate the serum and immediately frozen and stored at –85°C.

Microarray analysis of gene expression

Twelve hours after administration of LPS (i.p.; 1 mg/kg b.w.), total RNA from the perfused mouse hippocampus was isolated and analysed by using the Affymetrix Gene Chip Mouse genome 430 2.0. (Affymetrix Inc., Santa Clara, CA, USA), as described previously [21]. The data were normalized with the GC-RMA method and log2 transformed. Full microarray data (CEL files) are available in a public repository at: https://osf.io/x3jub/.

Cell culture experiment

Murine microglial BV2 cells were obtained from Elabscience Biotechnology Inc. (Houston, TX, USA) [6]. The cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 units/ml penicillin in a 5% CO_2 atmosphere at 37°C. The passages below the twentieth were used. The cells were frequently tested to avoid mycoplasma contamination.

JQ1 was dissolved in DMSO at a 10 mM stock solution and then it was diluted with a culture medium and added to cells at a 50 nM concentration. Thirty minutes after JQ1 the murine blood serum from con-

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trol animals or from LPS-treated animals was added to BV2 cells (final concentration: 2% v/v). After 12 h incubation, cells were washed twice with PBS, and RNA was isolated, as described below. In all experiments, the respective volume of vehicle was consequently added to corresponding groups.

PCR analysis of gene expression

Total RNA from the mouse hippocampus and from BV2 cells was extracted using TRI-reagent as described previously [44]. Reverse transcription and quantitative PCR were performed using pre-developed TaqMan Gene Expression Assays: *Cd33* (Mm00491152_m1), *Brd2* (Mm01271171_g1), *Brd3* (Mm01326697_m1), *Brd4* (Mm01350417_m1), *Tnf* (Mm00443258_m1), and *Gusb* (Mm01197698_m1) (Applied Biosystems, Foster City, CA, USA) [44]. The relative levels of mRNA were calculated using the ΔΔCt method. *Gusb* was used as a reference gene.

Statistics

Statistical analysis of data was performed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The distribution of the data was analysed using the Shapiro-Wilk normality test. *N* refers to the number of animals in the experimental group or to the number of independent experiments *in vitro*.

Results

Systemic injection of LPS is a widely used model of the systemic inflammatory response (SIR). Our previous studies demonstrated that SIR evoked by a moderate dose of LPS (1 mg/kg b.w.) induced acute but transient neuroinflammatory processes in the brain, including alterations in gene expression patterns, oxidative stress, and changes in the activity of several enzymes, among them Gsk-3β, Cdk5 and other kinases [15-21,34]. These molecular alterations were accompanied by short-lasting sickness behaviour and cognitive impairment [34]. In the present study, we focused on genes that were identified by GWAS studies as AD-linked genetic risk factors [4,5]. In our analysis, we also included genes that cause a fully penetrant monogenic form of AD: *Psen1*, *Psen2*, and *App*. As shown in Figure 1, our microarray data revealed that twelve hours after peripheral injection of LPS into a mouse, the mRNA level of several AD-GRF in the hippocampus was altered. The most pronounced change was noticed

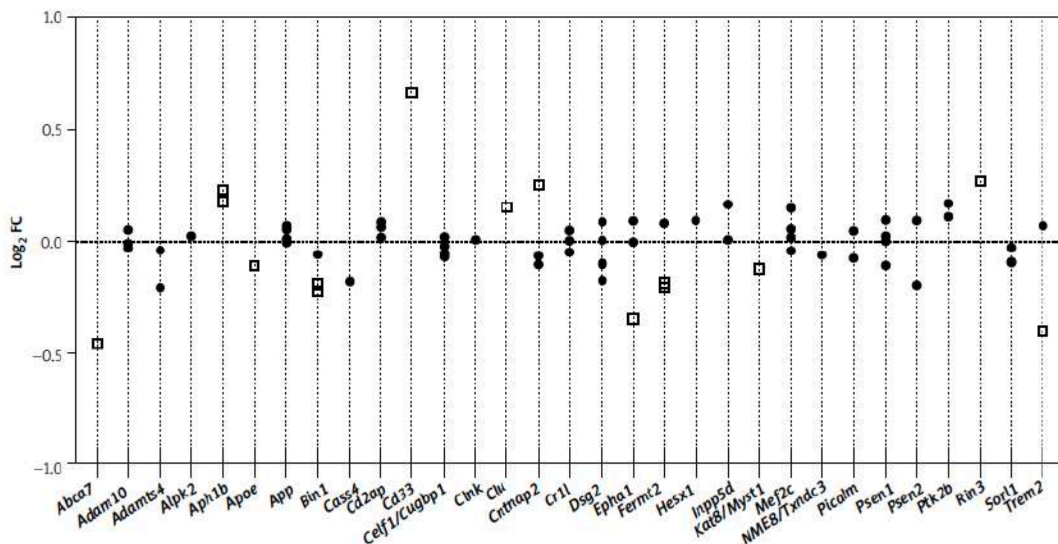

Fig. 1. Changes in mRNA levels of 31 genetic risk factors for Alzheimer's disease (AD) in the hippocampus of mice subjected to systemic inflammatory response (SIR) evoked by lipopolysaccharide (LPS). Microarray analysis was performed 12 hours after intraperitoneal administration of LPS. Each data point represents a mean value for a specific probe. The statistically significant change in expression ($p < 0.05$), compared to the control, was indicated by open squares. $N = 4$. FC – fold change.

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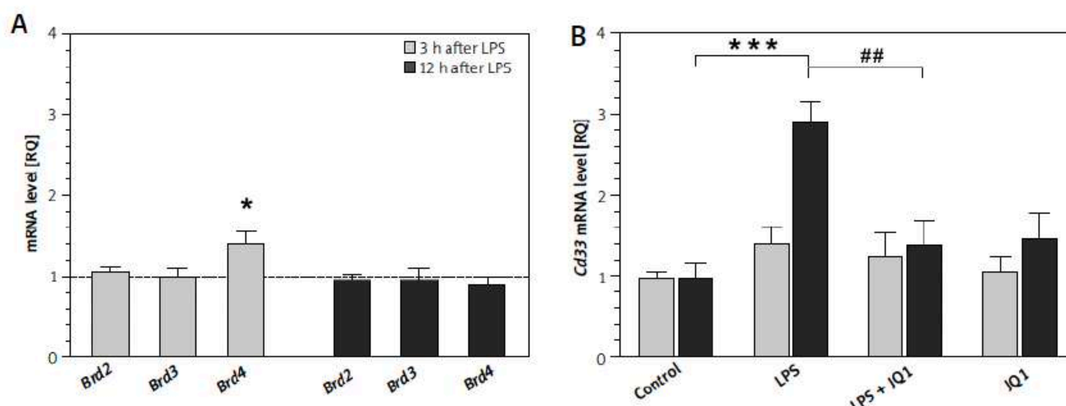

Fig. 2. Changes in mRNA levels of genes coding BET proteins and Cd33 in the mouse hippocampus 3 and 12 h after intraperitoneal injection of lipopolysaccharide (LPS). **A**) The impact of LPS on the transcription of *Brd2*, *Brd3*, and *Brd4* genes. The respective control level was presented as a dotted line. **B**) The effect of BET inhibitor, JQ1, on LPS-evoked alterations of *Cd33* expression in the hippocampus. The mRNA level was analysed by using the qPCR method. The data represent the mean values ± SEM from 6 animals. *,***$p < 0.05$, and $p < 0.001$, compared to the corresponding control, respectively; ##$p < 0.01$, compared to the LPS group.

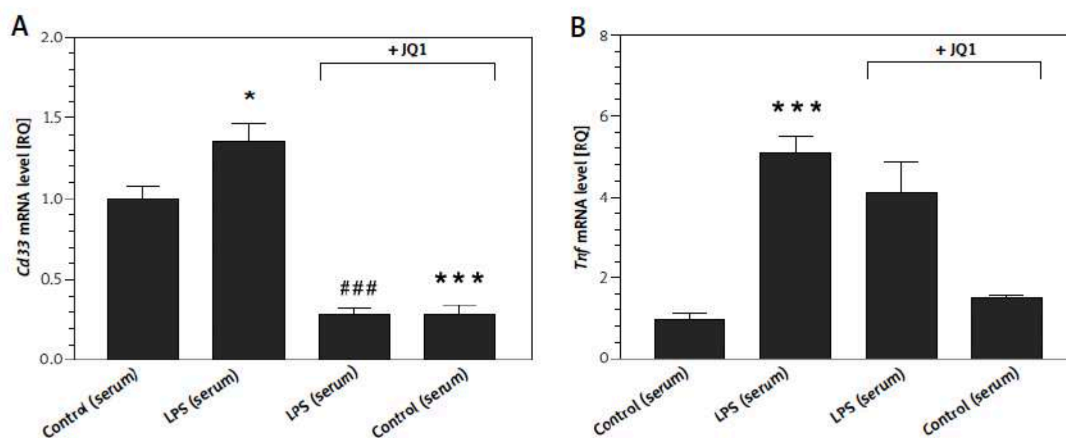

Fig. 3. The effect of BET proteins inhibitor, JQ1, on the mRNA level of *Cd33* and *Tnf* in the mouse microglial BV2 cells after 12 h incubation in the presence of blood serum from control and lipopolysaccharide (LPS)-treated mice. Mice received intraperitoneal injections of saline or LPS, then after 12 h blood was collected, and serum was prepared. BV2 cells were incubated in the presence of JQ1 (50 nM) for 30 min, then mice blood serum was added and incubation was continued for 12 h. The mRNA levels of *Cd33* (**A**) and the mRNA level of *Tnf* (**B**) were analysed by using the qPCR method. The data represent the mean values ± SEM from 5 independent experiments. *,***$p < 0.05$, and $p < 0.001$, compared to the corresponding control, respectively; ###$p < 0.001$, compared to the respective LPS group.

for the *Cd33* gene, whose expression was upregulated by 58% ($p < 0.001$).

The alterations in *Cd33* expression were verified on a separate set of animals by using the quantitative PCR method. Additionally, the effect of JQ1, a very selective inhibitor of BET proteins, was analysed. Our data demonstrated (Fig. 2A) that among the three brain-expressed isoforms of BET proteins (Brd2, Brd3, and Brd4), the expression of the *Brd4* gene increased three hours after injection of LPS. As shown in Figure 2B, three hours

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after administration of LPS, neither LPS nor JQ1 affected the mRNA level for *Cd33*. However, twelve hours after peripheral administration of LPS, the mRNA level for the *Cd33* gene was significantly increased in the hippocampus, probably in microglia cells. Moreover, JQ1 efficiently prevented LPS-evoked upregulation of *Cd33*.

To confirm that inflammatory conditions increase the expression of *Cd33* in microglial cells, in a separate experiment *in vitro*, we analysed the impact of JQ1 on the level of *Cd33* mRNA in stimulated mouse microglial BV2 cells. As demonstrated in Figure 3, stimulation of BV2 cells for twelve hours with blood serum from LPS-treated mice induced a substantial increase in the expression of *Cd33* and *Tnf* genes, compared to cells incubated with serum from control mice. At this time-point, JQ1 significantly reduced the mRNA level of *Cd33*, in both stimulated (79% decrease) and non-stimulated cells (72% decrease), but did not affect the expression of the *Tnf* gene.

Discussion

Our data demonstrated hippocampal expression of 29 genes that were identified as bearing single nucleotide polymorphisms affecting the risk of developing AD [4]. In addition, we examined genes that are responsible for a genetic form of AD: *Psen1*, *Psen2*, and *App*. We hypothesized that inflammation may evoke changes in the expression of these genes in the brain, which, in consequence, might contribute to the pathomechanism of AD. By using a mouse model of the systemic inflammatory response (SIR) evoked by peripheral administration of LPS we identified a significant increase in the mRNA level for *Cd33* in the hippocampus. To our knowledge, the change in CD33 expression in the brain due to SIR has not been previously reported, but it seems that it could promote molecular alterations leading to neurodegeneration.

It was recently suggested that bacterial endotoxins (LPS) derived from the gut microbiota or originating from invading bacteria may significantly contribute to stimulation or the overactivation of neuroinflammation in AD [26,36,61], however, the mechanism remains unclear. Therefore, the identification of specific molecules that mediate LPS-related neuroinflammation may potentially facilitate the development of novel targets for therapy in AD. This disease is the major cause of dementia in the elderly, but despite decades of extensive research, the primary trigger is still unknown, and we still do not have efficient curation. Epidemiological data, GWAS, and analysis of human AD brains suggested neuroinflammation as a driving force in the pathomechanism or even pathogenesis of AD [1,4,47].

