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Influence of genetic and environmental factors on central stress regulation and pain

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General Abstract

During the last century, there has been a long-lasting debate about the contribution of genetic and environmental factors to traits as well as psychiatric and somatic disorders (*i.e.* phenotypes) in humans, also known as the nature vs. nurture debate. Formal genetic studies (*i.e.* family studies including twin designs) are an appropriate way to disentangle the contribution of genetic and environmental factors to various phenotypes. Additionally, this approach enables estimating to which extent genetic as well as environmental factors overlap between different phenotypes. These studies have shown that for common diseases such as chronic pain and psychiatric disorders, genetic as well as environmental risk factors contribute in various degrees. Heritability estimates range between 30 - 80%. In the field of molecular genetics, the availability of large samples with genome wide genetic data allows quantifying the contribution of common genetic variation on the studied phenotypes in non-related subjects.

Overall, it has been established that both genetic and environmental factors contribute to most complex phenotypes and interact with each other. Understanding the risk factors underlying psychiatric and somatic disorders gives insight into their etiology and can stimulate new approaches for prevention and treatment.

In this thesis, four studies are presented which investigate environmental and genetic risk factors for phenotypes relevant to mental health and pain.

The first study (Chapter II) investigated how urban living and urban upbringing, well-known environmental risk factors for psychiatric disorders, may exert their effect on mental health using a brain imaging study. It is a robust but little understood epidemiological finding that individuals born and raised in cities are at a higher risk for psychiatric disorders than those born and raised on the countryside. For instance, city dwellers report higher rates of anxiety disorders and depression, and urban upbringing has been identified as a risk factor for schizophrenia. Increased social stress in an urban environment has been suggested to mediate those effects. In this study, two independent samples of healthy adults underwent two different social stress paradigms for functional magnetic resonance imaging (fMRI) -one developed in the frame of the present thesis (Scan*STRES*S)- to test brain activity in reaction to an acute social evaluative stressor. During stress processing, amygdala activation was associated with city living, while activity in the perigenual anterior cingulate cortex (pACC)

was associated with urban upbringing. As these findings were specific to those brain regions and specific to stress processing, they suggest that the established environmental risk factors city living and urban upbringing shape neural stress processing in specific neurodevelopmental phases.

In the second study (Chapter III), possible modulation of the effects of urban upbringing and city living on neural stress processing through genetic variation in the neuropeptide S (NPS) system was investigated using the Scan*STRESS* paradigm. In animals, pharmacological studies have shown that NPS has a strong anxiolytic effect and interacts with the regulation of the hypothalamus-pituitary-adrenal (HPA) axis. In humans, studies have linked genetic variation in the neuropeptide S receptor gene (*NPSR1*) to anxiety disorders and other stress-related phenotypes. The study findings demonstrated that a genetic variant in *NPSR1* (rs324981) modulates the effect of urban upbringing on activity in the right amygdala. This result implies that the NPS system is involved in the human stress response, and that it interacts with the environmental risk factor urban upbringing.

In the third study (Chapter IV), the effects of genetic variation in *NPSR1* on the neural and endocrine stress response were further investigated. For the regulation of the HPA axis as well as the prevalence of stress related disorders, strong differences between males and females are observed. Furthermore, it has been shown that genetic variants can influence HPA axis reactivity or the risk for psychiatric disorders in a sex specific-way. Sex-specific effects have also been reported for genetic variants in *NPSR1*. The study investigated the effects of *NPSR1* variants in 277 individuals which underwent the Trier Social Stress Test (TSST) and in 65 subjects measured with the Scan*STRESS* paradigm, taking sex-specific effects into account. Analysis revealed a sex-specific association of an allele combination (haplotype) of three functional SNPs (rs2530547, rs324981 and rs727162) with the cortisol response to the TSST, and a sex-specific effect of the variant tested in Chapter III (rs324981) on neural activation patterns. Those results indicate that sex might modulate the effects of genetic variation in NPS system genes on the human stress regulation.

In the fourth study (Chapter V), the influence of environmental and genetic risk factors on subjective chronic pain after the severe event of an amputation was studied. After the loss of a limb due to trauma or surgical removal the majority of individuals develop chronic phantom limb and/or residual limb pain. As the comparably rare event of an amputation hardly ever

happens in both members of a twin pair, the heritability of postamputation pain cannot be assessed with twin studies. The present study therefore aimed at investigating whether individual predisposition influences the development and phenotypical expression of postamputation pain in a sample of 122 individuals who had lost two limbs. For both studied pain types -phantom limb pain and residual limb pain- the presence and intensity of pain in one limb was strongly associated with the manifestation of the same pain type in the other limb and only moderately associated with the manifestation of the other pain type in the same or the other limb. This indicates the presence of specific as well as overlapping individual (potentially genetic) dispositions for each pain type. The results of this study, in line with previous findings from animal studies, suggest a strong genetic component for postamputation pain and may help define phenotypes for future genome-wide association studies of postamputation pain.

The work presented in this thesis (1) suggests a mechanism through which well-known environmental risk factors urban upbringing and city living exert their effects on the brain level, (2) indicates that this effect is modulated by genetic factors in NPS system genes, (3) indicates that the effects of genetic variation in genes of the NPS system on stress regulation are modulated by sex and (4) explores the influence of environmental factors and individual (potentially genetic) predisposition on the development of neuropathic pain.

