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The dual hit hypothesis of schizophrenia: Evidence from animal models

Cyprien G.J. Guerrin [a], Janine Doorduin [a], Iris E. Sommer [b], Erik F.J. de Vries [a],*

[a] *Department of Nuclear Medicine and Medical Imaging, University of Groningen, University Medical Centre Groningen, Hanzeplein 1, 9713, GZ, Groningen, the Netherlands*
[b] *Department of Biomedical Sciences of Cells and Systems, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713, GZ, Groningen, the Netherlands*

A B S T R A C T

Schizophrenia is a heterogeneous psychiatric disorder, which can severely impact social and professional functioning. Epidemiological and clinical studies show that schizophrenia has a multifactorial aetiology comprising genetic and environmental risk factors. Although several risk factors have been identified, it is still not clear how they result in schizophrenia. This knowledge gap, however, can be investigated in animal studies. In this review, we summarise animal studies regarding molecular and cellular mechanisms through which genetic and environmental factors may affect brain development, ultimately causing schizophrenia. Preclinical studies suggest that early environmental risk factors can affect the immune, GABAergic, glutamatergic, or dopaminergic system and thus increase the susceptibility to another risk factor later in life. A second insult, like social isolation, stress, or drug abuse, can further disrupt these systems and the interactions between them, leading to behavioural abnormalities. Surprisingly, first insults like maternal infection and early maternal separation can also have protective effects. Single gene mutations associated with schizophrenia did not have a major impact on the susceptibility to subsequent environmental hits.

1. Introduction

Schizophrenia is a major psychiatric disorder affecting approximately 0.7 % of the world's population. It is considered an important contributor to the global health economic and societal burden, due to high healthcare costs and the impact on the quality of life of affected individuals and their surroundings. The onset of schizophrenia occurs usually in early adulthood, with a more various age of onset in women. The disease is characterized by symptomatology classified into positive symptoms (hallucinations and delusions), negative symptoms (flattened emotion, social withdrawal, inability for goal-directed behaviour, and reduced motivation), mood symptoms, and cognitive deficits (Tandon et al., 2009). The onset and course of schizophrenia are not similar for men and women, with women having a later age of onset and more affective symptoms than men (Sommer et al., 2020). Since these differences are partly attributable to the protective effects of oestrogen, which may also play a role in rodents, rodent models often provide their findings for each sex separately.

The most common pharmacological treatment is antipsychotic medication which targets the dopaminergic neurotransmitter system and mainly ameliorates the positive symptoms of schizophrenia (Patel et al., 2014), though several countries are currently piloting non-pharmacological treatment. While antipsychotics are effective in treating acute psychosis and also reduce the chance for relapse when given as maintenance medication, important symptom clusters such as negative and cognitive symptoms do not benefit from this pharmacological approach. (Haddad and Correll, 2018; Tiihonen, 2016). Despite the extensive research on the pathophysiology of schizophrenia, the exact mechanisms underlying the syndrome remain unclear. A better understanding of the aetiology and pathophysiology of schizophrenia, particular of its molecular and cellular pathophysiology, is pivotal for the discovery of new therapeutic targets and the development of new drugs that can improve all symptoms.

A meta-analysis of genome-wide association studies showed that schizophrenia was associated with over 100 loci, related to dopamine synthesis, the immune system, glutamate receptors, and calcium channel regulation amongst others (Ripke et al., 2014). While none of these genes associated with schizophrenia has a significant impact on the risk to develop schizophrenia, several risk genes are often taken together in so called polygenic risk-scores that can increase the risk for specific aspects of schizophrenia, for example, treatment resistance (Gasse et al., 2019). Within a large population (the UK biobank) the polygenic risk

score for schizophrenia was found to be associated with cortical thinning (Alnæs et al., 2019).

In addition, pharmacological, brain imaging and post-mortem studies have observed increased presynaptic dopamine production (Howes and Murray, 2014), glutamatergic deviants, (Barch and Ceaser, 2012; Kantrowitz and Javitt, 2010; Moghaddam and Javitt, 2011), GABAergic deficits (Glausier and Lewis, 2017; Schmidt and Mirnics, 2014), low-grade immune activation (Doorduin et al., 2009; Fillman et al., 2016; Horváth and Mirnics, 2014), and the presence of increased oxidative stress (Do et al., 2015; Ng et al., 2008) as important contributing mechanism in schizophrenia. Alterations in these systems have repeatedly been associated with the symptoms of schizophrenia. For example, increased presynaptic dopamine synthesis has been associated with positive symptoms of schizophrenia (Stępnicki et al., 2018). The affected biological systems in schizophrenia likely interact with each other. For example, the dopaminergic system can be dysregulated by increased immune activation (Howes et al., 2013; Purves-Tyson et al., 2019), and increased immune activation can induce oxidative stress (Oskvig et al., 2012). Also, increased activation of the complement system can lead to higher microglia activation and more synaptic pruning (Druart et al., 2021; Germann et al., 2021). However, there is a gap in our understanding of the relationship between abnormalities in (the interaction between) these affected systems, and the behavioural symptoms of schizophrenia.

2. The dual hit hypothesis

Schizophrenia appears to have a multifactorial aetiology comprising a combination of genetic vulnerability, and environmental risk factors early and later in life (Modai and Shomron, 2016). These environmental factors alone seem to have a relatively weak impact on the individual and may not be sufficient to induce the development of schizophrenia. A concept called the dual hit hypothesis of schizophrenia proposes that genetic susceptibility (first hit) may prime the individual to become more responsive to environmental insults early or later in life (second hit) (Bayer et al., 1999; Maynard et al., 2001).

In addition to genetic factors, environmental factors early and later in life have been associated with an increased risk of developing schizophrenia. Early environmental factors include complications of delivery (caesarean section) and pregnancy (bleeding, preeclampsia and diabetes) (Cannon et al., 2002), abnormal foetal growth and development (Cannon et al., 2002), compromised prenatal environment (infection, malnutrition, antibiotic treatment and psychosocial stress during pregnancy) (al-Haddad et al., 2019a; Cattane et al., 2020; King et al., 2010), winter birth (Tochigi et al., 2004), childhood adversity and trauma (sexual, physical, and psychological abuse, neglect, parental death, bullying) (Varese et al., 2012), and growing up in an urban environment (Vassos et al., 2012). Later environmental factors include bullying at school and discrimination (Varese et al., 2012), early-onset drug abuse (Forti et al., 2014; Marconi et al., 2016), immigration (Bourque et al., 2011; Cantor-Graae and Pedersen, 2013), socioeconomic factors (Allardyce and Boydell, 2006; Byrne et al., 2004; Paksarian et al., 2015), social isolation and social defeat (social adversities, social trauma) (Stowkowy and Addington, 2012).

Epidemiological studies suggest that a combination of genetic, early and later environmental factors contribute to the development of schizophrenia (Stilo and Murray, 2019; Zwicker et al., 2018). These factors can have an additive effect (Stepniak et al., 2014) and each of these factors alone is usually not sufficient for disease induction (Stilo and Murray, 2010). A study by Guloksuz and colleagues observed an additive effect of genetic risk for schizophrenia and regular cannabis use, sexual abuse, emotional abuse, emotional neglect, and bullying (Guloksuz et al., 2019). A Danish study, including almost 1,000,000 individuals and about 10,000 schizophrenia patients, observed a significant synergic interaction between prenatal infection and peripubertal psychological trauma, which was stronger in men than in

women (Debost et al., 2017). A major limitation of epidemiological and clinical studies is that they cannot provide clues regarding the underlying mechanisms involved in the aetiology of schizophrenia. This is especially the case for studies on the dual hit hypothesis, as there are multiple variables for researchers to account for (e.g., severity of stress, the timing of exposure, number of stressors).

Animal studies enable the investigation of the mechanisms involved and allow for the assessment of preventive approaches and therapeutic interventions. Moreover, experimental variables (e.g., stimuli, severity, timing) can be controlled in rodent models. Therefore, several dual-hit rodent models, using different combinations of risk factors with different severities and at different ages (prenatal, early postnatal, adolescence, or adulthood), have been utilized for research on the aetiology of schizophrenia (Deslauriers et al., 2013; Gaskin et al., 2014; Giovanoli et al., 2013; Monte et al., 2017). Some models involve genetically selected animals to mimic the heritable vulnerability of patients, such as apomorphine-susceptible rats (Maas et al., 2020). Various

Table 1
Comparison between symptoms of schizophrenia and animal behaviour.

Symptoms of schizophrenia		Animal behaviour	Behavioural test
Positive	Hallucinations	Hallucination-like perceptions	Auditory startle response
	Delusions	Reality testing	Auditory conditioned stimulus paired with sucrose drinking
	Disorganized speech	N/D	N/D
	Abnormal motor behaviour	Hyperlocomotion, explorative behaviour	Open field test, drug-induced hyperlocomotion test
	Sensorimotor gating deficits	Sensorimotor gating deficits	Prepulse inhibition test
Negative	Avolition (emotional withdrawal, apathy, poor grooming, diminished emotional expression)	N/D	N/D
	Blunted affect (diminished facial and vocal expressions, poor eye contact)	N/D	N/D
	Alogia (avoid communication)		
	Asociality	Reduced social behaviour	Social interaction test, social preference test
	Anhedonia	Anhedonia-like behaviour	Sucrose preference test
	Anxiety, hypervigilance	Anxiety-like behaviour, hypervigilance	Open field test, elevated plus maze
	Mood problems, impulsivity, aggressivity	Learned helplessness, aggressive behaviour, vigilance behaviour	Forced swim test, general behaviour with peers (latency to attack, number of attacks)
Cognitive	Impaired verbal learning, goal-directed behaviour	N/D	N/D
	Working memory deficits, memory deficits	Working memory deficits, recognition memory deficits, fear motivated memory deficits	Novel object recognition test, Y-maze test, T-maze, Morris water maze test, fear motivated test
	Impaired attention	Impaired attention	Attentional learning test, latent inhibition test

N/D = not determined.

dual-hit models have mimicked behaviour related to schizophrenia (see Table 1) and showed variable vulnerability or resilience towards stress. Here, we review evidence supporting the dual-hit hypothesis of schizophrenia from studies in which animals were exposed to a combination of multiple environmental risk factors, or a combination of genetic and environmental risk factors. A summary of the genetic mutations and environmental stressors that have been investigated in these studies can be found in Fig. 1. To facilitate the interpretation of preclinical findings, a comparison between the symptoms of schizophrenia and the behavioural alterations in the dual hit rodent model of schizophrenia has been summarized in Table 1.

3. Environmental neurodevelopmental risk factors

Currently used dual-hit animal models consist of rodents exposed to the combination of two of the following factors: exposure to glutamate receptor antagonists, perinatal maternal infection or stress, neonatal maternal separation, social isolation, infection, or the administration of corticosterone, cannabis, or methamphetamine. Individual study findings can be found in Table 2.

