# Nurturing nature : testing the three-hit hypothesis of schizophrenia

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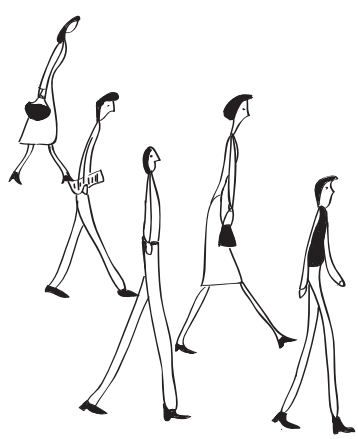
# Chapter 1

## General Introduction



# Table of Contents

This thesis describes our findings obtained with a rat model of schizophrenia, which is based on genetic predisposition for dopamine hyper-reactivity and exposure to environmental stressors during development. In the introduction, we report the current state of art of schizophrenia research by discussing the diagnostic classes, the basic neurobiology and the major thoughts (theories) on the pathogenesis of schizophrenia. We also report the necessity of the use of endophenotypes for proper translational research from human to animals (and back). Finally, we discuss findings in rodent research that are relevant to our animal model. In the end, we give the scope and outline of the thesis.

**Box 1. Introductory notes: based on [1, 2]**

Psychiatric disorders are very common in the general population with almost one in three people meeting the criteria for a psychiatric disorder diagnosis some time in life [3]. The term psychiatric disorder is used for a wide variety of mental disorders amongst which depression and psychosis.

Psychotic disorders. The lifetime prevalence of all psychotic disorders is approximately 3% [4]. There are several different types of psychosis, but when an individual experiences his first psychotic episode it is not always possible to determine which disorder is the cause. This is mainly because there are no specific diagnostic criteria yet, and each individual's experience of psychosis may differ even within the same diagnostic cluster.

The most common forms of psychosis are:

-Schizophrenia, defined by the presence of positive and/or negative symptoms for a period of at least six months.

-Bipolar Disorder, characterized by mood swings; patients may experience episodes of "highs" (mania) and "lows" (depression), and psychotic symptoms may appear during either phase. The psychotic thinking usually fits with the person's mood at the time.

-Major psychotic depression, characterized by major depression, together with psychotic symptoms. It is different from bipolar disorder in the sense that the individual does not experience any manic phases.

-Schizoaffective Disorder, a psychotic illness similar to bipolar and psychotic depression in that there are symptoms of a mood disorder and a psychotic disorder. The difference is that the symptoms of either psychosis or mood disturbance occur at the same time, but there is usually a period of time when there is psychosis alone without mood disturbance. Throughout the total duration of this psychotic illness, the mood disturbances represent a significant portion of illness time.

-Schizophreniform disorder, a psychotic disorder diagnosed when symptoms of schizophrenia are present for a significant portion of time within a one-month period, but signs of disruption are not present for the full six months required for the diagnosis of schizophrenia

-Brief psychotic disorder, which arises suddenly in response to severe stress. An individual experiencing this type of psychosis will usually make a fast recovery.

-Organic psychosis, which occurs as a result of a physical illness that disrupts normal brain function. Illnesses that have psychotic symptoms are for example brain tumors or AIDS. It is important to note that not all people who suffer from these physical illnesses will experience psychosis.

-Delusional disorder, characterized solely by delusions. Normally, only when the person begins to act on his delusional beliefs, it comes to the attention of others.

# 1. Schizophrenia

## 1.1 Neurobiology of schizophrenia

The pathophysiology of schizophrenia is characterized by several neurobiological abnormalities [5, 6]. Firstly, structural changes have been described: enlarged ventricles and reduced volume of a number of structures such as hippocampus, amygdala, and frontal and temporal cortices. Secondly, functional cortical deficits have been detected. Prefrontal and temporal lobe dysfunction is the most prominent, and is possibly related to the structural abnormalities. Thirdly, neuropathological studies did not find any evidence of gliosis to account for the structural deficits, but cytoarchitectural alterations suggest a certain type of failure in neuronal migration, orientation, or connectivity. The prefrontal cortex (PFC) and specifically Brodmann' s area 9 (dorsolateral part) display an abnormally high neuronal density and a reduced cortical thickness, indicating that the network in which cortical neurons are embedded may be reduced in the brain of schizophrenic patients [7]. The development of the circuitry linking cerebral cortex, thalamus and basal ganglia seems to be of vital importance in the pathophysiology of schizophrenia. Loss of cells in the thalamus may be primary or secondary to cortical or other subcortical pathology. This loss of thalamic cells potentially can lead to changes in thought processes [8]. Finally, several neurotransmitter systems appear to play a role in the expression of positive as well as negative psychotic symptoms. The evidence for alterations in the dopamine (DA) system is strong, but other neurotransmitters have also been implicated, including glutamate, serotonin (5-HT), and γ-aminobutyric acid (GABA) [1].

Taken together, cortico-striato-thalamic circuit, limbic and DA systems all appear to play a role. These are interconnected pathways and mediate different aspects of higher-level information processing, such as judgment, memory, planning, and motivation. Their involvement could arise in several pathogenesis mechanisms. One pathogenesis model suggests that neurodevelopmental abnormalities occur in utero, but the clinical manifestations of schizophrenia appear only after brain development is largely completed, in late adolescence [9]. This hypothesis dominates thinking about schizophrenia, but has several weaknesses as we will discuss below.

**Box 2. Schizophrenia**

Schizophrenia, coined by Eugen Bleuler in 1908 as the splitting of the mind [σχίζειν ("to split" in greek) + φρήν ("mind" in greek)] is one of the most common psychotic disorders and the seventh most costly medical illness to our society, affecting about 0.7% of the world's population (CI 95% 0.3-2.7%) with devastating consequences for affected individuals and their families [1, 12, 13]. Prevalence is greater in men throughout most of adulthood (male:female during early adulthood OR = 1.4), but is equal in both sexes by the end of the risk period. Men get also a more adverse version of the disease [14]. For men the onset of the symptoms is distinguishable at an age of 20-25, while the average age onset is 25-30 for a woman [15].

Schizophrenia as a syndrome: Schizophrenia has a syndromal appearance with a variety of presentations and the diagnosis is done commonly with the usage of Diagnostic and Statistical Manual (DSM; see Box 3) [2]. The symptoms and signs are very diverse, and they encompass the entire range of human mental activity. They include abnormalities in perception (hallucinations), inferential thinking (delusions), language (disorganized speech), social and motor behavior (disorganized behavior and abnormal or stereotyped movements), and initiation of goal-directed activity (avolition), as well as impoverishment of speech and mental creativity (alogia), blunting of emotional expression (flattened affect), and loss of the ability to experience pleasure (anhedonia). These symptoms and signs occur in patterns that may not overlap; one patient may have hallucinations and affective flattening, whereas another has disorganized speech and avolition [1, 16].

Patients with schizophrenia display also impairments in many different cognitive systems, such as memory, attention, and executive function. This is often referred to as a generalized deficit, and its existence provides additional support for the likelihood that the disorder is the result of a basic process such as a general impairment in the coordination of information processing [16, 17].

Prodromal phase: Schizophrenia has an onset in early adult life, but it is often difficult to define the exact onset due to the prodromal phase. This is a period of less specific phenomena, that are obvious in retrospect, which include withdrawal from previous roles, impairment in general functioning, behaviour that others see as odd, altered emotions, deterioration in personal hygiene, difficulties communicating with others, strange ideas, unusual perceptual experiences and restricted drive, initiative, interests or energy [15].

Prognosis: The psychotic symptoms vary in duration and incidence, with some patients having very rarely relapses, while others can have a prolonged version. The illness is not deadly itself, but other conditions like cardiovascular disease, metabolic syndrome, insulin resistance and suicide are a lot more frequent in schizophrenic patients [15, 18]. The earlier that schizophrenia is diagnosed and treated, the better the outcome of the person. Stressful environment can predict relapse in schizophrenic individuals, especially in the case of homes with negative expressed emotion [19].

Psychiatric comorbidity: To add more difficulty in the clinical differential diagnosis of schizophrenia, at least 50% of patients present comorbidity with depression at some of the stages of their illness. Depression in schizophrenia is associated with more frequent relapses and a poorer outcome. It is also associated with suicide and it is estimated that approximately 25-50% of patients attempt suicide at some point during the illness and 10% succeeds.

Treatment: The actual treatment is symptomatic and not totally successful. All drugs with established anti-psychotic effects decrease DA neurotransmission, but they are not effective often for the other schizophrenia symptoms [20]. Treatment options include [11, 15]:

A. Typical antipsychotics that mainly block the DA receptors have an effect on the positive symptoms, but

can give extrapyramidal side-effects such as impaired muscle control or cramps and endocrine side effects.
B. Atypical antipsychotics demonstrate similar efficiency regarding the positive symptoms as the typical antipsychotics, but with less extrapyramidal side effects and some efficacy for the treatment of the negative symptoms as well. However, side effects are seen within this group of drugs as well such as weight gain, sexual problems and indolence.
C. Mood stabilizers, antidepressants, anxiolytic, hypnotic, glycinergic drugs are often also used, as well as psychotherapy and social support.
Usually the patients need treatment continuously for a long period of time. It is necessary to develop new therapeutics targeting the pathogenesis and aetiology of the disease.

## 1.2 Neurochemistry of schizophrenia

Irregularities in the brain neurochemistry are clearly involved in the schizophrenia neurobiology; the increase or blockade of certain neurotransmitters with drugs (like amphetamine or Phencyclidine-PCP) can cause schizophrenia-like symptoms (e.g. amphetamine induces positive-like symptoms whereas PCP causes both positive and negative symptoms, and cognitive deficits) [10, 11].

DA imbalance was once thought to cause schizophrenia (DA hypothesis of schizophrenia). However, recent research indicates that neurotransmitters as GABA and glutamate are also involved [10]. The difficulty in the neurochemical theories of schizophrenia is that most brain processes involve changes in neurotransmitter levels, and neurotransmitters all interact with one another. Therefore, it is unrealistic to say that one particular neurotransmitter deficit can cause schizophrenia. With this limitation in mind, the focus in this thesis will be on the DA and stress system, their interaction, and their interaction with other systems.

### *1.2.1 DA system and schizophrenia*

Dopaminergic neurons can be found in a large number of areas in the brain, but four main circuits can be distinguished; the nigrostriatal, the mesolimbic, the mesocortical and the tuberoinfundibular pathways. The nigrostriatal pathway projects from the substantia nigra (SN) to the striatum (A9 neurons). The mesolimbic pathway is the dopaminergic pathway that begins in the ventral tegmental area (VTA) of the midbrain (A10 cell group) and connects to the limbic system via the nucleus accumbens (NAc), the amygdala, and the hippocampus as well as to the medial PFC. The mesocortical pathway is a neural pathway that connects the VTA A10 cells with the cerebral cortex, particularly the frontal lobes. The tuberoinfundibular pathway refers to A12 neurons in the arcuate nucleus of the mediobasal hypothalamus (the 'tuberal region') that project to the median eminence (the 'infundibular region') and control the release of prolactin [10, 11].