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Index of Publications

The present doctoral thesis includes a general introduction in Chapter I, and four other Chapters, which are published as 'Original Research Articles' in international peer-reviewed journals. The author is the first author of three of those articles. The articles are presented in their original published form, with the exception of formatting changes (e.g., figure labeling, references).

Content	**has been published as:**
Chapter II	Lederbogen, F.*, Kirsch, P.*, Haddad, L.*, **Streit, F.**, Tost, H., Schuch, P., Wüst, S., Pruessner, J. C., Rietschel, M., Deuschle, M., & Meyer-Lindenberg, A. (2011). City living and urban upbringing affect neural social stress processing in humans. Nature, 474(7352), 498-501. doi: 10.1038/nature10190 (Impact Factor 2015: 38.14)
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Index of Abbreviations

ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AVOI	Anatomical volumes of interest
BOLD	Blood-oxygen-level-dependent
bpm	Beats per minute
C	Celsius
cm	Centimeter
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
e.g.	For example
EPI	Echo planar imaging
ER	Error rate
fMRI	Functional magnetic resonance imaging
FWE	Family-wise error
FWHM	Full-width at half-maximum
GWAS	Genome wide association study
GR	Glucocorticoid receptor
HPA axis	Hypothalamus-pituitary-adrenal axis
HR	Heart rate
Hz	Hertz
ICC	Intra class correlation
i.e.	that is
M	Mean
MIST	Montreal Imaging Stress Test
MNI	Montreal Neurological Institute

min	Minutes
MRI	Magnetic resonance imaging
ms	Milliseconds
NPS	Neuropeptide S
NPSR1	Neuropeptide receptor 1
pACC	Perigenual anterior cingulate cortex
PET	Positron emission tomography
PLP	Phantom limb pain
PVN	Paraventricular nucleus
PFC	Prefrontal cortex
rACC	Rostral anterior cingulate cortex
RDoC	Research Domain Criteria
RLP	Residual limb pain
RNA	Ribonucleic acid
ROI	Anatomical regions of interest
s	Seconds
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
SECPT	Social Evaluated Cold-Pressor Test
T1	Longitudinal relaxation time
TE	Echo time
TR	Repetition time
TSST	Trier Social Stress Test

Chapter I - General rationale

Humans differ in various easy-to-observe physical characteristics such as height, weight, and hair color, but also with regard to how they behave and perceive their environment. In the same way, the risk to develop specific somatic or psychiatric disorders differs largely between individuals.

During the last century, it has been discussed if genetic or environmental factors underlie this variation, also termed the nature vs. nurture debate (Chakravarti & Little, 2003). While this dispute was fierce and often influenced by the ideological perspective of the discussants, nowadays –based on scientific findings– a consensus has been reached, agreeing that most phenotypes (*i.e.* observable characteristic of an organism) are influenced by both genetic and environmental factors (Plomin, DeFries, Knopik, & Neiderheiser, 2013). Furthermore, research has established that environmental and genetic factors often interact, meaning that a genetic predisposition for a phenotype might only or preferably manifest under specific environmental circumstances (Plomin et al., 2013).

For psychiatric disorders, it has been proposed that -especially in genetically vulnerable individuals- environmental stressors, such as aversive early life experiences, can shape the development of a dysfunctional neuronal and endocrine (hormonal) stress response (Akdeniz, Tost, & Meyer-Lindenberg, 2014; Chrousos, 2009). This in turn might impair the ability of an individual to appropriately react to stressful stimuli later in life, increasing the vulnerability for psychiatric disorders (de Kloet, Joels, & Holsboer, 2005). While extensive research has targeted the endocrine system in humans, there is still a lack of knowledge about how risk factors impact stress processing on the level of the human brain. The aim of the studies presented in Chapter II, III and IV was to investigate the effects of environmental and genetic risk factors for psychiatric disorders on the endocrine and neural stress regulation.

A neurological phenotype clearly caused by a traumatic external event is postamputation pain. After the traumatic event of the loss of a limb, the majority of individuals develop persistent and impairing pain in the missing limb (i.e. phantom limb pain) or the stump (i.e. residual limb pain) (Foell, Bekrater-Bodmann, Flor, & Cole, 2011). Phantom limb pain in particular has been proposed to reflect maladaptive neuronal changes in response to the traumatic external event (Flor, Nikolajsen, & Jensen, 2006). While evidence from animal studies suggests that individuals differ in their genetic susceptibility to develop pain after traumatic

injuries, this has not yet been studied in humans. The aim of the study presented in Chapter V was to investigate the contribution of an individual –potentially genetic- disposition to develop phantom limb or stump pain by examining a sample of subjects with two amputated limbs.

The interplay of environmental and genetic factors

In humans, scientists have investigated the relative contribution of genetic and environmental factors to various phenotypes with formal genetic approaches, i.e. family including twin studies (Plomin et al., 2013). These studies have shown that for phenotypes such as intelligence or temperament (Penke, Denissen, & Miller, 2007), pain disorders and pain processing (Nielsen, Knudsen, & Steingrimsdottir, 2012; Nielsen et al., 2008) and psychiatric disorders (e.g. bipolar disorder, schizophrenia, and major depression; see Cardno & Gottesman, 2000; McGuffin et al., 2003; P. F. Sullivan, Neale, & Kendler, 2000), the heritability estimates (*i.e.* the share of variance explained by the genetic background) range between 30% - 80%. While these numbers indicate a substantial genetic contribution, there is up to 70% of variance remaining, which can be largely attributed to environmental factors.

