Nurturing nature : testing the three-hit hypothesis of schizophrenia

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Chapter 6

General Discussion

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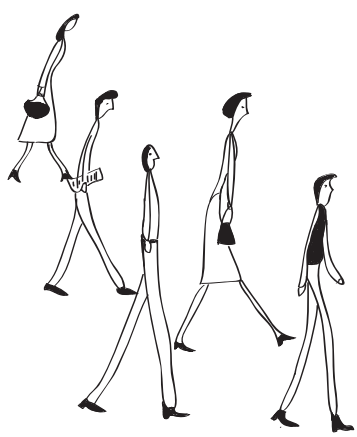

Contents

The goal of this thesis research was to study the interaction between schizophrenia-susceptibility and developmental stress in order to create an animal model of schizophrenia. For this purpose, we have used an already established rat 'schizophrenia' model which is characterized by a profound sensitivity to a dopamine agonist (apomorphine), the so-called apomorphine-susceptible (APO-SUS) rat line. The pharmacogenetic selection for apomorphine-susceptibility from an outbred Wistar rat population started in the late seventies of the past century by Alexander Cools in Nijmegen. In his previous research, the rat line was extensively characterized in neuroendocrine and behavioral response patterns. Rats of this APO-SUS line displayed increased fleeing in response to a threat, showed increased locomotor activity in a novel environment and displayed a peculiar neuroendocrine response to a psychosocial stressor. In response to stress, APO-SUS rats responded with a much higher ACTH response than their APO-UNSUS counterparts, while CORT levels were similar. This response pattern points to a new set-point in the HPA-axis characterized by adrenal hyporesponsiveness to ACTH and glucocorticoid (GC) feedback resistance and enhanced binding of CORT to the mineralocorticoid receptor (MR) in the limbic brain [1, 2].

In our studies, we have used the APO-SUS rat line to investigate if a history of early and/or late life stressors could aggravate schizophrenia-like phenotypes. As early-life experience two approaches were used: (i) the repeated prolonged maternal separation (MS) paradigm on postnatal days (pnd) 3 – 5, (ii) the natural occurring variation in maternal care expressed as bouts of licking and grooming (LG). As pre-pubertal social stressor we used post-weaning social isolation. As criteria for increased stress and psychosis susceptibility, we have measured, on the one hand, the fear response and GC feedback resistance and, on the other hand, social interaction, perseverative/ stereotypic behavior (apomorphine-induced gnawing), prepulse inhibition of acoustic startle (PPI) and working memory (T-maze spontaneous alternation).

The results are described in the Chapters 2-5. **Chapter 2** contains an in-depth exploration of the immediate endocrine effects of MS and stressful early-life experience during MS. We found that the rat pup readily adapts to repeated daily MS, but the magnitude of the stress response after MS is determined by the MS context. In **Chapter 3**, we raised the question whether stressful early-life experience during MS contributes to the programming of a fearful phenotype. We found that MS in combination with early-life stressful experience, occurring in isolation, programs a fearful individual through amygdala premature activation.

Chapters 4 & 5 were dedicated to test two influential hypotheses in the field of developmental origins of psychopathology: the "*three-hit*" (*cumulative stress*) hypothesis and the "*mismatch*" hypothesis. Briefly, the *three-hit* hypothesis states that cumulative exposure of genetically-susceptible individuals to environmental stress, during early

and late development generates a vulnerable phenotype for psychopathology. Individuals carrying the genetic background of apomorphine-susceptibility (APO-SUS) displayed increased stress and psychosis susceptibility after adversity in both the maternal environment and post-weaning social environment.

The *mismatch* hypothesis, states that if a *mismatch* occurs between the actual and the expected later life circumstances, based on experiences early in life, a vulnerable phenotype develops. Rats, without genetic-susceptibility to psychosis (Wistar), displayed increased psychosis susceptibility, when they encountered a radically different, in terms of stress, later social environment compared to their early maternal environment. Finally, we have tested the impact of GC-treatment on the expression of psychosis susceptibility since stress and GCs have been proposed to alter psychosis susceptibility. We discovered that individuals with increased psychosis susceptibility caused either by genetic background and/or life stress, are resistant to GC-regulation.

In the next sections, we will first discuss the animal model used, then the evidence in favor or against the *three-hit* and *mismatch* hypothesis and the use of GCs to alter schizophrenia endophenotypes. We conclude with a synthesis and future perspectives.

1. Immediate effects of early-life stress

1.1 CORT-desensitization phenomenon towards repeated MS

In Chapter 2 & 3, we have used a daily 8 hours MS paradigm. A single prolonged period of MS (≥8h) during the stress hypo-responsive period (SHRP) is needed for a large increase of basal ACTH and CORT [3-7]. Interestingly, we found that the newborn rodent's basal CORT response to 8h-MS on pnd 3 is abolished when the 8h-MS procedure is repeated the next day. This habituation or adaptation of the pup to the experience of repeated MS is not due to a time course shift of the MS-induced CORT response [3]. CORT desensitization is a robust phenomenon since it occurs irrespective of genotype (Wistar or Long Evans rats) and context (home or novel).