3.1. Administration of glutamate receptor antagonists or methamphetamine combined with social isolation rearing

Administration of glutamate receptor antagonists, such as MK-801 and phencyclidine (PCP), or methamphetamine, either early in life or during adulthood, has been used as a model of schizophrenia. Glutamate receptor antagonists stimulate the release of dopamine and induce behavioural and mechanistic alterations related to schizophrenia. This model has been combined with social isolation rearing (SIR), an environmental risk factor of schizophrenia, to develop a model, which would potentially represent the complexity of schizophrenia more accurately (Table 2).

Glutamate receptor antagonists (PCP or MK-801) administration early in life (PND7−21) to SIR exposed rats induced a synergistic detrimental effect on hyperlocomotion, memory, prepulse inhibition (PPI), and social behaviour in adulthood (PND63−90) (Gaskin et al., 2016, 2014; Hamieh et al., 2021; Lim et al., 2012; Liu et al., 2017; Watson et al., 2016), but not at a younger age (PND50) (Liu et al., 2017). In addition to the behavioural deficits, the combination of both hits synergistically induced brain changes, such as alterations in GABA receptor signalling, dopamine receptor density and reduction in the volume of the medial prefrontal cortex (Gaskin et al., 2016; Gilabert-Juan et al., 2013; Shortall et al., 2020). In contrast, no synergistic effects of SIR and MK-801 administration on behaviour and molecular and cellular mechanisms were observed when the glutamate receptor antagonist was given later in life (PND56−63) (Ashby et al., 2010; Hawken et al., 2013; Hickey et al., 2012; Simpson et al., 2012, 2010).

Combining methamphetamine injection during adolescence (PND35−50) with SIR (PND21−77) did not exacerbate the PPI deficits, social withdrawal, and hyperlocomotion induced by either hit alone (Strauss et al., 2014). The levels of noradrenaline, serotonin, and dopamine in the striatum and the frontal cortex were not modified in any group, except for the single hit-induced increase in dopamine in the frontal cortex, which was reduced if the two hits were combined (Strauss et al., 2014).

These results suggest that SIR in combination with administration of a glutamate receptor antagonist early in life can have a synergistic detrimental effect on behaviour, molecular and cellular processes, which was not observed when the drugs were given later in life. The administration of the glutamate receptor antagonist early in life seems to disrupt neurodevelopmental processes. These processes include synaptogenesis, myelination, synaptic pruning, shape neural and axonal connections (Babikian et al., 2011; Chini and Hanganu-Opatz, 2021), which are known to be altered in schizophrenia (Flynn et al., 2003; Germann et al., 2021; Osimo et al., 2019).

3.2. Maternal immune activation in combination with peri-adolescent risk factors

Inflammation during pregnancy, induced by maternal infection, might act as a primer to make the offspring more susceptible to the effect of stress later in life. This section focuses on the potential priming effect of maternal inflammation on the response of the juvenile offspring to a stressor, such as unpredictable mild stress, restraint stress, social isolation, methamphetamine or cannabis administration (Table 2).

Poly I:C injection during pregnancy has been used as a model of maternal immune activation (MIA). While some studies observed that the combination of MIA in pregnant rodents with various stressors in the juvenile offspring was able to induce PPI deficits and hyperlocomotion (Deslauriers et al., 2014, 2013; Giovanoli et al., 2013), another study did not observe such synergistic effects (Yee et al., 2011). The combination of both hits did not exacerbate the anxiety-like behaviour, or the altered latent inhibition induced by either hit alone (Giovanoli et al., 2013).
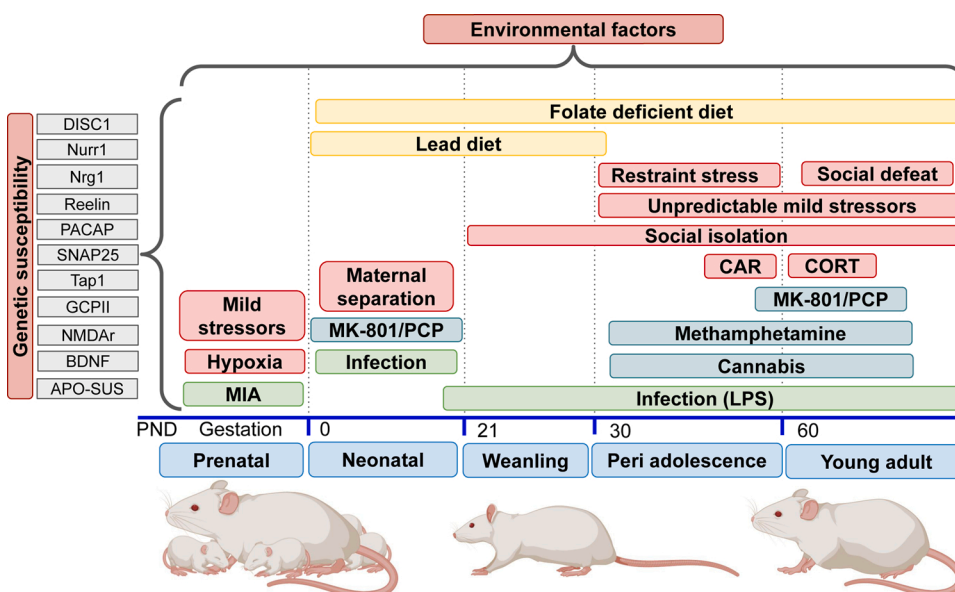
Fig. 1. Risk factors used in animal studies to investigate the dual-hit hypothesis of schizophrenia.

BDNF = Brain-derived neurotrophic factor, CAR = conditioning avoidance training, CORT = corticosterone, DISC1 = disrupted-in-schizophrenia, GCPII = Glutamate carboxy-peptidase II LPS = lipopolysaccharide, MIA = maternal immune activation, NMDAr = N-methyl-D-aspartate receptor, Nrg1 = Neuregulin1, Nurr1 = nuclear receptor-related 1 protein, PACAP = Pituitary adenylate cyclase-activating peptide, PCP = phencyclidine, PND = postnatal day, SNAP25 = Synaptosomal-associated protein-25, TAP1 = Transporter associated with antigen processing 1. Blue box: substance administration; Green box: infection; Red box: stressors; Yellow box: diet factors.

Table 2

Evidence of behavioural, molecular, and cellular alterations in environmental dual-hit animal models of schizophrenia.

First hit	Second hit	Synergistic behavioural effects	Synergistic molecular effects	Rescue experiments	Species	Reference
Social isolation rearing + administration of glutamate receptor antagonist or methamphetamine						
SIR PND23	PCP PND 7.9.11, 10 mg/kg	PND63-90: ↓PPI, fear motivated memory, reversal learning, social behaviour ↑locomotion	PND63: ↓Genes glutamate metabolism pathway, GABA receptor signalling, dopamine receptor signalling, glutamate release to 5-HT$_6$ antagonist, GABA cell number in the HP	Lamotrigine (glutamate release inhibitor, 10-15 mg/kg, IP, 1 h before behaviour), MMPIP (mGlu$_7$ antagonist, 10 mg/kg, IP, 30 min before behaviour), cariprazine (D$_2$/D$_3$ receptor partial agonists, 0.3 mg/kg, IP, 30 min before behaviour), aripiprazole (D$_2$ receptor partial agonists, 3 mg/kg, IP, 30 min before behaviour), and clozapine (D$_{1-4}$ receptor antagonist, 3 mg/kg, IP, PND63-90), but not SB-399885 (5-HT$_6$ antagonist, 10 mg/kg, IP, 30 min before behaviour), rescued the dual hit effect on brain and behaviour	Rats Lister-Hooded ♂	(Gaskin et al., 2016, 2014; Hamieh et al., 2021; Shortall et al., 2020; Watson et al., 2016)
SIR PND21	MK-801 PND7-10 once daily, 0.2 mg/kg IP	PND77-84-91: ↓PPI, recognition memory ↑locomotion	N/D	Clozapine (D$_{1-4}$ receptor antagonist 5 mg/kg, IP, PND77/PND84-94) rescued the dual hit effect on behaviour	Rats Sprague-Dawley ♂	(Lim et al., 2012)
SIR PND21	MK-801 PND7-21 once daily, 0.5 mg/kg IP	PND50: No synergistic effect on recognition memory, locomotion, PPI, anxiety-like behaviour	N/D	N/D	Rats Sprague-Dawley ♂	(Liu et al., 2017)
SIR PND21	MK-801 PND7, 1 mg/kg IP	N/D	PND77: ↓Volume of the mPFC ↑SIR/MK-801-induced decrease in GABA cells, ErbB4r expression in the mPFC	N/D	Rats Lister-Hooded ♂	(Gilabert-Juan et al., 2013)
SIR PND21	MK-801 PND56-63, 0.5 mg/kg IP twice daily	PND70: No synergistic effect on locomotion, social and anxiety-like behaviour, defensive burying, spatial memory, polydipsia	PND70: No synergistic effect on the single hit induced increased GABA$_a$ receptor expression and GABA transporter 1 activity in the HP and the FC	N/D	Rats Sprague-Dawley ♂	(Ashby et al., 2010; Hawken et al., 2013; Hickey et al., 2012; Simpson et al., 2012, 2010)
SIR PND21-77	METH PND35-50 ↑twice daily dose (0.2-6.0 mg/kg)	PND78: No synergistic effect on the single hit induced deficits in PPI, social behaviour, and hyperlocomotion	PND78: ↓SIR/METH-induced increase in dopamine in the FC No synergistic effect on noradrenalin and serotonin in the STR and FC, dopamine in the STR	N/D	Rats Wistar ♂	(Strauss et al., 2014)
Maternal infection + peri adolescent risk factors						
MIA poly I:C IP GD15 (20 mg/kg)	Restraint stress PND33-35 2 h/day	24 h post RS: ↓PPI	24 h post RS: ↑Oxidative stress in the PFC ↑MIA-induced increase dopamine D$_2$ receptors in the STR and PFC ↓GABA neurons in the STR and PFC	α-lipoic acid (antioxidant, IP, 50 mg/kg, 3 h before each stressor) prevented brain and behavioural alterations	Mice C57BL/6♂	(Deslauriers et al., 2014, 2013)
MIA poly I:C IV GD9 (1 mg/kg)	PND30-40, electric foot shock, restraint stress, swimming stress, water deprivation, repeated home cage changes	PND41-45: No synergistic effect PND70-100: ↓PPI, ↑locomotion in response to amphetamine/MK-801 No synergistic effect on anxiety-like behaviour and latent inhibition	PND41: ↑Activated microglia and IL-1β-cells in the HP and PFC ↑ IL-1β, TNF-α in plasma PND70: ↑Dopamine in the HP ↓GABA neurons (parvalbumin cells) in the vDG Restored GABA (reelin cells) in the dCA1-3	Minocycline (microglia activation inhibitor, 30 mg/kg per day, PND30-40) prevented brain and behavioural alterations except for anxiety	Mice C57BL/6♂♀	(Giovanoli et al., 2016, 2014, 2013)
MIA poly I:C IV GD15 (4 mg/kg)	PND27-29, forced swimming, elevated platform, restraint stress	PND60-70: No synergistic effect on the MIA-induce PPI deficits and increase anxiety-like behaviour	PND69: No synergistic effect on plasma pro-inflammatory cytokines	N/D	Rats Sprague-Dawley ♂	(Yee et al., 2011)
MIA poly I:C IP GD15 (10 mg/kg)	SIR PND22	PND50-130: Restored the SIR-induced impaired associative memory and reversal learning, reduced social behaviour, hyperlocomotion No synergistic effect on PPI	PND 90: Restored the SIR-induced elevated cytokine levels and mTOR activation in the HP and FC	N/D	Rats Lister-Hooded ♂	(Goh et al., 2020)
MIA poly I:C IP	SIR PND21	PND50: ↓Exploratory behaviour	N/D	Clozapine (D$_{1-4}$ receptor antagonist, IP, PND43-50 +		(Deslauriers et al., 2016)