While it has been established that the environment as well as genetic factors contribute to phenotypes, an additional suggestion to the nature vs. nurture debate is that both factors interact: i.e. that disorders are especially common in genetically vulnerable individuals exposed to adverse environments (Ottman, 1996; Uher, 2014). In the field of psychiatry, the works of Caspi and colleagues linked specific genetic variation to an increased vulnerability to adverse environments. For example, in a widely acclaimed study they could show that the experience of stressful life events increased the risk for depression and suicide attempts, especially in individuals carrying a specific variant in the gene coding for the serotonin transporter (Caspi et al., 2003). However, in light of inconsistent replications, the general validity of reported gene x environment interactions has been challenged (e.g. Duncan & Keller, 2011) and is currently a matter of debate (Caspi, Hariri, Holmes, Uher, & Moffitt, 2010; Dick, 2011).

The identification of the specific genes contributing to the moderate to high genetic heritability of various phenotypes has been one major challenge of genetics. In the beginning of molecular genetic research, candidate genes were selected based on the results of linkage

analyses in multiplex families and/or their biological plausibility and then investigated in candidate-gene-association studies (Harrison & Weinberger, 2005).

During the last two decades, driven by technological advances in combination with large recruitment efforts, genome wide association studies have been performed for countless phenotypes, investigating the effects of hundreds of thousands of genetic variants (i.e. single nucleotide polymorphisms; SNPs) covering the whole genome in samples ten thousands of subjects large (Hirschhorn & Daly, 2005; Manolio, 2013). These studies represent another, systematic approach to reliably identify single variants (and the assigned genes) associated with various phenotypes and/or disorders. Additionally, this approach allows the estimation of variance explained by common genetic variants given that samples of sufficient size have been investigated (Yang et al., 2010; Yang, Lee, Goddard, & Visscher, 2011). In the case of psychiatric and psychological phenotypes, a comparably large share of variance in the investigated phenotype could be explained by common genetic variants, for example for schizophrenia (Consortium, 2014) and neuroticism (de Moor et al., 2015). In general, molecular genetic studies revealed that most disorders and traits represent genetically complex phenotypes, with thousands of genetic variants individually contributing, each with mainly small to modest effects (Manolio, 2013; Patrick F. Sullivan, Daly, & O'Donovan, 2012).

Intermediate phenotypes

A main goal of biological psychology research is to determine how specific (genetic or environmental) risk factors exert their effects on psychological well-being or disorders. One approach, which has been promoted for this, is the investigation of intermediate phenotypes: i.e. mechanism-related observable phenotypes associated with disorders (Goldman & Ducci, 2007). Examples of intermediate phenotypes of interest are neuropsychological test results, the startle reaction, Electroencephalography (EEG) measures, and functional or structural measures in positron emission tomography (PET) and magnetic resonance imaging (MRI) studies. Such biological measures potentially represent more specific entities in closer proximity to their underlying biological causes than syndromal and often heterogeneous diagnoses (Meyer-Lindenberg & Weinberger, 2006). Due to this, it is assumed that specific genetic or environmental risk factors show stronger associations with intermediate phenotypes than diagnoses (Goldman & Ducci, 2007). Furthermore, it has been proposed that mechanistic phenotypes might help disentangle subgroups of individuals with the same psychiatric

diagnosis and might even guide the reshaping of diagnostic classification (e.g. Research Domain Criteria (RDoC) (Insel et al., 2010). This is important because psychiatry needs to further develop the currently applied diagnostic systems to enable more specific treatment which targets the underlying mechanisms in each affected individual (Tamminga et al., 2014). Translational research can profit from this approach as well, as closer similarity between humans and animal models can be assumed for some intermediate phenotypes. For example, the startle reaction or the regulation of the hypothalamic pituitary adrenal (HPA) axis (see below) can be studied in animals as well as in humans, while most psychiatric diagnostic criteria cannot be applied as easily in animals (Gould & Gottesman, 2006). On the downside, studying intermediate phenotypes in humans requires a bigger effort than solely assessing a psychiatric diagnosis.

The stress response

One psychobiological mechanism which has received a lot of attention as a possible underlying biological pathway for the development of psychiatric disorders is the stress response system. Organisms, including humans, survive by maintaining a complex dynamic equilibrium (homeostasis) (McEwen & Gianaros, 2010). Internal or external forces, so-called stressors, challenge this equilibrium which necessitates the individual to react with the goal to reestablish homeostasis (Chrousos & Gold, 1992). Stressors include physiological stimuli such as a cold environment, internal signals (e.g. a reduction in blood sugar), and psychological elements such as the perception of the environment as potentially harmful or threatening (Chrousos & Gold, 1992). The stress reaction, with the goal of reestablishing homeostasis, comprises various adaptions on a physiological, psychological and behavioral level. The most known reaction pattern facing a threat was termed the "fight-or-flight" reaction by Walter Cannon (Cannon, 1935).

The HPA axis

The major endocrine component of the stress reaction is the HPA axis (Chrousos, 2009). The paraventricular nucleus (PVN) in the hypothalamus integrates signals from various brain regions processing and evaluating the threat, i.e. from the amygdala, the hippocampus, and the prefrontal cortex (PFC) (Ulrich-Lai & Herman, 2009). In the case of threat appraisal, the stress reaction initiates with the release of Corticotropin-releasing hormone (CRH) into the pituitary gland. Subsequently, adrenocorticotropic hormone (ACTH) is released from the pituitary gland into the blood stream, triggering the release of cortisol from the adrenal glands. Within the frame of minutes to hours, cortisol exerts a cascade of effects in the

periphery and the brain, initiating first the reaction to the stressor and then the recovery from the stress reaction. This includes effects on the immune system, on the metabolism and on arousal, attention and memory processes (Chrousos, 2009). Additionally, mediated by the glucocorticoid receptor (GR), cortisol exerts negative feedback on the activation of the HPA axis on various levels, on the adrenal gland, the pituitary, and the hippocampus amongst others, leading to a normalization of circulating cortisol levels after the acute stress reaction (Herman et al., 2003).