1.2. Enhanced adrenal stress sensitivity after repeated MS

Single MS causes increased adrenal sensitivity to heterotypic stressors and exogenous ACTH [7-9]. In our study this enhanced adrenal sensitivity to stressors was maintained after repeated absence of the dam, under conditions that the basal HPA-axis activity had adapted and CORT levels were similar in non-separated controls. However, the pups stayed on alert and retained the ability to respond to stressors with an increase in ACTH and CORT levels [3]. We have studied stress sensitivity in our model in two different MS-conditions, in which the rat pups were either with their siblings together in the home cage ('home separation') or isolated and housed individually for 8h in a novel cage ('novel separation'). The two separation procedures had a different outcome in response to a subsequent 30min novelty-stressor. Rat pups separated in a novel environment apparently adapted to this condition, because the additional

novelty-stress could not any longer trigger a CORT response, while the repeated "home separation" condition did. Apparently, the previous experience of maternal absence in a novel environment not only prepares the pups for the transient maternal absence, but also for the experience of "novelty" itself (Chapter 2). Adrenal MCR-2 protein content was enhanced after repeated MS in home environment. TH protein level, an indirect measure of medullary-catecholamine response to maternal absence [9], displayed an increase over the basal levels after a third home-MS (Chapter 2). Elsewhere it was reported that this increase in adrenal activity coincides with an activation in locus coeruleus (LC) implicating the LC-NE neurons as modulators of neonate adrenal stress sensitivity [10].

1.3 Repeated MS mechanism and premature stress-induced amygdala activation.

Although the mechanism underlying the effects of repeated MS has been explored previously, there were no sufficient explanations [3]. Effects of mineralocorticoid receptor antagonism and PVN c-Fos expression data suggested the involvement of a central mechanism [3]. In fact, a neonatal learning mechanism could have played a key role and our objective in Chapter 3 was to specifically link our findings with the ontogeny of the amygdala-dependent fear aversion [11]. When the dam is present in the nest, adversity towards the pups will be negligible and attachment to the dam care-giver is expected to develop irrespective the quality of maternal behavior [12]. Until approximately pnd 10, pups exhibit preference to novel odors, even if they are paired with negative stimuli [13, 14]. In the post-sensitive period, odor-avoidance behavior appears and is associated with CORT-dependent neural processes in amygdala and piriform cortex [13-16]. Interestingly, if during the "sensitive" period the dam is away, then the odor aversion neuronal system is activated prematurely and aversive memories can be formed as long as the CORT levels are elevated in blood and in amygdala [15, 16].

In our experiments, the pups were at an age (pnd 3-5 during the SHRP) that permitted formation of memories only during long-term absence of the dam. The pups may have learned to predict the return of the mother and, thus, the reinstatement of maternal care. After being separated from their mothers for the first time, pups experience high amounts of CORT (Chapter 2 & 3). Hence after the first separation period CORT priming of the amygdala is possible, but yet the outcome is profoundly diverging as a function of the MS environment. At pnd 5, the pups that had stayed in the home cage (HOME SEP) responded to a mild novelty-stressor with an enhanced CORT response (Chapter 3), since in this condition their adrenal cortex was primed for hyper-responsiveness (Chapter 2). In contrast, NOVEL SEP pups displayed enhanced amygdala c-Fos expression and ACTH release. Interestingly, these pups, while showing the central stress-induced activation, did not show a peripheral adrenocortical response to stress confirming the notion that the adrenals' hyporesponsiveness actually accounts for the SHRP [9, 17].

1.4 Maternal care after repeated MS.

Artificial feeding during maternal absence acts as an inhibitory factor to the neonate's basal and stress-induced adrenal activity [4, 18-22], and artificial stroking can inhibit the central activation of limbic and hypothalamic brain areas [19, 20, 23]. An altered pattern of maternal care after home or novel MS might lead to a greater suppression of the HPA-axis and result in blunted stress activation after the third 8h-MS. We observed the following: (i) home and novel separated groups received overall similar care, which was reduced compared to that of the controls. Licking & Grooming (LG) was in the low range of an undisturbed large Wistar population (Chapter 3 & 5); (ii) post-separation bouts of LG were higher for novel separated than for home separated pups(Chapter 2); (iii) separation did not induce an increase in active nursing (AN) upon reunion (Chapter 2). Taking these data together, it can be concluded that experimentally-induced maternal absence induces an altered pattern of care characterized by reduced average care and increased variation in time.

1.5 Summary of findings.

In Table 1, we summarize our findings on pups with a history of MS in their subsequent neuroendocrine response towards maternal absence or to stressors, compared to naïve pups. Immediately after MS, the greatest central and peripheral activation of the stress system occurs for naïve rat pups. The repeatedly separated pups, irrespective of MS-context, readily adapt to MS. We favor the neonatal learning explanation for this robust phenomenon, since the pups in this age are able to recognize environments that they have experienced before. The pup's learning and the blunted peripheral CORT response might be facilitated by the daily dam's maternal care response.