Table 2 (*continued*)

First hit	Second hit	Synergistic behavioural effects	Synergistic molecular effects	Rescue experiments	Species	Reference
GD12 (20 mg/kg)		↑Aggressive behaviour, helplessness No synergistic effect on PPI, social and anxiety-like behaviour		30 min before behaviour) rescued the dual hit effect on behaviour	Mice C57BL/6♂	
MIA LPS SC GD15-16 (100 µg/kg)	METH PND 35-50 escalating dose 0.2-6 mg/kg twice daily	PND62-64: No synergistic effect on recognition memory, PPI, and social behaviour	PND65: ↓MIA-induced increase in TNF-α in plasma No synergistic effect on the single hit-induced reduction in noradrenaline and serotonin, and increase in dopamine, DOPAC and oxidative stress in the FC PND55: ↓5-HT$_{1A}$ receptor density, altered gene transcription involved in GABAergic and glutamatergic neurotransmission in the EC PND65-90: ↑5-HT$_{1A}$ receptors in the CA1 PND70-90: Restored MIA-induced decrease in the number of spontaneously active dopaminergic neurons in the VTA	N-acetylcysteine (150 mg/kg, SC, PND 51-64) rescued the dual hit effect on brain and behaviour	Rats Sprague-Dawley ♂	(Swanepoel et al., 2018)
MIA poly I:C IV GD15 (4 mg/kg)	Cannabis (HU210) PND35-50 100 µg/kg	N/D		N/D	Rats Wistar ♂	(Dalton et al., 2012; Hollins et al., 2016)
GD15 4.0 mg/kg poly I:C	Cannabis (THC) PND45-55 increasing dose 2.5-10 mg/kg	N/D		N/D	Rats Sprague-Dawley ♂	(Lecca et al., 2019)
Early post-natal or later in life infection + later risk factors						
Poly I:C PND5-7 IP 2 mg/kg	PND40-48, restraint stress, electric foot shock, swimming stress, water deprivation, restraint stress	PND60: ♂♀↓Social behaviour (<♂), ♀memory, PPI ♂♀↑Locomotion (<♀)	PND60: ♂♀↑Oxidative stress in the STR and PFC	N-acetylcysteine (220 mg/kg, oral gavage, PND 30-59) prevented brain and behavioural alterations	Rats Wistar♂ ♀	(Monte et al., 2020, 2017)
LPS PND3-5 IP 0.05 mg/kg	PND85-87, restraint stress, social isolation	PND85: ♂♀↑Vigilant behaviour, ♂anxiety	PND85: Blunted corticosterone response to hide box/OFT	N/D	Rats Wistar ♂ ♀	(Walker et al., 2009)
Poly I:C PND38-48 IP 20 mg/kg, 5 times	SIR PND25	PND60-80: No synergistic effect on PPI, recognition memory, spatial memory, social interaction	N/D	N/D	Rats Wistar ♂	(Lukasz et al., 2013)
Maternal separation (MS) + later risk factors						
MS 24 h PND3	SIR PND21	No synergistic effect on the MS-induced passive strategy in the forced swim test	No synergistic effect on the MS-induced increase in basal CORT levels	N/D	Rats Wistar ♂♀	(Vargas et al., 2016)
MS 24 h PND9	SIR PND21	PND 69: Prevented single hit-induced reduction in PPI	N/D	N/D	Rats Wistar ♂	(Ellenbroek and Cools, 2002a)
MS PND3-10 3 h daily	CAR PND49-56 /PCP (3 mg/kg) after CAR	PND49-56: MS + CAR: Restored MS-induced PPI deficits MS + PCP: ↑anxiety MS + CAR + PCP: ↓PPI	N/D	N/D	Rats Sprague-Dawley ♂♀	(Chen et al., 2011)
MS 24 h PND9	100 mg corticosterone SC implant weeks8-10	Week12: ↓Short/long term spatial memory, No synergistic effect on working memory, Restored single hit-induced reduction in PPI	Week12: ↓BDNF in the HP	N/D	Rats Wistar ♂	(Choy et al., 2009, 2008)
MS PND2-14, 3 h daily	50 mg/L corticosterone weeks8-10	N/D	Week16: ♂↑ D$_2$r, D$_3$r, BDNF expression and protein levels in mPFC	N/D	Rats Wistar ♂♀	(Hill et al., 2014)
MS 1 h daily, PND7-14	PND40-45, 30 min of restraint stress	N/D	PND46: ↑CaMKIIβ expression in the striatum	N/D	Rats Long-Evans ♂	(Novak et al., 2013)
MS PND2-14 3 h daily	METH 5 mg/kg SC PND60-70 4times daily	N/D	PND77: ↓♂(not♀) dopamine transporter in the STR	N/D	Rats Long-Evans ♂♀	(Hensleigh and Pritchard, 2015)
MS PND2-14 3 h daily	METH 1 mg/kg IP PND 33-36	N/D	PND52: ↓Proteins involved in cytoskeletal modification, energy metabolism, intracellular signalling, protein degradation, cellular growth in the Nac	N/D	Rats Sprague-Dawley ♂	(Dimatelis et al., 2012)
MS PND2-14 3 h daily	Cannabis (CP55,940), IP 0.2 mg/kg weeks8-10	Week 12: ↑♂ (not♀) Anhedonia No synergistic effect on single-hit induced changes	N/D	N/D	Rats Wistar ♂♀	(Klug and van den Buuse, 2012)

Table 2 (*continued*)

First hit	Second hit	Synergistic behavioural effects	Synergistic molecular effects	Rescue experiments	Species	Reference
MS PND1-14 3 h daily	Cannabis (dronabinol) 5-10 mg/kg IP PND35-48	in PPI, memory, locomotion, anxiety-like behaviour PND63-90: Prevented maternal separation-induced increase in consumption of morphine	PND63-90: Restored maternal separation/cannabis-induced ↓preproenkephalin mRNA (precursor of an endogenous analgesic) in the STR	N/D	Rats Long-Evans ♂	(Morel et al., 2009)
MS 24 h PND9	Cannabis (THC) PND35-45 (increasing dose 2.5-10 mg/kg)	PND65: ♀ (not♂): Prevented THC-induced decrease in recognition memory, restored maternal separation-induced increase in aggressive behaviour No synergistic effect on the single hit induced reduction in social behaviour and increased helplessness	PND82:♀Prevented THC-induced reduction in NMDA receptor density in the HP, restored maternal separation-induced increase in NMDA receptor density and reduction in D_2r density in the STR ♂ Restored maternal separation-induced reduction in D_1r density in the FC ♂♀No synergistic effect on the single hit-induced increase in the dopamine D_2r density in the FC and dopaminergic D_1r in the NAc	N/D	Rats Sprague-Dawley ♂♀	(Zamberletti et al., 2012)

Abbreviations: ♂ = male, ♀ = female, ↑ = increase, ↓ = decrease, 5-HT = serotonin, CaMKIIβ = Calcium/calmodulin-dependent protein kinase II beta, CAR = Conditioning avoidance training, CUMS = chronic unpredictable mild stress, DA = dopamine, DG = dentate gyrus, EC = entorhinal cortex, FC = frontal cortex, GD = gestational day, GSH = glutathione, HP = hippocampus, HT = hypothalamus, IDO = Indoleamine 2,3-dioxygenase, IP = intra peritoneal, IV = intra venous, KC = keratinocyte derived chemokine, LPS = lipopolysaccharide, METH = methamphetamine, MIA = maternal immune activation, MIP = macrophage inflammatory protein, MK-801 = dizocilpine, MS = maternal separation, mTOR = mammalian target of rapamycin, NA = noradrenalin, NAc = nucleus accumbens, N/D = not determined, PCP = phencyclidine, PFC = prefrontal cortex, PND = postnatal day, PPI = pre pulse inhibition PS = prenatal stress, RS = restraint stress, SC = subcutaneous, SIR = social isolation rearing, THC = tetrahydrocannabinol.

However, the combination of MIA and juvenile stressors caused a short-lasting (PND41), but not long-lasting (PND70−100), exacerbation of immune markers in plasma, hippocampus, and prefrontal cortex (Giovanoli et al., 2014, 2013; Yee et al., 2011). Furthermore, the combination of both hits increased dopamine levels in the hippocampus, increased oxidative stress and dopaminergic D_2 receptors, and reduced GABA neurons in the striatum, and prefrontal cortex (Deslauriers et al., 2014, 2013; Giovanoli et al., 2014). The combination of both hits also reduced GABA neurons in the ventral dentate gyrus but did not affect the increased GABA neurons in other hippocampal regions, or the increase in dopamine levels and the decrease in serotonin in the nucleus accumbens, observed in animals exposed to either hit alone (Deslauriers et al., 2014, 2013; Giovanoli et al., 2014).

MIA was protective against the negative effects of 4 weeks of SIR on hyperlocomotion, memory, and social behaviour (Goh et al., 2020). Furthermore, the combination of both hits did not alter PPI or anxiety-like behaviour but reduced exploratory behaviour and increased aggressive behaviour and helplessness (Deslauriers et al., 2016; Goh et al., 2020). In addition to the behavioural alterations, MIA reduced the SIR-induced elevated cytokine levels and mTOR activation in the hippocampus and frontal cortex (Goh et al., 2020).

MIA combined with methamphetamine exposure (PND35−50) did not exacerbate the single hit-induced deficits in PPI, memory and social behaviour, alterations in neurotransmitters (noradrenaline, serotonin, and dopamine) and increased oxidative stress in the frontal cortex (Swanepoel et al., 2018). However, the combination of both hits reduced the MIA-induced increase in the pro-inflammatory cytokine TNF-α in plasma (Swanepoel et al., 2018).