CD33 (Siglec-3) is a member of the Siglec family (sialic acid-binding immunoglobulin-like lectins), which in the brain is expressed mainly in microglial cells [14,25]. In humans, due to alternative splicing, two isoforms of CD33 are synthesised: a full-length protein hCD33M (M = 'Major'; 90%), and devoid of exon 2 short protein hCD33m (m = 'minor'; 10%) [25]. There is compelling evidence that CD33 affects the pathology associated with Aβ accumulation in AD by impairing Aβ clearance by microglia active during neuroinflammation [30]. A level of sialoglycan ligand for CD33, $(RPTP\zeta)^{S3L}$, is about twofold higher in the brains of AD patients than in the age-matched healthy controls [28]. Also, a large family-based GWAS indicated *CD33* as one of the top-level AD risk-related genes [3,58]. Two single nucleotide polymorphisms (SNP) in the *CD33* gene are considered crucial: rs3865444, which is located upstream of *CD33*, and rs12459419, which is located within the second exon [3]. The risk allele of rs3865444 evokes higher cell surface expression of CD33 and is related to the decline of cognitive functions [7,52]. The occurrence of the minor (protective) rs3865444 polymorphism was related with a decreased level of functional CD33 protein and with decreased levels of insoluble Aβ in the brains of AD patients [30]. The common allele rs12459419 favours the synthesis of a full-length CD33M, whereas the protective allele favours the synthesis of a short CD33m isoform. Interestingly, that 'protective' allele was suggested to be derived, human-specific, and reflect evolutionary pressure for longevity in humans [53]. Also, rs3826656 and rs2455069 have been associated with a higher risk of AD [3,55]. In AD brains, the level of CD33 is increased and correlates with a higher Aβ level and cognitive decline. Moreover, experimental studies demonstrated that CD33 reduces the clearance of $A\beta_{42}$ by microglia; therefore, it was suggested that CD33 protein may play a crucial role in the pathomechanism of AD, and its attenuation may be beneficial [31]. Indeed, the inactivation of CD33 in genetic mouse models of AD decreased levels of insoluble $A\beta_{1\text{-}42}$ in the brain and alleviated Aβ plaque pathology [29,30]. Moreover, expression of the human full-length variant of CD33 in 5×FAD mice increased Aβ pathology in the brain, but the protective variant had the opposite effect [24].

An important question arises about the impact of aging on the brain expression of CD33, which could potentially play a key role in the pathomechanism of AD. Unfortunately, the available data are inconsistent. In BALB/c mice, aging had no impact on *Cd33* expression level (GEO Profiles:12080108) [27]. Also, studies on human post-mortem brain tissues gave varying results (GEO profiles 5718268 and 117386467) [41,42], therefore, additional research is necessary to answer this question.

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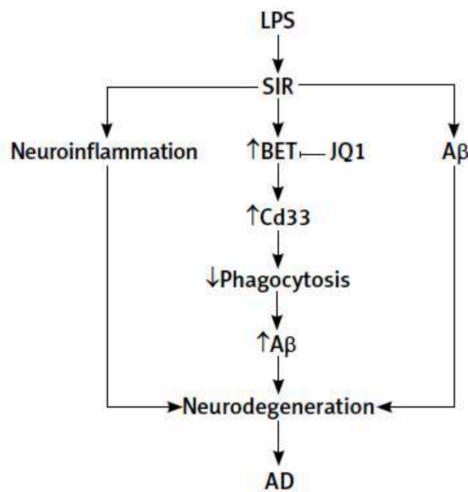

Fig. 4. Systemic inflammatory response (SIR), a trigger of CD33, could lead to neurodegeneration and Alzheimer's disease (AD). Lipopolysaccharide (LPS)-evoked SIR induces neuroinflammatory processes [16,20], accumulation of Aβ [37,56], and a BET-dependent increase in the expression of the *Cd33* – AD risk factor in the hippocampus. These processes may contribute to neurodegeneration and could be associated with AD. Enhanced expression of CD33 leads to inhibition of microglial phagocytosis and, in consequence, an increase in Aβ load in the brain. JQ1, an inhibitor of BET proteins, prevents SIR-related upregulation of *Cd33* expression.

Another important issue is the similarity between human and murine CD33. The fundamental physiological differences between humans and mice are evident, but still, a relatively large part of the human genome (about 40%) has a homologous locus in the mouse genome [38]. The human *CD33* and mouse *Cd33* genes have a similar structure and chromosomal position, and their protein sequence identity is 62% within extracellular domains [25]. The murine Cd33 lacks the characteristic ITIM (immunoreceptor tyrosine-based inhibitory motif) domain, which suggests that murine Cd33 may not precisely replicate the action of human CD33. Even though gene control systems are similar in mice and humans, some RNA expression diversity exists [11,40]. The previous reports demonstrated some variances in expression patterns and ligand recognition between human and murine CD33 [8]. However, both human and mouse CD33 inhibited phagocytosis of Aβ [30]. Therefore, future research should be performed to confirm our observations in human cells.

Because inhibitors of BET proteins were demonstrated to efficiently attenuate several LPS-evoked changes [2,32], the second goal of this study was to analyse the impact of JQ1, an inhibitor of BET proteins, on LPS-changed expression of AD-GRF genes in the hippocampus during systemic inflammation. In mice, JQ1 is well tolerated even after chronic treatment, and it efficiently enters the brain (AUC_{brain}/AUC_{plasma} = 98%) [43,45,48]. Our previous data indicated that in mouse microglia *in vitro* JQ1 reduced the expression of the *Cd33* gene by 83% [44]. In the current study, it was observed that JQ1 reduced the level of *Cd33* mRNA in the mouse hippocampus during SIR, but had no effect in the corresponding control. We can assume the cerebral action of JQ1, therefore, its inhibitory effect on microglial phagocytosis [44] cannot be completely excluded. Based on these data, we propose that inhibitors of BET proteins may prevent inflammation-evoked changes in the expression of the *Cd33* gene and, therefore, may be used to attenuate CD33-dependent signalling.

In summary, our study demonstrated the upregulation of the *Cd33*, a well-established genetic risk factor for AD, in the mouse hippocampus during LPS-evoked systemic inflammation. Moreover, our data indicated that an inhibitor of BET proteins prevented the activation of the *Cd33* gene in hippocampal cells during SIR. These results suggest that inhibitors of BET proteins may be suitable for prevention or for slowing down the progression of neuroinflammatory processes, which may be critical events in brain function and in the pathogenesis/pathomechanism of AD (Fig. 4).

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Datasets/data availability statement

The data supporting the findings of this study are available on request from the corresponding author. Full microarray data (CEL files) are available in a public repository at: https://osf.io/x3jub/.

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Disclosures

The study was approved by the II Local Ethics Committee for Animal Experimentation in Warsaw (permission WAW2/060/2020).

The authors report no conflict of interest.

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BET protein inhibitor JQ1 reduces inflammation and hippocampal amyloid-β level without altering Tau phosphorylation in LPS-challenged adult wild-type mice

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Corresponding Author:	Grzegorz Arkadiusz Czapski, PhD Mossakowski Medical Research Institute Polish Academy of Sciences POLAND
First Author:	Marta Matuszewska
Order of Authors:	Marta Matuszewska
	Magdalena Cieślik, PhD
	Dorota Sulejczak, PhD
	Anna Wilkaniec, PhD
	Grzegorz Arkadiusz Czapski, PhD
Abstract:	Introduction A growing body of evidence highlights the role of infection and inflammation in the progression of Alzheimer's disease (AD). In this study, we aimed to analyze the impact of JQ1, an inhibitor of bromodomain and extraterminal domain (BET) proteins, which are key readers of the epigenetic acetylation code, on AD-related gene expression changes and biochemical alterations in the hippocampus during a lipopolysaccharide (LPS)-induced systemic inflammatory response in mice. Methods JQ1 and LPS were administered intraperitoneally to adult male wild-type C57BL/6J mice. Changes in selected general and brain-specific parameters were measured for up to 12 h. Results Our results demonstrated that inhibition of BET proteins reduced LPS-induced sickness behavior and time-dependent elevation of proinflammatory signaling. LPS did not significantly alter amyloid-β (Aβ) levels; however, a significant reduction in Aβ load was observed in JQ1-treated animals overall, suggesting that BET proteins play a crucial role in regulating Aβ levels in the brain. At the same time, JQ1 treatment did not affect LPS-induced increases in phospho-Tau levels. Discussion Our results suggest that inhibiting BET proteins, in addition to their anti-inflammatory action, may be an effective strategy for reducing Aβ levels in the brain. However, a mechanistic explanation of this phenomenon requires further investigation.

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Cover Letter

DEPARTMENT OF CELLULAR SIGNALLING
MOSSAKOWSKI MEDICAL RESEARCH INSTITUTE
POLISH ACADEMY OF SCIENCES
A. Pawinskiego 5, 02-106 Warsaw, Poland
Tel: +48 22 6086600
E-mail: gczapski@imdik.pan.pl

22 Sep 2025, Warsaw

Dear Editor,

Enclosed you will find the research article under the title "BET protein inhibitor JQ1 reduces inflammation and hippocampal amyloid-β level without altering Tau phosphorylation in LPS-challenged adult wild-type mice" by Marta Matuszewska, Magdalena Cieślik, Dorota Sulejczak, Anna Wilkaniec, and Grzegorz A. Czapski for consideration for publication in the Experimental Neurology.

The bacterial endotoxin lipopolysaccharide (LPS) has been implicated in influencing, and potentially triggering and accelerating, neuropathological alterations in Alzheimer's disease (AD). LPS-induced activation of the innate immune system involves changes in gene expression patterns. Therefore, controlling LPS-evoked alterations may represent an efficient strategy to attenuate the progression of AD. In this study, we utilized a murine experimental model of acute LPS-evoked systemic inflammation to investigate the role of bromodomain and extraterminal domain (BET) proteins, which are readers of the acetylation code, in regulating molecular processes in the brain. **The novelty and biggest significance of our study** lie in demonstrating that inhibitor of BET proteins, in addition to its anti-inflammatory effects (both peripheral and central), significantly reduces the levels of Amyloid-β peptide in the brain of non-transgenic mice. This observation requires additional confirmation in human cells, but potentially may stimulate new research and development of novel therapeutic strategies in AD.

All authors have contributed to the work and agree with the presented findings. All authors have approved the manuscript for submission. The material submitted for publication has not been previously reported and is not under consideration for publication elsewhere. The manuscript follows all journal policies.

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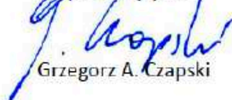
The authors declare no conflict of interest.

We think that our manuscript fits well within the scope of the Experimental Neurology. I would very much appreciate your opinion thereon.

Respectfully yours,



Grzegorz A. Czapski

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Highlights

Highlights

- BET protein inhibitor JQ1 tested in murine endotoxemia model
- Endotoxemia drives robust Brd4 upregulation in the hippocampus
- JQ1 lowers LPS-driven cytokines and sickness behavior
- Tau phosphorylation remains unaffected by JQ1 under endotoxemic conditions
- JQ1 significantly reduces amyloid-beta accumulation in the hippocampus

Manuscript

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1 Research Article

2

3 **BET protein inhibitor JQ1 reduces inflammation and hippocampal amyloid-β level**

4 **without altering Tau phosphorylation in LPS-challenged adult wild-type mice**

5

6 Marta Matuszewska[a]

7 Magdalena Cieślik[a]

8 Dorota Sulejczak[b]

9 Anna Wilkaniec[a]

10 Grzegorz A. Czapski[a]

11

12 [a] Department of Cellular Signalling, Mossakowski Medical Research Institute, Polish

13 Academy of Sciences, Warsaw, Poland;

14 [b] Department of Experimental Pharmacology, Mossakowski Medical Research Institute,

15 Polish Academy of Sciences, Warsaw, Poland

16

17 Correspondence: Grzegorz A. Czapski

18 Mossakowski Medical Research Institute, Polish Academy of Sciences

19 Pawińskiego 5

20 02-106 Warsaw

21 Poland

22 Email: gczapski@imdik.pan.pl

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23 **Abstract:**
24 Introduction
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26 progression of Alzheimer's disease (AD). In this study, we aimed to analyze the impact of
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28 key readers of the epigenetic acetylation code, on AD-related gene expression changes and
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30 systemic inflammatory response in mice.
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36 behavior and time-dependent elevation of proinflammatory signaling. LPS did not
37 significantly alter amyloid-β (Aβ) levels; however, a significant reduction in Aβ load was
38 observed in JQ1-treated animals overall, suggesting that BET proteins play a crucial role
39 in regulating Aβ levels in the brain. At the same time, JQ1 treatment did not affect LPS-
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45

46 **Keywords:** lipopolysaccharide; systemic inflammation; neuroinflammation; JQ1
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Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and a leading cause of dementia in the aging population. The classical neuropathological hallmarks of AD are extracellular amyloid-β (Aβ) aggregates (senile plaques) and intracellular neurofibrillary tangles (NFTs), which are composed of hyperphosphorylated Tau protein. Early-onset AD (EOAD), accounting for less than 5% of all cases, is caused by mutations in the genes encoding presenilin 1 (PSEN1), presenilin 2 (PSEN2), and amyloid-β (Aβ) precursor protein (APP), resulting in accelerated Aβ deposition (Chartier-Harlin et al., 1991; Sherrington et al., 1995). In contrast, the vast majority of cases are sporadic, late-onset AD (LOAD), wherein complex interactions among genetic susceptibility, environmental factors, and immune responses contribute to disease onset and progression.