The maternal mediation hypothesis might not be the sole mechanism explaining the effects of early-life stress on the HPA-axis reactivity. Instead it is proposed that the combination of environmental adversity and the maternal repertoire determines the lasting alterations on the offspring's HPA-axis response [24, 25]. Pup's stress reactivity is proposed to be a function of the additive effects of the environmental stress (per se) and maternal care response to environmental stress, acting as two independent factors [24, 25]. We were also able to underline a dichotomy between the effects on the pup's central and peripheral (adrenal) stress reactivity. We propose that the pup's central response shows a U-shape relationship with maternal environment adversity, whereas the peripheral response an inverted U-shape relationship (Fig. 1).

Table 1.

HPA-axis reactivity of pnd 5 pups that were naïve or repeatedly separated in home (HOME SEP) or novel context (NOVEL SEP).

Measurements			Adversity of the maternal environment								
			+			++			+++		
			1st SEP			HOME SEP			NOVEL SEP		
			Basal levels	Post MS: basal levels	Post MS: stress reactivity	Basal levels	Post MS: basal levels	Post MS: stress reactivity	Basal levels	Post MS: basal levels	Post MS: stress reactivity
Central	Limbic	c-Fos	+	++	++	+	nm	+	+	nm	+++
	PVN	c-Fos	+	++	+++	+	+	++	nm	nm	nm
	Pituitary	ACTH	+	++	++	+	+	+	+	++	++
Peripheral	Adrenal	CORT	+	++	+++	+	+	++	+	+	+
		MC2-R	+	+	nm	+	+++	nm	+	+	nm
		c-Fos/TH	+	+	++	+	+++	+++	+	+	+
Maternal Care		Response to adversity	+	++	na	+	++	na	+	+++	na
		OVERALL	+++	++	na	++	+	na	++	++	na

Note. Ratings are subjective. Strength of effect: + to +++, nm: not measured, na: not applicable. The table is based on data of this thesis and from literature [3, 5, 9, 20, 26, 27]. First separated (1st SEP) had no previous history of treatments on pnd 3 & 4 and repeatedly separated pups were exposed to 8h-MS on pnd 3 & 4 in a home (HOME SEP; home separated) or novel context (NOVEL SEP; novel separated). The data presented reflect the changes if MS occurs in the beginning of the stress hyporesponsive period (SHRP) at pnd 3-5. The expected changes can be different if the experiments are performed before (pnd 1-3), in the middle (pnd 5-12), in the end (pnd 12-14) or even after (pnd 14-21) the SHRP [4, 28-31].

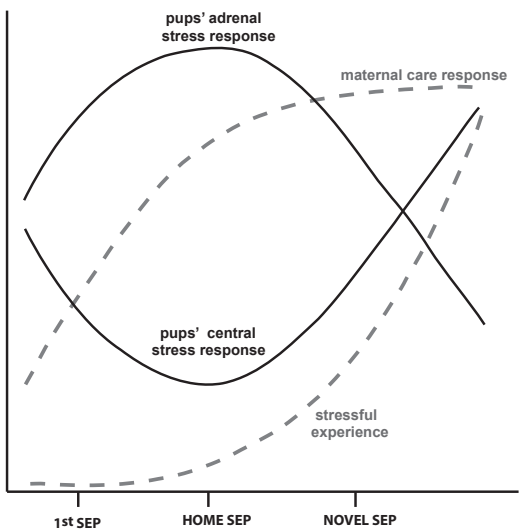

Figure 1. Illustration of possible relationship of pup's postnatal day (pnd) 5 stress reactivity (central and peripheral) with the adversity of the maternal environment [based on data included in Table I and inspired from [24]]. The increase of the adversity of the maternal environment (1st SEP →HOME SEP→NOVEL SEP) increases the stressful experience for the dam and the pups, which leads to an increased maternal care response of the dam (in an effort to maintain the overall levels of maternal care). Both these factors influence the pups' stress response. Interestingly, the pups' central stress response has a U-shape relationship with early-life adversity; the peripheral adrenal stress response has an inverted U-shape relationship. First separated (1st SEP) had no previous history of treatments on pnd 3 & 4 and repeatedly separated pups were exposed to 8h-MS on pnd 3 & 4 in a home (HOME SEP; home separated) or novel context (NOVEL SEP; novel separated). x-axis: adversity of the maternal environment, y-axis: the 4 different variables (stressful experience, maternal care response, pups' central stress response, pups' adrenal stress response) in arbitrary units.

2. Long term effects of early-life stress

Adversity of the maternal environment experimentally-induced (in the form of MS) or just naturally occurring (variations of maternal care) can predict altered social behavior, altered learning & memory, sensorimotor gating and susceptibility to drugs of abuse [32-42]. Below, we summarize the long-term effects of the early-life conditions we have studied.

2.1 HOME SEP (Repeated MS)

HOME SEP animals maintain a remarkable enhanced stress-induced CORT response from the first postnatal week into adulthood. Their enhanced CORT response occurs in the face of attenuated stress-induced ACTH release indicating a profound adrenal hyper-responsiveness (Chapter 2 & 3). The elevated CORT secretion of HOME SEP, that already became manifest at baseline, may explain the poor retention of the freezing response that they also display (Chapter 3). The increased scanning they show during fear acquisition indicates a more active coping style with evaluation and appraisal of the stressful electric shock and also predicts poor retention of the freezing response [43].