In various somatic and psychiatric disorders, impaired feedback mechanisms and a dysregulation of the HPA axis have been described and proposed as an intermediate phenotype. In depression for example, increased basal cortisol levels and a reduced reactivity of the HPA axis have been attributed to impaired feedback mechanisms (Chrousos, 2009; de Kloet et al., 2005; Holsboer, 2000).

To investigate the stress reaction in humans in an experimental setting, various paradigms have been developed with the aim to reliably elicit a HPA axis response. This goal has been achieved most successfully by paradigms, which combine uncontrollable and unpredictable challenges with social evaluation of the participant's behavior (Dickerson & Kemeny, 2004). Examples for established paradigms are the Trier Social Stress Test (TSST) (Kirschbaum, Pirke, & Hellhammer, 1993), the Social Evaluated Cold-Pressor Test (SECPT) (Schwabe, Haddad, & Schachinger, 2008), and the Montreal Imaging Stress Test (MIST) (Dedovic et al., 2005).

Genetic and environmental contributions

In the general population, large intra-individual differences in stress vulnerability and HPA axis regulation exist (Kudielka, Hellhammer, & Wüst, 2009), and it has been proposed that this variability is driven by both genetic and environmental factors. A moderate to high heritability has been shown for subjective stress perception (Federenko et al., 2006; Rietschel et al., 2014) as well as HPA axis regulation (Federenko, Nagamine, Hellhammer, Wadhwa, & Wüst, 2004; Wüst, Federenko, Hellhammer, & Kirschbaum, 2000), indicating a contribution of genetic as well as of environmental factors. These findings are complemented by research in animal models, which show short and long-term alterations of HPA axis regulation induced by environmental factors (Francis & Meaney, 1999), as well as altered HPA axis regulation in genetically different mouse strains (Anisman, Hayley, Kelly, Borowski, & Merali, 2001) and knock-out mice (Hartmann et al., 2012; Timpl et al., 1998). In humans, the involvement of specific genetic variants in the regulation of the HPA axis has primarily been shown in genes coding for proteins involved in the regulation of the HPA axis, such as *FKBP5* (Ising et al.,

2008), the *GR* (Kumsta et al., 2007), or the CRH receptor 1 gene (*CRHR1*; Bradley et al., 2008). The impact of specific environmental factors, such as pre- or post-natal stress, has been demonstrated in several studies investigating HPA axis regulation (e.g. Entringer, Kumsta, Hellhammer, Wadhwa, & Wust, 2009). In addition, numerous studies could demonstrate that the effect of environmental risk factors is modulated by variation in genes such as *FKBP5* (Roy, Gorodetsky, Yuan, Goldman, & Enoch, 2010) or *CRHR1* (Bradley et al., 2008) in terms of gene x environment interactions.

Brain Stress Processing

Alterations in brain regions moderating stress appraisal and HPA axis activity in the response to acute stress should accompany the HPA axis changes that have been found in individuals with somatic or psychiatric disorders. Functional magnetic resonance imaging (fMRI), one approach to investigate neural processing in humans was applied in the three studies presented in Chapters II, III and IV. In these three studies, the specific method used was blood-oxygen-level dependent (BOLD) imaging, which is most commonly applied in fMRI studies. In BOLD fMRI, neural activation in the whole brain is assessed by measuring brain perfusion changes as a proxy: increased neuronal activation leads to an increased blood flow carrying oxygenated hemoglobin in the respective brain regions. Hemoglobin has different magnetic properties when it is oxygenated or deoxygenated, and this fMRI technique works by measuring the resulting changes in the magnetic signal in the respective brain regions. It is important to note that the nature of this signal is relative, so different conditions must be contrasted in order to determine blood flow changes, e.g. stress vs. control condition (Huettel, Song, & McCarthy). Since its first application in humans in the early 1990s (Kwong et al., 1992), BOLD fMRI has developed into an established and widely applied tool of neuroscience (Stelzer, Lohmann, Mueller, Buschmann, & Turner, 2014) due to being a non-invasive technique which functions without the use of radioactive tracers, and allows for a good spatial resolution (Logothetis, 2008).

As a non-invasive neuroimaging technique, fMRI is a valuable tool to study the effects of risk factors for psychiatric disorders on neural functioning (Meyer-Lindenberg & Tost, 2012; Meyer-Lindenberg & Weinberger, 2006). Numerous studies have demonstrated that brain activation in several cognitive and emotional tasks is modulated by specific genetic (Esslinger et al., 2009), and environmental risk factors (Javanbakht et al., 2015; Taylor, Eisenberger, Saxbe, Lehman, & Lieberman, 2006), as well as by altered HPA axis regulation (Bogdan & Hariri, 2012; Burghy et al., 2012).