The amyloid cascade hypothesis, which postulates Aβ accumulation as the primary driver of neurodegeneration, has been increasingly challenged by numerous unsuccessful clinical trials targeting Aβ (Armstrong, 2014; Kepp et al., 2023; Weinstock, 2024). Even though anti-Aβ antibody-based therapy was finally approved (Jucker and Walker, 2023), its modest benefits and numerous disadvantages highlight the need to explore alternative hypotheses. Growing evidence indicates that neuroinflammation is not merely a bystander but rather a prerequisite for neurodegeneration in AD. For example, cognitively resilient individuals with high Aβ and Tau loads exhibit reduced neuroinflammatory markers, highlighting the modulatory role of inflammation in AD susceptibility (Barroeta-Espar et al., 2019; Perez-Nievas et al., 2013).

Systemic inflammation, such as that induced by bacterial or viral infections, is a well-established risk factor for accelerated AD progression (Bu et al., 2015; Douros et al., 2021; Sun et al., 2022b). Early observations suggested a detrimental role for the inflammation-induced release of potentially neurotoxic mediators (McGeer and McGeer, 1998). Peripheral administration of lipopolysaccharide (LPS), a potent endotoxin derived from Gram-negative bacteria, triggers robust microglial activation, cytokine release, blood–brain barrier (BBB) dysfunction, and transcriptional alterations in the brain (Czapski et al., 2016; Ifuku et al., 2012; Wang et al., 2018b). Rodent studies have demonstrated that both acute and chronic LPS exposure lead to AD-like neuropathology,

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79 including Aβ accumulation and Tau hyperphosphorylation (Czapski et al., 2016; Hossain
80 et al., 2018; Ifuku et al., 2012; Kahn et al., 2012; Lee et al., 2008; Wang et al., 2018b), and
81 that these effects may persist long after the initial immune challenge (Wang et al., 2020).
82 Indeed, chronic neuroinflammation and progressive neurodegeneration have been
83 observed following systemic LPS administration in mice (Holmes et al., 2009), with
84 analogous mechanisms implicated in human AD progression (Cunningham et al., 2009).

85 Although AD is characterized by slow progression, acute systemic inflammation
86 may act as a mechanistic "first hit," initiating or amplifying neurodegenerative cascades
87 (Cunningham et al., 2009). This notion is supported by evidence that systemic LPS
88 challenges induce lasting cognitive impairments and neuroinflammatory reprogramming
89 (Qin et al., 2007; Teeling and Perry, 2009). While such acute models in young, wild-type
90 mice do not fully recapitulate the complexity of AD, they are valuable for dissecting early
91 immune–brain interactions and identifying potential therapeutic targets (Cunningham et
92 al., 2009; Lee et al., 2008).

93 One proposed mechanism involves CD33, a microglial immunomodulatory
94 receptor that inhibits the phagocytosis of Aβ. Genetic variants of CD33 are associated with
95 LOAD (Griciuc et al., 2013), and its upregulation has been linked to increased Aβ burden
96 and impaired microglial clearance. Our recent findings demonstrate that systemic LPS
97 elevates hippocampal Cd33 expression in mice (Czapski et al., 2024), suggesting that
98 recurrent peripheral inflammation may potentiate amyloid pathology via this axis.

99 Epigenetic regulation plays a critical role in orchestrating these neuroimmune
100 responses. Bromodomain and extraterminal domain (BET) proteins—including Brd2,
101 Brd3, and Brd4—function as readers of acetylated histones and interact with transcription
102 factors such as NF-κB to regulate the expression of proinflammatory genes (Martella et al.,
103 2023; Wang et al., 2023). BET dysregulation has been implicated in LPS-induced
104 neuroinflammation (Wang et al., 2020), and BET inhibition using the selective inhibitor
105 JQ1 has demonstrated potent anti-inflammatory effects in both peripheral immune cells
106 and the central nervous system (Belkina et al., 2013; Gilan et al., 2020; Liu et al., 2021;
107 Wang et al., 2020; Wang et al., 2018a; Wienerroither et al., 2014). Notably, JQ1 treatment
108 downregulates Cd33 expression, suppresses LPS-induced cytokine production, and impairs
109 microglial phagocytosis in vitro (Czapski et al., 2024; Matuszewska et al., 2022).

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110 Furthermore, BET proteins have been implicated in neuronal transcriptional regulation and
111 memory processes (Korb et al., 2015), suggesting that BET inhibition may confer both
112 immunomodulatory and neuroprotective benefits relevant to early AD. While this acute
113 inflammatory model does not replicate the full chronicity and complexity of human AD, it
114 provides a controlled framework to elucidate initial molecular and epigenetic events that
115 may prime the brain for later neurodegenerative processes.

116 Given these findings, we aimed to investigate the effects of a single JQ1
117 administration on neuroinflammatory and AD-related markers in young mice subjected to
118 systemic LPS-induced inflammation. This approach facilitates the study of early
119 transcriptional and epigenetic responses that may underlie the initiation of AD-like
120 pathology and offers insights into the therapeutic potential of BET inhibitors in disease
121 prevention.

122 **Material and methods**

123 ***Animals***

124 Experiments were conducted on 3-month-old male C57BL/6J mice supplied by the
125 Animal House of the Mossakowski Medical Research Institute, Polish Academy of
126 Sciences (Warsaw, Poland). A total of 72 animals were randomly allocated into eight equal
127 groups. No exclusion criteria were established a priori. The animals were maintained under
128 standard conditions, with controlled temperature (22°C ± 10%) and humidity (55% ± 10%).
129 All animal experiments were approved by the II Local Ethics Committee for Animal
130 Experimentation in Warsaw (permission WAW2/060/2020). This study was performed in
131 accordance with the relevant guidelines and regulations. All efforts were made to minimize
132 animal suffering and to reduce the number of animals. All manipulations were performed
133 gently and promptly to reduce stress.

134 ***Experimental design***

135 The JQ1 solution was prepared as described previously (Jostes et al., 2017). Briefly,
136 it was dissolved in DMSO and mixed at a 1:10 ratio with a 10% solution of 2-
137 hydroxypropyl-β-cyclodextrin. LPS (from *E. coli* serotype O55:B5; toxicity 1.5×10^7
138 EU/mg; Sigma, St. Louis, MO, USA) was dissolved in saline.

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139 All the treatments were performed in a random order in the morning. Animals
140 (three-month-old males) were transported to the laboratory room and, after 30 min
141 acclimatisation, intraperitoneally injected with JQ1 (50 mg/kg b.w. or 300 µL of the vehicle
142 in respective groups) and, 30 min later, with LPS (i.p.; 1 mg/kg or 50 µL of the vehicle in
143 respective groups) (Belkina et al., 2013). Sickness behavior was estimated every 3 h. After
144 3 or 12 h, the animals were anesthetized by isoflurane inhalation and decapitated (Fig. 1a).

145 ***Analysis of sickness behavior***

146 The severity of sickness behavior was estimated using a simplified Murine Sepsis
147 Score (MSS) (Shrum et al., 2014). In brief, the animals were observed by an investigator
148 blinded to the treatment groups, and changes in appearance, level of consciousness,
149 activity, response to stimuli, and eye condition were ranked using a scale of 0 to 4 (0
150 representing normal, 4 indicating severe alteration). In contrast to the original MSS
151 analysis protocol described by Shrum et al. (36), we omitted the assessment of respiration
152 rate and respiration quality due to interpretation challenges.

153 ***Analysis of serum cytokine levels (multiplexing assay)***

154 Blood was collected at 3 or 12 h after LPS administration. Immediately after clot
155 formation, the samples were centrifuged at 1000 × g for 5 min to separate the serum, which
156 was then immediately frozen and stored at -85° C until analysis. Serum cytokine levels
157 were determined using a Bio-Plex Pro Mouse Cytokine 23 assay kit following the
158 manufacturer's instructions (Bio-Rad) using a Luminex-200 apparatus, before the analysis,
159 the samples were diluted four fold with a dedicated sample diluent.

160 ***Analysis of mRNA levels (qPCR)***

161 Total RNA was extracted from mouse hippocampi using TRI reagent (Thermo
162 Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA quality and yield
163 were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific,
164 Inc.). Potential DNA contamination was digested using DNase I (Sigma-Aldrich)
165 according to the manufacturer's protocol. Reverse transcription was performed using a
166 High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher
167 Scientific, Inc.) according to the manufacturer's protocol. The cDNA was stored at -20°C

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168 until use. Quantitative PCR was performed on Applied Biosystems 7500 Real-Time PCR
169 System by using pre-developed TaqMan Gene Expression Assays *Arg1*
170 (Mm00475988_m1), *Abca7* (Mm00442646_m1), *Bin1* (Mm00437457_m1), *Cd2ap*
171 (Mm00815310_s1), *Clu* (Mm01197002_m1), *Cr1l* (Mm00785297_s1), *Il1b*
172 (Mm00434228_m1), *Il6* (Mm00446190_m1), *Nos2* (Mm00440502_m1), *Picalm*
173 (Mm00525455_m1), *Rin3* (Mm00617220_m1), *Tnf* (Mm00443258_m1), *Trem2*
174 (Mm04209424_m1), *Zyx* (Mm00496120_m1). Relative mRNA levels were calculated
175 using the ΔΔCt method, with *Hprt* (Mm00446968_m1) as a reference gene.

176 ***Analysis of protein level and phosphorylation (Western blotting)***

177 Protein immunoreactivity analysis was performed as previously described with
178 modifications (Gąssowska-Dobrowolska et al., 2021). Briefly, tissue samples were
179 homogenized in RIPA buffer, and the protein concentration was determined using a BCA
180 Protein Assay Kit (Thermo Fisher Scientific), with bovine serum albumin as a standard.
181 Samples were mixed with Laemmli buffer and heated at 95°C for 5 min. After SDS-PAGE,
182 the proteins were transferred to a nitrocellulose membrane under standard conditions and
183 used for immunochemical analysis, followed by chemiluminescent detection.
184 Densitometric analysis was performed using TotalLab4 software (NonLinear Dynamics
185 Ltd., Newcastle upon Tyne, UK) using size-marker-based verification. Glyceraldehyde-3-
186 phosphate dehydrogenase (GAPDH) was used to normalize the data. The anti-Tau
187 antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). The rabbit anti-
188 pTau(Ser199/202) antibodies and rabbit anti-GAPDH antibodies were from Sigma Aldrich
189 (St. Louis, MO, USA). The mouse anti-pTau(Ser396) and rabbit anti-pTau(Ser416) were
190 from Cell Signalling Technology (Danvers, MA, USA). The secondary antibodies used
191 were anti-mouse IgG (GE Healthcare) and anti-rabbit IgG (Sigma-Aldrich). The Tau
192 phosphorylation sites were identified according to the 2N4R isoform (441 kDa) sequence.
193 Details of Western blotting reagents and conditions are presented in Supplementary Table
194 1.