Their playful behavior was intact which suggests that their lack of freezing response towards contextual fear is rather a cognitive impairment than an affective deficit and it possibly has a hippocampal origin, given also the poor control of their CORT stress release (Chapter 3). This was also confirmed in a low-stress spatial memory (T-maze spontaneous alternation), where they showed reduced spontaneous alternation. Finally, their startle response, sensorimotor gating and DA-susceptibility were normal (Chapter 3 & 5).

2.2 NOVEL SEP (Repeated MS + postnatal social isolation)

The phenotype of the NOVEL SEP offspring persisted into adulthood in its enhanced amygdala c-Fos activation and ACTH release. The central stress response pattern matched their fear conditioning behavior which indicates increased emotional memory performance (Chapter 3). NOVEL SEP individuals initiated less frequently play-fighting, which can also be interpreted as a fearful phenotype (Chapter 3). They also displayed enhanced acoustic startle response, reduced prepulse and increased susceptibility to preservative behavior (apomorphine induced gnawing), while their working memory was intact (Chapter 3 & 5).

2.3 Summary of findings

In Table 2, we summarize the long term effects of the two repeated MS paradigms used

in this thesis on later behavior. Phenotypes induced by MS resemble endophenotypes of schizophrenia linked to a wide spectrum of clinical symptoms (positive, cognitive, affective, and negative). The relationship between early-life experience and long-term outcome is complex. Taken together, the data suggest that the social and environmental context in which the adverse early-life experience is perceived is an important factor: HOME SEP rats have behavioral deficits mainly in the cognitive domain (↓working and emotional memory). NOVEL SEP rats display behavioral alterations in all domains: positive/ cognitive (↑stereotypy, ↑gating), cognitive/affective (↑emotional memory), and negative symptoms (↓ social interaction).

Table 2.
Schizophrenia endophenotypes of HOME SEP and NOVEL SEP rats (comparison group: Wistar NON SEP/ Med LG after post-weaning social rearing)

Domains of schizophrenia endophenotypes	Domains of clinical symptoms	HOME SEP	NOVEL SEP
		Social Rearing	Social Rearing
Stereotypy	Positive	Basal	↑
Sensorimotor gating	Positive/ cognitive	Basal	↓
Working Memory	Cognitive	↓	Basal
Emotional Memory	Cognitive /Affective	↓	↑
Social Interaction	Negative	Basal	↓

Note. Non separated (NON SEP/ Med LG) had no previous history of treatments and repeatedly separated pups were exposed to 8h-MS on pnd 3 4 & 5 in a home (HOME SEP; home separated) or novel context (NOVEL SEP; novel separated).

Table 3.
Schizophrenia endophenotypes in APO-SUS individuals exposed to developmental stress (comparison group: Wistar Med LG after post-weaning social rearing)

Domains of schizophrenia endophenotypes	Domains of clinical symptoms	1 HIT APO-SUS Med LG	2 HIT APO-SUS Low LG	3 HIT APO-SUS Low LG
		Social Rearing	Social Rearing	Isolation Rearing
Stereotypy	Positive	↑↑	↑↑	↑↑
Sensorimotor gating	Positive/Cognitive	Basal	↓	↓↓
Working Memory	Cognitive	Basal	Basal	↓

Note. Low or Med LG: rats with history of low or medium levels of Licking & Grooming (LG) the first week of life. Social and isolation rearing: postweaning housing in groups or in social isolation, respectively.