Cannabis administration during adolescence (PND35−50), as a second hit, altered the density of serotonin receptors, and the transcription of genes involved in GABAergic and glutamatergic neurotransmission in MIA exposed rats (Dalton et al., 2012; Hollins et al., 2016). However, cannabis administration during adolescence (PND45−55) blocked the MIA-induced decrease in the number of spontaneously active dopaminergic neurons in the ventral tegmental area, but not their firing rate (Lecca et al., 2019). Unfortunately, none of the studies have investigated whether the molecular effects of the combination of MIA and cannabis

administration during adolescence are correlated with behavioural abnormalities.

Altogether, these results suggest that maternal infection during pregnancy can be protective or prime the individual to become more responsive to adverse events later in life, such as exposure to stress or drug abuse. These results parallel the suggestion that a certain level of adversity in humans may increase resilience (Seery et al., 2013).

3.3. Early post-natal infection followed by exposure to risk factors later in life

To investigate the priming effect of infection during the third trimester of pregnancy in humans, occurring ex-utero in rodents, poly I:C was injected on PND5−7 followed by a second hit consisting of a combination of five unpredictable mild stressors on PND40−48 (Table 2) (Monte et al., 2020, 2017). Offspring displayed a synergistic adverse effect of the two hits, characterized by changes in PPI (females only), memory (females only), locomotion and social behaviour (more pronounced in males) and increased oxidative stress in the prefrontal cortex and the striatum on PND 60 (Monte et al., 2020, 2017). LPS infection on PND3−5 combined with three mild stressors on PND85−87 induced a blunted corticosterone response and increased anxiety-like behaviour in the offspring when compared to either single hit alone (Walker et al., 2009). Interestingly, combining multiple poly I:C injections later in life (PND38−48) with SIR had no synergistic effect on PPI, memory and social behaviour thus suggesting that early life, but not the peripubertal period, is a window of vulnerability during which the individual is more susceptible to infections. Taken together, these results also suggest that infections early in life can prime the individual to be more responsive to stressors later in life. In addition, male and female offspring seem to respond differently to the combination of both hits as the males displayed more social disruptions and females more cognitive deficits.

3.4. Maternal separation in combination with exposure to risk factors later in life

Childhood trauma, another risk factor of schizophrenia, can be mimicked in animals using maternal separation as a first hit (Table 2). Social isolation after weaning did not exacerbate the maternal separation-induced passive coping strategy and increased basal corticosterone levels in the plasma (Vargas et al., 2016). Furthermore, maternal separation alone induced PPI deficits which were absent if four weeks of SIR or conditioning avoidance training (PND49−56) were performed as a second hit (Chen et al., 2011; Ellenbroek and Cools, 2002a). Interestingly, the positive effects of conditioning avoidance training were abolished by PCP administration on the same day, as a third hit (Chen et al., 2011). PCP administration also increased the maternal separation-induced anxiety-like behaviour (Chen et al., 2011). Combining maternal separation with the administration of the stress hormone corticosterone from week 8–10 reduced the PPI deficits observed in animals exposed to either hit alone, decreased spatial memory but did not alter working memory (Choy et al., 2008). In addition to the behavioural deficits, the combination of maternal separation and corticosterone increased brain-derived neurotrophic factor, dopamine D_2 and D_3 receptors expression in the medial prefrontal cortex, decreased brain-derived neurotrophic factor mRNA levels in the hippocampus but did not modify the dopaminergic system in the striatum (Choy et al., 2009, 2008; Choy and van den Buuse, 2008; Hill et al., 2014). Maternal separation combined with restraint stress during adolescence (PND40−45) synergistically increased the expression of the calcium/calmodulin-dependent protein kinase II beta in the striatum (Novak et al., 2013).

Maternal separation combined with the administration of methamphetamine (PND60−70) decreased the expression of the dopamine transporter compared to single hits alone (Hensleigh and Pritchard, 2015). Moreover, maternal separation or the administration of methamphetamine (PND33−36) induced a reduction in proteins related to cytoskeletal modifications, energy metabolism, intracellular signalling, protein degradation, and cellular growth in the nucleus accumbens, which was exacerbated if both hits were combined (Dimatelis et al., 2012).

Maternal separation and cannabis administration during adulthood (week 8−10) induced a synergistic dual hit effect by increasing anhedonia but did not aggravate the single hit effects on anxiety, locomotion, working and recognition memories and PPI (Klug and van den Buuse, 2012). However, maternal separation and cannabis administration (THC or dronabinol) during adolescence was mutually protective, as maternal separation prevented the behavioural and molecular (glutamatergic and dopaminergic systems) alterations induced by cannabis administration and vice versa (Morel et al., 2009; Zamberletti et al., 2012).

Taken together, these results support the concept that a first developmental insult sensitizes the animals to a subsequent hit, but also show that each hit can negate the effects of the other. Maternal separation combined with the administration of methamphetamine, or a glutamate receptor antagonist induced a synergistic detrimental effect on brain processes. On the other hand, a mutually protective effect between maternal separation and other second hits (SIR, conditioning avoidance training, corticosterone, and cannabis) on behaviour and brain functions was observed.

4. Genetic predisposition combined with environmental risk factors

Genetically vulnerable individuals have a higher susceptibility to environmental stressors, resulting in a higher risk to develop schizophrenia (Uher, 2014). This part summarizes the preclinical studies that support this observation. Genetic rodent models of schizophrenia have been generated by downregulating the expression of genes related to schizophrenia in humans. To explore the dual hit hypothesis, these mutant animals were exposed to at least one neurodevelopmental stressor. Individual study findings can be found in Table 3.

4.1. Disrupted-in-schizophrenia 1 gene

The disrupted-in-schizophrenia 1 (DISC1) gene is a synaptic protein involved in neurodevelopment, neuro-signalling, and synaptic functioning and is implicated in schizophrenia (Johnstone et al., 2011; St Clair et al., 1990). Pregnant mice with a DISC1 mutation were more responsive to infection by poly I:C as a greater increase in pro-inflammatory cytokine IL-6 was observed when compared to wild-type mice (Lipina et al., 2013). The combination of DISC1 genetic predisposition and MIA or neonatal immune activation (poly I:C PND2−6) or SIR or lead-containing diet from birth induced positive, negative, and cognitive behavioural alterations, which were not observed when only one hit was applied (Table 3) (Abazyan et al., 2014; Fry et al., 2020; Ibi et al., 2010; Lipina et al., 2013). Moreover, the combination of DISC1 genetic mutation and neonatal immune activation resulted in a reduction in the number of parvalbumin-positive GABA cells in the medial prefrontal cortex (Ibi et al., 2010), which is also observed in schizophrenia patients (Kaar et al., 2019). Furthermore, the combination of the DISC1 gene mutation with a lead-containing diet increased the size of the lateral ventricles (week 12) (Abazyan et al., 2014). However, nine-weeks old DISC1 mutant mice exposed to a 20-day social defeat protocol displayed no synergistic or additive effects on behaviour (Table 3) (Haque et al., 2012). Dominant-negative DISC1 mutation in astrocytes combined with cannabis during adolescence (PND30−51), but not adulthood (PND70−91), synergistically reduced memory and parvalbumin-positive presynaptic inhibitory boutons in the CA3 region, and increased the nuclear factor kappa B cyclooxygenase-2 (NF-κB−COX2) proinflammatory signalling and glutamate secretion in the hippocampus (Jouroukhin et al., 2019). No effect of amphetamine administration during adolescence was found. The synergistic effect was not observed if the DISC1 mutation was performed in neurons, suggesting that individual vulnerability to cannabis is mediated by astrocytes. Taken together, these results suggest that mice with a DISC1 mutation are more susceptible when the environmental factors occur early, but not later, in life.

4.2. Nuclear receptor-related 1 gene

The nuclear receptor-related 1 (Nurr1) protein is an orphan nuclear receptor critical for the development and survival of mesencephalic dopaminergic neurons and was suggested to be a potential susceptibility gene in schizophrenia (Buervenich et al., 2000; Zetterström et al., 1997). Like DISC1 mutated pregnant mice, pregnant mice with a mutation in Nurr1 had a more severe MIA response, characterized by an increase in IL-6 and IL-6/IL-10 ratio, which was not observed in wild-type mice exposed to MIA (Vuillermot et al., 2012). The offspring of mice with a Nurr1 mutation and exposed to MIA or SIR displayed exaggerated alterations in PPI, attention and locomotion in adulthood (Eells Misler and Nikodem, 2006; Vuillermot et al., 2012). These behavioural alterations were associated with a reduction in dopaminergic neurons in the prefrontal cortex and dopaminergic D_2 receptors in the nucleus accumbens (Eells Misler and Nikodem, 2006; Vuillermot et al., 2012). Nurr1 heterozygous mice, exposed to the parasite *Toxoplasma gondii* on PND120 displayed hyperlocomotion but had normal PPI and anxiety-like behaviour, 6 weeks after exposure to the infection (Eells et al., 2015). These results suggest that mice with a Nurr1 mutation are more susceptible to the exposure of an early, but not later in life environmental factor.

4.3. Neuregulin 1 gene

Neuregulin (Nrg1) is involved in the regulation of the expression and activation of neurotransmitter receptors and synaptogenesis (Stefansson

Table 3
Evidence of behavioural, molecular, and cellular alterations in genetic and environmental dual-hit model of schizophrenia.