195 ***Analysis of BET proteins level (ELISA)***

196 Brd2, Brd3, and Brd4 levels were quantitatively determined using an enzyme-
197 linked immunosorbent assay (ELISA) kit (Abbexa Ltd. Cambridge, UK). Briefly, the

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198 mouse hippocampi were rinsed with ice-cold PBS to remove excess blood. The tissues
199 were finely chopped and homogenized on ice in PBS using a tissue homogenizer, and the
200 samples were sonicated. The homogenates were centrifuged at 10,000 × g for 5 min and
201 the supernatants were collected. Protein concentration was determined using the BCA
202 assay with bovine serum albumin as the standard. The samples were diluted to a
203 concentration within the acceptable range of 0.01 mol/L in PBS (pH 7.2). Fresh samples
204 were used to prevent protein degradation and denaturation. The ELISA was performed
205 according to the manufacturer's instructions. The optical density was measured
206 spectrophotometrically at 450 nm using a Multiskan GO Microplate Spectrophotometer
207 (Thermo Fisher Scientific, Inc.). The results were normalized to protein levels.

208 ***Analysis of Amyloid-β level (ELISA)***

209 Quantitative determination of mouse $A\beta_{1-40}$ and $A\beta_{1-42}$ levels was performed using
210 commercially available ELISA kits (Thermo Fisher Scientific, Inc.). Briefly, mouse
211 hippocampi were homogenized in cold 5 M guanidine-HCl/50 mM Tris (pH 8.0).
212 Homogenates were placed in a laboratory rocker at room temperature for 4 h. Then, the
213 samples were diluted tenfold with cold PBS containing a 1× protease inhibitor cocktail and
214 centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was transferred to clean
215 microcentrifuge tubes and used for further analysis, according to the manufacturer's
216 protocol. The results were normalized to protein levels. The entire assay, including the
217 standard curve, was completed within one day.

218 ***Curated Analysis of Published mRNA Expression Data***

219 In July 2024, the NCBI GEO database (Edgar et al., 2002) was searched for data
220 concerning the impact of JQ1 on human gene expression. Data deposited by Baek and co-
221 workers (Baek et al., 2021), accessible through the GEO Series accession number
222 GSE155408 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155408), were
223 utilized for a curated analysis of selected genes.

224 ***Statistical Analysis***

225 The group size was calculated with G*Power software, which calculates the
226 minimum required group size based on the size of the Cohen d effect. Standard assumptions

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227 adopted: test power = 0.95, significance level = 0.01, groups of equal size. Based on our
228 results from previous experiments with LPS we obtained desired n=9. Statistical analyses
229 were performed using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA,
230 USA). The distribution of data was analyzed using the Shapiro-Wilk test. Outliers were
231 determined using Grubs test and removed if $p<0.05$. The results are expressed as mean ±
232 SEM. Depending on the experimental design, data were analyzed using Student's t-test or
233 one-way analysis of variance (ANOVA) with Bonferroni post-hoc test with correction for
234 multiple comparisons. Statistical significance was set at $P < 0.05$. N refers to independent
235 samples (i.e., various animals).

236 **Results**

237 Intraperitoneal administration of lipopolysaccharide (LPS) is a commonly used
238 experimental method for inducing a systemic immune response. This response
239 encompasses acute innate mechanisms in the peripheral tissues and central nervous system.
240 In the current study, we administered LPS at 1 mg/kg b.w. (1.5×10^7 U/kg b.w.) (Czapski
241 et al., 2013; Czapski et al., 2007; Czapski et al., 2006; Czapski et al., 2004; Czapski et al.,
242 2010; Czapski et al., 2016; Jacewicz et al., 2009). This dose evoked a rapid, substantial,
243 and transient response, which was significantly lower than the lethal dose for mice
244 (approximately 50 mg/kg body weight) (Back et al., 2000; Li et al., 2018). However, this
245 dose is high enough to induce a broad range of brain pathologies, including oxidative stress,
246 followed by the activation of PARP-dependent AIF-mediated necroptosis, changes in
247 kinase activity, or inflammatory signaling(Czapski et al., 2013; Czapski et al., 2007;
248 Czapski et al., 2006; Czapski et al., 2010; Czapski et al., 2016; Czapski et al., 2020). To
249 evaluate whether a single administration of a BET protein inhibitor modulates the systemic
250 and central inflammatory responses induced by LPS, we examined a range of peripheral
251 and central variables. At the behavioral level, the response to LPS-evoked endotoxemia is
252 described as a sickness behavior, which includes changes in body temperature, apathy, loss
253 of social interest, and lack of appetite (Harden et al., 2015). We implemented a murine
254 sepsis score (MSS) assessment for semi-quantitative determination of sickness behavior
255 (Shrum et al., 2014). As presented in Fig. 1b, LPS induced a rapid and persistent increase
256 in the level of MSS, which began 3 h after injection and was observed until the end of the

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257 experiment, 12 h after exposure (all components of MSS are presented separately in
258 Supplementary Fig. 1). In addition, LPS induced a decrease in body weight, which was
259 indistinct 3 h after injection (Fig. 1c) but was evident at 12 h (Fig. 1d). In animals pretreated
260 with JQ1, the increase in MSS was attenuated and tended to decrease. However, JQ1 did
261 not affect the LPS-evoked changes in body weight.

262 Although the anti-inflammatory properties of JQ1 are well established, its effects
263 on the levels of specific inflammatory cytokines in blood serum have not been thoroughly
264 investigated. Therefore, we used a multiplexing method to simultaneously analyze the
265 levels of 23 inflammation-related signaling compounds in the mouse serum. Given that no
266 significant changes in the examined parameters were observed in the statistical analysis
267 between the 3-hour and 12-hour time points in the control group receiving the vehicle, we
268 decided to combine these groups into a single unified control group. As expected, LPS
269 induced a rapid increase in the serum levels of nearly all the 23 cytokines tested (Fig. 2).
270 Specifically, the levels of 22 cytokines were elevated at 3 h post-administration and 21
271 remained elevated at 12 h. Applying the BET protein inhibitor to animals that were not
272 challenged with LPS did not affect their serum cytokine/chemokine profile. However, in
273 the LPS group, JQ1 pretreatment resulted in the augmentation of IL-1α, IL-2, IL-12p70,
274 GM-CSF, and RANTES at 3 h post-LPS, and IL-5, Il-6, IL12p40, Eotaxin, MCP-1, and
275 RANTES at 12 h post-LPS. These results indicate that the BET protein inhibitor had a
276 highly selective effect on the expression of genes related to inflammation. This finding
277 suggests that the mechanism by which JQ1 reduces sickness behavior may involve more
278 than just a reduction in cytokine levels. The complete dataset used to generate the heat map
279 is presented in Supplementary Table 2. The levels of all cytokines exhibited a strong
280 positive correlation with either MSS or with each other (Supplementary Fig. 2).

281 Our previous studies have shown that the hippocampus is highly sensitive to
282 peripheral proinflammatory signaling (Czapski et al., 2013; Czapski et al., 2010; Czapski
283 et al., 2016; Czapski et al., 2024; Czapski et al., 2020). Therefore, we analyzed whether
284 LPS-induced systemic inflammatory response induces alterations in this structure. First,
285 we measured the levels of BET proteins in the hippocampi of animals treated with LPS
286 using commercial ELISA assay kits. Although our previous paper (Czapski et al., 2024)
287 reported a significant increase in mRNA levels for *Brd4* 3 hours after LPS injection, the

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288 current study did not show a corresponding change in Brd4 levels at this early time point
289 (data not shown). However, a significant increase in Brd4 protein levels was observed 12
290 h after LPS treatment (Fig. 3).

291 Next, we analyzed the impact of LPS and JQ1 on processes related to inflammatory
292 signaling in the hippocampus. Because the principal mode of action of BET inhibitors is
293 through the modulation of gene expression, we focused on the quantitative analysis of the
294 mRNA levels of selected genes, which seemed more reliable than measuring protein levels.
295 We limited our analysis to the canonical markers of cytotoxic/cytoprotective states, *Il1b*,
296 *Il6*, *Tnf*, *Nos2*, and *Arg1*. Similar to the upregulation of cytokines in the blood serum, the
297 transcription of selected inflammation-related genes was also elevated during the systemic
298 inflammatory response at both 3 and 12 h after LPS administration (Fig. 4a and 4b,
299 respectively). The increased expression of these genes suggests that microglia are activated
300 toward the proinflammatory/cytotoxic (often referred to as 'M1') phenotype.
301 Simultaneously, the expression of Arg1, a marker of the microglia's anti-
302 inflammatory/cytoprotective phenotype (often referred to as 'M2'), remained unchanged
303 in the systemic inflammatory response group. We observed an evident inhibitory effect of
304 JQ1 on the expression of proinflammatory genes 3 h after LPS treatment. However, this
305 effect was no longer apparent after 12 hours. JQ1 treatment alone did not affect the
306 expression of any of the tested genes under the control conditions.

307 The sickness behavior index (MSS) and inflammatory gene expression data from
308 the animals in all experimental groups were subjected to correlation analysis. As expected,
309 MSS was positively correlated with the mRNA levels of *Il1b*, *Il6*, and *Tnf*; however, the
310 correlation was moderate, likely due to the relatively low number of data pairs
311 (Supplementary Fig. 3).

312 As shown previously, using the same dose of LPS, the neuroinflammatory
313 processes in the brain activated by the systemic inflammatory response significantly
314 increase the phosphorylation of Tau protein at Ser396 (Czapski et al., 2016). In the present
315 study, we confirmed and extended this observation. As presented in Fig. 5, 12 h after
316 peripheral administration of LPS, the level of Tau protein in the hippocampus was not
317 altered, but phosphorylation of Ser 396 and Ser 416 was significantly increased, whereas
318 phosphorylation of Ser 199-202 demonstrated a strong tendency to increase ($p=0.05$).

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319 Pretreatment with JQ1 alone did not affect Tau levels or phosphorylation and had a
320 negligible effect on the phosphorylation of this protein induced by a systemic inflammatory
321 response.

322 However, JQ1 significantly reduced the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the
323 hippocampus of wild-type mice (Fig. 6a, 6b). This effect was evident in both control and
324 LPS-treated animals. Notably, peripheral administration of LPS did not affect Aβ levels in
325 the hippocampus. The levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the hippocampi of the animals in all
326 experimental groups were subjected to correlation analysis. As shown in Fig. 6c, the levels
327 of Aβ1-40 and Aβ1-42 were highly positively correlated, which may suggest a common
328 mechanism of JQ1 action for these two forms of Aβ. Because ELISA quantification relies
329 on a standard curve, the confidence in quantifying our samples could be limited due to the
330 very low concentrations, which are close to the lowest point on the standard curve. To
331 mitigate this limitation, each sample was analyzed in duplicate (two technical replicates).
332 The strong correlation between $A\beta_{1-40}$ and $A\beta_{1-42}$ levels increased the confidence in the
333 reliability of our results.

334 Our previous study showed that JQ1 significantly attenuates phagocytic function in
335 murine microglia in vitro (Matuszewska et al., 2022). We demonstrated that the
336 phagocytosis of fluorescently labeled $A\beta_{1-42}$ by BV2 cells was reduced after treatment with
337 JQ1, and the expression of several phagocytosis/endocytosis-related genes was altered in
338 BV2 cells exposed to JQ1. Moreover, our recent in vivo study, conducted under the same
339 experimental conditions demonstrated that JQ1 reduced LPS-induced *Cd33* expression in
340 the hippocampus but had no effect on the transcription of the *Cd33* gene in mice not
341 exposed to endotoxemia (Czapski et al., 2024). Therefore, we hypothesized that alterations
342 in phagocytosis- or endocytosis-related genes might cause JQ1-evoked reduction in Aβ
343 load in the hippocampus. Thus, this study focused on investigating changes in the
344 expression of genes previously implicated in the AD pathomechanism. As shown in Fig.
345 7, JQ1 did not affect the mRNA levels of any of the tested phagocytosis/endocytosis-
346 related genes in the mouse hippocampus at either 3 h or 12 h after administration.

347 Additionally, since the impact on amyloidogenesis is a potential mechanism of JQ1
348 action, we investigated the protein levels of β-secretase (BACE1) and γ-secretase (Aph1,

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349 Presenilin 1 and 2, PEN2, Nicastrin). However, we did not find any impact of LPS or JQ1
350 on the expression of those proteins (data not shown).