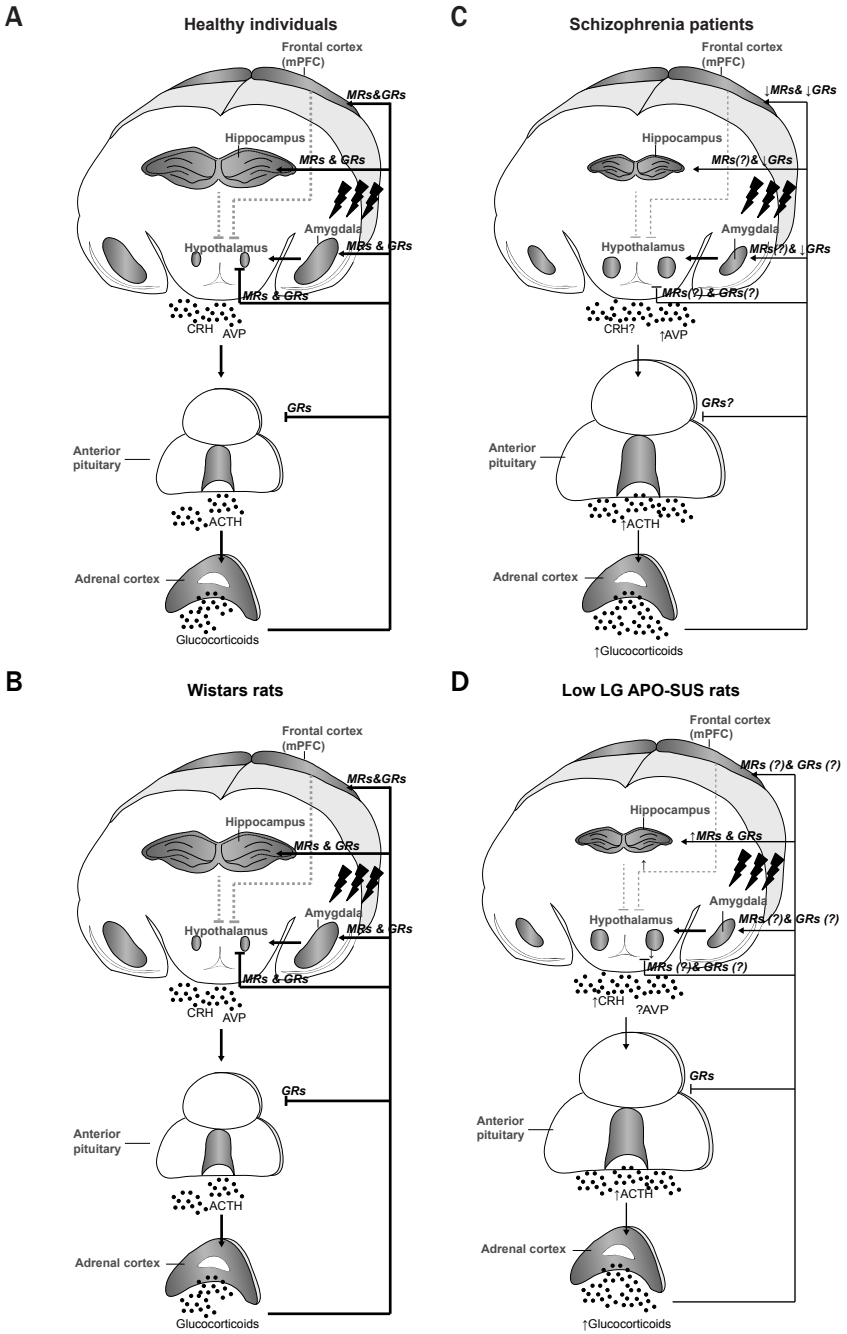

Figure 2. HPA-axis stress reactivity in healthy individuals (A, adapted from [53]) and common Wistar rats (B). The brain detects a threat (physiological or psychological); as a consequence a coordinated physiological response involving autonomic, neuroendocrine, metabolic and immune system components is activated. A key system in the stress response that has been extensively studied is the hypothalamus-pituitary-adrenal (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 prefrontal cortex (mPFC) exert a counteractive negative influence. Following activation of the stress 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 individuals with schizophrenia (C) and in Low Licking & Grooming (LG) APO-SUS rats (D), limbic structures are probably smaller in volume and display altered functions. At the same time, hypothalamus and pituitary are larger in volume possibly because of inefficient negative feedback (GC resistance). In stress conditions, 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 because of CORT resistance. When stress passes over a certain threshold, the amygdala can get activated and then the HPA-axis output will be higher than in healthy individuals since the negative feedback is impaired.

3. The *three-hit* (*cumulative stress*) hypothesis for schizophrenia

In Chapter 4, we tested the *three-hit* hypothesis for psychopathology in rats genetically-susceptible for schizophrenia-like phenotypes (APO-SUS rats).

3.1 Hit 1: Genetic predisposition for apomorphine susceptibility and GC resistance

We showed that APO-SUS' APO-gnawing, apart from being extremely increased, was CORT-resistant, since CORT injected one hour prior to testing had no effect. Socially reared APO-SUS rats did not display lower basal PPI compared to their paternal strain, the Wistar rats. Furthermore, APO-SUS' PPI performance was not sensitive to CORT injected 10min prior to testing, which further establishes their CORT resistance [44]. In most of the previous studies, testing was performed after days of social isolation and this might have influenced the result [45, 46]. APO-SUS displayed reduced freezing in response to social threat [2] and to foot-shock (Chapter 4). Their endocrine response to stress is characterized by an enhanced ACTH-response, blunted prolactin-response and normal CORT-response, indicating GC-resistance while their hippocampal MR capacity [2, 47, 48] and protein levels are higher (Chapter 4). Finally, early traces of the adult APO-SUS phenotype can be found in the neonatal endocrine response, the adrenal TH levels and in the developmental somatic markers. ACTH response to novelty at pnd 5 were enhanced, while adrenal basal TH protein levels were increased compared to Wistars. APO-SUS rat pups eye-opening was delayed for a day and they gained less body weight than the WH pups throughout life (in line with possible increased DRD_2 neurotransmission). These findings supported previous reports on the altered developmental trajectory of APO-SUS rat line [49].

3.2 Hit 2: Naturally occurring adverse maternal environment

The effect of cross-fostering in reversing the phenotype of APO-SUS rats had implied in the past a poor naturally occurring maternal environment [50]. APO-SUS, indeed, displayed an inherent altered maternal behavior: (i) lower levels of LG, AN & PN, (ii) more time away from the nest or engagement in self-orientated behaviors.

To disentangle the effects of altered maternal care from the genotype effect or genotype dependent reduction of maternal care, we decided to study the effects of naturally occurring variation of maternal care within the APO-SUS rat population. Low LG APO-SUS offspring were compared to High LG offspring. The Low LG rats displayed a much enhanced endocrine stress response (Prolactin, ACTH, CORT) to a conditioned emotional stressor and displayed basal PPI deficits, while their short-term memory was enhanced (Chapter 4). This observation showed that the most severe phenotype within the APO-SUS individuals is linked to adverse early-life experience. The strength of our approach was that we have replicated our findings in two generations.