Consumption of drugs like amphetamine, cocaine or apomorphine, can induce paranoid psychosis, resembling schizophrenia in many aspects and when administered to schizophrenic patients, these drugs can induce a worsening of symptoms, especially

psychotic and thought disturbance [21, 22]. Neurochemical imaging with $^{18}$F-dopa and $^{11}$C-raclopride showed that schizophrenic patients, during an acute psychotic state, had an increase in DA synthesis, DA release and resting state synaptic DA concentrations [23]. The density of DA receptors (DR) is not necessarily changed in schizophrenia, but their occupancy by DA. This enhanced extent of occupancy could potentially be explained by the increased DA release, but animal studies have also revealed the possibility for an increased proportion of DRs with high affinity state for DA (i.e. high affinity $DRD_2$ receptors) [24]. Furthermore, some clinical observations showed that cognitive impairment was linked to DA abnormalities in the PFC [23] and that, in animal models where DA depletion was induced in the PFC, cognitive deficits could explain negative-like symptoms [25].

**Box 3. Diagnostic criteria for schizophrenia according to Diagnostic and Statistical Manual (DSM) of Mental Disorders IV-TR [2] and the corrections proposed in the draft of DSM V (http://www.dsm5.org).**

A. Characteristic symptoms: Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated). At least one of these should include 1-3.

1. Delusions
2. Hallucinations
3. Disorganized speech
4. Grossly abnormal psychomotor behavior, such as catatonia
5. Negative symptoms, i.e., restricted affect or avolition/asociality

B. Social/occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Duration: Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. Schizoaffective and mood disorder exclusion: Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either (1) no major depressive or manic episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. Relationship to a pervasive developmental disorder: If there is a history of autistic disorder or another pervasive developmental disorder, or other communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

Taken together, in schizophrenia, there is hyperactivity in the mesolimbic dopaminergic pathway, while the mesocortical pathway to PFC and other frontal cortical regions would be in a hypo-dopaminergic state. The former would explain the positive symptoms and the latter may explain negative, cognitive and affective symptoms according to the integrated DA hypothesis of schizophrenia [11, 15]. Interestingly the dopaminergic alterations could be secondary to N-methyl-D- Aspartate (NMDA) glutamate receptors in the descending cortico-brainstem glutamate pathway [11].

Effective therapeutic doses of antipsychotic drugs correlate with the blockage of $DRD_2$ receptors [10, 20, 21]. However, although $DRD_2$ antagonism is beneficial for positive symptoms, it can cause lack of pleasure for reward by the blockade in the mesolimbic pathway, secondary or worsening of negative, cognitive or affective symptoms by the blockade in the mesocortical pathway, extrapyramidal symptoms and tardive dyskinesia by the blockade in the nigostriatal pathway, and increased prolactin levels with galactorrhea, obesity, and amenorrhea by the blockade in the tuberoinfundibular pathway [11].

### *1.2.2 Stress system, stress response and schizophrenia*

Physical and emotional stressors induce a variety of autonomic nervous system and endocrine responses (i.e. Hypothalamic–pituitary–adrenal axis; HPA-axis). HPA-axis is comprised by the hypothalamus, the anterior pituitary and the adrenal cortices, which are involved in an interaction that controls the stress response in the organism. Parvocellular neurons in the paraventricular nucleus (PVN) in the hypothalamus synthesize corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These in turn are released in the portal vessel system and act on the anterior pituitary gland and stimulate this gland to release pro-opiomelanocortin (POMC), which is cleaved to the product adrenocorticotropic hormone (ACTH). ACTH is released to the blood stream and when reaching the adrenals it binds to Melanocortin receptors Type 2 (MCR-2) in the zona reticularis of the cortex of these organs. The binding leads to a fast production and secretion of glucocorticoids (GCs), which will act back on the hypothalamus and the pituitary in a negative feedback loop dampening further release of hormones and controlling the levels of GCs. The amygdala is involved in stimulation of the HPA-axis activity in rats, while the hippocampus and the PFC play an inhibitory role in its regulation [26-28].

GCs (cortisol for humans/ corticosterone for rodents; CORT) bind to two kinds of receptors: the high affinity mineralocorticoid receptor (MR) expressed predominantly in the limbic structures and the widely distributed low affinity glucocortiocoid receptor (GR). MR in the limbic structures, particularly the hippocampus is in charge of the maintenance of a basal state of stress system activity and is involved in initial phase of the stress response, while GR facilitates the termination of the stress response by negative feedback. An imbalance in these receptor mediated actions could lead to an inadequate regulation of the stress response, emotional disturbances and cognitive

alterations affecting the susceptibility to mental disease [27-29].

HPA-axis dysregulation has been associated with behavioural and cognitive deficits and brain changes across a large range of disorders [30]. Four lines of evidence suggest a link between HPA-axis activity and psychotic symptoms [31]:

(a) Illnesses associated with elevated CORT (e.g., Cushing's syndrome) or the exogenous administration of GCs often induce psychotic or manic symptoms that sometimes persist even after remission [32, 33].

(b) Patients with schizophrenia or other psychotic disorders manifest HPA-axis dysregulation (Fig. 1):

- Elevated basal CORT (& ACTH) secretion can be present in schizophrenia patients at different phases of their illness. Drug-naïve first episode patients have consistently elevated basal CORT levels. Psychotic symptoms and differences in perceived daily stress of the patients can be confounding factors in these studies. Antipsychotic medication can influence directly HPA-axis basal activity or indirectly by improving clinical symptoms [31, 34].

- A reduction in the volume of hippocampus and amygdala, brain regions important for the HPA-axis regulation, is a consistent finding in imaging studies of schizophrenia [35, 36]. However, in bipolar disorder or in schizophrenia different disease phases different findings were reported for the amygdala volume ranging from volume reduction to enlargement [37, 38]. Interestingly early-life stress or later chronic stress can induce similar changes in the volume of hippocampus and amygdala [26, 39-41]. Less information is known about PFC, hypothalamus and pituitary volumes in schizophrenia. However, a decrease of PFC and increases of PVN and pituitary volumes have been already reported [42, 43].

- Reduced MR and GR number. Webster and colleagues compared depressed, bipolar, and schizophrenia patients with controls. In frontal cortex, GR mRNA levels were decreased in layers III–VI in the subjects with depression, bipolar disorder and schizophrenia. In inferior temporal cortex, GR mRNA levels were decreased in layer IV in all three diagnostic groups. In the entorhinal cortex, GR mRNA levels were decreased in layers III and VI in the bipolar group. In hippocampus, GR mRNA levels were reduced in the dentate gyrus, CA4, CA3 and CA1 in the schizophrenia group. In the subiculum, GR mRNA levels were reduced in the bipolar group [44]. In PFC, MR mRNA expression in bipolar disorder and schizophrenia was reduced compared with control subjects [45] and GR mRNA expression was also reduced in the basolateral/ lateral nuclei of the amygdala in both diseases [46]. Overall, these decreases of receptors for GCs, could be involved in an aberrant negative feedback control of the HPA-axis. The dysfunctional negative feedback control possibly leads to an excessive HPA-axis stress responsiveness.

- Altered response to stress. Schizophrenic patients show enhanced CORT responses to physical stressors and blunted CORT responses to psychosocial stressors. These

findings imply differences in the appraisal and coping to stress as well [31, 34, 47]. Key areas for stress appraisal include amygdala, hippocampus and PFC, which display alterations in volume in schizophrenia. In a subset of persons with schizophrenia (those with polydipsia) elevated levels of AVP have been also reported in response to psychological stress, which are related to hippocampal-mediated impairment in the regulation of HPA-axis and deficit in central oxytocin activity [48].

- Depression comorbidity. In addition, a hyperactive HPA-axis is associated with depression, commonly seen among the schizophrenic patients (50%) strengthening further the probability of the axis's involvement in the disease [15, 19].

(c) Synergistic relation between GCs and DA.

- GCs and stress increase striatal DA release that in turn reduces $DRD_2$ receptor transcripts [49, 50]. Early-life stress or chronic stress can induce a GC-dependent DA system hyper-reactivity [50-55].

- GCs help 'energize' goal-directed behaviour and thus help to mentally cope with a stressful situation. CORT facilitates the development of amphetamine self-administration. Rats predisposed to develop amphetamine self-administration are marked by a highly reactive mesolimbic DA system, higher basal and stress induced CORT release and lower activity of hippocampal GR and MR [53, 56-60].

- GR is expressed in most cell types of the reward circuitry: midbrain DA neurons and their targets, including dopaminoceptive neurons of the NAc, caudate putamen, and PFC. Pharmacologic approaches and brain-selective GR-gene inactivation have demonstrated the role of GR in the facilitation of cocaine self-administration and locomotor sensitization [53, 56, 61, 62].

- DA is able to increase HPA-axis activity through $DRD_1/D_2$ receptors expressed in CRH secreting parvocellular neurons of the PVN [63]. Dopaminergic neurons of the tuberinfundibular system exert a tonic inhibition on prolactin release [64, 65]. Finally, DA can also influence melanocyte stimulating hormone (MSH) and endorphins synthesis through regulation of POMC in the intermediate lobe of the rat pituitary [66].

(d) Stress-exposure & sensitization.

Factors implicated in the etiology of schizophrenia, particularly perinatal/postnatal factors and life trauma, can contribute to HPA-axis dysregulation [26, 31, 67-69]. It is also known that stress-vulnerability is higher in schizophrenic patients, who respond with more negative emotions to everyday stressors [70]. This liability may very well have a genetic component (diathesis), as it is at least partially shared by family members. But also environment seems to be involved, since earlier stress exposure can increase stress sensitivity [71]. An overall stress sensitization that leads to HPA-axis enhanced reactivity may trigger a cascade of events resulting in neural circuit dysfunction that would eventually lead to the DA system dysregulation causing psychotic symptoms ("*diathesis-stress* model of psychosis", "affective pathway to psychosis") [70-72]. Finally,

previous stressful experience (like childhood maltreatment) and later environmental exposure (like canabis use) can increase the risk for psychosis through cross-sensitisation [73].