351 **Discussion**

352 Activation of innate immune mechanisms is a component of virtually all
353 neurodegenerative conditions (Castro-Gomez and Heneka, 2024). Pattern recognition
354 receptor-mediated inflammatory responses are critical during both prodromal and clinical
355 phases of these diseases. Recent data have highlighted the importance of the bacterial
356 endotoxin, LPS, as a factor that may significantly contribute to or even promote
357 neurodegeneration. The proposed 'endotoxin hypothesis' of AD suggests that LPS may be
358 a crucial factor in triggering the pathological cascade leading to neurodegenerative injury
359 (Brown, 2019; Brown and Heneka, 2024). Sepsis-associated encephalopathy, which occurs
360 in approximately 70% of patients with severe sepsis, is related to long-lasting brain
361 dysfunction, including cognitive deficits and post-traumatic stress disorder with increased
362 anxiety (Grünewald et al., 2024; Iwashyna et al., 2010; Sipilä et al., 2021). Fine-tuning of
363 inflammatory signaling is increasingly being considered as a promising strategy to change
364 the trajectory of neurodegeneration. Therefore, identification of potential therapeutic
365 targets to attenuate LPS-induced brain dysfunction and cognitive changes is necessary to
366 promote healthy aging. BET proteins, the key players in the epigenetic control of gene
367 transcription, have been proposed for inflammation-targeted treatment (Clayton et al.,
368 2024; Martella et al., 2023).

369 Our study employed a mouse model of LPS-induced mild systemic inflammatory
370 response, based on previous studies, in which we demonstrated that intraperitoneal
371 injection of LPS (1.5×10^7 U/kg body weight) evoked transient but robust and reproducible
372 biochemical and molecular changes in the hippocampus of experimental animals (Czapski
373 et al., 2013; Czapski et al., 2010; Czapski et al., 2016; Czapski et al., 2024; Czapski et al.,
374 2020; Jacewicz et al., 2009). Moreover, we observed temporary symptoms of sickness
375 behavior lasting up to 48 h and long-lasting cognitive impairments (Czapski et al., 2013;
376 Czapski et al., 2007; Czapski et al., 2016; Czapski et al., 2020; Jacewicz et al., 2009). It is
377 important to note that our model is an acute inflammatory response, not a model of the AD
378 itself. However, BET inhibitors influenced some processes that are also relevant to AD.

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379 In this study, we used a small-molecule broad-spectrum inhibitor of BET proteins, JQ1.
380 JQ1 exerts its effects by competitively binding to the acetyl-lysine recognition pocket of
381 BET proteins, thereby preventing the binding of acetylated proteins and disrupting the
382 recruitment of transcriptional complexes (Filippakopoulos et al., 2010). JQ1 was well
383 tolerated without overt signs of toxicity or weight loss at a dose of 50 mg/kg in mice
384 (Filippakopoulos et al., 2010). Pharmacokinetic analysis of JQ1 demonstrated its good
385 permeability across the blood-brain barrier (AUC_{brain}/AUC_{plasma} = 98%) after a single
386 intraperitoneal dose of 50 mg/kg in male mice (Matzuk et al., 2012). However, it cannot
387 be confidently excluded that some systemic interactions account for the effects observed
388 in the brain.

389 The response of an organism to infection is complex and multiphase. A component
390 of this response is a phenomenon known as sickness behavior (Dantzer, 2023), a universal
391 and non-specific pattern of symptoms that develops during activation of the immune
392 system. Fever, sleepiness, lethargy, anorexia, reduced social interest, etc. - all these
393 symptoms are related to suppressed animal activity. They are considered to promote the
394 fight against infection (energy repartition, metabolic reprogramming (O'Neill et al., 2016))
395 and prevent the spread of infectious agents in the population (Dantzer, 2023). The
396 mechanism of sickness behavior in response to infectious agents is related to the immune
397 system-dependent release of proinflammatory mediators, especially interleukin-1β
398 (Dantzer et al., 1998), which affects brain function. Further research is required to gain a
399 detailed understanding of the molecular mechanisms involved in this phenomenon. Our
400 study investigated the role of BET proteins in sickness behavior, recognizing their crucial
401 role in regulating numerous genes and pathways associated with immunity (Wang et al.,
402 2021). Numerous studies have demonstrated that BET inhibitors block excessive
403 production of inflammatory mediators and reduce the cell surface expression of chemokine
404 receptors (Maksylewicz et al., 2019; Sanchez-Ventura et al., 2019; Wasiak et al., 2023).
405 Following these studies, our results demonstrated that JQ1 significantly reduces LPS-
406 evoked sickness behavior in mice, which is directly related to its anti-inflammatory effect,
407 as shown by correlation analysis. However, we observed that JQ1 treatment affected only
408 a select group of proinflammatory genes in a time-dependent manner. Intraperitoneal
409 administration of JQ1 decreased serum levels and hippocampal gene expression of

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410 proinflammatory mediators in the LPS-treated group but did not affect the basal levels of
411 these cytokines. We expect that a more powerful analysis (on larger experimental groups)
412 or more extensive analysis (at more time points) could confirm this effect. Moreover, it
413 would be reasonable to analyze the impact of JQ1 on long-lasting changes, such as LPS-
414 evoked cognitive impairment, in future studies. The mechanisms underlying the effect of
415 JQ1 on sickness behavior likely involve a reduction in cytokine levels but could also
416 include other processes. These effects may include a decrease in vascular inflammation,
417 modulation of the complement cascade, and normalization of cerebral blood flow (Huang
418 et al., 2017; Wasiak et al., 2017; Yang et al., 2018).

419 The mechanism underlying the anti-inflammatory action of JQ1 is related to its
420 effect on gene expression. Because BET proteins interact with a broad spectrum of
421 transcription and elongation factors, their inhibition may affect various molecular
422 pathways. Previous studies have shown that JQ1 suppresses LPS-induced microglial
423 activation via the MAPK/NF-κB signaling pathway (Wang et al., 2018a). Activation of
424 NF-κB and subsequent expression of inflammatory genes require acetylation of the RelA
425 subunit at lysine-310 (Chen et al., 2002).

426 A notable finding of the current study was that a single peripheral administration of
427 JQ1 decreased hippocampal levels of native murine Aβ in wild-type mice. This effect was
428 evident for $A\beta_{1-40}$ and $A\beta_{1-42}$ both under control conditions and in LPS-treated animals. In
429 the extraction protocol, we used a guanidine hydrochloride-containing buffer, assuming
430 that we could measure the total level of Aβ in both the soluble and insoluble forms in the
431 tissue. The amount of endogenous Aβ in wild-type mice is significantly smaller than that
432 in transgenic animals overexpressing human Aβ; therefore, to assess Aβ levels, we used an
433 ELISA assay, which has been previously demonstrated to be a specific and accurate
434 method for measuring low levels of endogenous Aβ in wild-type mice (Lu et al., 2016;
435 Peng et al., 2021). Although rodents do not naturally develop AD, an increase in Aβ levels
436 is typically observed in aged wild-type animals (Drummond and Wisniewski, 2017;
437 Fukumoto et al., 2004; Hoh Kam et al., 2010; Krstic et al., 2012; Oakley et al., 2006).
438 However, with the exception of a single study by Ahlemeyer et al. (Ahlemeyer et al., 2018),
439 extracellular deposits of Aβ have not been observed in the brains of naïve wild-type
440 rodents. Immunological challenges—whether single prenatal or combined prenatal and

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441 postnatal (at 12 months)—have been shown to induce the formation of plaque-like
442 structures in 15-month-old wild-type mice. However, the density of these "plaques"
443 remained significantly lower compared to those observed in transgenic Alzheimer's disease
444 model mice (Krstic et al., 2012). Also, administering LPS to rodents increased Aβ levels
445 in the brain; however, multiple injections of LPS were necessary (Hossain et al., 2018;
446 Ifuku et al., 2012; Kahn et al., 2012; Lee et al., 2008). In contrast, a single, but very high
447 dose of LPS increased soluble Aβ levels in the rat brain (Wang et al., 2018b). In our study,
448 the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ did not change 12 h after peripheral administration of LPS.
449 However, regardless of LPS-induced systemic inflammation, the administration of JQ1
450 significantly decreased Aβ levels in the hippocampus. Our previous studies have
451 demonstrated that JQ1 significantly reduces *Cd33* gene expression in the microglial BV2
452 cell line (Matuszewska et al., 2022) and in mouse brains (Czapski et al., 2024). This
453 suggests that the observed effect of JQ1 may involve Cd33, potentially linked to the
454 activation of Aβ scavenging via microglial phagocytosis. Therefore, this study focused on
455 other phagocytosis/endocytosis-related genes previously implicated in the AD
456 pathomechanism (Jansen et al., 2019). First, we performed a curated analysis of the
457 expression data deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and
458 accessible through the GEO Series accession number GSE155408
459 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155408) (Table 1). The
460 authors of that dataset, Baek and co-workers, used RNA sequencing to analyze the impact
461 of JQ1 on gene expression in the human microglial HMC3 line (Baek et al., 2021). For
462 comparison, we have included a summary of our previously published analysis of murine
463 microglia (Matuszewska et al., 2022). The effect of JQ1 varied between the two cell lines.
464 We hypothesize that these differences may be attributed to the effects of varying JQ1 doses
465 and incubation times, as well as the testing of different organisms. Therefore, a mechanistic
466 explanation for the anti-Aβ activity of JQ1 in the hippocampus requires further research.
467 We propose that two other pathways may be involved in the anti-amyloid activity
468 of JQ1. JQ1 might exert anti-amyloid effects by activating autophagy. Previous studies
469 have demonstrated that JQ1 can stimulate autophagy by modulating the AMPK-mTOR-
470 ULK1 signaling pathway (Li et al., 2020). Notably, dysregulation of autophagy has been
471 implicated in the pathogenesis of AD (Di Meco et al., 2020). Consequently, activation of

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472 autophagy has emerged as a promising therapeutic approach for removing and clearing Aβ
473 deposits (Friedman et al., 2015; Rahman et al., 2020). Indeed, numerous autophagy
474 activators have been shown to effectively reduce Aβ levels in various experimental models
475 (Kong et al., 2020; Ordóñez-Gutiérrez et al., 2018; Pierzynowska et al., 2019). A second
476 potential pathway involves the inhibition of the NF-κB transcription factor, a key player in
477 the pathogenesis of AD (Sun et al., 2022a). Notably, BET protein inhibitors attenuate the
478 expression of NF-κB-regulated genes, thereby contributing to their anti-inflammatory
479 effects. Furthermore, NF-κB inhibition has been shown to reduce Aβ production in cells
480 overexpressing APP (Paris et al., 2007). Several NF-κB inhibitors have been demonstrated
481 to decrease the levels of $A\beta_{1-40}$, $A\beta_{1-42}$, sAPPβ, and APP-CTFβ, suggesting that they
482 diminish β-secretase-mediated cleavage of APP. It is noteworthy that the promoter of the
483 gene encoding BACE1, the aspartyl protease responsible for β-site cleavage of APP and
484 subsequent Aβ production, contains a binding site for NF-κB (Rossner et al., 2006).
485 However, our experimental model did not show any increase in hippocampal Aβ levels
486 during inflammation, suggesting that JQ1-mediated inhibition of inflammation-induced
487 NF-κB and subsequent BACE1-dependent Aβ production is unlikely.

488 The anti-amyloidogenic effect of JQ1 in our experimental model appears to be
489 specific to BET proteins, as preliminary data from another model (currently under
490 preparation for publication) indicate that OTX-015, another BET inhibitor, exhibits a
491 similar attenuating effect on Aβ levels in the brains of wild-type mice. However, BET
492 inhibitors do not affect Tau phosphorylation in the hippocampus. Our previous study
493 demonstrated that systemic inflammation enhances Tau phosphorylation at Se 396 by
494 cyclin-dependent kinase 5 (Czapski et al., 2016). In the current study, we confirmed and
495 extended this observation by demonstrating increased phosphorylation of Tau at Ser 199-
496 202, Ser 396, and Ser 416 in the hippocampus during systemic inflammation. However,
497 JQ1 did not affect Tau phosphorylation at any of the sites tested. The opposite effects of
498 JQ1 were observed after chronic treatment of 3×Tg mice (Magistri et al., 2016). In the
499 triple transgenic AD model, due to the presence of three human transgenes carrying
500 mutations, *APP*(K670N/M671L; Swedish mutation), *PS1*(M146V), and *MAPT*(P301L),
501 mice developed Aβ and Tau pathology followed by cognitive deficits. Mice treated with
502 JQ1 for 15 weeks showed reduced Tau hyperphosphorylation at Ser 396 in the

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503 hippocampus and frontal cortex; however, JQ1 did not affect the level of soluble Aβ
504 (Magistri et al., 2016). We propose that distinct molecular pathways may be responsible
505 for Tau hyperphosphorylation in the transgenic 3×Tg and LPS-challenged mice.