The exaggerated ACTH release combined with CORT resistance in the Low LG APO-SUS offspring have remarkable similarities with the HPA-axis dysregulation seen in schizophrenic individuals [51]. These similarities on HPA-axis dysregulation are summarized in Figure 2. Previously, for Long Evans rats, an epigenetically driven reduction in hippocampal GR protein levels observed in Low LG offspring was used as main explanation for the HPA-axis dysregulation [32, 52]. However, the hippocampal MR and GR protein levels between the Low LG and High LG APO-SUS offspring were the same in our experiments (Chapter 4).

3.3 Hit 3: Unfavorable social environment.

An unfavorable social environment (isolation rearing) caused a PPI deficit in APO-SUS rats (Med LG rats were used) and also reduced T-maze spontaneous alternation. Further, we observed that the Low LG rats were very vulnerable to the effects of isolation rearing, while the High LG offspring were protected. Low LG APO-SUS rats displayed a very severe phenotype after isolation rearing that included: total absence of basal PPI, working memory impairment and further enhancement of the gnawing response to apomorphine.

3.4 Summary of our findings.

Our data of Chapter 4 are summarized in Table 3 and support a *three-hit* hypothesis of psychopathology: genetically predisposed individuals (Hit 1: APO-SUS) exposed to both early-life adversity (Hit 2: Low LG) and a later psycho-social stressor (Hit 3: Isolation rearing) display a severe schizophrenia-like phenotype.

4. The *mismatch* hypothesis of schizophrenia

In Chapter 5, we tested the *mismatch* hypothesis for psychopathology in non-genetically-selected Wistar rats:

4.1 Low LG and High LG Wistar offspring after social rearing

Low maternal care history did not reduce PPI, but it did increase acoustic startle. Apomorphine induced gnawing was also not affected by maternal care history. Taken together, the data showed that naturally occurring low maternal care in Wistar does not show the same effect as in outbred Long Evans [40]. Therefore genetic background influences the sensitivity of the pups to early-life adversity.

4.2 Low LG and High LG Wistar offspring after isolation rearing.

According to the developmental *mismatch* hypothesis [54, 55], an organism develops a phenotype that is adaptive for the expected future environment. This phenotype will be adaptive if the actual environment *matches* with the expected one; it will be maladaptive if the actual environment *mismatches*. When Low LG Wistar offspring were housed in a post-weaning social isolation, PPI was enhanced and APO-gnawing reduced (since there was "*match*" → resilience). In contrast, when High LG individuals were housed in isolation rearing, disruption of PPI appeared and APO-gnawing higher than controls (since there was "*mismatch*" → vulnerability). Accordingly, post-weaning social environment interacts with early-life experience in defining the outcome for psychosis susceptibility, a finding that is in support of the *mismatch* hypothesis.

5. Glucocorticoid challenge of rats with low and high psychosis susceptibility

5.1 Genetically-selected rats.

APO-SUS rats, overall, are not sensitive to acute effects of CORT on their PPI and APO-gnawing performance. Within the APO-SUS rat population, Low LG individuals were, particularly, CORT-resistant, whereas the High LG individuals with enhanced basal PPI were sensitive to a CORT-induced disruption of PPI.

5.2 Non genetically-selected rats.

For Wistar rats, overall, PPI and APO-gnawing is sensitive to acute CORT injections. Within the Wistar population, Low LG individuals were, particularly, CORT-sensitive, whereas the High LG individuals were not sensitive to a CORT-induced disruption of PPI.

5.3 Summary of findings.

Taken together, individuals with enhanced psychosis susceptibility (Low LG APO-

SUS offspring, High LG Wistar offspring) were CORT resistant qualifying GC resistance as characteristic phenotype of schizophrenia. In contrast, individuals with reduced psychosis susceptibility (High LG APO-SUS offspring, Low LG Wistar offspring) were sensitive to the CORT effects.

6. Synthesis of the *three-hit* & *mismatch* hypothesis (differential susceptibility)

Our results in genetically-susceptible rats (APO-SUS rat line) and non-susceptible rats (parent common Wistar rat) had opposing outcomes as a function of environmental influence. In order to incorporate all these results in one model we did a comparison of the basal sensorimotor gating between the two rat populations under all the environemntal conditions in order to test the theoretical models presented by Belsky et al. [56-58] describing the possibilities for differential influence of the environment (models of susceptibility to environment). These models are depicted in Figure 3 (A, B, C, D) and state the following:

Model a - differential susceptibility (Fig. 3A): Non-susceptible to environment individuals are not affected by changes of the environment, but susceptible individuals display both enhanced negative outcome after increasing the negative environmental input and enhanced positive outcome in a positive environment. The slope of the susceptible subgroup should be significantly different from zero and at the same time significantly steeper than the slope of the non-susceptible subgroup.

Model b - absence of susceptibility (Fig. 3B): the two subgroups show different outcomes, but variation in the environmental factor does not affect the outcome.