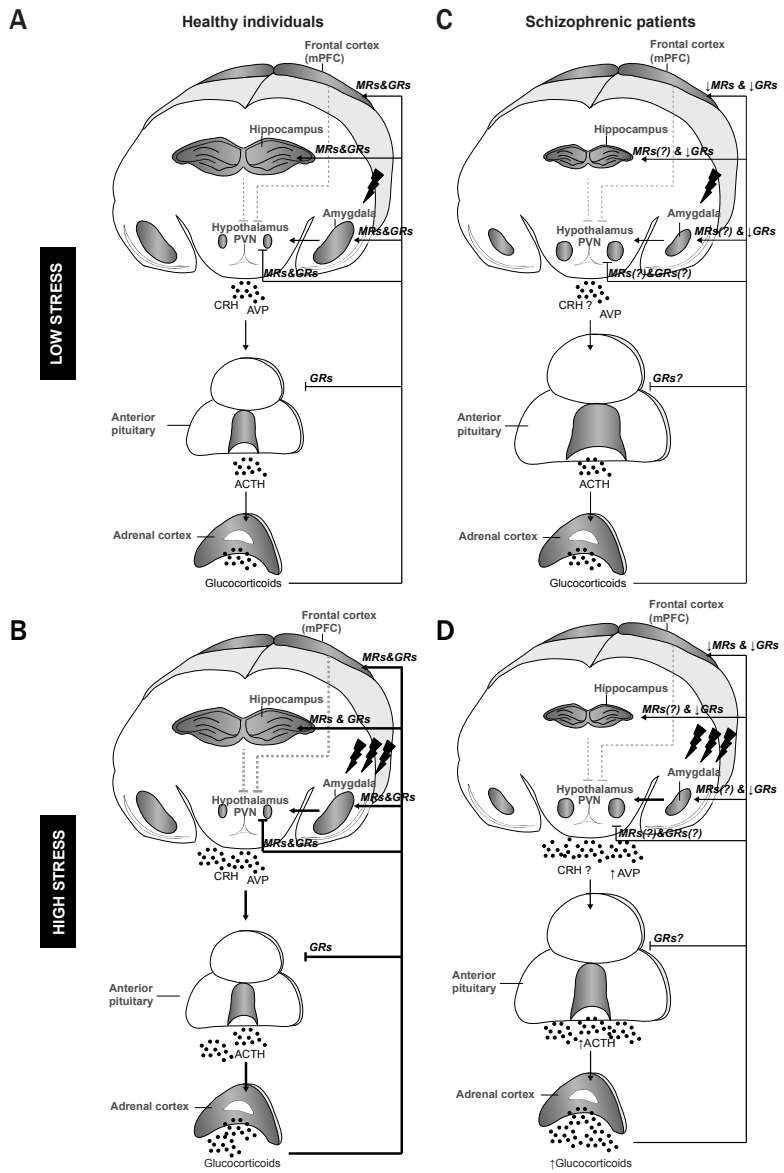



Figure 1. HPA-axis reactivity in healthy individuals (A,B; adapted from [26]). The brain detects a threat (physiological or psychological); a coordinated physiological response involving autonomic, neuroendocrine, metabolic and immune system components is activated. A key system in the stress response that has been extensively studied is the hypothalamus-pituitary-adrenal axis (HPA-axis). Neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus release corticotrophin releasing hormone (CRH) and arginine-vasopressin (AVP). This triggers the

subsequent secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, leading to the production of glucocorticoids (GCs) by the adrenal cortex. In addition, the adrenal medulla releases catecholamines (adrenaline and noradrenaline) (not shown). The responsiveness of the HPA-axis to stress is in part determined by the ability of GCs to regulate ACTH and CRH release by binding to two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Whereas the amygdala exerts a positive influence over the HPA-axis, the hippocampus and medial PFC (mPFC) exert a counteractive negative influence. Following activation of the system, and once the perceived stressor has subsided, feedback loops are triggered at various levels of the system: a direct negative feedback on the hypothalamus and pituitary level (continuous inhibitory lines) and indirect through activation of hippocampus and mPFC (discontinuous inhibitory lines). In low stress conditions (A), the activation of the HPA-axis and the subsequent activation of the negative feedback is lower than in high stress conditions (B). HPA-axis dysregulation in individuals with schizophrenia, limbic structures are smaller in volume and display altered function. In the same time, hypothalamus and pituitary are larger in volume possibly because of inefficient negative feedback (GC resistance). In low stress conditions (C), the amygdala, responsible for the stress anticipation of psychosocial stimuli, is activated to a less extent (than in healthy individuals), and the negative feedback is also less efficient. Overall, there is not a detectable difference in hormonal levels. However, when stress passes over a certain threshold (high stress conditions; (D)), the amygdala can get activated and then the HPA-axis output will be higher than in healthy individuals since the negative feedback is impaired.

## 1.3 Etiology of schizophrenia

### *1.3.1 Risk factors*

Epidemiological studies have already shown that both genetic and environmental factors play an important role in the precipitation of schizophrenia. There is an inheritable predisposition to the disorder, since it runs in families (OR: 9.8) [74]. Although liability to schizophrenia is highly heritable (RR: 81%), concordance between identical twins is almost 50% (Fig. 2), suggesting a role for non-shared environmental or stochastic influences, as well as environmental effects shared by members of a twin pair [74-76].

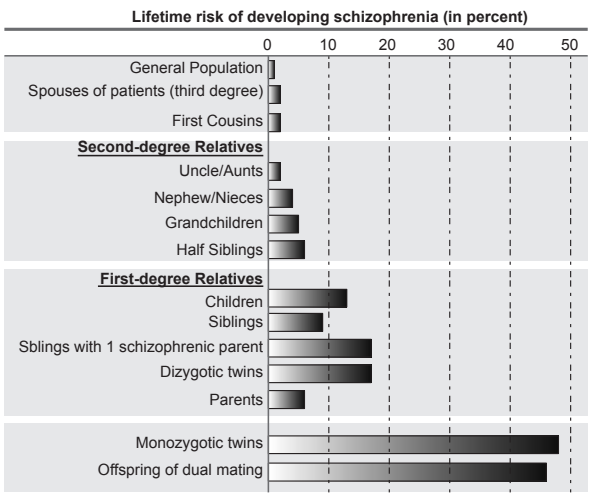



Figure 2. Lifetime risk of developing schizophrenia when a family member has the disorder. The closer the relative is, the higher the chance is of developing schizophrenia. Adapted from literature [77].

A number of early neurological insults and later life stressors confer additional risk for schizophrenia and other neurological disorders. While many such factors have been studied, obstetric complications and migrant/minority status illustrate two of the largest effects on increasing schizophrenia known at this time [OR=2.0 (obstetric complications); RR=2.9 (migration)] [78-80].

### *1.3.2 Etiological theories of schizophrenia*

Schizophrenia probably occurs as a consequence of "*multiple hits*", which include some combination of inherited genetic factors and external, non-genetic, factors that affect the regulation and expression of genes governing brain function or that injure the brain directly [16, 81, 82]. The major etiological theories for the causes of schizophrenia will be discussed.

#### *1.3.2.1 Genetics of schizophrenia*

While schizophrenia is highly heritable, its genetics is complex and multigenic. Genetic variation linked to schizophrenia includes single nucleotide mutations or polymorphisms, copy number variations and cytogenetic abnormalities. Linkage studies (which identify regions of the genome where schizophrenia genes might be found) suggested regions in several chromosomes (1p/q, 2q, 3p-q, 5q, 6p/q, 8p, 10p/q, 13q, 16p-q, 17p-q and 22q), including regions that reached genome-wide significance [83-85]. However, even in the most convincing cases, the risk haplotypes appear to display small effect sizes (OR: 2.5), and, although this is difficult to determine, do not appear to explain fully the linkage findings. Association studies revealed a complex genetic background with multiple genes of modest effect (max OR: 1.5), possibly interacting to produce the phenotype. The most promising candidate genes with supporting evidence are summarized in Table 1 [74, 86, 87].

The latest GWAS studies have revealed a strong associations of variants of zinc-finger binding protein gene (ZNF804A - SzGene rank 7, grade C) gene with schizophrenia (and with bipolar disorder) and of reelin gene with schizophrenia in females (RELN - SZgene: rank 22, grade C) [84, 88]. Interestingly, the presence of one of ZNF804A variants influences the functional connectivity between dorsolateral PFC and hippocampus in healthy individuals [89]. This type of connectivity is abnormal in schizophrenia [90]. Genome wide data also revealed schizophrenia associations with variants across the major histo-compatibility complex (MHC) region at chromosome 6p [SzGenes: rank: 1, 2, 4, 5, 17,23, grade A-C), with an intronic marker of transcription factor 4 (TCF4 - SZgene: rank 7, grade A) and with a marker upstream of neurogranin gene (NRGN-SZgene: rank 3, grade A) [84, 88].

The importance of DA in the PFC for the pathogenesis of schizophrenia was confirmed by the discovery of COMT as a susceptibility gene in genome wide association studies and as part of 22q11.2 microdeletions in velo-cardio-facial syndrome (syndrome with 25% prevalence of schizophrenia)[93]. COMT is one of the degrading enzymes of DA

Table 1
Ranking and grading of schizophrenia susceptibility genes [74, 86, 87] according to SZgene database (http://www.szgene.org) [88].

| Gene | | Role | Locus | Altered expression in schizophrenia | SZgene ranking | SZgene grade |
|---|---|---|---|---|---|---|
| ***DRD$_4$*** | *Dopamine receptor $D_4$* | G protein-coupled receptor | 11p15.5 | Not known | **10** | **A** |
| ***DRD$_2$*** | *Dopamine receptor $D_3$* | G protein-coupled receptor | 11q23 | Yes, ++ | **11** | **C** |
| ***DAOA (G72/G30)*** | D-amino acid oxidase activator | Glutamatergic transmission | 13q33.2-34 | Not known | **12** | **A** |
| ***HTR2A*** | Serotonin receptor 2A | G protein-coupled receptor | 3q14-21 | Yes, ++ | **16** | **A** |
| ***DTNBP1*** | Dystrobrevin-binding protein 1 (Dysbindin) | Protein-complex component | 6p22.3 | Yes, ++ | **20** | **C** |
| ***DISC1*** | Disrupted in schizophrenia 1 | Neurite outgrowth & cortical development | 1q42.1 | Not known | **25** | **B** |
| ***NRG1*** | Neuregulin 1 | Signalling | 8p12 | Yes, + | **26** | **C** |
| ***Akt1*** | V-akt murine thymoma oncogene homolog 1 | Serine-threonine protein kinase | 14q32.32 | Yes, ++ | **28** | **B** |
| ***RGS4*** | Regulator of G-protein signalling 4 | Deactivation of G protein subunits | 1q23.3 | Yes, ++ | **29** | **C** |
| ***PPP3CC*** | Protein phosphatase 3 catalytic subunit gamma isozyme | Calcium-dependent, calmodulin-stimulated protein phosphatase | 8p21.3 | Yes, + | **30** | **C** |
| ***COMT*** | Catechol-O-methyltransferase | Degradation of catecholamines | 22q11.21 | Yes, + | **36** | **C** |
| ***DAO*** | D-amino-acid oxidase | Peroxisomal enzyme | 12q24r | Not known | **40** | **C** |
| **SLC18A1** | solute carrier family 18 (vesicular monoamine), member 1 | Vesicular monoamine transporter | 8p21.3 | Not known | **43** | **C** |

Note. Strength of evidence: 0 to ++ (ratings are subjective). SZgene ranking and grading based on HuGENet (Human Genome Epidemiology Network) interim criteria for the assessment of cumulative evidence of genetic associations [91, 92].

and it gets a special role in PFC where DA-transporters are not expressed. In fact, animal studies have shown that COMT is responsible for more than 60% of DA degradation in PFC. DA activity in PFC is very important for cognition. This effect of DA, and its disruption, is possibly responsible for the deficits in attention and executive functioning commonly found in patients with schizophrenia [94].