The Scan*STRESS* paradigm (Streit et al., 2014), a social stress paradigm for application in MRI, was developed in the frame of this thesis. Within this paradigm, the stress characteristics uncontrollability and social evaluation, which have been demonstrated to elicit HPA axis responses (Dickerson & Kemeny, 2004), are operationalized via two cognitive tasks: arithmetic and mental rotation. The subject has to solve those tasks under time pressure, with speed and difficulty being adapted to the subject's individual performance. Additionally, a social component is introduced by the presence of an expert panel which monitors the subjects and provides negative visual and verbal feedback irrespectively of the subject´s actual performance (for details see Chapter III).

In Chapter II, III and IV, data is presented which has been generated via the application of this paradigm to investigate the effects of environmental and genetic risk factors for psychiatric disorders on acute stress processing.

Urbanicity: an environmental risk factor for psychiatric disorders

In the first study of the present thesis (Chapter II), the association of the environmental risk factors city living and urban upbringing with neural stress processing was investigated. In large epidemiological studies, it has been established that the risk for schizophrenia is increased in persons who grow up in an urban environment (Pedersen & Mortensen, 2001; van Os, Pedersen, & Mortensen, 2004), while depression and anxiety disorders are more prevalent in people currently living in cites compared to people living in the countryside (Peen, Schoevers, Beekman, & Dekker, 2010). It has been suggested that besides increased pollution and noise, increased social stress and social defeat might mediate these effects (Akdeniz, Tost, & Meyer-Lindenberg, 2014).

In the first study, the effect of urban upbringing and current urbanicity on neural processing of acute stress was assessed in two independent samples of healthy adults (Lederbogen et al., 2011). The discovery sample was investigated using the MIST (Dedovic et al., 2005), while in the replication sample the Scan*STRESS* paradigm (Streit et al., 2014) was applied. In both samples urban upbringing modulated the neural activity in the perigenual anterior cingulate cortex (pACC), while current city living was associated with increased amygdala activation. These findings were specific to stress and limited to the ACC and amygdala. The specificity of the results was confirmed in a working memory task or an emotional face matching task where no effect of current or early urbanicity on brain activation was shown. These results suggest that stress processing in the ACC and amygdala is shaped by the environmental risk factor urbanicity in specific phases of the life.

Neuropeptide S – a regulator of anxiety and stress

In the second study of the present thesis (Chapter III), the goal was to assess if the effects of the environmental risk factors urban upbringing and current urbanicity on acute stress processing are modulated by a genetic variant in the neuropeptide S receptor gene (*NPSR1*).

Neuropeptide S (NPS) has raised interest, because it has been found to act as an anxiolytic agent and at the same time increase arousal in animal models (Xu et al., 2004). Additionally, it was demonstrated that NPS strongly interacts with the HPA axis (Smith et al., 2006). In humans, genetic variation in the neuropeptide S receptor gene (*NPSR1*) has been linked to psychiatric disorders - mainly anxiety disorders (e.g. Domschke et al., 2011). A recent study showing an association between the functional SNP rs324981 (A/T) in *NPSR1* and the HPA reaction to social stress (Kumsta, Chen, Pape, & Heinrichs, 2013), implied NPS is in involved in stress regulation in humans. The effects of early urbanicity on schizophrenia have been shown to be strongest in individuals carrying a familial risk for the disorder (van Os et al., 2004), which indicates gene x environment interaction for this risk factor. In the case of NPS, genetic variation in *NPSR1* has been demonstrated to modulate the environmental risk for psychiatric and somatic disorders (Bruce et al., 2008; Klauke et al., 2014; Laas et al., 2014). Therefore, *NPSR1* presented itself as a promising candidate gene to test for a gene x environment interaction with urbanicity in the presented study.

In the second study, the modulation of the neural and endocrine stress response by rs324981 and its interaction with the environmental risk factors early and current urbanicity was investigated in the replication sample of the first study.

The effect of urban upbringing on right amygdala activation was modulated by the genotype of rs324981. An increased amygdala activation under stress was observed in homozygous carriers of the T allele, while homozygous carriers of the A allele showed an inverse association. The results of this study support the view that genetic predisposition moderates the influence of the environmental risk factor early urbanicity and imply the involvement of the NPS system in the stress regulation in humans.

Sex specific effects of neuropeptide S receptor gene variants

In the third study of the present thesis (Chapter IV), the influence of genetic variation in *NPSR1* on the endocrine and neural stress regulation was further investigated, taking potential sex specific-effects into account. Sex differences in HPA axis regulation, e.g. a reduced cortisol response to psycho-social stress in females, are a well-established finding (Kudielka

& Kirschbaum, 2005). Stress related psychiatric disorders such as anxiety or depression (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014; Ferrari et al., 2013) show an increased prevalence in females, and an earlier age at onset is commonly reported in male schizophrenia patients (Ochoa, Usall, Cobo, Labad, & Kulkarni, 2012). It has been hypothesized that the observation that some genetic risk variants exert differential effects in males and females might be partially due to different hormonal concentrations in the cellular environment (Weiss, Pan, Abney, & Ober, 2006). In case of *NPSR1*, such sex x genotype interactions have been reported for several psychiatric phenotypes (e.g. Domschke et al., 2011; Laas et al., 2014, 2015; Okamura et al., 2007).