Model c - contrastive susceptibility (Fig. 3C): the slopes of the two susceptible subgroups are significantly different from zero but run in opposite directions (susceptibility & "inverse susceptibility").

Model d – dual risk (Fig. 3D): represents a fan-shaped interaction, with the environmental variation affecting the outcome of the susceptible subgoup in just one direction; an increased negative environment worsens the outcome, but positive environment does not have an effect.

Our data are presented in Figure 3E: While the negative environment was increasing (from point 1 → point 3 in the x-axis; Fig. 3E), we observed that APO-SUS rats' basal PPI was decreasing (confirming the *three-hit* or *cumulative stress* hypothesis; [59-61]) and, at the same time, was significantly different from the PPI of Wistar rats. Actually, the basal PPI of Wistar rats was increasing as the negative environment increased (in support of the *mismatch* hypothesis; [54, 55, 62]). Our findings support the theoretical model c, since the two rat populations were significantly different from each other

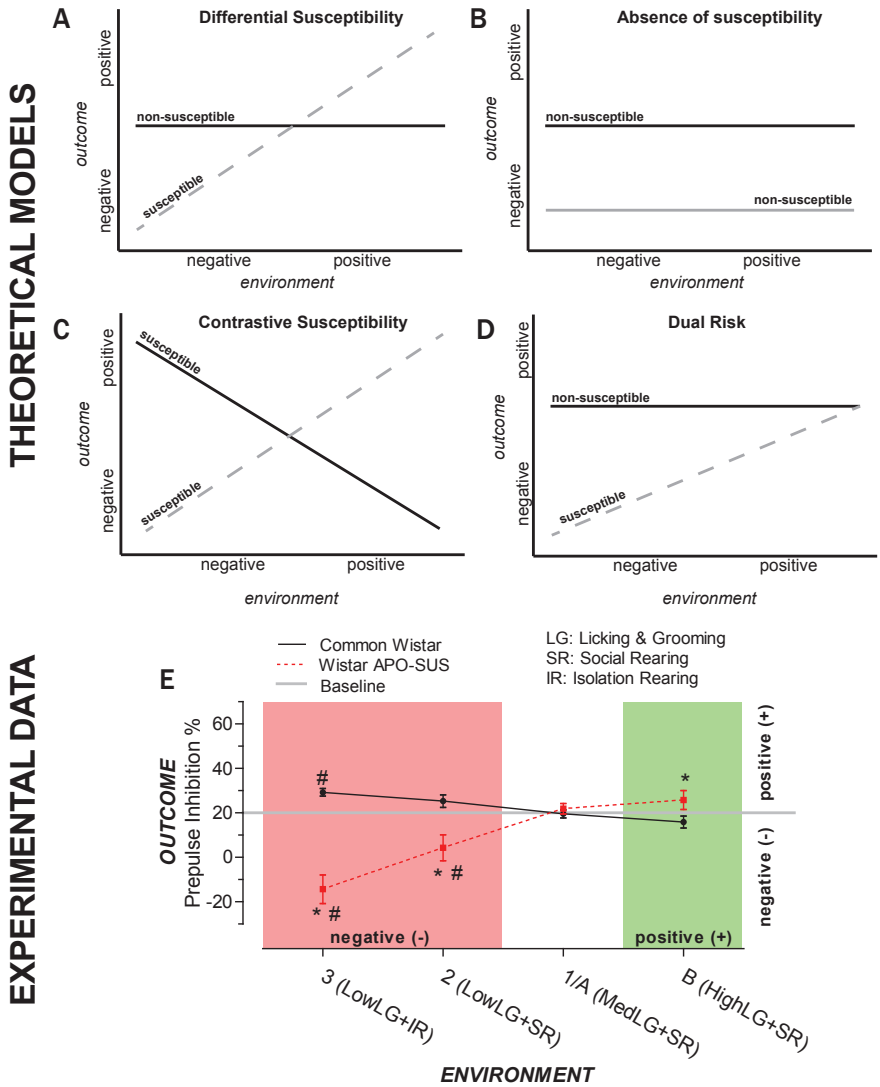

Figure 3. Graphical display of different theoretical models of susceptibility to environmental influence (adapted from [56]). The x-axis indicates variation in the environmental factor from negative to positive; the y-axis indicates the outcome from negative to positive; and the lines (continuous and discontinuous) depict two subgroups of individuals differing on their susceptibility to environment. Model a - differential susceptibility (A); Model b - absence of susceptibility (B); Model c - contrastive susceptibility (C); Model d – dual risk (D). In (E) our experimental data from Chapters 4 & 5. Negative or positive outcome as a function of the variation of the environment (negative to positive) for two rat populations (Common Wistar strain: black continuous line; Wistar APO-SUS rat line: red discontinuous line). The two rat populations displayed the same baseline (depicted as a straight grey line). Low, Med or High LG: rats with history of low, medium or high levels of Licking & Grooming (LG) the first week of life. Social and isolation rearing: postweaning housing in groups or in social isolation, respectively. Outcome presented is the average pre-pulse inhibition of acoustic startle using 4 prepulse intensities (2, 4, 8, 16 dB[A] over background) and one pulse intensity (120 dB[A]). Data presented as MEAN ± SEM. * vs. Common Wistar: at point B (High LG+SR): $F_{1,41}$=4,297; p=0.045, at point 2(Low LG+SR): $F_{1,41}$=12,232; p=0.001, at point 3(Low LG+IR): $F_{1,26}$=72,617; p<0.001. # vs. point 1/A (Med LG+SR): for Common Wistar: $F_{3,135}$=4,263; p=0.007, for Wistar APO-SUS: $F_{3,107}$=12,498; p<0.001.

after exposure to negative environment and also different from baseline (depicted as a straight line; Fig. 3E) but in opposite directions(APO-SUS: susceptibility, Wistar: "inverse susceptiblity").