Genetic mutation	Second hit	Synergistic behavioural effects	Synergistic molecular effects	Rescue experiments	Species	Reference
Disrupted-in-schizophrenia (DISC1)						
DISC1 heterozygous	MIA: poly I:C (2.5 mg.kg) GD9	Week 16: ↓PPI, latent inhibition, recognition memory, social behaviour	N/D	Anti-IL-6 antibody (100 μg, co-injected with poly I:C) prevented behavioural alterations	Mice C57BL/6 J ♂	(Lipina et al., 2013)
DISC1 heterozygous	Neonatal immune activation: poly I:C 5 mg/kg SC daily PND2-6	Week 8: ↓Short term recognition memory, HP-dependent fear memory, social behaviour ↑locomotion, MK-801-induced hyperactivity	Week 8: ↓Parvalbumin-positive cells in the mPFC but not in the HP	N/D	Mice C57BL/6N♂	(Ibi et al., 2010)
DISC1 heterozygous	Social isolation week 5-8	Week12: ↑ Psychotic-like behaviour (increased licking for water in response to an auditory conditioned stimulus paired with sucrose solution)	N/D	N/D	Mice C57BL/6♂	(Fry et al., 2020)
DISC1 heterozygous	Lead diet from birth	Week 12-24: ♀ ↓PPI ♂♀↑MK-801-induced hyperlocomotion	Week12-24: ♀ ↑ Size of the lateral ventricles	N/D	mDISC1 mice ♂♀	(Abazyan et al., 2014)
DISC1 heterozygous	RSD daily week9-11	Week 9-11: Day 17-21: ↑ Anxiety No synergistic effect on anhedonia, helplessness, social behaviour, PPI, and latent inhibition	N/D	N/D	Mice C57BL/6♂	(Haque et al., 2012)
DISC1 dominant-negative in astrocytes	Cannabis (THC), 8 mg/kg, SC, daily, PND30-51	PND72: ♂♀↓recognition memory (<♂) No synergistic effect on fear conditioning	PND72: ♂↑ NF-κB-COX2 proinflammatory signalling in astrocytes, and glutamate secretion, in the HP ↓ Parvalbumin-positive presynaptic inhibitory boutons (not density or size) in the CA3	NS398 (COX-2 inhibitor, 10 mg/kg, daily SC 30 min before THC injections PND30-51) prevented cognitive and brain alterations	Mice C57BL/6 j ♂♀	(Jouroukhin et al., 2019)
Nuclear receptor related1 protein (Nurr1)						
Nurr1 heterozygous	Poly I:C GD17 2 mg/kg	PND75-120: ↓PPI, attentional learning ↑Locomotion	PND75-120: ↓Dopamine D₂R density in the NAc, dopamine cell number in the mPFC	N/D	Mice C57BL/6 ♂	(Vuillermot et al., 2012)
Nurr1 heterozygous	SIR PND21	Week 15: ↓PPI	Week 18: ↓DA in the NAc	N/D	Nurr1-null mice, N/S	(Eells Misler and Nikodem, 2006)
Nurr1 heterozygous	200 μg T. gondii PND120	Week 6 after infection: ♂♀↑Locomotion	N/D	N/D	Mice C57BL/6♂♀	(Eells et al., 2015)
Neuregulin1 (NRG1)						
Nrg1 heterozygous	MIA 5 mg/kg poly I:C IP GD9	PND35-45: Normal social behaviour PND90-135: No synergistic effect on the singe-hit induced reduction in social behaviour, PPI, and working memory	N/D	N/D	Mice C57BL/6 ♂♀	(O'Leary et al., 2014)
Nrg1 heterozygous	Cannabis (THC) 5-10 mg/kg IP acute 30 min before behaviour on week 22	Week 22: 30 min after THC injection: ↑ PPI, anxiety No synergistic effect on THC-induced reduction in social behaviour and locomotion	N/D	N/D	Mice C57BL/6♂	(Boucher Arnold et al., 2007)
Nrg1 heterozygous	Cannabis (CP55,940) 10 mg/kg IP daily 15 days from week 22	Week 22: Day1: ↑PPI Day7-15: ↑Anxiety, normal PPI	N/D	N/D	Mice C57BL/6 ♂	(Boucher et al., 2011)
Nrg1 heterozygous	Cannabis (THC) 10 mg/kg IP PND31-52	PND31: ↑PPI ↓ Locomotion PND53: Prevented THC-induced reduction in social behaviour, normal PPI	PND51: ↑NMDA receptor in the HP Prevented THC-induced reduction in 5-HT₂ₐ receptor density in the insular cortex, caudate and putamen nuclei, Cg, ventral pallidum and CB₁ receptor binding in the SN	N/D	Mice C57BL/6 ♂	(Long et al., 2013)
Reelin						
Reelin heterozygous	50 mg/L, corticosterone weeks6-9	Week 11: ♂♀↓Spatial working memory, but no change on recognition memory and social interaction	N/D	N/D	Mice C57BL/6♂♀	(Schroeder et al., 2015)

Table 3 (*continued*)

Genetic mutation	Second hit	Synergistic behavioural effects	Synergistic molecular effects	Rescue experiments	Species	Reference
Reelin heterozygous	Prenatal hypoxia (9% oxygen) GD17 2 h	♂ Restored corticosterone-induced PPI reduction Week 24: No synergistic effect on PPI, working memory, locomotion, anxiety-like behaviour	Week 24: No synergistic effect on blood flow in the HP and PFC	N/D	Mice C57BL/6, N/S	(Howell and Pillai, 2016)
Brain derived neurotrophic factor (BDNF)						
BDNF heterozygous	Cannabis (CP55.940) 6-9week IP 0.4 mg/kg	Week11: No synergistic effect on working memory, recognition memory, and PPI	N/D	N/D	Mice C57BL/6♀♂	(Klug and van den Buuse, 2013)
BDNF heterozygous	methamphetamine 6-9week IP escalating dose (1-4 mg/kg)	Week11-14: No synergistic effect on locomotion and PPI	N/D	N/D	Mice C57BL/6♀♂	(Manning and van den Buuse, 2013)
BDNF heterozygous	25 mg/L corticosterone, weeks6-9	Week11: ♂↓Working memory (not ♀)	Week11: ♂↑NMDA receptor in the dHP, but no change in BDNF mutation-induced reduction in NMDA receptor density in the vHP	N/D	Mice C57BL/6♀♂	(Klug et al., 2012)
Other genetic mutations						
Tap1 KO mice	Influenza A virus PND3-4	Month5-6: ↓PPI	PND7-13-24: ↑IDO in the brain PND13: ↑Kynurenic acid in the brain	N/D	Mice C57BL/6♂	(Asp et al., 2010)
GCPII heterozygous	Folate deficient diet PND0-sacrifice	PND90: ♂♀ Restored single hit-induced reduction in motor coordination, cognition, social interaction (no change in PPI observed)	N/D	N/D	Mice C57BL/6♂♀	(Schaevitz et al., 2012)
Ppp1r2-cre/fGluN1 knockout (NR1 KO)	SIR PND21	Week8-12: ♂ ↑Anxiety, anhedonia	Week8-16: ♂♀↓Parvalbumin neurons in mPFC and S1 cortex ↑ROS Impaired antioxidant defence system in the mPFC	Apocynin (antioxidant, 7.5microg/mL drinking water, PND14-sacrifice) prevented brain and behavioural alterations	Mice C57BL/6NTac ♂♀	(Jiang et al., 2013)
PACAP-null homozygous	4weeks old; 2 weeks of social isolation	Week6: ↑Locomotion, aggressiveness Week8: ♂↓PPI, social interaction	N/D	N/D	CD1 mice ♂	(Ishihama et al., 2010)
SNAP25 mutant	Prenatal stress GD11-17, restraint stress, open field, forced swim, cage change, social stress	No synergistic effect on locomotion, memory, and anxiety-like behaviour	N/D	Clozapine (D$_{1-4}$ receptor antagonist, 5 mg/kg, IP, 20 min before PPI) and haloperidol (D$_2$ receptor antagonist, 0.3 mg.kg, IP, 20 min before PPI) rescued the PPI deficits	Mice C57BL/6 ♂	(Oliver and Davies, 2009)
Apomorphine susceptible (APO-SUS)						
APO-SUS	Cocaine, 20 mg/kg IP or amphetamine 1 mg/kg IP	Month3: 15 min after drug injection: ↓PPI	N/D	Remoxipride (D$_2$ receptor antagonist, 5 mg/kg, IP, 30 min before PPI) rescued the PPI deficits	APO-SUS rats ♂	(van der Elst et al., 2006, 2007)

Abbreviations: ♂ = male, ♀ = female, ↑ = increase, ↓ = decrease, 5-HT = serotonin, APO-SUS = apomorphine susceptible, D$_2$r = dopamine D$_2$ receptor, DA = dopamine, DG = dentate gyrus, FC = frontal cortex, GCPII = Glutamate carboxypeptidase II, GD = gestational day, HP = hippocampus IDO = Indoleamine 2,3-dioxygenase, IP = intra peritoneal, IV = intra venous, MIA = maternal immune activation, NAc = nucleus accumbens, N/D = not determined, NF-κB−COX2 = Nuclear factor-kappa B-cyclooxygenase-2, NMDAr = N-methyl-ᴅ-aspartate receptor, PFC = prefrontal cortex, PND = postnatal day, PPI = pre pulse inhibition, ROS = reactive oxygen species, RSD = repeated social defeat, SC = subcutaneous, SIR = social isolation rearing, SNAP25 = synaptosomal-associated protein-25, SN = substantia nigra, Tap1 = transporter associated with antigen processing 1, THC = tetrahydrocannabinol.

et al., 2002), and is associated with an increased susceptibility for schizophrenia. Pregnant Nrg1 mutant mice did not have an exacerbated response to maternal infection (normal IL-6 levels) (O'Leary et al., 2014). No dual hit effect of Nrg1 mutation and MIA was observed on working memory, PPI and social behaviour in the offspring on PND35 or PND90 (O'Leary et al., 2014). Nrg1 mutant mice chronically exposed to cannabis during adolescence (PND31−52) or adulthood (PND154−198) displayed short term increased PPI, which quickly normalized on the seventh day of injection, and increased anxiety-like behaviour, but no changes in locomotion (Boucher Arnold et al., 2007, 2011; Long et al., 2013). Nrg1 mice were more resistant than wild-type animals to the negative effect of THC administration during adolescence (PND31−52) on social behaviour (Long et al., 2013). Furthermore, Nrg1 also prevented the THC-induced reduction in serotonin receptor-2$_A$ and cannabinoid receptor-1 density in the brain. However, the combination of Nrg1 and THC exposure increased the NMDA receptor density in the

hippocampus (Long et al., 2013). Overall, Nrg1 mutation does not appear to enhance the response to MIA and may even make the mice more resistant to the negative effects of cannabis administration during adolescence.

4.4. Reelin glycoprotein gene

Reelin glycoprotein is involved in synaptic plasticity and brain development and has been associated with schizophrenia (Ishii et al., 2016). In reelin-mutant mice, corticosterone administration (week 6−9) reduced spatial memory but neither hit alone nor their combination affected recognition memory or social interaction (Schroeder et al., 2015). Moreover, the deleterious effects of corticosterone on PPI in wild-type mice were normalized in reelin-mutant male mice (Schroeder et al., 2015). Six months old reelin-mutant mice exposed to prenatal hypoxia (2 h, GD17) did not have any significant changes in PPI,

working memory, locomotion, and anxiety-like behaviour compared to wild-type mice and normoxia controls (Howell and Pillai, 2016). These results suggest that some neurodevelopmental stressors do not induce a synergistic effect in the reelin-mutant mice. In fact, the combination of a reelin mutation and environmental factors can even be mutually protective.

4.5. Brain-derived neurotrophic factor gene

Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in brain development, neuroplasticity, neurotransmission, and cognition and is implicated in the pathophysiology of schizophrenia (Autry and Monteggia, 2012). BDNF gene mutation and cannabinoid (CP55,940) or methamphetamine exposure (week 6−9) did not induce a synergistic effect on memory and PPI (Klug and van den Buuse, 2013; Manning and van den Buuse, 2013). In contrast, exposure of 6–9-week-old BDNF-mutant mice to corticosterone induced a reduction in working memory, accompanied by an increased NMDA receptor density in the dorsal hippocampus but not by a further exacerbation of the BDNF-induced reduction in NMDA receptor density in the ventral hippocampus (Klug et al., 2012). Taken together, these results suggest stress exposure (mimicked by corticosterone administration) might have some synergistic effects with BDNF gene mutation, but no synergistic effects were observed for substance abuse (methamphetamine or cannabis) in BDNF-mutant mice.