506 Finally, our results demonstrated that only Brd4 is upregulated in the hippocampus
507 under inflammatory conditions. This result is in agreement with the data of Wang et al.
508 (Wang et al., 2020) that showed the selective upregulation of Brd4 in macrophages.
509 Importantly, Brd4 plays a crucial role in controlling innate immune response to LPS (Bao
510 et al., 2017). Compared to wild-type mice, Brd4-KO mice challenged with LPS displayed
511 reduced expression of proinflammatory cytokine genes and were resistant to LPS-induced
512 sepsis. However, selective gene silencing revealed that all three BET proteins regulate
513 inflammatory gene expression in murine macrophages (Belkina et al., 2013). However, the
514 action of JQ1 is not necessarily limited to Brd4, as Brd2 and Brd3—although not
515 upregulated—may still play a role in regulating gene expression. Consequently, their
516 inhibition might also influence JQ1's effect in our experimental conditions. It is important
517 to note that BET proteins have overlapping, yet distinct, roles in regulating gene
518 expression. The differences arise from their specific domains and interaction partners. For
519 example, our previous study demonstrated that silencing of the Brd2 and Brd4 genes in
520 BV2 microglia reduced phagocytic activity to a greater extent than silencing of the Brd3
521 gene (Matuszewska et al., 2022). Moreover, the impact on phagocytosis-related gene
522 expression was also specific to the BET protein. Because of the pivotal role of BET proteins
523 in gene expression, the knock-out of Brd2 and Brd4 genes is lethal (Gyuris et al., 2009;
524 Houzelstein et al., 2002; Shang et al., 2009), so the complex analysis of the role of
525 particular BET proteins in vivo is currently not possible.

526 The limitation of this study is that it used a mouse model of systemic inflammation
527 to explore aspects of human diseases. As previously suggested, inflammatory responses in
528 mouse models show a limited correlation with human conditions, and the underlying
529 regulatory mechanisms may differ significantly (Diehl and Boyle, 2018; Seok et al., 2013).
530 However, other studies have indicated that some genomic responses during an
531 inflammatory challenge show significant similarities between humans and mice, even
532 though distinct regulatory mechanisms may be involved in the conserved transcriptional
533 responses (Shay et al., 2013; Takao and Miyakawa, 2015). Moreover, although our results

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534 enhance the understanding of acute and dynamic changes in Aβ levels and their
535 pharmacological regulators, the use of young mice limits the extrapolation of our findings
536 to the chronic processes involved in Alzheimer's disease. Therefore, further long-term
537 studies are needed to assess their clinical relevance. Furthermore, although our attempts to
538 elucidate the mechanism by which JQ1 reduces Aβ levels have been inconclusive, we have
539 ruled out the impact on β- and γ-secretases, as well as the inhibition of NF-κB or
540 phagocytosis. Future studies should explore other potential mechanisms, such as
541 autophagy. Another limitation of our study is that we only tested male animals to minimize
542 the variability associated with the estrous cycle in females. However, as AD is more
543 prevalent in females, future studies should investigate both sexes to obtain more
544 comprehensive results. Additionally, our study focused on acute changes, limiting our
545 analysis to the short-term effects of JQ1 on animal behavior. Given that we previously
546 observed cognitive impairment in mice persisting up to 7 days after LPS administration in
547 this same model, future studies should investigate whether BET inhibition can mitigate
548 these long-term effects.

549 **Conclusion**

550 Our results indicate that inhibiting BET proteins is an effective strategy for
551 attenuating the inflammatory response in both the periphery and the brain, including the
552 alleviation of sickness behavior. Moreover, the inhibitor of BET proteins, JQ1, has been
553 demonstrated to decrease the brain level of Aβ, suggesting that inhibition of BET proteins
554 may be considered a potential anti-inflammatory and anti-amyloidogenic treatment.
555 Inhibitors of BET proteins affect the expression of numerous genes, and their long-term
556 effects may be extensive. Nevertheless, their widespread use in clinical trials for cancer
557 highlights the potential of BET proteins as promising therapeutic targets.

558 **Ethics approval**

559 All experiments conducted on animals were approved by the II Local Ethics Committee
560 for Animal Experimentation in Warsaw (permission WAW2/060/2020), and were carried
561 out following the EU Directive 2010/63/EU for animal experiments, and complied with
562 ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All efforts
563 were made to minimize animal suffering and to reduce the number of animals.

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564 **Consent for publication**

565 Not applicable.

566 **Data Availability**

567 The data supporting the findings of this study are available in the RepOD repository at
568 https://repod.icm.edu.pl/dataset.xhtml?persistentId=doi:10.18150/I3B5JT

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572 **Declaration of interests**

573 The authors declare that they have no known competing financial interests or personal
574 relationships that could have appeared to influence the work reported in this paper.

575 **Acknowledgments**

576 Not applicable.

577

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Figure captions

Figure 1: The effect of JQ1 on sickness behavior after LPS-evoked systemic inflammation.

(a) Experimental design. Mice were injected with JQ1 (50 mg/kg b.w.) and after 30 min with LPS (1 mg/kg b.w.). Each group received corresponding volumes of vehicles. Sickness behavior was analyzed up to 12 hours post-injection. The level of cytokines in serum and mRNA level in the hippocampus was analyzed 3 and 12 h post-injection. The Aβ load was determined 12 h post-injection. (b) Sickness behavior (SB) index (MSS) was determined every 3 hours after administration of LPS until the end of the experiment. The total body weight change was determined at 3 h (c) and 12 h (d) after administration of LPS. **,*** $p<0.01$ and 0.001, comparing to respective group: corresponding time point control in (b) or bracket-indicated group in (d). ### $p<0.001$, comparing to respective (corresponding time point) LPS-treated group, n=8-17 (b) and n=7-9 (c and d). Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test.

Figure 2: The effect of JQ1 on cytokine levels in blood serum after LPS-evoked systemic inflammation.

Mice were injected with JQ1 (50 mg/kg body weight) and after 30 min with LPS (1 mg/kg body weight). The respective groups received the respective vehicle volumes. Cytokine levels in blood serum were determined using the multiplexing method and are presented as a heatmap. The control groups at 3 and 12 h post-injection did not differ statistically; therefore, they were combined into one common control group. Statistical analyses were performed on the raw data before normalization. *,**,*** $p<0.05$, 0.01 and 0.001, compared to control. #, ##,### $p<0.05$, 0.01, and 0.001, respectively, compared to the LPS-treated group, n=3-8. Statistical analyses were conducted using one-way ANOVA followed by the Bonferroni post hoc test.

Figure 3: The effect of LPS-evoked systemic inflammation on the level of BET proteins in the murine hippocampus.

Mice were injected with LPS (1 mg/kg body weight) or the respective volumes of vehicle. The levels of BET proteins in the hippocampus were determined 12 h post-injection using

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609 ELISA and normalized to the total protein level. ** $p<0.01$, compared to control, n=5-6.
610 Statistical analyses were conducted using the Student's t-test (Brd2, Brd3) or Mann-
611 Whitney test (Brd4), selected based on data distribution.
612
613 **Figure 4: The effect of LPS and JQ1 on expression of inflammation-related genes in**
614 **hippocampus.**
615 Mice were injected with JQ1 (50 mg/kg body weight) and after 30 min with LPS (1 mg/kg
616 body weight). The respective groups received the respective vehicle volumes. The mRNA
617 levels in the hippocampus were determined using qPCR at 3 h (a) and 12 h (b) after LPS
618 administration. *,**,*** $p<0.05$, 0.01, and 0.001, comparing to respective control. ##, ###
619 $p<0.01$, and 0.001, respectively, compared to the respective LPS-treated group, n=5-9 (a)
620 and 7-9 (b). Statistical analyses were conducted using one-way ANOVA followed by the
621 Bonferroni post hoc test. RQ, relative quantification.
622
623 **Figure 5: The effect of JQ1 on Tau level and phosphorylation in hippocampus after**
624 **LPS-evoked systemic inflammation.**
625 Mice were injected with JQ1 (50 mg/kg body weight) and after 30 min with LPS (1 mg/kg
626 body weight). The respective groups received the respective vehicle volumes. The level
627 and phosphorylation of Tau in the hippocampus were determined using Western blotting
628 with Gapdh as a loading control. The immunoreactivity of Tau (a) was normalized to the
629 Gapdh level, and the immunoreactivity of phospho-Tau (b-d) was normalized to the total
630 Tau level. * $p<0.05$, Statistical analyses were conducted using one-way ANOVA followed
631 by the Bonferroni post hoc test. n=5-6 (a,d) and n=6 (b,c). (e) Typical images were
632 presented. n.s. – not significant,
633
634 **Figure 6: The effect of JQ1 on Amyloid-β level in hippocampus 12 h after LPS-evoked**
635 **systemic inflammation.**
636 Mice were injected with JQ1 (50 mg/kg body weight) and after 30 min with LPS (1 mg/kg
637 body weight). The respective groups received the respective vehicle volumes. The levels
638 of $A\beta_{1-40}$ (a) and $A\beta_{1-42}$ (b) in the hippocampus were determined using ELISA. **,***
639 $p<0.01$ and 0.001, n=5-6. Statistical analyses were conducted using one-way ANOVA

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640 followed by the Bonferroni post hoc test. (c) Analysis of the correlation between $A\beta_{1-40}$
641 and $A\beta_{1-42}$ in the hippocampus. Dotted lines represent 95% confidence intervals.
642
643 **Figure 7: The effect of JQ1 on expression of phagocytosis-related genes in**
644 **hippocampus.**
645 Mice were injected with JQ1 (50 mg/kg body weight) and NaCl after 30 min, as described
646 above. The control group received the respective vehicle volume. Hippocampal mRNA
647 levels were determined at 3 h (a) and 12 h (b) post-injection using qPCR. n=6-8 (a) and 6-
648 9 (b). Statistical analyses were conducted using one-way ANOVA followed by the
649 Bonferroni post hoc test. RQ, relative quantification.
650

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651 **Tables**

652 **Table 1** The curated analysis of the effect of JQ1 on the expression of phagocytosis-related
653 genes in BV2 and HMC3 lines

654 The curated analysis of the RNA sequencing data by Baek and co-workers (Baek et al.,
655 2021) that have been deposited in NCBI's GEO database and are accessible through GEO
656 Series accession number GSE155408
657 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155408). A summary of the
658 qPCR data by Matuszewska and co-workers (Matuszewska et al., 2022) was added for
659 comparison purposes. nc, not changed; nd, not detected. Details presented in
660 Supplementary Table 3.

	murine BV2 line (Matuszewska et al., 2022) (50 nM JQ1, 24 h)	human HMC3 line (Baek et al., 2021) (500 nM JQ1, 4 h)
Abca7	nc	upregulation
Bin1	nc	downregulation
Cd2ap	nc	nc
Cd33	downregulation	nd
Clu	nc	upregulation
Cr1l	nc	nc
Picalm	nc	nc
Rin3	nc	downregulation
Trem2	downregulation	nc
Zyx	nc	nc

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663 **Glossary:** Ab, antibody; AD, Alzheimer's disease; Aβ, amyloid-beta; BCA, bicinchoninic
664 acid; BET, bromodomain and extraterminal domain proteins; DMSO, dimethyl sulfoxide;
665 EOAD, early onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; LPS,
666 lipopolysaccharide; MSS, murine sepsis score; nc, not changed; nd, not detected; NFT,
667 neurofibrillary tangles; NSAID, non-steroidal anti-inflammatory drugs; RIPA,
668 radioimmunoprecipitation assay; SP, sickness behavior.