When the positive environment increased (from point A → point B in the x-axis; Fig. 3E), APO-SUS rats displayed higher basal PPI than the Wistars, which did not really change performance. This supports the theoretical model a (APO-SUS: susceptiblity, Wistar: "absence of susceptibility").

Taking these data together, the APO-SUS line with the reactive dopaminergic alleles obeyed Belski' s "for better or worse" susceptibility principle of model a [56-58]. The parental Wistar strain theoretically comprises of rats from the whole range of susceptibility and, hence, if the extremes are represented equally in our rat population a straight line was expected for the relationship between the outcome parameter (PPI) and the environmental variation. However, our Wistar rats displayed a mixture of outcomes randing from "non-susceptibility" to "inverse suscetibility".

In this respect, it would be of interest to test:

- A greater variety of environmental inputs and especially vary the positive environment more (including post-weaning social enrichment for example).

- Different common outbred rat populations with possible variation in the distribution of susceptible and non susceptibe individuals.

- The APO-UNSUS line. Our prediction is that the APO-SUS rats respond negatively to negative environment and positively to positive environment ("susceptibility"). Inversely, the APO-UNSUS rats are expected to be the mirror image responding positively to negative environment and negatively to a positive environment ("inverse susceptibility"; [1, 50, 63]).

7. Future perspectives

7.1 Programming of psychopathology by early-life stress through amygdala priming

Alterations of the maternal environment destabilize the SHRP and enhance susceptibility to stressors. In our early-life stress animal models, psychosis susceptibility coincided with a specific dysregulation of the HPA-axis: hyper-reactivity of brain and pituitary to stress in the face of GC resistance. Exposure to stressors early in life, like a novelty experience in social isolation during maternal absence, can program the amygdala fear pathway for life with long-lasting consequences for both fear-related behavior and psychosis susceptibility (Chapter 3 & 5). We have provided strong evidence that amygdala and HPA-axis are biological substrates for psychosis susceptibility induced by environmental stressors experienced in early development [51, 64].

Developmental plasticity of the amygdala is a very promising substrate for understanding stress and psychosis susceptibility. Pharmacological manipulations in that region during maternal absence (e.g. administration of GR or MR-antagonists) may help to investigate further the role of CORT in the premature activation by stressful

experience. Future research could also investigate the learning capacities of pups during maternal absence by manipulating early-life environmental cues.

7.2 *Three-hit* hypothesis of psychopathology

Early-life adversity programs in interaction with predisposing genes enhanced emotional and stress reactivity in later life. This was particularly the case if the APO-SUS rats were exposed to early-life adversity and a pre-pubertal psychosocial stressor. Under these extreme conditions an enhanced ACTH response occurred, while corticosterone levels were not much different from control. Hence the APO-SUS phenotype has aggravated as marked by a profound corticosterone feedback resistance and altered corticosteroid receptor function. These changes in neuroendocrine markers were associated with a strongly impaired prepulse inhibition and working memory. These markers are also often deficient in patients suffering from schizophrenia. The findings demonstrate how cumulative exposure to adversities (multiple hits) may precipitate enhanced vulnerability to mental health problems, but only with a particular dopaminergic genetic background that enhances the susceptibility to the environment.

The *three-hit* animal model of schizophrenia has face validity and etiological validity and therefore qualifies as an appropriate model to investigate the disease mechanism. Using this model it will be feasible to examine epigenetic variations in gene expression networks characteristic for the high risk individuals (i.e. APO-SUS Low LG offspring). Possibly, the genes altered in expression by the epigenome may provide novel targets for treatment of schizophrenia.

Our model has also translational opportunities. Dopaminergic receptor susceptibility is clearly a genetic background worth investigating in parallel in rat and human since it heightens susceptibility to stressful experience [65]. Our findings call for:

- Control of environmental inputs to raise the validity of GWA studies. The design of Genome x Environment-Wide Association studies for mental disorders is therefore very promising and challenging [66]. In my view such studies should always be coupled with hypothesis-driven gene-by-environment interaction studies.

- Apart from animal models, rare human syndromes could be of help in that respect. For example, patients suffering from Cushing's disease may very well be a human model of chronic exposure to cortisol excess.

- Individually tailored family intervention programs [65]

7.3 Developmental *mismatch* hypothesis

For non susceptible rats, a *match* of early-life experience with later life living conditions can enhance resilience to psychopathology, while with a *mismatch* the opposite occurs and vulnerability is enhanced. These findings underscore the amazing plasticity of the brain: *nothing is written in stone [67].*

8. References

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