4.6. Other genetic mutations

The transporter associated with antigen processing 1 (TAP1) gene, a gene involved in the expression of MHC class I of the immune system, has been associated with schizophrenia (McAllister, 2014). TAP1 knock-out mice exposed to influenza A virus early in life (PND3−4) displayed reduced PPI in week 22 and activation of the kynurenine pathway, suggesting an increased conversion of tryptophan into kynurenic acid, rather than serotonin (Asp et al., 2010).

The one-carbon metabolic and glutamatergic pathways are connected. Folate is a substrate of the former and its depletion, often observed in schizophrenic patients, increases glycine and homocysteine concentrations that can act as agonists of the NMDA receptor and therefore increase glutamatergic neurotransmission (Deth et al., 2008; Sugden, 2006). Glutamate carboxypeptidase II (GCPII) is an enzyme involved in the one-carbon metabolic pathway. GCPII catalyses the production of glutamate by cleaving derivatives of folate. GCPII depletion, often observed in schizophrenic patients, would result in a reduction of the NMDA receptor activity (Guilarte et al., 2008). Investigating how folate deficiency and GCP II depletion, two risk factors of schizophrenia, interact may allow a better understanding of the underlying mechanisms of schizophrenia. While heterozygous GCPII mice fed with a folate-deficient diet (dual hit animals) performed similar to control mice, mice exposed to a single hit alone showed altered motor coordination, cognition and social behaviour (Schaevitz et al., 2012). These results suggest that the folate-deficient diet and the GCPII mutation are mutually protective (Schaevitz et al., 2012). A possible explanation is that folate deficiency increases the activation of the NMDA receptor and results in an increase in glutamate release, while a mutation in GCPII will decrease the glutamate release and induce a hypoactivity of the NMDA receptor. Therefore, the combination of both hits is mutually compensatory and results in normalization of the NMDA receptor activity.

NMDA receptor hypofunction on GABAergic interneurons has been associated with schizophrenia (Cohen et al., 2015; Nakazawa et al., 2012). Postnatal deletion of NMDA receptors in cortical interneurons combined with SIR in male mice resulted in increased anxiety-like behaviour, and anhedonia, but no additional alterations in working memory or PPI deficits. In addition to these alterations, increased oxidative stress, an impaired antioxidant defence system, and a reduced

number of interneurons in the medial prefrontal cortex were observed in the mutant mice exposed to SIR, but not in mice exposed to only one hit (Jiang et al., 2013).

Pituitary adenylate cyclase-activating peptide (PACAP), a susceptibility gene associated with schizophrenia, is a neuropeptide regulating cell proliferation, differentiation, and metabolism (Hashimoto et al., 2007). Four-weeks old homozygous PACAP-null mutant mice exposed to 2 weeks of social isolation displayed increased baseline locomotion and aggressiveness after the isolation, but no increase in the PPI deficits induced by social isolation alone (Ishihama et al., 2010).

Synaptosomal-associated protein-25 (SNAP25) is involved in neurotransmitter release and is implicated in schizophrenia (Lewis et al., 2003). Mutant mice expressing a defective SNAP25 protein exposed to prenatal stressors (GD11−17) displayed synergistic alterations in PPI and social behaviour. However, the combination of both hits did not modify exploratory behaviour, anxiety, and memory (Oliver and Davies, 2009).

In summary, single gene mutations in one of the many genes associated with schizophrenia do not have a major impact on the susceptibility to a second hit. This parallels the human situation where a single risk gene has hardly any impact on the individual risk to develop schizophrenia. Only many genes acting in concert in polygenic risk profiles will impact an individual's risk status. While it is difficult to breed rodents with a large number of mutated genes, it may be more practical to selectively breed rodents on basis of specific traits. One of such traits could be apomorphine susceptibility (van Schijndel et al., 2011).

The apomorphine susceptible (APO-SUS) rats are selected based on their exaggerated behavioural response to apomorphine and then bred together. Their phenotypic counterparts, the apomorphine unsusceptible (APO-UNSUS), are bred based on their lack of response to apomorphine. APO-SUS rats display behavioural features relevant to schizophrenia, such as decreased PPI (van der Elst et al., 2006), increased drug sensitivity and exploratory behaviour (Cools et al., 1993; Ellenbroek and Cools, 2000), reduced latent inhibition and sucrose preference (Ellenbroek et al., 1995; van Vugt et al., 2014), and cognitive and memory deficits (Maas et al., 2020; Tuinstra et al., 2000). Furthermore, APO-SUS rats are more susceptible to amphetamine and cocaine administration as these drugs strongly reduced PPI in APO-SUS rats but did not affect APO-UNSUS rats (van der Elst et al., 2006; Van Der Elst et al., 2007). In addition to behavioural abnormalities, APO-SUS rats display hyperactive HPA axis, elevated dopamine D2-receptor binding, and hypomyelinated GABAergic interneurons in the prefrontal cortex (Maas et al., 2020; Rots et al., 1996), features similar to those observed in schizophrenia patients (Du et al., 2013; Flynn et al., 2003; Hakak et al., 2001; Selten et al., 2016). These results suggest that the APO-SUS model is an idiopathic model of schizophrenia, thus allowing the study of the mechanisms underlying the aetiology of schizophrenia. Future studies should investigate the effects of early and later environmental stressors in this model. We predict a synergistic effect on behaviour and brain alterations related to schizophrenia.

5. Integration of the effects of dual hit exposure on brain mechanisms

In this review, we aimed to establish a clear picture of the combined effects of the developmental, environmental, and genetic risk factors of schizophrenia on behaviour and molecular mechanisms in animal models. Taken together, the studies suggest that combining several risk factors in animal models offers a stronger face validity for schizophrenia as it induces a broad range of behavioural abnormalities, some of them similar to those observed in patients suffering from schizophrenia. Each risk factor may affect different molecular and cellular mechanisms, such as GABAergic, glutamatergic, and dopaminergic neurotransmission, the immune system and oxidative stress. However, we also saw several combinations in which one risk factor seems to nihilate the effect of the

other one. In such situations, adverse circumstances in early life seem to have a protective effect on stressors that follow. Apparently, in some instances, stress tolerability can be increased by early life adversity. This is an interesting concept to further study in humans, supporting the common saying "what doesn't kill you makes you stronger." The next part of the review aims to integrate the effects that risk factors, alone or combined, have on molecular and cellular mechanisms in the brain and how they interact with each other (Fig. 2).

5.1. The immune system

The immune system plays a role in the pathophysiology of schizophrenia, not only as a defence against infections but also for its pivotal role in brain development when microglia, complement and other immune components determine the velocity and timing of brain connectivity and maturation (Thion et al., 2018).In humans, maternal infection and early life adversity, such as childhood trauma or infection, are thought to make the immune system of the individuals more responsive to stressors later in life, thereby increasing the risk of developing schizophrenia (Khandaker et al., 2013, 2012).

Increased levels of the pro-inflammatory cytokines IL-6, TNFα, IFN, IL-1, and acute-phase protein C-reactive protein, have repeatedly been reported in early-stage schizophrenia patients (Fond et al., 2020). PET imaging studies investigating the involvement of neuroinflammation in schizophrenia, however, gave conflicting results (Conen et al., 2020; Reis Marques et al., 2018). Some studies showed low to moderate levels

of neuroinflammation in schizophrenia patients, whereas other studies did not detect any microglial activation at all. This discrepancy could be due to methodological issues, the sensitivity of the technique, or differences in the patient population and disease stage. In post-mortem brain tissue, the expression of pro-inflammatory genes was found to be increased, although effect sizes are modest (Van Kesteren et al., 2017). The immune system mediates the interaction between neurons and glial cells, regulating neurodevelopmental processes such as synaptic pruning and neurogenesis, as well as neurotransmission (Dietz et al., 2020). In animals, several neurodevelopmental hits, such as prenatal or early life stress, or injection with LPS or poly I:C, were shown to prime the immune system to be more responsive to inflammatory challenges or stressors later in life (Diz-Chaves et al., 2013, 2012; Giovanoli et al., 2016, 2013; Lipina et al., 2013). Genetic mutations relevant to schizophrenia, such as the DISC1 mutation, can increase the vulnerability of the immune system (Lipina et al., 2013; Vuillermot et al., 2012), although single gene effects are usually weak. Several genes located on the short arm of chromosome 6 all act on complement factor 4, inducing a more susceptible complement factor 4a, which is an important step in opsonisation of synapses for pruning and may also be a trigger for microglia activation in schizophrenia (Germann et al., 2021; Kim et al., 2021). Furthermore, the immune system can be affected by environmental risk factors, including infection. Early life environmental stressors, such as maternal separation, and adolescent/adulthood stressors, such as restraint stress and chronic unpredictable stressors, can also trigger the activation of the immune system. These stressors can
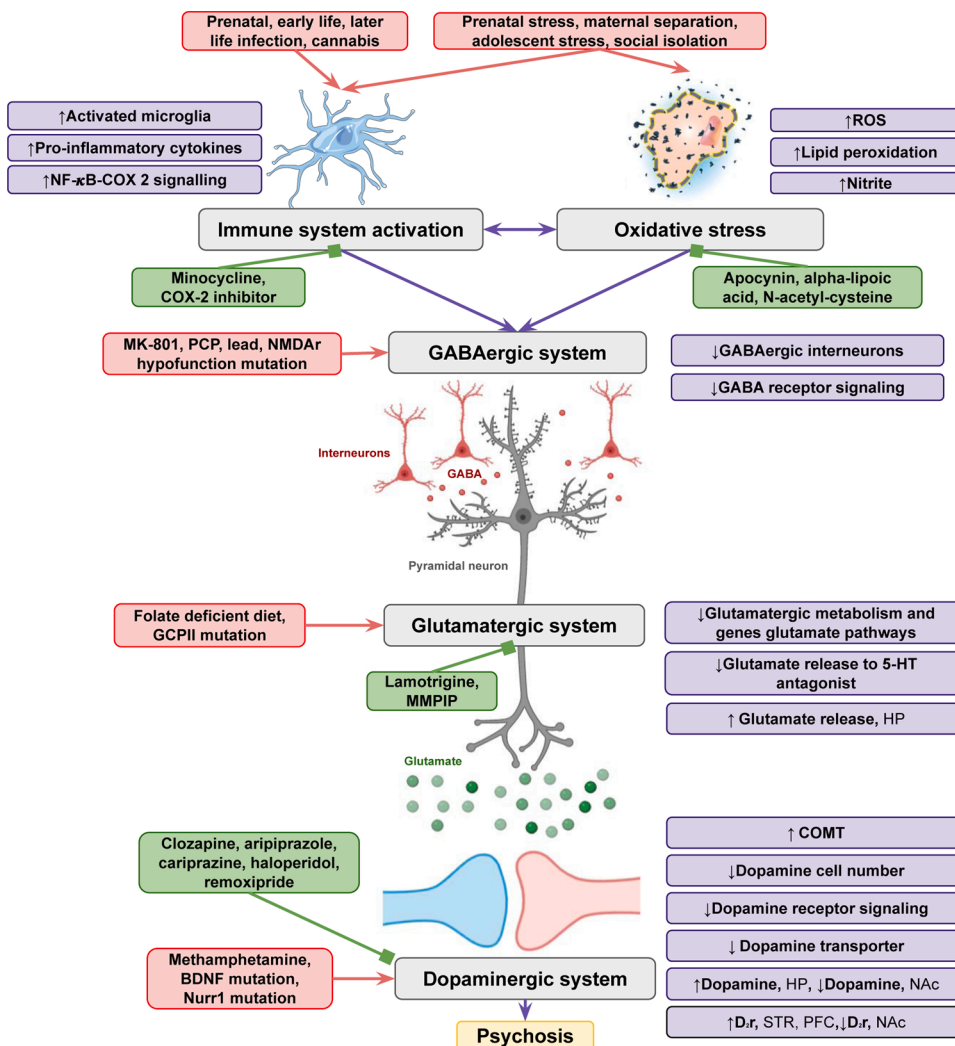