Together with COMT, genetic variance in other DA related genes (e.g. $DRD_4$, $DRD_2$ , Akt1, RGS4, SLC18A1-Table 1) acts to confer risk for schizophrenia by regulating synapse DA levels or postsynaptic cellular signaling (canonical or non canonical) [95].

### *1.3.2.2 The neurodevelopmental hypothesis of schizophrenia*

Brain development starts antenatally and continues in childhood and adolescence, and can depend on a combination of biological events and psychosocial factors [96, 97]. Early abnormalities may have adverse effects on neurodevelopment and aberrant neural circuitry which eventually lead to psychopathology. Disorders characterized by this process, which typically manifest during the first few years of life, have traditionally been referred to as neurodevelopmental disorders; examples are autism and phenylketonuria [12, 97].

A neurodevelopmental model of schizophrenia pathogenesis was proposed [98]. Evidence that abnormal brain development contributes to schizophrenia is rich: (a)

schizophrenia is characterized not only by psychotic symptoms, which have their onset in adolescence and early adulthood, but in many cases there are abnormalities (cognitive, behavioral and morphological) dating back to early childhood; (b) history of obstetric adversities in high-risk families; (c) absence of evidence of neurodegeneration in postmortem tissue; and (d) association of developmental pathologic conditions (like autism) with adult emergence of psychosis and related phenomena. Daniel Weinberger, in his classic paper on brain development and schizophrenia, stated that schizophrenia is "not the result of a discrete event or illness process at all, but rather one end of the developmental spectrum that for genetic and/or other reasons 0.5% of the population will fall into"[9, 81].

The neurodevelopmental model has proved extremely influential in schizophrenia research, and numerous studies have provided more supportive evidence. The neurodevelopmental model predicted successfully widespread brain abnormalities occurring long before the clinical symptoms, as well as in unaffected siblings. These early brain abnormalities can potentially interfere with the normal maturation of the brain. Brain imaging of schizophrenic patients show indeed widespread brain changes, including structural alterations such as enlarged lateral and third ventricles as well as reductions in cortical gray matter in frontal, thalamic, limbic structures [99]. Interestingly, patients suffering from early-onset schizophrenia display a particularly big reduction in cortical gray matter that seems to be an exaggeration of the normal brain development [99]. Candidate genes for schizophrenia are often highly expressed during developmental periods of different brain regions [86]. However, despite this, the key pathophysiological disruptions in the disorder, their precise relationship with the proposed etiological factors is still not clearly understood [97].

Non-genetic pre- and perinatal factors exert a big influence in the early brain development and are risk factors for schizophrenia. Many such factors have been studied: infections or obstetric complications during pregnancy or delivery, exposure to toxins or radiation prenatally, maternal stress and malnutrition during pregnancy, birth during the winter, increased paternal age etc. [100].

*1.3.2.3 Nature-nurture theory: diathesis-stress, genetics-epigenetics, mismatch hypothesis, differential susceptibility*

- *Diathesis-stress* model (*multiple hit - cumulative stress* hypothesis)

A revised *diathesis-stress* model for psychopathology (Fig. 3A), originally proposed by the son of Eugen Bleuler (Manfred Bleuler) and David Rosenthal, see schizophrenia as a result of a complex interaction between thousands of genes and multiple adverse environmental risk factors, none of which on their own causes schizophrenia [81, 101, 102]. According to this *multiple-hit* model individual differences in behavior have thus an inheritable background (genetic vulnerability- *diathesis)* that its influence is potentiated by experience of adverse environmental factors (*stress*). This model predicts

two individuals: the vulnerable one that will be sensitive to negative effects of negative environment and the resilient one that will be not sensitive; those two categorical groups will be not different when the environment is positive [103, 104].

- Genetic-epigenetics

Genetic variation in combination with external non-genetic factors has an effect on the regulation and expression of genes influencing brain functions. The biological basis of this is the epigenome. Epigenetics refers to functionally relevant modifications to the genome that do not involve a change in nucleotide sequence. Such modifications include chemical marks that regulate the transcription of the genome e.g. DNA methylation and chromatin remodeling. There is now evidence that environmental events can directly modify the epigenetic state of the genome modified in sensitive developmental periods, but also in adulthood (possibly to a lesser extent) leading to changes in gene expression and neural function. These studies define a biological interplay between environmental signals and the genome in the regulation of individual differences in behavior, cognition, and physiology. Interestingly, the epigenetic status of an individual can be transferred through generations [106-108].

Several recent studies described gene-by-environment interactions relevant to the risk for schizophrenia, including interactions between genetic liability and prenatal exposure to infection, urban birth, maternal depression as well as between cannabis use and a COMT polymorphism [109-113]. Several researchers have concluded that schizophrenia is likely due to epigenetic alternations, in DNA methylation and histone acetylation/phosphorylation, initiated by environmental stressors [105, 114, 115].

- Predictive adaptive response (developmental *mismatch* hypothesis)

In an evolutionary perspective, these epigenetic changes should not be interpreted as defects, but as the molecular mechanisms for predictive adaptive responses [116]. Specific phenotypic variations in our species, especially largely prevalent variants, are programmed by the environment and may be adaptive phenotypes in certain environmental conditions [18]. Developmental plasticity evolved to *match* an organism to its environment, and a *mismatch* between the phenotypic outcome of adaptive plasticity and the current environment increases the risk of disease (*mismatch* hypothesis). These considerations point to epigenetic processes as a key mechanism that underpins the developmental origins of disease [116]. The *mismatch* hypothesis for schizophrenia would accommodate also the comorbidity of schizophrenia with other "thrifty" phenotypes like cardiovascular diseases, metabolic syndrome and metabolic syndrome, which all share early developmental aetiology [18]. It is intriguing to think of schizophrenia as a maladaptive phenotype in the modern industrial context, which fits with epidemiological data of urbanicity, migration, minority status and social stress as risk factors for schizophrenia [117].

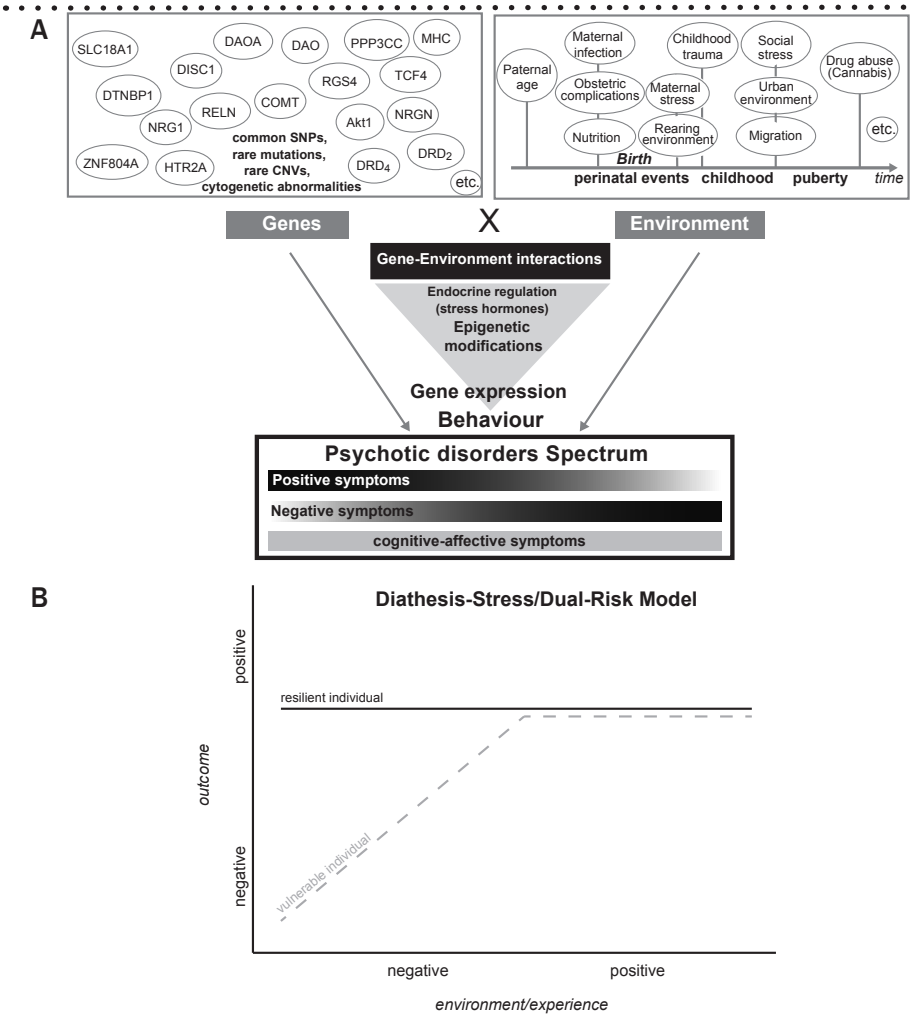



Figure 3. (A) A diathesis-stress model leading to psychotic disorder (inspired from [105]). Genetic variation is combined with multiple adverse environmental factors (that are timed) and result in epigenetic modifications and, eventually, gene expression changes that will lead to negative changes in behavior and clinical symptoms. (B) Graphical display of the diathesis-stress (dual-risk susceptibility model; adapted from [103, 104]). The x-axis indicates quality of the environment/ experience from negative to positive. The y-axis indicates the outcome from negative to positive. The lines depict two categorical subgroups that differ in their responsiveness to a negative environment: the "vulnerable" group shows a negative outcome when exposed to a negative environment, while the "resilient" group is not affected by it. No differences between the two groups emerge in a positive environment.