The study investigated the effects of *NPSR1* variation in a sample of 277 individuals who underwent the TSST, and in 65 individuals assessed with the Scan*STRESS* paradigm. In the TSST sample, the effects of three functional SNPs (rs2530547, rs324981 and rs727162) were investigated with a haplotype-based approach, taking the effect of specific allele combination into account. In the Scan*STRESS* sample, rs324981 was analyzed. In the TSST sample, a significant effect of the overall haplotype structure on area under the curve cortisol levels was observed, and the follow-up analysis revealed a sex-specific effect of a specific haplotype. In the Scan*STRESS* sample, a significant interaction of rs324981 genotype x sex was observed on the neural activation in a cluster close to the parahippocampal gyrus (whole-brain corrected). Those exploratory findings in two cohorts suggest that the NPS system influences the endocrine and neural stress response in humans, which might represent a potential mechanism by which genetic variation could influence the risk for psychiatric disorders in a sex-specific way.

Post amputation pain

In the fourth study (Chapter V), the interplay of genetic and environmental factors was addressed in regard to a different phenotype. Specifically, the study aimed to investigate whether individual -potentially genetic- factors influence the manifestation of phantom and residual limb pain after an external traumatic event (i.e. the loss of a limb).

Phantom limb pain and residual limb pain

Postamputation pain represents a model for other, more common pain types. It is of high interest since, in contrast to many other chronic pain disorders, a clear event constitutes the beginning of the pain phenomena (at least in traumatic cases). After the loss of a limb, the majority of patients develop phantom limb pain (pain in the missing limb) and/or residual

limb pain (pain in the remaining limb) (Ephraim, Wegener, MacKenzie, Dillingham, & Pezzin, 2005; Foell et al., 2011; Sherman, Sherman, & Parker, 1984). Even though the two pain types can appear independently of each other, they show a modest association and co-occur with increased probability (Foell et al., 2011). Research investigating the mechanisms involved in the etiology of postamputation pain has identified processes such as neural plasticity, pain memory processes and neurological aspects (Foell et al., 2011; Jutzeler, Curt, & Kramer, 2015).

Genetic and environmental contributors of postamputation pain

Twin studies investigating the heritability of other pain-related disorders such as migraine, back pain, and chronic widespread pain, or related phenotypes such as pain sensitivity, have demonstrated that both genetic as well as environmental factors contribute to their manifestation (Nielsen et al., 2012; Nielsen et al., 2008). In addition to estimating the individual contribution of genetic and environmental factors, the formal genetic studies allow estimation of the overlap of genetic and environmental factors between different traits and disorders (Neale & Cardon, 1992). In the case of pain, the results of twin studies suggest a genetic overlap between different pain disorders and between pain disorders and depression (Ligthart et al., 2014; Pinheiro et al., 2015).

Given that twin samples are difficult to recruit for post amputation pain, as there are hardly any twin pairs in which both twins have lost a limb, the fourth study used an alternative approach to assess individual susceptibility for phantom and residual limb pain. Out of a bigger sample of amputees recruited in the frame of the PHANTOMMIND study at the Central Institute of Mental Health in Mannheim (Bekrater-Bodmann et al., 2015), 122 amputees with two amputated limbs were selected to study the intra-individual component of the aforementioned pain types, and their overlap.

The analysis revealed only a modest association between the different pain types in the same limb or between both limbs. On the other hand, each pain type showed a high intra-individual concordance between the two amputated limbs. This was observed both for the life-time presence of the respective symptoms, as well as for the currently perceived pain intensity. From these results it can be drawn that there is a strong individual component to develop the specific pain types, which might be driven by a genetic vulnerability.

The results suggest that the two pain types are distinct entities with only a modest overlap in etiology. Therefore, a separation of phantom and residual limb pain might strongly benefit research as well as treatment outcomes. Future studies should take this into account and further explore the overlap or respective distinctiveness between pain types.

Summary and discussion

This thesis comprises four studies which investigated the effects of genetic and environmental factors on the central stress response and postamputation pain.

In studies 1 and 2, it was demonstrated that the environmental risk factor urbanicity modulates central stress processing and that this effect is modulated by genetic variation in *NPSR1*. Furthermore, study 3 indicated that the genetic variation in *NPSR1* exerts sex-specific effects on the stress response. Studying genetic variation is one way to investigate whether a specific system is involved in a phenotype or a disorder when there is no corresponding pharmacological agent available for administration. The results of this thesis suggest that NPS influences stress processing in humans. In rodent studies, NPS has been administered via nasal spray (Lukas & Neumann, 2012; Medina, Ji, Gregoire, & Neugebauer, 2014). If this treatment would become available for application in human studies as it is the case for oxytocin or insulin (Cardoso, Kingdon, & Ellenbogen, 2014; Ferreira de Sa et al., 2014), it could be used in neuroimaging studies to further explore the role of NPS in central stress processing. This would also facilitate translation of results between humans and animal models.

Also, in the frame of this thesis, the Scan*STRESS* paradigm was developed and validated as a reliable stressor as well as an fMRI measure that can be applied in other studies. Recently, Akdeniz and colleagues (2014) applied this paradigm and demonstrated altered stress processing in second generation migrants, a group with an increased schizophrenia risk. However, considering the limited sample sizes, replication of the presented results is wanted in larger samples, with the same as well as other social stress paradigms. In the future, it would be of high interest to investigate further high-risk groups for psychiatric disorders, for example subjects with prodromal symptomatology, or subjects with an increased familial risk.

In study 4, evidence for an individual disposition to develop specific types of post amputation pain was presented, based on data from subjects with two amputated limbs.