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Figure 1

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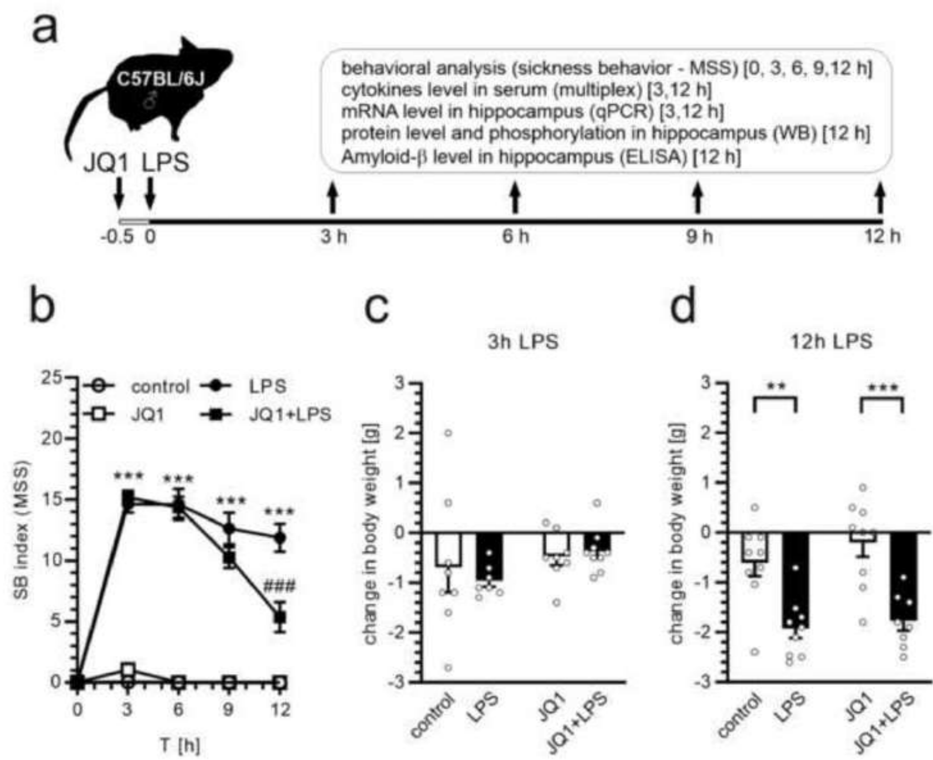

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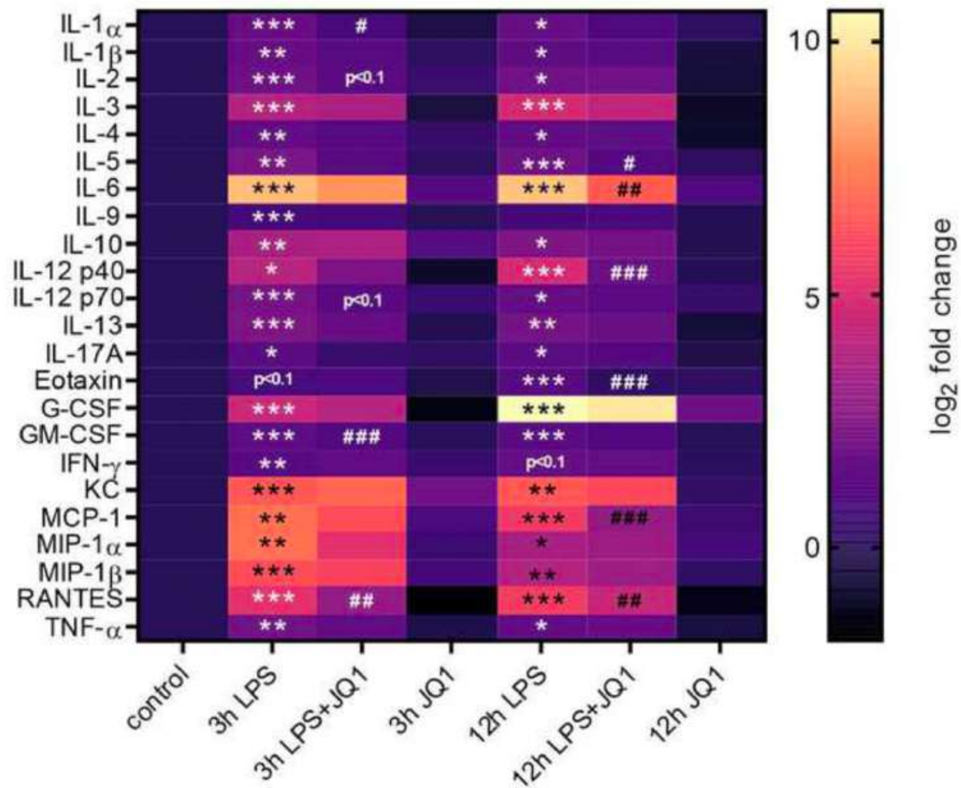

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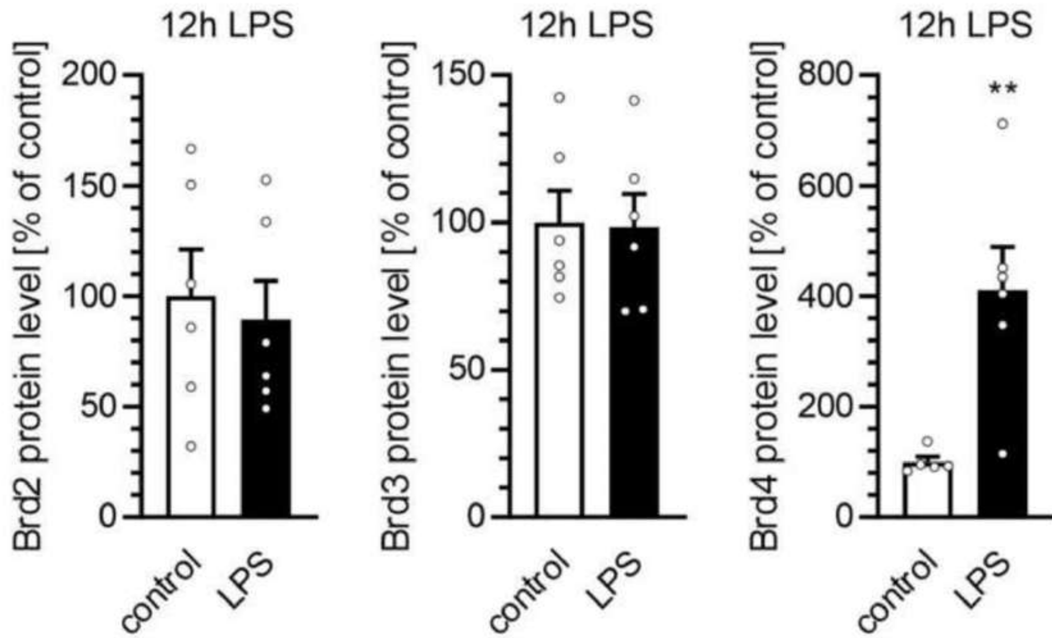

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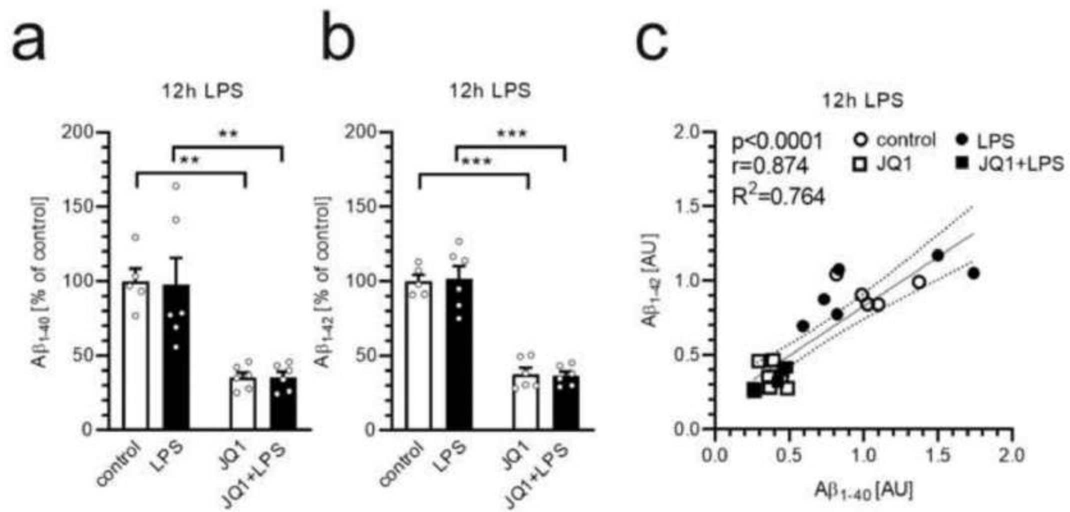

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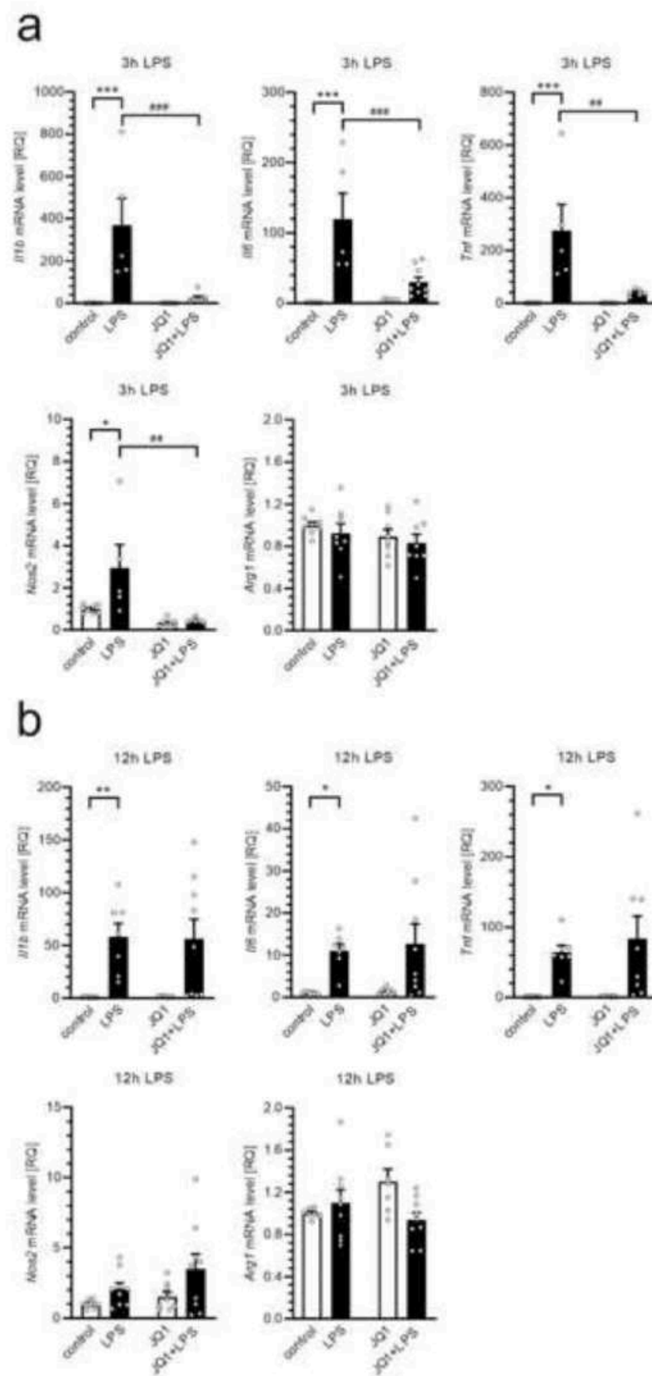

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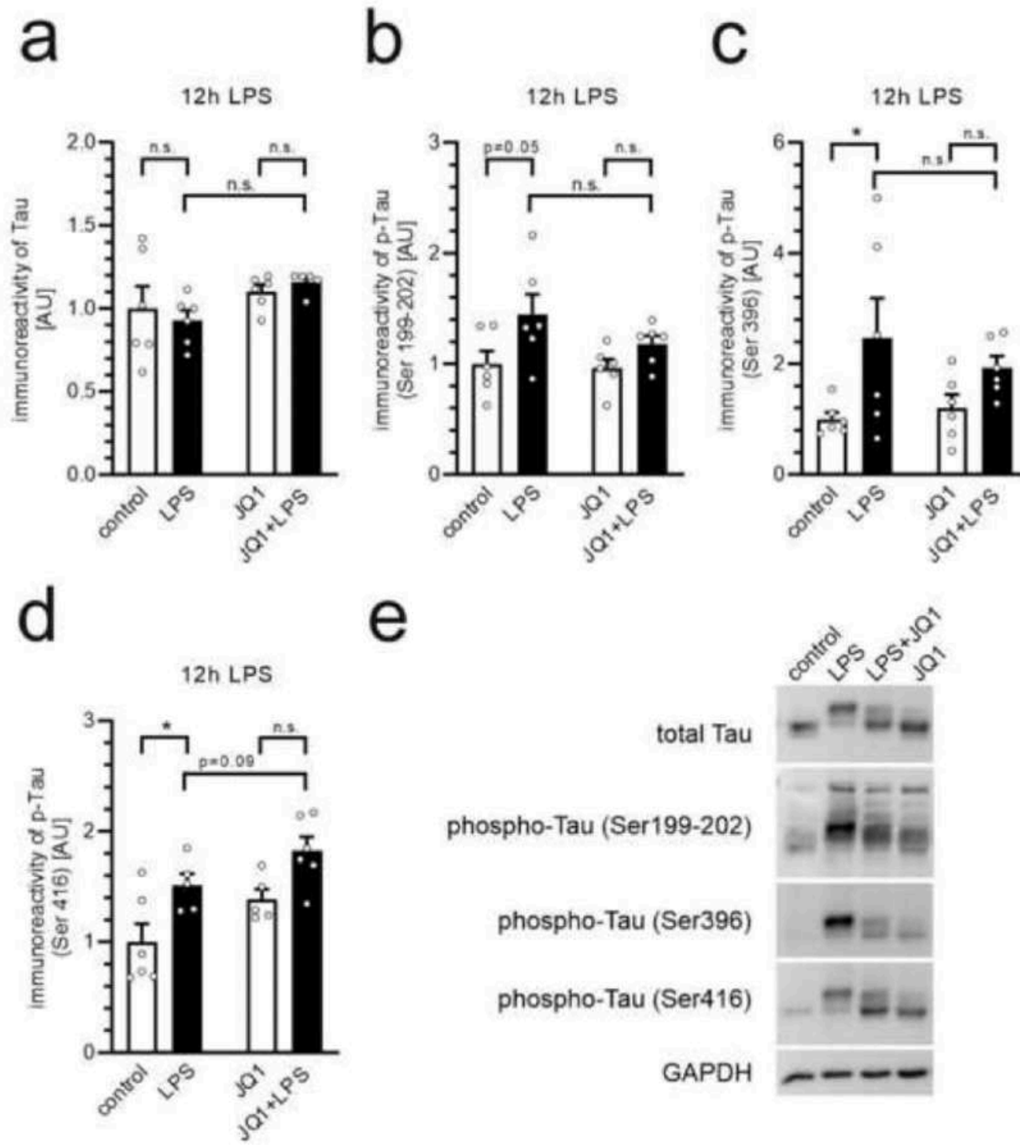