- Differential susceptibility (to environmental influence)

According to the *nature-nurture* theory things are plastic since the interaction of genes and environment can lead to both increased and decreased risk for disease [118]. It actually predicts the following conditions (Fig. 4):

1. Individuals with genetic susceptibility to schizophrenia will be sensitive to environmental influence overall: After environmental adversity (accumulation of stress), their risk for disease will increase (according to the *cumulative stress* hypothesis). However, they will be also sensitive to the beneficial effects of the environment.

2. Individuals without genetic susceptibility will not be sensitive to environmental influence overall: After environmental adversity and as stress accumulates their risk for disease will be the same or even decrease (because of *matching* conditions according to the *mismatch* hypothesis). Additionally, they will not be sensitive to the beneficial effects of the environment.

These predictions and recent data from gene-by-environment interaction studies supported a "differential susceptibility to environmental influence" (Fig. 4); susceptibility not only to negative but also to positive environment [104, 118, 119]. A $DRD_4$ repeat polymorphism, for example, could moderate the association between maternal unresolved trauma and infant attachment disorganization; children having the 7-repeat allele displayed increased attachment disorganization when maternal unresolved trauma was high and decreased attachment disorganization when maternal unresolved trauma was low [120]. Evolutionary-developmental psychologists characterized aptly the non genetically-susceptible to psychopathology children as "dandelion kids" since they are hardly influenced by the environment and genetically-susceptible children as "orchid kids" since they are sensitive to bad environment, where they cannot develop properly and to good environment, where they can "flourish" [103, 121, 122].

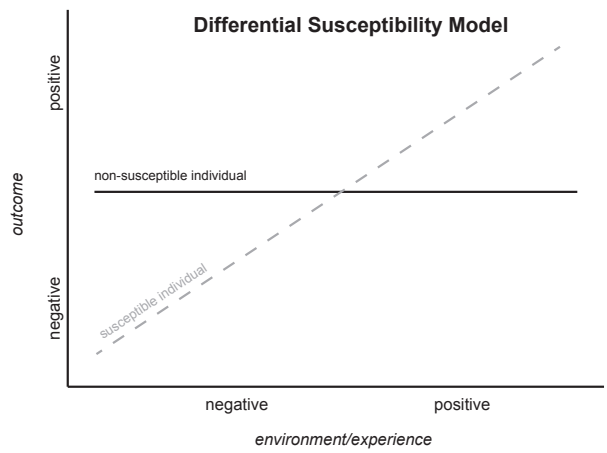



Figure 4. Graphical display of the differential susceptibility model (adapted from [103, 104]. The x-axis indicates quality of the environment/ experiences from negative to positive. The y-axis indicates the developmental outcome from negative to positive. The lines depict two categorical groups that differ in their responsiveness to the environment: the "plastic" susceptible group is disproportionately more affected by both negative and positive environments compared to the "fixed" non-susceptible group.

# 2. Schizophrenia endophenotypes



## 2.1 Endophenotypes and schizophrenia endophenotypes

An alternative approach to classification of heterogeneous disorders is to define endophenotypes [75, 123]. An endophenotype is a heritable trait that is generally considered to be more highly associated with a gene-based neurological deficit than a disease phenotype itself. Endophenotypes are quantitative traits that may not be readily apparent in routine clinical examinations of affected individuals, yet may reflect neurobiological features underlying the disease. Valid endophenotypes will associate with schizophrenia in population studies. They will be present (though less prominent) in the first degree family members of probands with schizophrenia, and will be found at similar levels in both members of twins discordant for schizophrenia. A variety of potential endophenotypes have been associated with schizophrenia, though none has yet been confirmed in large, unselected samples of at-risk individuals [124]. The use of endophenotypes had also great translational advantages in animal research [125]. Some of these traits will be reviewed with their equivalents in the rat that were used in this thesis.

## 2.2 Stereotypy and perseverative behavior

Stereotypy and perseverative behavior are considered to be endophenotypes, as well as symptoms, of schizophrenia [126, 127], and could affect working memory performance. Set-shift is necessary for successful working memory performance. In fact, studies have shown that patients with schizophrenia are often impaired on both reversal learning and extra-dimensional set-shifting. Patients engage in perseverative behavior on the Wisconsin Card Sorting Test (WCST), which is considered to be a measure of PFC activity, as well as on a measure designed specifically to assess stereotypy (Stereotypy Test Apparatus) [126]. First-degree relatives of schizophrenia probands demonstrate perseverative behavior on the WCST, further suggesting its relevance as a measureable endophenotype [128].

Assessing perseverative behavior is relatively simple in animal models. Rats given psychostimulants often exhibit locomotor hyperactivity, and at higher doses they exhibit stereotypy or perseverative behaviour [129, 130]. Hyperlocomotion and stereotyped behavior, often seen in pharmacologic animal models, are thought to model the positive symptoms of schizophrenia [131, 132].

## 2.3 Sensorimotor gating

Schizophrenic patients process stimuli differently, which may correlate with their neurological abnormalities. Sensorimotor gating is a process whereby excess or trivial information is screened out of awareness (i.e., gated out), permitting the individual to focus on the more important stimuli in the environment [17, 133, 134]. A simple neural

circuit of stimulus processing is the startle reflex, reaction to intense sensory stimuli [135, 136]. Sensorimotor gating permits the lowering of the startle response when the strong stimulus is preceded by a weak stimulus. This reduction (Fig. 5), called prepulse inhibition of the startle response (PPI), occurs if the interval between the prepulse and pulse is small (10 to 500 ms), whereas when it is higher the opposite will occur, prepulse facilitation (PPF) [17, 133, 137, 138]. As the PPI has as a consequence the reduction of behavioral responses to disruptive inputs by regulating the motor system and/or pre-motor system, PPI is thought to reflect sensorimotor gating [136]. Schizophrenic patients (including first episode patients), unaffected relatives as well as individuals with schizotypy show impairments in PPI compared to healthy individuals, qualifying PPI as a valid endophenotype. However, it is important to note that PPI deficits are not found only in schizophrenia [137, 139].

The PPI test is thought to relate more to the thought disorder within the positive symptoms given the fact that drugs that increase DA neurotransmission disrupt it and this effect can be reversed by $DRD_2$-antogonism [135]. Since this process is impaired in schizophrenia patients and can be measured in animals with an identical method, it has received enormous attention in the field of schizophrenia animal models [140]. During the test procedure, the animal is placed in an acoustic startle box (a small chamber; Fig. 5) and exposed to acoustic stimuli. A loud stimulus (pulse) elicits a startle response. However, when a weaker stimulus (prepulse) is given in a certain time interval before the pulse the startle response is inhibited [138].

Impairments in the PPI ability involves several areas of the brain and has been linked to schizophrenia-like anatomical (processes in the forebrain), neurochemical,

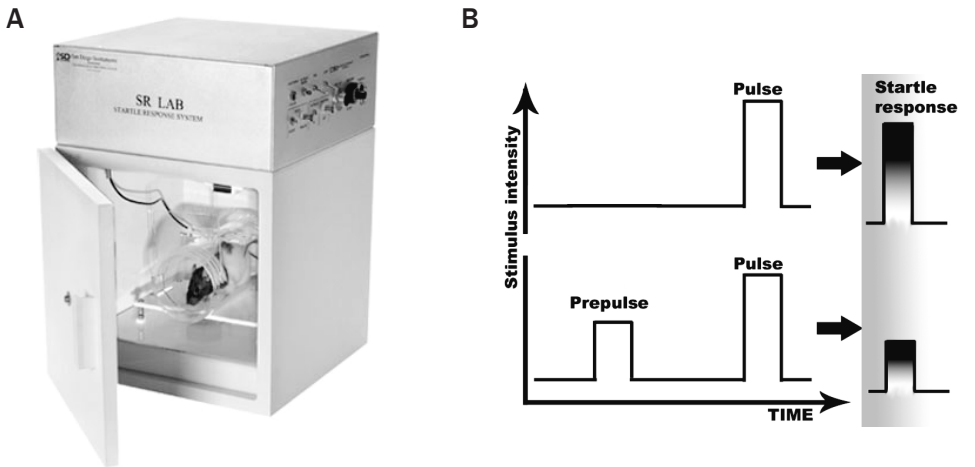



Figure 5. (A) An example of a rodent acoustic startle box (SR-LAB, San Diego Instruments, San Diego, CA). (B) Schematic view of the prepulse inhibition concept: normally a stimulus (pulse) elicits a startle response. However, when a weaker stimulus (prepulse) is given in a certain time interval before the pulse the startle response is inhibited (In Wikipedia. Retrieved Sept.2011, from http://en.wikipedia.org/wiki/Prepulse_inhibition).

developmental and genetic deficits [141]. In rodents, sensorimotor gating and specifically PPI are mainly regulated by cortical structures as the temporal cortex and medial PFC, which was found by various studies in which experimental manipulations (lesions, local-intracerebral-systemic infusions of receptor or transmitter-specific pharmacologic agents or neurotoxins) of these brain areas proved to be correlated with PPI disruption [135].

## 2.4 Working memory

Working memory is conceptualized as "the ability to actively hold information in the mind needed to complete complex tasks such as reasoning, comprehension and learning". Working memory tasks are those that require the goal-oriented active monitoring or manipulation of information or behaviors in the face of interfering processes and interactions. The cognitive processes involved include the executive and attention control of short-term memory, which provide for integration, processing, disposal, and retrieval of information [142]. Many studies have found impairments in working memory in patients with schizophrenia and their unaffected siblings [143]. Studies have suggested that patients with schizophrenia may have deficiencies in active rehearsal processes, whereas passive stimulus maintenance processes and long-term memory networks may remain unaffected. Spatial working memory may be one possible endophenotype of schizophrenia. Two meta-analyses that examined working memory in patients with schizophrenia have suggested working memory deficits, regardless of modality, although there may be more consistent impairments in visual working memory [131, 144, 145].

Spatial working memory is easily assessed in animal models of schizophrenia using spatial working memory tasks. Many animal models, including pharmacologic, neurodevelopmental and genetic models, have shown impairments in these working memory tasks [131, 146]. The Morris water maze is the most commonly used task in behavioral neuroscience research and can easily be configured to test spatial working memory in rats [147]. In this procedure, rats are placed in a pool of opaque water where there is a hidden platform located slightly below the water's surface. The rat is then released into the water from various points around the periphery of the pool and must navigate towards the platform on the basis of the spatial cues in the room. However, the water maze is rather stressful for the rats since it involves swimming (e.g. elevation of CORT) at a water temperature of 25°C [148]. Other tests of working memory include tests of alternation in mazes of different sizes and shapes with or without reward.