Fig. 2. Schematic representation of the impact of risk factors on different systems. 5-HT = serotonin, BDNF = Brain-derived neurotrophic factor, COMT = Catechol-O-methyltransferase, D_2r = dopamine D_2 receptor, GABA = gamma-Aminobutyric acid, GCPII = Glutamate carboxypeptidase II, HP = hippocampus, MK-801 = dizocilpine, NAc = nucleus accumbens, NF-κB−COX2 = Nuclear factor-kappa B-cyclooxygenase-2, NMDAr = N-methyl-D-aspartate receptor, Nurr1 = nuclear receptor-related 1 protein, PCP = phencyclidine, PFC = prefrontal cortex, ROS = reactive oxygen species, STR = striatum. Green box = inhibiting drugs, purple box = deficits observed following a combination of two risk factors, red box = risk factor for schizophrenia.

activate the HPA axis and induce oxidative stress (Choy and van den Buuse, 2008; Giovanoli et al., 2013). In humans, the risk to develop schizophrenia is significantly increased if the individual was exposed to the combination of prenatal infection and peripubertal psychological trauma (Debost et al., 2017).

MIA primes the immune system and consequently a second hit (adolescent stressors) can result in an increased inflammatory response, characterized by an increase in activated microglia in the hippocampus and prefrontal cortex and pro-inflammatory cytokines in the hippocampus, prefrontal cortex and plasma, when compared to either hit alone (Giovanoli et al., 2013). In addition, stressors can exacerbate oxidative stress in the striatum and prefrontal cortex of animals exposed to MIA or early life infection (Deslauriers et al., 2014; Monte et al., 2020, 2017). Note that the timing of the stressor was found to be important as the synergistic effect was only observed if the dual hit was applied in the juvenile period, but not in early adulthood (Giovanoli et al., 2013).

Anti-inflammatory treatment can be used to further understand the involvement of the immune system in schizophrenia. The PPI deficits induced by MIA in DISC1 mutant mice could be reversed by co-injecting an anti-IL-6 antibody with the administration of poly I:C, suggesting that the effects of MIA are IL-6 dependent (Lipina et al., 2013). In addition, a COX-2 inhibitor injected in aDN-DISC1 mutant mice during the adolescent exposure to cannabis could prevent the appearance of cognitive and molecular abnormalities during adulthood (Jouroukhin et al., 2019). Furthermore, minocycline, commonly used to inhibit microglia activation, injected in MIA mice during the juvenile exposure to stressors could prevent the appearance of schizophrenic-like behaviour (except anxiety-like behaviour) and molecular alterations during adulthood, suggesting a crucial role of inflammation in the development of behavioural abnormalities (Giovanoli et al., 2016). In humans, a large meta-analysis of clinical studies demonstrated that treatment with some anti-inflammatory drugs such as minocycline, combined with regular antipsychotic treatment, can have beneficial effects on the severity of symptoms (Çakici et al., 2019).

Inflammation and oxidative stress are two interconnected mechanisms that are suggested to be involved in the pathophysiology of schizophrenia. Activated inflammatory cells produce reactive oxygen species that induce oxidative stress, which in turn activate pathways that amplify inflammation (Mittal et al., 2014; P., C. et al., 2014). Using an antioxidant treatment in individuals that potentially have a primed immune system can help to increase our understanding of the role of oxidative stress. Alpha-lipoic acid, an antioxidant, moderated the increase in oxidative stress and prevented the behavioural alterations induced by prenatal activation of the immune system in combination with juvenile stressors (Deslauriers et al., 2014). In animals exposed to early-life infection and stressors at a juvenile age, N-acetyl-cysteine administration reduced oxidative stress to control levels and reversed the majority of the behavioural deficits in male and female offspring, except for the PPI deficits induced by the dual hit in male offspring (Monte et al., 2020). N-acetyl-cysteine administration could prevent not only the MIA-induced oxidative stress and activation of the immune system but also the behavioural alterations induced by prenatal infection and methamphetamine administration during adolescence (Swanepoel et al., 2018). The same compound could also rescue prefrontal hypomyelination and cognitive deficits in the APO-SUS rat model of schizophrenia (Maas et al., 2021). In schizophrenia patients, N-acetyl-cysteine treatment was found to have a significant beneficial effect on symptom severity (Çakici et al., 2019).

In conclusion, these findings support the hypothesis that genetic susceptibility or an early life event may prime the immune system of the individual to become more susceptible to stressors later in life. The negative effects of the primed immune system on behaviour can be blocked by agents that reduce inflammation or oxidative stress, thus confirming the involvement of these systems.

5.2. The GABAergic and glutamatergic systems

The GABAergic and glutamatergic systems are altered in schizophrenia. A meta-analysis of post-mortem studies found that patients with schizophrenia have a reduction in prefrontal and hippocampal GABAergic interneurons (Kaar et al., 2019; Nakazawa et al., 2012; Zhang and Reynolds, 2002). In addition, some people abusing NMDA receptor antagonists, such as PCP or ketamine, experience psychotic symptoms similar to those experienced during a psychotic episode (Farber, 2003). The dysfunction of the NMDA receptor on GABAergic interneurons induces disinhibition of the excitatory pyramidal neurons, resulting in hyperexcitation, more release of glutamate, and increased release of striatal dopamine, resulting in psychotic symptoms (Howes et al., 2015; Kesby et al., 2018; Lisman et al., 2008; Uno and Coyle, 2019). These systems can be affected or sensitized in an animal model with genetic mutations or by pharmacological approaches (PCP, MK-801, lead, non-competitive NMDA receptor blockers) in early or later life. It is possible to challenge these modified systems in animals to determine if they result in an increased risk to develop schizophrenia-like symptoms.

Mice exposed to SIR were more susceptible to glutamate receptor antagonists early in life, as the combination of both hits synergistically decreased GABA receptor signalling, glutamate metabolism, and dopamine receptor density in the hippocampus (Gaskin et al., 2016; Shortall et al., 2020). This effect was not observed if the exposure of SIR mice to glutamate receptor antagonists occurred during young adulthood, suggesting that the GABAergic system must be altered early in life to become sensitive to stressors and that SIR alone is not enough to modify the GABAergic system (Simpson et al., 2010; Watson et al., 2016). Mice with a DISC1 mutation treated with lead, an antagonist of the NMDA receptor, showed a synergistic increase in the size of the lateral ventricles (Abazyan et al., 2014), a feature also observed in schizophrenic patients. Another way to induce NMDA receptor hypofunction on GABAergic interneurons is the use of Pp1r2-cre/fGluN1 knockout mice. Exposing the knockout mice to SIR resulted in a synergistic decrease in parvalbumin-positive interneurons in the prefrontal cortex (Jiang et al., 2013). The combination of MIA and juvenile stressors also resulted in a reduction of GABAergic interneurons in the ventral dentate gyrus, striatum, and prefrontal cortex (Deslauriers, 2013; Deslauriers et al., 2014; Giovanoli et al., 2013, 2014). DISC1 mutant mice exposed to neonatal immune activation had a synergistic behavioural alteration and a reduction in GABAergic interneurons in the prefrontal cortex, suggesting that inflammation alters the GABAergic interneurons (Ibi et al., 2010).

Antioxidant drugs can be used in these models to better understand the effect of oxidative stress on the GABAergic and glutamatergic system. Apocynin, an antioxidant and ROS scavenger, could reverse the negative effects of SIR on the GABAergic interneuron-specific NMDA receptor hypofunction in a mouse model, suggesting that the high vulnerability of corticolimbic interneurons to oxidative stress may be responsible for the induction of a synergistic dual hit effect (Jiang et al., 2013). Interestingly, this dual hit model displayed no microglial alterations, possibly because the NMDA receptor hypofunction occurs only after microglia activation in the developmental stage of schizophrenia has resolved (Jiang et al., 2013). The antioxidant alpha-lipoic acid restored the reduced number of GABA neurons and prevented the behavioural alterations induced by prenatal activation of the immune system and juvenile stressors (Deslauriers et al., 2014). In animals exposed to early-life infection and stressors at a juvenile age, administration of the antioxidant N-acetylcysteine was found to increase the number of GABAergic interneurons in the hippocampus and to reverse the majority of the behavioural deficits (Monte et al., 2020). Post-mortem studies observed that oxidative stress is increased in schizophrenic patients and that GABAergic interneurons in patients are more susceptible to this oxidative stress (Hardingham and Do, 2016).

Drugs can also be used to modify the activity of the GABAergic and

glutamatergic systems. Calbindin-positive GABAergic interneurons located in the hippocampus express serotonin 5-HT$_6$ receptors which can be inhibited with a 5-HT$_6$ antagonist, resulting in blocking of the inhibitory effect of the interneurons and consequently an increase in glutamate release. SB-399,885, a 5-HT$_6$ antagonist could reverse the negative effects of SIR on recognition memory, but it did not affect animals exposed to both SIR and glutamate receptor antagonist in early life (Shortall et al., 2020). Rats exposed to a glutamate receptor antagonist early in life and SIR have a reduced number of GABA interneurons expressing 5-HT$_6$ receptors, thus reducing the inhibitory effect of 5-HT$_6$ antagonists (Shortall et al., 2020). This suggests that the combination of SIR-induced oxidative stress further exacerbates the alterations of the GABAergic system. Lamotrigine, a sodium channel blocker that reduces glutamate release and MMPIP, a mGlu$_7$ antagonist, could reverse the synergistic effect of SIR and exposure to a glutamate receptor antagonist early in life (Gaskin et al., 2016; Shortall et al., 2020).

Overall, these results suggest that GABAergic system alterations early, but not later, in life increase the individual susceptibility to stressors later in life by increasing glutamate release, that the immune system and oxidative stress have a detrimental effect on GABAergic interneurons, and that these mechanisms are involved in schizophrenia.