As twin studies cannot be easily applied in the study of postamputation pain, this approach generated pioneering evidence for a genetic component in the development of these specific pain phenotypes. The findings, indicating a partially independent etiology of phantom limb and residual limb pain, support that both should be assessed separately in research and treatment settings. In a next step, further studies are already planned using larger samples of single limb amputees, including the one collected in the PHANTOMGENE project, a subproject of the PHANTOMMIND study (Bekrater-Bodmann et al., 2015). These samples will be investigated using molecular genetic approaches. Specific risk variants, which can be

identified in such large genetic studies, will then be used to direct follow-up studies in smaller, more thoroughly phenotyped samples, to assess potential intermediate phenotypes of postamputation pain, such as fMRI data mapping neural plasticity or pain sensitivity measures (Flor, Diers, & Andoh, 2013; Jutzeler et al., 2015; Tracey, 2011). Furthermore, identified genes could also be studied in animal models and targeted with pharmacological components in a translational approach. GWAS data could be used to dissect the factors underlying phantom limb and residual limb pain, as well as to assess the genetic overlap that those pain phenotypes have with other risk factors, e.g. diabetes risk or depression.

In summary, the here described studies represent promising approaches to investigate stress processing and pain phenotypes and their underlying genetic and environmental risk factors. Future studies utilizing larger samples should further investigate these risk factors and their interplay.

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Chapter II - City living and urban upbringing affect neural social stress processing in humans

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Abstract

More than half of the world's population now lives in cities, making the creation of a healthy urban environment a major policy priority (Dye, 2008). Cities have both health risks and benefits (Dye, 2008), but mental health is negatively affected: mood and anxiety disorders are more prevalent in city dwellers (Peen, Schoevers, Beekman, & Dekker, 2010) and the incidence of schizophrenia is strongly increased in people born and raised in cities (Krabbendam & van Os, 2005; Mortensen et al., 1999; Pedersen & Mortensen, 2001; van Os, Pedersen, & Mortensen, 2004). Although these findings have been widely attributed to the urban social environment (Krabbendam & van Os, 2005; Peen et al., 2010; Selten & Cantor-Graae, 2005; van Os, Kenis, & Rutten, 2010), the neural processes that could mediate such associations are unknown. Here we show, using functional magnetic resonance imaging in three independent experiments, that urban upbringing and city living have dissociable impacts on social evaluative stress processing in humans. Current city living was associated with increased amygdala activity, whereas urban upbringing affected the perigenual anterior cingulate cortex, a key region for regulation of amygdala activity, negative affect (Pezawas et al., 2005) and stress (Diorio, Viau, & Meaney, 1993). These findings were regionally and behaviourally specific, as no other brain structures were affected and no urbanicity effect was seen during control experiments invoking cognitive processing without stress. Our results identify distinct neural mechanisms for an established environmental risk factor, link the urban environment for the first time to social stress processing, suggest that brain regions differ in vulnerability to this risk factor across the lifespan, and indicate that experimental interrogation of epidemiological associations is a promising strategy in social neuroscience.

Manuscript

Urbanization, a process that started in North America and Western Europe but is now mainly occurring in developing nations, is a major socio-ecological change confronting mankind. By 2050, 69% of humans will live in urban areas (Dye, 2008). Although city dwellers, on average, are wealthier and receive improved sanitation, nutrition, contraception and health care[1], urban living is also associated with increased risk for chronic disorders, a more demanding and stressful social environment and greater social disparities. The biological components of this complex landscape of risk and protective factors remain largely uncharacterized. Some of the best-established effects of urbanization concern mental health. Meta-analyses show that current city dwellers have a substantially increased risk for anxiety disorders (by 21%) and mood disorders (by 39%) (Krabbendam & van Os, 2005). For the major brain disorder, schizophrenia, incidence is about doubled in subjects born and brought up in cities (Peen et al., 2010), with evidence of a dose–response relationship (Pedersen & Mortensen, 2001) that probably reflects causation (Peen et al., 2010). Genetically vulnerable individuals are more at risk (van Os et al., 2004); in agreement with the assumption that schizophrenia represents a neurodevelopmental disorder (Weinberger, 1987). Importantly, urbanicity effects on schizophrenia later in life are minor (Krabbendam & van Os, 2005; Pedersen & Mortensen, 2001), providing an epidemiological dissociation between current and early life urbanicity effects, which are associated with mood and anxiety disorders and schizophrenia, respectively. Because longitudinal studies indicate that urbanicity effects on mental illness are causal and not mediated by other epidemiological variables (Peen et al., 2010; van Ockenburg et al., 2016; van Os et al., 2010), attempts to explain these associations must consider the specifics of the urban situation affecting the brain (Andreas Meyer-Lindenberg, 2010). Increased social evaluative threat (Dickerson & Kemeny, 2004), including social defeat and chronic social stress, might constitute such a factor (Selten & Cantor-Graae, 2005). Consequently, many authors have proposed that social stress processing in the urban environment underlies the greater risk for mental illness (Krabbendam & van Os, 2005; Peen et al., 2010; Selten & Cantor-Graae, 2005; van Os et al., 2010), and contributes to the manifestation of these disorders in adults. To test experimentally the hypothesis that urban living and upbringing modulate neural processing of acute social evaluative stress, we studied the neural responses of healthy German volunteers undergoing such stress during functional magnetic resonance imaging (fMRI). We confirmed our findings in a second study using a different social stress paradigm and then tested for cognitive specificity by ascertaining the