A T maze test can be used to assess working memory. The T-maze is an elevated or enclosed apparatus in the form of a T and is placed horizontally. Animals are started from the base of the T and allowed to choose one of the goal arms abutting the other end of the stem. If two trials are given in quick succession, on the second trial the rodent tends to choose the arm not visited before, reflecting memory of the first choice. This is called 'spontaneous alternation'. This tendency can be reinforced by making the animal

hungry and rewarding it with a preferred food if it alternates. One brain area that both spontaneous and rewarded alternations are depend from is the hippocampus [149].

## 2.5 Social interaction

Social withdrawal is not strictly considered to be a schizophrenia endophenotype, but rather a negative symptom of schizophrenia. It is often assessed in animal models of schizophrenia. Studies have shown that adult patients with schizophrenia have a reduced social network and deficits in social competence and social skills, including the misperception of affective information [150, 151]. Social anhedonia and social cognition deficits have also been observed in patients and in the relatives of schizophrenia probands [152, 153].

Rodents are typically social animals and tend to approach unfamiliar conspecifics. As such, the social interaction test in animal models of schizophrenia is designed to measure social impairments similar to those in human patients [154, 155]. Tests can include observations of animal dyads in their home cages or in a novel arena or while one animal's movements are limited by placing it under a wire cup. Certain developmental periods (e.g. adolescence social play period) are especially important for developing social interaction skills and can also be used as a sensitive tool to measure early social deficits. Regardless of the test used when studying social behavior, the basic expectation using an animal model of schizophrenia is a reduction in social behavior. Pharmacologic, genetic and neurodevelopmental animal models of schizophrenia have consistently found a reduction in social behavior [156].

## 2.6 Emotional learning and emotional response

There is evidence that fear conditioning is deficient in the schizophrenic patient. In a discriminative aversive conditioning test, schizophrenic patients failed to develop a discrimination towards aversively reinforced trials, whereas healthy matched individuals acquired a differential response to reinforced vs. unreinforced trials [157]. In another study, schizophrenics displayed a continuous deficit in emotional learning even after remission of symptoms, while performance on non-emotional learning tasks was improved and no longer different from controls. The deficit in emotional learning co-occurred with a diminished right amygdala volume [36]. Conditioned inhibition, is demonstrated when the meaning of conditioned stimulus (CS) is qualified by another (conditioned inhibitor). Whilst the CS presented alone reliably predicts the outcome (unconditioned stimulus, US), when presented in conjunction with the conditioned inhibitor the otherwise expected US will not occur. In a study investigating the relationship between conditioned inhibition and schizotypy scores (personality trait marking liability for schizophrenia), Migo and colleagues found a negative correlation between these two elements, suggesting impaired learning capabilities in those susceptible to develop schizophrenia [158].

Investigating these different forms of emotional learning deficits in animal models of

schizophrenia could allow more in depth investigation of the mechanisms underlying the cognitive and affective deficits seen in schizophrenia. Fear conditioning in rats has been used, throughout the literature, to study fear formation, recall and extinction together with the relevant brain circuits [29, 159]. It involves the pairing of a neutral CS with an aversive US. After few trials, the CS elicits the same response as the aversive stimulus. The aversive stimulus can be visual, auditory, tactile, gustatory or olfactory. Measuring the freezing behavior of animals in response to the conditioned stimulus can allow one to determine whether fear conditioning was acquired or not [29, 159]. Fear conditioning is also considered to be stress inducing [29, 159]. Accordingly, the stress the animal experiences elicits an emotional neuroendocrine response that starts in few seconds (adrenergic response) and can last several hours (HPA-axis response) This response can be measured long after conditioning in the absence of US, just like freezing [160, 161].

# 3. Animal models for psychosis with etiological validity

## 3.1 Validity criteria for animal models

Generating animal models of human neuropsychiatric disorders is extremely challenging given the subjective nature of many symptoms, the lack of biomarkers and objective diagnostic tests, and the early state of the relevant neurobiology and genetics. Progress in understanding pathophysiology and in treatment development would benefit greatly from improved animal models [155]. Sets of criteria are proposed for creating and assessing animal models of human mental disorders: construct/ etiological validity (theoretical rationale), population validity (only a subgroup of a population should display the disease-like phenotype), face validity (phenomenological similarity), predictive validity (animal behaviour in the test predicts human condition)[162].

## 3.2 Genetic animal models of schizophrenia

### *3.2.1 General approach*

Genetic animal models of schizophrenia were based on selective breeding, random mutagenesis, screening for phenotypes of interest, transgenic animals (e.g. knockouts, knock-ins, overexpression), and virally mediated gene delivery to discrete brain regions [155]. Here we will focus on a rat strain created with selective breeding for DA susceptibility that shows a striking phenotypic similarity with schizophrenia (face validity).

### *3.2.2 Apomorphine susceptible rat line (i.e. APO-SUS rats)*

Apomorphine is a relatively non-selective dopaminergic agonist (agonist of the $DRD_1$ and $DRD_2$ receptors) and, like psychomimetic drugs, can induce symptoms in healthy humans such as hyperactivity, stereotypy, ataxia and impaired social behaviors, which resemble the positive symptoms of schizophrenia [163]. The administration of apomorphine in rats results in stereotypical behavior like hyperactive locomotion and compulsive gnawing in (striatum) DA sensitive rats [Fig. 6 & Ref.[130, 164]]. When testing Wistar rats on apomorphine susceptibility Cools and colleagues found that in several behavioral traits the Wistar rats showed a bimodal distribution (Fig. 6) with a significant number of rats had a very high gnawing response (>500 counts) while a similar proportion of rats showed almost no gnawing response (≤10 counts) [165, 166]. These two significantly different groups were called NON-GNAW (≤10) and GNAW (>500). Only a small proportion of the tested rats had an intermediate response (11-500 counts). The gnawing score was used to select the nine highest scoring males and females and the nine lowest scoring males and females and breed them. This breeding continued for several generations. Thus, they selectively bred for apomorphine susceptibility and, eventually, generated the apomorphine susceptible (APO-SUS) and apomorphine unsusceptible (APO-UNSUS) rat lines [165, 166].

The APO-SUS rats have a remarkable genetic predisposition for schizophrenia-related features. These rat lines show several abnormalities ranging from behavioral, central nervous system, endocrine and immunological alterations to reduced susceptibility to several diseases like arthritis and reduced tumor formation. For more details in relation to the hyperactive DA system, hyper-reactive HPA-axis and GC-resistance see Table 2. Genetic variation differences between the APO-SUS and APO-UNSUS rats have been investigated as well. So far, the following genetic differences were reported: a gene-dosage imbalance of Aph-1b (component of γ-secretase)[167], genetic variants in topoisomerase II binding sites [168], cluster of variations in myosin 1b gene [168], various intragenic copy number variations (CNVs)[169] and genetic variants in the corticosteroid-binding-globulin (CBG) gene [170]. The functional implications of APO-SUS genetic-background in relation to DA and GCs cellular signaling are missing.

## 3.3 Early-life stress animal models

In altricial species, like rodents, development continues in the postnatal period. Thus, prenatal stress (e.g. dam's restraint stress) and disruption of the stress hyporesponsive period by repeated MS have been used to evaluate the effect of early-life experiences on individual differences in the neuroendocrine activity and behavior later in life (etiological validity) [188]. Here, we will focus on the postnatal early-life adversity models and especially those applied within the stress-hyporesponsive period.

### *3.3.1 Stress-hyporesponsive period (SHRP)*

SHRP is a developmental period [postnatal days (pnd) 1-10 mice; 3-14 rats], in which

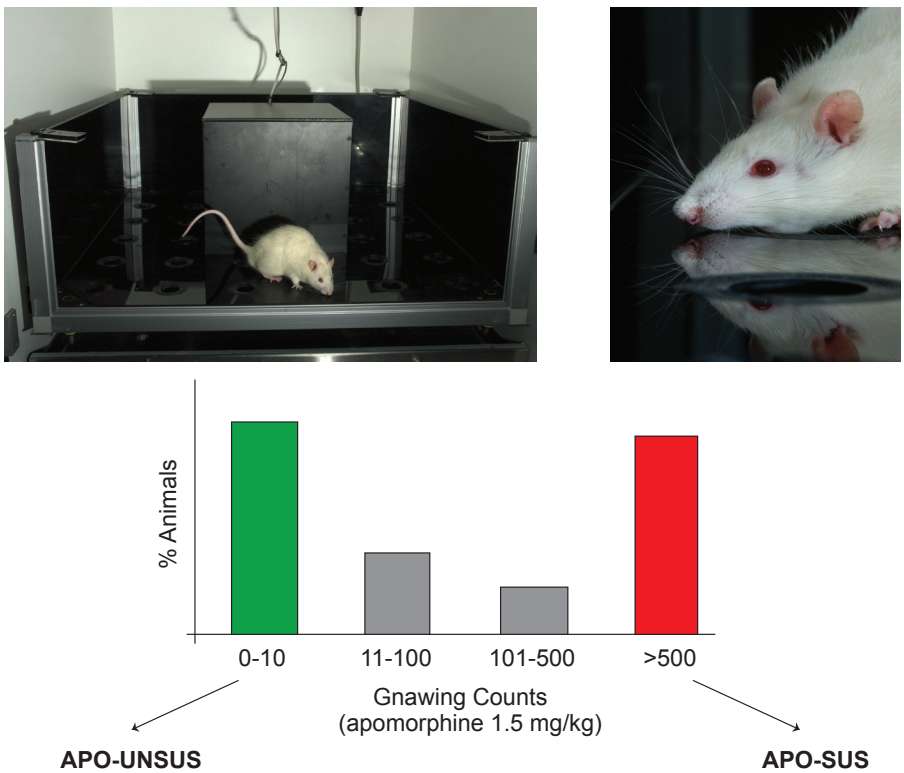



Figure 6. The gnawing box (A) and a close-up of a Wistar rat stereotypically gnawing (B) the concentric ridges of a hole after an apomorphine injection (photos courtesy of D.Saaltink & N. Daskalakis). (C) Schematic representation of the generation of the APO-SUS and UNSUS rat model. Rats from the Wistar population were injected with the dopamine $DRD_1$/ $DRD_2$ receptor agonist apomorphine and tested for their gnawing responses. Rats with a low gnawing score (<10 times per 45min) and rats with a high Gnawing score (>500 times per 45min) were selected and used for breeding of the apomorphine-unsusceptible (APO-UNSUS) and apomorphine-susceptible (APO-SUS) rat lines, respectively (based on [166]).

the elevation of CORT in response to mild stressors is attenuated. For the neonate's stress hypo-responsiveness a lot of factors have been implicated like: adrenal inhibition/ insensitivity [189-192], enhanced GR-mediated negative feedback [193-195], inhibition of the brain renin-angiotensin system [196, 197], CRH-1 and CRH-2 receptor system function [198-201], $\alpha_2$ adrenoreceptor control of pituitary secretion [202] and central action of metabolic factors [203-206]. Yet, the most proximal cause for the transient hyporesponsiveness to stress in the adrenal secretion of CORT