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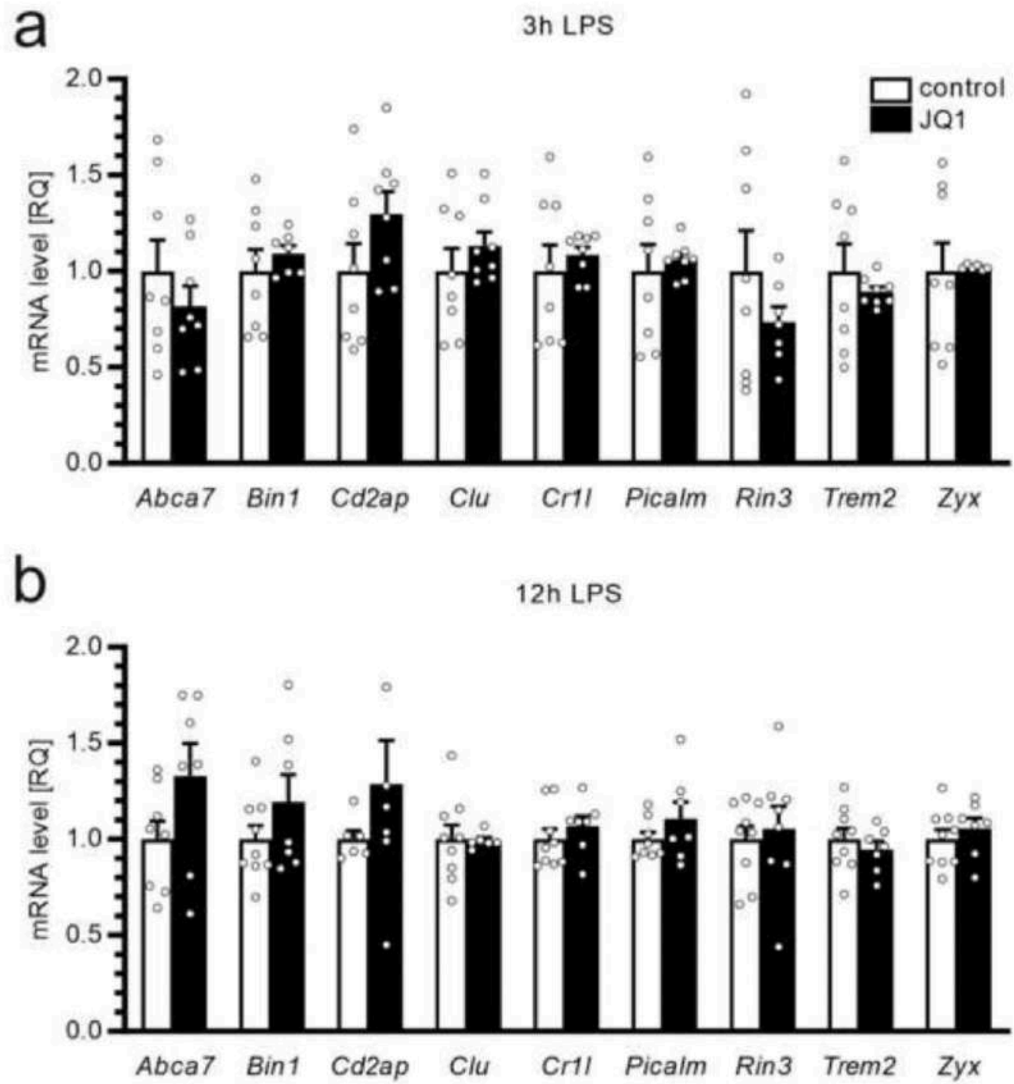

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Declaration of Interest Statement (Editable version)

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Title page (with Author Information)

Research Article

BET protein inhibitor JQ1 reduces inflammation and hippocampal amyloid-β level without altering Tau phosphorylation in LPS-challenged adult wild-type mice

Marta Matuszewska[a]
Magdalena Cieślik[a]
Dorota Sulejczak[b]
Anna Wilkaniec[a]
Grzegorz A. Czapski[a]

[a] Department of Cellular Signalling, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland;
[b] Department of Experimental Pharmacology, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Correspondence: Grzegorz A. Czapski
Mossakowski Medical Research Institute, Polish Academy of Sciences
Pawińskiego 5
02-106 Warsaw
Poland
Email: gczapski@imdik.pan.pl

http://rcin.org.pl

Oświadczenia autorów

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
mgr inż. Marta Matuszewska

e-mail: mmatuszewska@imdik.pan.pl

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Oświadczenie współautora

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- PUBLIKACJA 1.
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- PUBLIKACJA 2.
  Czapski, G.A.; **Matuszewska, M.**; Cieślik, M.; Strosznajder, J.B. Inhibitor of bromodomain and extraterminal domain proteins decreases transcription of Cd33 in the brain of mice subjected to systemic inflammation; a promising strategy for neuroprotection. Folia Neuropathol. 2024, 62(2), 127–135, doi: 10.5114/fn.2024.138140.

- PUBLIKACJA 3.
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- PUBLIKACJA 4.
  Matuszewska, M.; Wilkaniec, A.; Gąssowska-Dobrowolska, M.; Cieślik, M.; Olech-Kochańczyk, G.; Pałasz, E.; Gawinek, E.; Strawski, M.; Czapski, G.A. Inhibition of BET proteins modulates amyloid-beta accumulation and cognitive performance in middle-aged mice prenatally exposed to maternal immune activation. Front. Mol. Neurosci. 2025, 18, 1619583, doi: 10.3389/fnmol.2025.1619583.

Wkład obejmuje planowanie, tworzenie koncepcji oraz założeń merytorycznych prac, adaptację metod badawczych do warunków eksperymentalnych, realizację badań, analizę i interpretację wyników, przygotowanie manuskryptów i odpowiedzi na recenzje.

Marta Matuszewska

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr hab. Grzegorz Czapski, prof. IMDiK

e-mail: gczapski@imdik.pan.pl

Warszawa, 23.09.2025 r.

Oświadczenie współautora

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Wkład obejmuje opiekę merytoryczną i nadzór nad realizacją badań, udział w planowaniu i wykonywaniu badań, tworzeniu koncepcji badań i założeń merytorycznych badań, analizie i interpretacji wyników, redakcji i korekcie manuskryptów oraz odpowiedzi na recenzje, a także pozyskiwanie środków na badania oraz zarządzanie projektem.

Wyrażam zgodę na wykorzystanie wyżej wymienionych publikacji w przewodzie doktorskim mgr inż. Marty Matuszewskiej.

Grzegorz Czapski

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Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Magdalena Cieślik

e-mail: mcieslik@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

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Wkład obejmuje udział w tworzeniu koncepcji badań i założeń merytorycznych prac, wykonywaniu doświadczeń, udział w korekcie manuskryptów.

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Magdalena Cieślik

Magdalena Cieślik

http://rcin.org.pl

Warszawa, 08.08.2025 r.

dr Marcin Strawski
Wydział Chemii UW
ul. Pasteura 1, 02-093 Warszawa
marcin@chem.uw.edu.pl

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

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Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Anna Wilkaniec

e-mail: awilkaniec@imdik.pan.pl

Warszawa, 08.08.2025 r.

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- Matuszewska, M.; Cieślik, M.; **Wilkaniec, A.**; Strawski, M.; Czapski, G.A. The Role of Bromodomain and Extraterminal (BET) Proteins in Controlling the Phagocytic Activity of Microglia In Vitro: Relevance to Alzheimer's Disease. Int. J. Mol. Sci. 2023, 24(1), 13, doi: 10.3390/ijms24010013.

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Wkład obejmuje udział w tworzeniu koncepcji badań i założeń merytorycznych prac, adaptacja metod badawczych do warunków eksperymentalnych, udział w wykonywaniu doświadczeń, udział w korekcie tekstu manuskryptów.

Wyrażam zgodę na wykorzystanie wyżej wymienionych publikacji w przewodzie doktorskim mgr inż. Marty Matuszewskiej.

A. Wilkaniec

Anna Wilkaniec

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
prof. dr hab. Joanna Strosznajder

e-mail: jstrosznajder@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

- Czapski, G.A.; Matuszewska, M.; Cieślik, M.; **Strosznajder, J.B.**
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Joanna Strosznajder

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr hab. Magdalena Gąssowska-Dobrowolska

e-mail: mgassowska@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

- Matuszewska, M.; Wilkaniec, A.; **Gąssowska-Dobrowolska, M.**; Cieślik, M.; Olech-Kochańczyk, G.; Pałasz, E.; Gawinek, E.; Strawski, M.; Czapski, G.A. Inhibition of BET proteins modulates amyloid-beta accumulation and cognitive performance in middle-aged mice prenatally exposed to maternal immune activation. Front. Mol. Neurosci. 2025, 18, 1619583, doi: 10.3389/fnmol.2025.1619583.

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Magdalena Gąssowska-Dobrowolska

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
mgr Elżbieta Gawinek

e-mail: egawinek@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

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Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Marty Matuszewskiej.

Elżbieta Gawinek

Elżbieta Gawinek

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Gabriela Olech-Kochańczyk

e-mail: gkochanczyk@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

- Matuszewska, M.; Wilkaniec, A.; Gąssowska-Dobrowolska, M.; Cieślik, M.; **Olech-Kochańczyk, G.**; Pałasz, E.; Gawinek, E.; Strawski, M.; Czapski, G.A. Inhibition of BET proteins modulates amyloid-beta accumulation and cognitive performance in middle-aged mice prenatally exposed to maternal immune activation. Front. Mol. Neurosci. 2025, 18, 1619583, doi: 10.3389/fnmol.2025.1619583.

Wkład obejmuje udział w wykonywaniu doświadczeń (analiza immunohistochemiczna), adaptację metod badawczych do warunków eksperymentalnych, udział w korekcie tekstu manuskryptu.

Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Marty Matuszewskiej.

Gabriela Olech-Kochańczyk

Gabriela Olech-Kochańczyk

http://rcin.org.pl

Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Ewelina Pałasz

e-mail: epalasz@imdik.pan.pl

Warszawa, 08.08.2025 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

- Matuszewska, M.; Wilkaniec, A.; Gąssowska-Dobrowolska, M.; Cieślik, M.; Olech-Kochańczyk, G.; **Pałasz, E.**; Gawinek, E.; Strawski, M.; Czapski, G.A. Inhibition of BET proteins modulates amyloid-beta accumulation and cognitive performance in middle-aged mice prenatally exposed to maternal immune activation. Front. Mol. Neurosci. 2025, 18, 1619583, doi: 10.3389/fnmol.2025.1619583.

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Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Marty Matuszewskiej.

Ewelina Pałasz

Ewelina Pałasz

http://rcin.org.pl