5.3. The dopaminergic system

The dopaminergic system has long been known to be involved in the pathophysiology of schizophrenia. Imaging studies have observed that increased presynaptic dopamine production in the striatum is associated with positive symptoms in schizophrenic patients (Howes and Murray, 2014; Stępnicki et al., 2018). To investigate the involvement of the dopaminergic system, it is possible to use methamphetamine, a drug inducing the release of dopamine, or genetic mutations, such as the Nurr1 mutation, which is a mutation of an orphan nuclear receptor critical for the development and survival of mesencephalic dopaminergic neurons. The altered dopaminergic system can be challenged by stressors, which could result in an increased risk to develop schizophrenia. Alternatively, animals can be selected and bred for their susceptibility to apomorphine or other dopamine release enhancers (van Schijndel et al., 2011).

SIR-exposed animals were less responsive to the effect of methamphetamine, as they had a lower dopamine release in the frontal cortex, but not striatum, compared to control animals (Strauss et al., 2014). Combining both maternal separation and corticosterone administration (mimicking stress) during adulthood prevented the occurrence of the PPI deficits induced by amphetamine or apomorphine, which was not observed for either hit alone, suggesting desensitization of the dopaminergic system caused by a stress-induced hyperdopaminergic state in animals exposed to the combination of both hits (Choy et al., 2008). The combination of two hits was necessary to pass a certain threshold for the desensitization of the dopaminergic system. In addition, maternally separated offspring administered with methamphetamine during young adulthood displayed reduced dopamine transporter levels, a protein responsible for the reuptake of dopamine, in the striatum (Hensleigh and Pritchard, 2015). Furthermore, the combination of maternal separation and methamphetamine administration resulted in a reduction in proteins involved in cytoskeletal modification, energy metabolism, intracellular signalling, protein degradation, and cellular growth in the nucleus accumbens (Dimatelis et al., 2012). Mutant Nurr1 mice were also more susceptible to the priming of the immune system and oxidative stress. SIR in Nurr1 mice induced a decrease in dopamine in the nucleus accumbens (Eells Misler and Nikodem, 2006). In addition, maternal immune activation in Nurr1 mutant mice had a synergistic detrimental effect on the dopaminergic system, as the mice had a reduced dopamine D$_2$ receptor density in the nucleus accumbens, and a reduced dopamine cell number, and increased COMT in the prefrontal cortex (Vuillermot et al., 2012). Prenatal priming of the immune system using MIA and juvenile stressors resulted in increased dopamine in the

hippocampus, and increased dopamine D$_2$ receptors in the striatum and prefrontal cortex (Deslauriers, 2013; Deslauriers et al., 2014; Giovanoli et al., 2013, 2014).

Studies using antioxidant treatment may increase our understanding of the effect of oxidative stress on the dopaminergic system. Alpha-lipoic acid, an antioxidant, could not only restore the altered dopaminergic system but also prevented the behavioural alterations induced by prenatal activation of the immune system and juvenile stressors (Deslauriers et al., 2014). Likewise, N-acetyl-cysteine administration could prevent alterations in the serotoninergic and dopaminergic systems in the frontal cortex, and the behavioural alterations induced by prenatal infection and methamphetamine administration during adolescence (Swanepoel et al., 2018).

To further investigate the role of the dopaminergic system, drugs can be used to modify its activity. Clozapine, an antipsychotic drug binding to, amongst others, dopamine D$_2$ receptors, could prevent the synergistic detrimental effect of the combination of SIR and prenatal priming of the immune system on behaviour, except for social behaviour, thus suggesting that interventions targeting the dopaminergic system can counteract the synergistic effects induced by inflammation and oxidative stress (Deslauriers et al., 2016). Clozapine and the D$_2$ receptor antagonist haloperidol could reverse the disruptive effects on PPI of prenatal stress on SNAP25 mutant mice (Oliver and Davies, 2009). Furthermore, clozapine and the D$_3$-preferring dopamine D$_2$/D$_3$ receptor partial agonists cariprazine or aripiprazole could reverse the negative effects of glutamate receptor antagonists exposure early in life combined with SIR (Hamieh et al., 2021; Lim et al., 2012; Watson et al., 2016). The latter drugs injected in healthy rats, reduced the natural forgetting, and increased novel object recognition. Remoxipride, a D$_2$ receptor antagonist could reverse the disruptive effects on PPI of cocaine on APO-SUS rats (van der Elst et al., 2006). Clozapine, haloperidol, cariprazine, and aripiprazole have been used in clinical trials and showed therapeutic efficacy in schizophrenic patients (Essali et al., 2009; Garnock-Jones, 2017; Leucht et al., 2020; Siskind et al., 2016).

In conclusion, these results suggest that genetic mutations in the dopaminergic system increase its susceptibility to neurodevelopmental and environmental insults, and that oxidative stress and the immune, GABAergic, and glutamatergic systems interact with the dopaminergic system likely leading to behavioural alterations.

6. Concluding remarks

The dual hit models allow investigation of the cellular and behavioural modifications induced by different genetic, behavioural, and environmental factors. The models have face, construct, and predictive validity. In addition, the dual hit models allow the investigation of different risk factors and the determination of their effects, which could help to better understand the underlying mechanisms. Animal studies have indicated that inhibiting the immune system (including oxidative stress) and the GABAergic, glutamatergic, and dopaminergic neurotransmitter systems could be useful approaches to counteract the detrimental effects of the dual hits on behaviour and could help to understand how certain processes and interactions can lead to the development of schizophrenic symptoms. However, the complexity of schizophrenia extends to other systems, such as the glucose metabolism pathway. Schizophrenia is associated with altered brain bioenergetics (Dean et al., 2016; Pinacho et al., 2016) and abnormal glucose metabolism (Zhang et al., 2015), which could be explained by an instability of the astrocyte-neuron compartment. Astrocytes supply energy to neurons by taking up glucose from blood vessels and converting it into lactate via glycolysis or glycogenolysis (Chechik et al., 1987). Deficits in the astrocyte-neuron compartment can induce a leakage in glucose that will negatively affect neuronal activity, activate microglia, and alter behaviour and cognition (Chechik et al., 1987; Churchward et al., 2018; Sullivan et al., 2017). Regarding the dual hit model, a recent hypothesis proposes that a first hit may dysregulate and prime the glucose

signalling pathway, thus making it more vulnerable to an environmental insult that would promote a pathological response via the astrocytic glycogenolysis signalling pathway (Roosterman and Cottrell, 2021). DN-DISC1 mutation in astrocytes reduced the levels of the glucose transporter 4, diminished glucose uptake, and reduced the production of lactate by astrocytes, which was associated with affective symptoms and deficient memory that could be rescued by systemic lactate treatment (Jouroukhin et al., 2018). Combining astrocyte DISC1 mutation with cannabis exposure during adolescence synergistically altered the immune, GABAergic and glutamatergic systems, and recognition memory (Jouroukhin et al., 2019). Furthermore, expression of the same genetic mutation in neurons produced no synergistic effect on brain and behaviour, thus suggesting a specific role of astrocytes in the increased susceptibility to an environmental insult. This remains preliminary work and future research should focus on investigating the impact of genetic risk variants in astrocytes in combination with environmental insults on the glucose metabolism pathway.

It is important to keep in mind that schizophrenia is more complex than a single genetic mutation or one neurodevelopmental or environmental stressor. Future studies could investigate the effects of neurodevelopmental and environmental stressors in animal models that have been generated by natural selection for susceptibility, rather than having one specific mutation (Ellenbroek and Cools, 2002b). We predict a synergistic effect on behaviour and brain alterations if neurodevelopmental stressors were applied to such a model. In support of this hypothesis, pregnant mice with certain genetic susceptibilities (DISC1 or Nurr1 mutations) had a higher inflammatory response to MIA which resulted in a primed immune system in the offspring that is overreacting to stressors later in life (Lipina et al., 2013; Vuillermot et al., 2012). On the other hand, if the pregnant mice did not have an exaggerated inflammatory response, the offspring did not have a hyperreactive immune system and therefore did not display a synergistic dual hit effect (O'Leary et al., 2014).

Future studies could also investigate the impact of preventive interventions, using either pharmacological or non-pharmacological interventions, on different molecular systems. The dual hit models offer the possibility to test preventive approaches as it is possible to intervene before the second hit is applied, thus allowing investigation of the effect of drugs activating or inhibiting specific pathways or enzymes related to these systems. Developing therapeutic strategies is important to alleviate the effect of a combination of risk factors. Adolescence involves many neurodevelopmental changes, such as neurogenesis, myelination, and synaptic pruning, in both humans and rodents and is, therefore, a vulnerable period (Hefner and Holmes, 2007; Spear, 2013; Yoo et al., 2013). The brain is plastic at that age and is, therefore, more responsive to pharmacological and non-pharmacological approaches to reduce the effect of stress, priming of the immune system and genetic predisposition. However, while injecting drugs during early development is possible in rodents and allows investigation of the mechanisms involved, the translation to humans would not be as simple, because pharmacological interventions before disease onset are ethically challenging in humans and could induce other long-term complications. Therefore, non-pharmacological interventions to alleviate the effects of prenatal stress have raised a lot of interest. In humans, child intervention programs, consisting of a broad array of activities to enhance children's development, can reduce the risk associated with compromised development of the brain due to early life adversity (DiPietro et al., 2002; Ramer and Ramey Landesman, 1998). Physical exercise can help to improve health (Carek et al., 2011). Voluntary running-wheel (RW) exercise at PND30−60 could reverse the abnormal behaviour after prenatal maternal infection in mice (Andoh et al., 2019). Other non-pharmacological interventions could be dietary interventions with for example probiotics, vitamin D, omega-3 fatty acids, choline, iron, or zinc supplementation. All these supplements were shown to be essential for normal foetal development (Brown, 2011; Luan et al., 2018; Mattei and Pietrobelli, 2019; Szeligowski et al., 2020). For example, maternal probiotics and vitamin D supplementation were shown to prevent the deleterious effects of MIA in the offspring (Hsiao et al., 2013; Luan et al., 2018; Wang et al., 2019). Another study showed that supplementing the diet of MIA offspring with polyunsaturated fatty acids from weaning onward could prevent the development of symptoms (Basil et al., 2018; Li et al., 2015). Using this type of supplementation early in the life of offspring might help the offspring to become more resilient. Such interventions could easily be translated to humans.

Summarizing, the preclinical studies on the dual hit hypothesis show that first insults can prime or be protective against adversity later in life. Early disruption of the immune, GABAergic, glutamatergic, or dopaminergic system increases the susceptibility to adverse events later in life, such as social isolation, stress, or drug abuse. Surprisingly, animal studies also found protecting effects of several first insults like maternal infection and early maternal separation. This is an interesting concept that may have human equivalents. Single gene mutations in one of the many genes associated with schizophrenia do not have a major impact on the susceptibility to environmental hits and may even increase resilience. Still, essential pieces of the puzzle are missing, and future studies should therefore further investigate the underlying molecular and cellular mechanisms behind the combination of several hits, as well as the interaction between them to better understand the aetiology of schizophrenia.

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