Prolonged MS (>8h), implemented within first two weeks of life, causes neonatal mice and rats to emerge from the SHRP and display elevated basal and stress-induced levels of CORT [189]. These observations showed that central neural mechanisms required to elicit and control an endocrine response following stress appear to be effective early in development [194, 195, 207, 208], but various components of the

Table 2.
Phenotype of APO-SUS rat line (vs. APO-UNSUS rats)

| Measurement | | Conditions | | Ref. |
|---|---|---|---|---|
| | | Basal | Challenged | |
| ***Behaviour: Increased behavioural response to dopamine, Active stress coping style, Sensorimotor gating deficits, Memory deficit*** | | | | |
| Gnawing | | Nd | APO: ↑ gnaw | [166, 171] |
| Locomotor activity | | ↑ | Open field: ↑loco<br>DEXAMPH: ↑loco<br>Ergometrine (in ventr.striatum): ↑ loco<br>Picrotoxin (in superior colliculus.): ↑ explosive running<br>Picrotoxin (in SN): ↑ lack of control<br>Phenylephrine (in ventr.striatum): ↑ loco | [166, 172, 173] |
| Fear | | | Social defeat: Freezing: ↓; Fleeing: ↑ | [166] |
| Learning & Memory | | | Stress: Active avoidance: ↓; Water-maze: ↓ | [172, 174] |
| Startle<br>& Sensorimotor gating | | Startle: Socials: nd / Adult isolate: ↑<br>PPI: Socials: nd / Adult isolate: ↓ | COC: Startle: nd/ PPI: ↓ *(reversal by $DRD_2$ antagonism)*<br>AMPH: Startle: nd/ PPI: ↓ *(reversal by $DRD_2$ antagonism)* | [175-178] |
| Latent Inhibition | | Conditioned taste aversion paradigm: ↓ | | [175] |
| ***Dopamine system: Hyperactivity*** | | | | |
| Nigro-striatal | SN<br>Striatum | TH mRNA: ↑<br>Norandrenaline IR: ↓<br>$DRD_1$ mRNA: ↑/ binding: nd<br>$DRD_2$ mRNA: nd/ binding: ↑ | | [166] |
| Meso-limbic | Nucl. Accumbens | TH IR network&varicosities: ↑<br>TH mRNA: nd<br>Extracellular DA: nd | Stress<br>TH IR network&varicosities: ↑<br>Extracellular DA: ↑ | [179-181] |
| Tuberoinfundubular | Arcuate Nucl. | TH mRNA: ↑ | | [179-181] |
| ***Stress system: Hyper-reactivity*** | | | | |
| Hippocampus | | DynB: DynB IR: ↑, $DynB^+$ intra/infra-pyramidal mossy fiber terminal fields length: ↓<br>GR: mRNA: nd, binding: nd, affinity: nd, protein: nd<br>MR: mRNA: nd, binding: ↑ / affinity: nd, MR protein | Stress<br>DynB IR: ↓ | [161, 166, 172, 182, 183] |
| Hypothalamus (PVN) | | CRH mRNA: ↑<br>Synaptic density: ↑ | Stress:<br>Fos IR: ↓ | [160, 184, 185] |
| Pituitary | | MR binding: ↑ | | [182] |
| Prolactin | | Nd or ↓ | Stress: ↓ | [161] |
| ACTH | | ↑ or nd | Stress: ↑↑<br>ADX: ↑↑ | [160, 161, 170] |
| CORT | | Total: nd<br>Free: ↓ (↑ CBG / CBG SNP) | Stress:<br>Total: nd or ↑/ Free: ↑ | [160, 161, 170] |
| ***Developmental deviations & Stress system dysfunction precedes dopamine system dysfunction*** | | | | |
| Development | | digits separation, anogenital-distance, rooting-reflex, body-displacement (pnd 4) : delay; eye opening: earlier | | [186] |
| pnd 10 | | PVN CRH mRNA: nd; PVN GR mRNA: ↑; ACTH: nd; CORT: nd; Hip. MR mRNA: nd; Hip. GR mRNA: nd; Adrenal weight: nd; Thymus weight: nd; Striatum $DRD_1$& $DRD_2$ mRNA: nd | | [187] |
| pnd 18 | | PVN CRH mRNA: nd; PVN GR mRNA: nd; ACTH: ↑; CORT: nd; Hip.MR mRNA: nd; Hip.GR mRNA: ↑; Adrenal weight: nd; Thymus weight: ↑; Striatum $DRD_1$& $DRD_2$ mRNA: nd | | |

Abreviations. ACTH: adrenocorticotropic hormone, AMPH: amphetamine, APO: apomorphine, CBG: corticosteroid-binding globulin, Coc: Cocaine, CORT: corticosterone, CRH: Corticotropin-releasing hormone, DA: dopamine, DEXAMPH: dexamphetamine, $DRD_1$&$D_2$: dopamine receptors $D_1$ & $D_2$, DynB: dynorphin B, Fos: FBJ osteosarcoma oncogene, GR: glucocorticoid receptors, Hip.: hippocampus, IR: immunoreactivity, LI: latent inhibition, MR: mineralocorticoid receptors, nd: not different, pnd: postnatal day, PPI: prepulse inhibition, PVN: paraventricular nucleus, SN: substantia nigra, SNP: Single-Nucleotide Polymorphism, TH: Tyrosine hydroxylase.

dam's behavior (mostly feeding and tactile stimulation) seem capable of inhibiting or dampening the HPA-axis response; namely tactile stimulation is able to inhibit the central effects (on CRH and ACTH) and feeding is able to inhibit the peripheral effects of MS (CORT increase) [189, 205, 209-213].

The human HPA-axis development is in concordance with rats (even though rats are prematurely born) since the axis is not yet fully developed at birth and goes through a

comparable SHRP during months 6 to 12, while human babies are dependent on their caregivers for normal development, and adverse experiences in this period can have a long lasting impact [214].

### *3.3.2 Transitions from attachment learning to aversion learning in infancy*

Rat pups are dependent on maternal care for survival and show a sensitive period of facilitated odor preference-learning to maternal odor, under the age of pnd 10. During this period, pups show an attenuated learning of fear, and remain with the dam. This absence of avoidance learning is crucial for forming dam-pup attachments [215]. After pnd 10 (post-sensitive period), a short term maternal absence allows odor avoidance-learning [216]. Elevation of CORT levels is crucial for transition to aversion. To prematurely trigger this aversion to noxious stimuli CORT needs to activate the amygdala [217].

### *3.3.3 Maternal care model*

Most common experimental manipulations are focusing on the dam-pup relationship. Some models work with the naturally occurring variations of maternal care and in other models maternal absence periods are experimentally induced. In Long Evans rats variations in maternal care in the form of licking and grooming (LG) were found to permanently affect the number of hippocampal GR, possibly through epigenetic changes occurring the first week of life which involve DNA methylation in the GR promoter region [218]. This lasting modulation of the GR has in turn consequences for HPA-axis reactivity later in life and is linked to hyper-reactivity for the Low LG offspring and hypo-reactivity for the High LG offspring [219]. In addition to the programming of neuroendocrine response pattern, emotional reactivity and cognition [220-223]. Interestingly, Low LG offspring display high acoustic startle and deficits in PPI [224].

### *3.3.4 Maternal Separation (MS) model*

The MS model is commonly applied during the SHRP. The MS manipulations have been applied as a function of strain and gender of the animals, and were varied in frequency and duration as well as the age of the pup and the time point within light cycle [188, 225]. Also the environmental context such as the housing in groups or in isolation, inside the home cage or in a novel environment and the ambient temperature were varied in several ways [225, 226]. The most commonly used separation protocols are: a 15min long separation including handling of the pups (i.e. neonatal handling), repeated daily separations for about 3 to 8h (i.e. R-MS), and a maternal deprivation (MD) model where the separation lasts for a single 24h period (i.e. 24h-MD) [188].

In the short-term, brief MSs (typical for neonatal handling) do not activate the HPA-axis the first time, but when repeated sensitisation of the CORT response occurs in parallel with adrenal growth [227]. Prolonged separations activate, slowly the first time,

the HPA-axis, which becomes responsive to stress. However, when repeated the stress system adapts and basal CORT levels do not rise anymore upon multiple separations but remains responsive to stress [228]. In the long-term, the early-life manipulations induce lasting alterations in the neuroendocrine activity. Maternally separated individuals, as compared to handled individuals or controls (animal facility reared), display an increased response to stress, enhanced emotional reactivity and impaired cognitive performance. MS can result also in enhanced acoustic startle, lower PPI and latent inhibition [229].

The sensitivity to early-life traumas varies per individual. Early-life traumas interact with pre-existing genetic susceptibility to stress and disease [230]. The genotype (strain) can influence clearly the effect of the environment in animal models in various developmental stages as is manifested by conditions such as prenatal stress, cross-fostering procedures, maternal care variations, MS and isolation rearing [171, 231-237]. In Table 3, we highlight the importance of genetic background for the outcome of MS on different aspects of adult behavior relevant for animal models of schizophrenia. These include measures of stress and emotional reactivity, fear and anxiety, emotional memory performance and cognitive performance, startle reactivity and PPI. We selected for this summary studies with two widely used outbred rat strains: Wistars and Long Evans. Long Evans rats seem to be most sensitive to the effects of early-life

Table 3

Comparison of Wistar and Long Evans male rats in the long term outcome of MS applied the first two weeks of life

| Strain | pnd(s) | ELS Type | Control group | HPA-R | Anxiety | Emotional Learning | Cognition | Startle | PPI/LI/ SG | Drug-R | *Ref.* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **WISTAR** | 3 | 24h-MD | AFR, EH | nd | nd | ↑ | ↓ | nd or ↑ | ↓ | Apo: ↑ | *[54, 148, 229]* |
| | 4 | 24h-MD | AFR | ↑ | nd | ↑ | ↑ | nd | Nd | | *[244-246]* |
| | 6 | 24h-MD | EH | | | | | ↓ | ↓ | | *[229]* |
| | 9 | 24h-MD(or 12hMD + 12hMD) | EH, AFR | ↑ hip.GR:↓ | nd or ↑ | ↑ | ↑ or nd or ↑ | nd or ↑ | ↓ or nd | Apo: nd or ↑ Amph: nd | *[229, 234, 244-252]* |
| | 1-10 | RMS (3h/d) | NH | | nd | ↓ or nd | | | | | *[253]* |
| | 1-13 | RMS (2x3h/d) | AFR | | nd | | | | | | *[254]* |
| | 2-14 | RMS (3h/d) | NH | ↓ | | ↓ | | | | | *[255]* |
| | 2-14 | RMS (3h/d) | EH | nd | | nd | | | | | *[255]* |
| | 3-12 | RMS (3h/d) | AFR | ↑ | ↑ | | | | | | *[256]* |
| **LONG EVANS** | 3 | 24h-MD | NH, EH | hip.MR&GR :↓ | | | | | | | *[257]* |
| | 5 | 24h-MD | EH | ↑ | ↑ | | | | | | *[258]* |
| | 14 | 24h-MD | EH | ↑ | ↑ | | | | | | *[258]* |
| | 1-14 | RMS (3h/d) | NH | | | | | | | Amp: ↑ | *[259]* |
| | 2-14 | RMS (3h/d) | AFR, EH, NH | ↑ hip.MR&M R/GR:↑ FC GR: ↓ | ↑ | | ↓ | ↑ | | Amp: ↑ Coc: ↑ | *[51, 260-266]* |

Note. We selected studies using two widely used outbred Wistar and Long Evans rat strains. Early-life stress protocols reviewed here were restricted to the first two weeks of life. Concerning the MS we review only the studies with whole litter design because extra littermate separation could add more complexity to the comparison. Adult testing refers to adult males. Abreviations. AFR: animal facility reared, Amp: amphetamine, Apo: apomorphine, Coc: Cocaine, EH: early handling, GR: glucocorticoid receptors, HPA: hypothalamus-pituitary-adrenal axis, LI: latent inhibition, 24h-MD: 24h maternal deprivation, MR: mineralocorticoid receptors, nd: not different, NH: not handled, pnd: postnatal day, PPI: prepusle inhibition, R: response, hip.: hippocampus RMS: repeated maternal separation, SG: sensory gating.

manipulations and display all expected phenotypic characteristics of a vulnerable phenotype. Maternally separated Wistar rats are however often not different from the control rats and therefore the Wistar strain may be considered rather resilient to early-life manipulations. This gene dependent vulnerability of the Long Evans strain versus resilience of the Wistar towards early-life stress has been demonstrated previously also in humans [230]; for example the S-carriers of the 5-HT transporter gene promoter display increased depression after stressful life events including childhood abuse [238].

## 3.4 Late development and early adulthood stress models

The rapid development of the brain during early neonatal life is thus a very susceptible period for lasting changes brain maturation induced by environmental factors. However, a second period of rat brain development of almost equal importance is the post-weaning period (juvenility-puberty-early adulthood). To study the effects of stress during the post-weaning period different manipulations have been used.

### *3.4.1 Social isolation rearing*

Postweaning social isolation is a reliable and chronic psychosocial stressor in the study of schizophrenia is. This kind of isolation if lasting for at least approximately 30 days starting the 4th week of life can induce behavioral disruption as well as neuroanatomical and neurochemical changes comparable to schizophrenia. The behavioral changes include impaired PPI, cognitive impairments and social dysfunctions [239-243]. Studies have shown that the behavioral responses in rats as a result of the isolation are followed by raised levels of high affinity $DRD_2$ receptors, which have been suggested to be involved in the positive symptoms of schizophrenia [24]. Weiss and colleagues studied the effects of isolation rearing on PPI in three different rat strains (Wistar, Sprague-Dawley and Lister hooded). Although they found a significant lowering effect of isolation on PPI in the Sprague-Dawley and Lister hooded rats, the isolated Wistar rats did not show a reduction in PPI [236]. This is an indication of gene-by-environment interaction in this model.

### *3.4.2 Chronic stress*

Other approaches to accomplish increased later-life stress are chronic administration of high doses of the stress hormone (CORT) or exposure to daily stressors, preferably in a chronic unpredictable paradigm. Chronic stress has shown to affect brain structure and functions, and can cause atrophy and decreased branching of the dendrites of hippocampal neurons as well as reduced neurogenesis in the dentate gyrus [267]. Most importantly, the procedures of chronic CORT administration or chronic unpredictable stress can enhance schizophrenia-related behaviours in the rat [268].

## 3.5 Combination of various models

APO-SUS genotype + environmental stress: Ellenbroek and colleagues studied the

effects of maternal care in APO-SUS rats by crossfostering APO-SUS rat pups with APO-UNSUS dams. The APO-SUS rat pups were raised by their own mother as controls, raised by another APO-SUS mother or cross fostered by an APO-UNSUS mother. In adulthood, the apomorphine induced gnawing was scored. The cross fostering group displayed a significantly lower apomorphine-induced gnaw score as compared with the control group. APO-UNSUS pups raised by APO-SUS dams were not sensitive to the effects of cross-fostering [171]. Inversely, APO-UNSUS were sensitive to the effects of MS (24h-MD), while the APO-SUS were not [171]. These findings indicated that APO-SUS dams might show less maternal care behaviors like LG than APO-UNSUS dams. Accordingly, it is postulated that APO-SUS genes are linked to a low maternal care trait. The notion that the APO-SUS genetic background shows a particular feature of interaction with environmental stress was also revealed in the epigenetic differences found in specific brain areas of APO-SUS and APO-UNSUS offspring (differences found in methylation variation in cerebellum and hypothalamus, but not hippocampus) [168].

Multiple environmental stressors: Environmental factors in early-life or later-life can influence the development of schizophrenia. However, the early-environmental factors can also affect how an individual respond to the late environmental factors. This kind of environment-environment interactions form the basis of the developmental *mismatch* hypothesis.

(a) Low maternal care early-life history + Adult acute stress: Champagne and colleagues studied how variations in LG can influence learning under stress during adulthood. Long Evans offspring were divided in a Low LG or High LG group. At adulthood both groups were examined in contextual fear conditioning which reflects learning capacity under conditions of high stress. The results showed that the adult Low LG offspring had under stressful conditions a better memory performance than the High LG group. In addition synaptic plasticity was reduced in hippocampal slices of the adult Low LG as compared to slices of the High LG group, but enhanced if the slices were exposed to high concentrations of CORT. These results are in agreement with the developmental *mismatch* hypothesis [221].

(b) MS + Isolation rearing: Ellenbroek & Cools showed that Wistar rats that suffered MD had a decreased PPI. However, if the maternally deprived rats were reared in post-weaning social isolation (*match*), the MD did not lead to a decreased PPI, but instead PPI was returned to normal levels [269].

(c) MS + Chronic stress: Choy and van den Buuse studied how early and later life stress affects the schizophrenia phenotype of adult Wistar rats. 24-hMD was used as early-life stressor and the chronic administration of CORT later in life was used to mimic chronic stress. They showed less disruption of PPI by apomorphine or amphetamine in rats that had undergone both MD and CORT treatment [247-249]. Similarly, maternally deprived rats showed an adaptive behavioral response also to chronic unpredictable stress characterized by less anxiety, better cognition and enhanced hippocampal plasticity compared to control rats [250].

# 4. Scope and outline of the thesis



## 4.1 Rationale

Human studies have demonstrated that genotype in interaction with adverse life experiences timed during early and late brain development determine interindividual differences in vulnerability to schizophrenia. Animal models addressing these interactions are needed for the study of the disease mechanisms and for the discovery of novel drug targets. For this purpose, several different rat and mouse strains with various genetic backgrounds have been used and they were often exposed to different life stress paradigms (MS, naturally occurring variations in maternal care, isolation rearing).

## 4.2 Objective

The objective of the research described in this thesis was to develop an animal model for schizophrenia in order to identify novel drug targets. For this purpose we have investigated the effect of interactions between genetic predisposition for enhanced dopamine susceptibility, early-life experience and later-life stressors on the development of schizophrenia endophenotypes in rats.

To achieve this objective the following hypotheses were tested:

1. Early-life adversity programs individuals' psycho-neuro-endocrine response to stress and psychosis susceptibility.
2. *Three-hit* (*cumulative stress*) hypothesis of schizophrenia i.e. environmental adversity during early and late brain development in interaction with predisposing genetic susceptibility creates a phenotype vulnerable to schizophrenia.
3. *Mismatch* hypothesis of schizophrenia i.e. a *match* of early-life adversity with later-life stressful conditions can lead to resilience to psychosis; and a *mismatch* to enhanced vulnerability to psychosis.

## 4.3 Outline of the thesis:

i. In **Chapter 2**, we describe the immediate endocrine effects of maternal absence in rats. During SHRP the adrenal CORT secretion shows little responsiveness to stressful stimuli while central components of the stress system still respond. MS causes over several hours a slow activation of CORT secretion. However, the pups rapidly adapt to repeated maternal absence. If the separation is repeated the next day, CORT does not rise anymore, but the pup continues to respond to a novelty-stressor. In our study we investigated the effects of novelty-stress during maternal absence, maternal care and also of genotype on this phenomenon. For this purpose we varied the degree of novelty in the separation environment, monitored maternal care and used two different rat strains.

ii. In **Chapter 3**, we describe the long-lasting impact of early-life stress on stress and emotional reactivity. Maternal absence destabilizes SHRP and enhances susceptibility to stressors in early postnatal life, leading to stress vulnerability in adulthood. In our study, we tested the hypothesis that a stressful experience non-shared between peers during repeated MS, rather than the maternal absence per se, leads through amygdala priming to the programming of an adult fearful phenotype.

iii. In **Chapter 4**, we test the *three-hit* (*cumulative stress*) hypothesis of schizophrenia. In particular, we test that early-life adversity in interaction with genetically-selected "reactive" dopaminergic alleles may create a phenotype vulnerable to schizophrenia, which can be further deteriorated with additional stressors in later life. To examine this in our study, APO-SUS rats, genetically-selected for profound DA susceptibility were exposed as pups to reduced maternal care as early-life adversity during the first week of life. A subgroup of animals was also exposed to a pre-pubertal psychosocial stressor. We measured stress-related and schizophrenia-like phenotypes in order to demonstrate that after cumulative exposure to early and later-life adversity these phenotypes co-precipitate provided that the dopamine genetic susceptibility is present.

iv. In **Chapter 5**, we test the *cumulative stress* and *mismatch* hypotheses for schizophrenia in non genetically-selected rats. In particular, we investigate if the relationship of early-life adversity and psychosis susceptibility depends on the later-life social context.

v. In **Chapter 6**, the experimental data are discussed and placed in the *nature-nurture* conceptual framework highlighting possible pathogenetic mechanisms for schizophrenia and the special role of GCs. We conclude with a synthesis and future perspectives.

vi. In the **Addendum** we evaluate how individual components of the mild early-life experience of neonatal handling (i.e. novelty exposure and tactile stimulation) are critical for adult memory performance and basal adrenocortical function.

# 5. References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