effect of urbanicity on brain activation during cognitive processing without stress. Importantly, our subjects did not have a mental disorder nor were they at high risk for one; the link to these illnesses from the environmental risk factor that we studied is established by the epidemiological evidence discussed earlier. In our first (discovery) study, we used the Montreal Imaging Stress Task (MIST; Dedovic et al., 2005), a social stress paradigm where participants solve arithmetic tasks under time pressure. Difficulty was varied adaptively to keep success rates—visually presented on a 'performance scale'—at between 25–40%. Study investigators provided further negative feedback after each test segment through headphones. Subjective stress levels were measured before and after the session using a visual analogue scale, and effects of the MIST on salivary cortisol, heart rate and blood pressure were recorded repeatedly. Urbanicity was quantified as follows (Mortensen et al., 1999): city with more than 100,000 inhabitants (3); town with more than 10,000 inhabitants (2); and rural area (1). For urban upbringing, these numbers were multiplied by the number of years living in the area up to age fifteen and added. Thirty-two participants with rural as well as urban upbringing and habitation entered the final analysis (Supplementary Table II-1a). City dwellers did not differ in subjective health, depressed mood, social support, or personality dimensions. Baseline circadian cortisol measures were normal (Lederbogen et al., 2010). The MIST increased cardiovascular and hormonal measures (Supplementary Figure II-1a and Supplementary Table II-2), indicating that stress was successfully induced. Stress-related brain activations (compared to a control condition without social evaluative threat) were most prominent in the right temporoparietal junction (t = 9.53, P = 0.001, all significance values are family-wise error (FWE) corrected for multiple comparisons), anterior cingulate cortex (ACC) and posterior cingulate cortex (anterior: t = 7.91, P < 0.001; posterior: t = 8.09, P < 0.001), insular cortex (right: t = 8.18, P < 0.001; left: t = 6.69, P = 0.003) and hypothalamus (right: t = 7.12, P < 0.001; left: t = 6.86, P = 0.002, see Supplementary Figure II-1b and Supplementary Table II-3 for complete list). Differential brain activation correlated significantly with the test induced rise in cortisol for hippocampus (right: r = -0.59, P = 0.041; left: r -0.59, P = 0.040) and amygdala (right: r = -0.61, P = 0.016; left: r = -0.55, P = 0.048), confirming previous reports (Pruessner et al., 2008). Although current and early life (birth to age 15) urbanicity shared moderate variance (r = 0.37, P < 0.05), their neural effects were fully distinct. Current urban living was associated with amygdala activity (t = 3.6; Figure II-1a), which increased stepwise from subjects living in the country to those living in small towns, and was highest in city dwellers (r = 0.55, P < 0.001; Figure II-1b).

In contrast, urban upbringing was associated with differential activity in the perigenual ACC (pACC, t = 3.8; Figure II-2a), increasing linearly with highest activation in participants entirely brought up in cities (r = 0.56, P < 0.001; Figure II-2b). Results were not explained by demographic or clinical variables (see Supplementary Methods). These effects were regionally specific as no other brain regions showed any urbanicity association at exploratory thresholds (P < 0.001, uncorrected). To test whether our findings were related to specific aspects of the sample or task, we studied a second cohort of 23 participants (characteristics in Supplementary Table II-1b) with a modified stress paradigm. Subjects performed two cognitive tasks (arithmetic and mental rotation) while being continuously visually exposed to disapproving investigator feedback through video (see Supplementary Methods). This experiment fully replicated the findings from the previous sample: city living was again specifically associated with activity in the amygdala and was highest in city dwellers (t = 3.3, P < 0.05, FWE corrected; Figure II-1c, d), whereas a cluster within the pACC (t = 4.0, P < 0.05, FWE corrected; Figure II-2c) showed a significant linear correlation with urban upbringing (r = 0.64, P < 0.001; Figure II-2d). Post hoc, to study the effect of current urbanicity in a larger and better distributed sample, we additionally tested 24 predominantly town and rural dwellers. The analysis of the combined sample again confirmed the effect (Supplementary Figure II-2). As before, no other brain regions showed urbanicity associations even at exploratory thresholds. These findings indicated that specifics of the procedure did not confound the urbanicity effects. Because acute social stress interacts with cognitive processing (Dickerson & Kemeny, 2004), the question arose as to whether the observed effect of urbanicity in these experiments was related to social evaluative stress per se or to the cognitive tasks used.

To investigate this issue, we studied a sample of 37 healthy adults from an ongoing study (Supplementary Table II-1b; Esslinger et al., 2009) during a working memory and an emotional face matching task, both without stress. Even at a threshold of P < 0.01, uncorrected, there were no voxels within the ACC or amygdala whose activations were correlated to current or early life urbanicity, indicating that our findings were indeed related to social stress with a degree of specificity. In a secondary analysis, we increased the sample size for this experiment to 80 (Supplementary Table II-1c), with power to detect an effect of similar size to the discovery study of 0.99. Again, no significant associations with urbanicity were observed. In this initial examination of the effects of urbanicity, no neural circuit was hypothesized a priori. Nevertheless, the regions identified, controlling for false positives, can plausibly be related to previous epidemiological observations. In the amygdala, activity

during social stress was specifically related to city living. The amygdala, which among other functions signals negative affect and environmental threat (LeDoux, 2000), has been strongly implicated in anxiety disorders, depression, and other behaviours that are increased in cities, such as violence (Meyer-Lindenberg et al., 2006). Conversely, urban upbringing showed a distinct, but equally regionally specific effect on the pACC, a major part of the limbic stress regulation system (LeDoux, 2000) that exhibits high neuronal glucocorticoid receptor

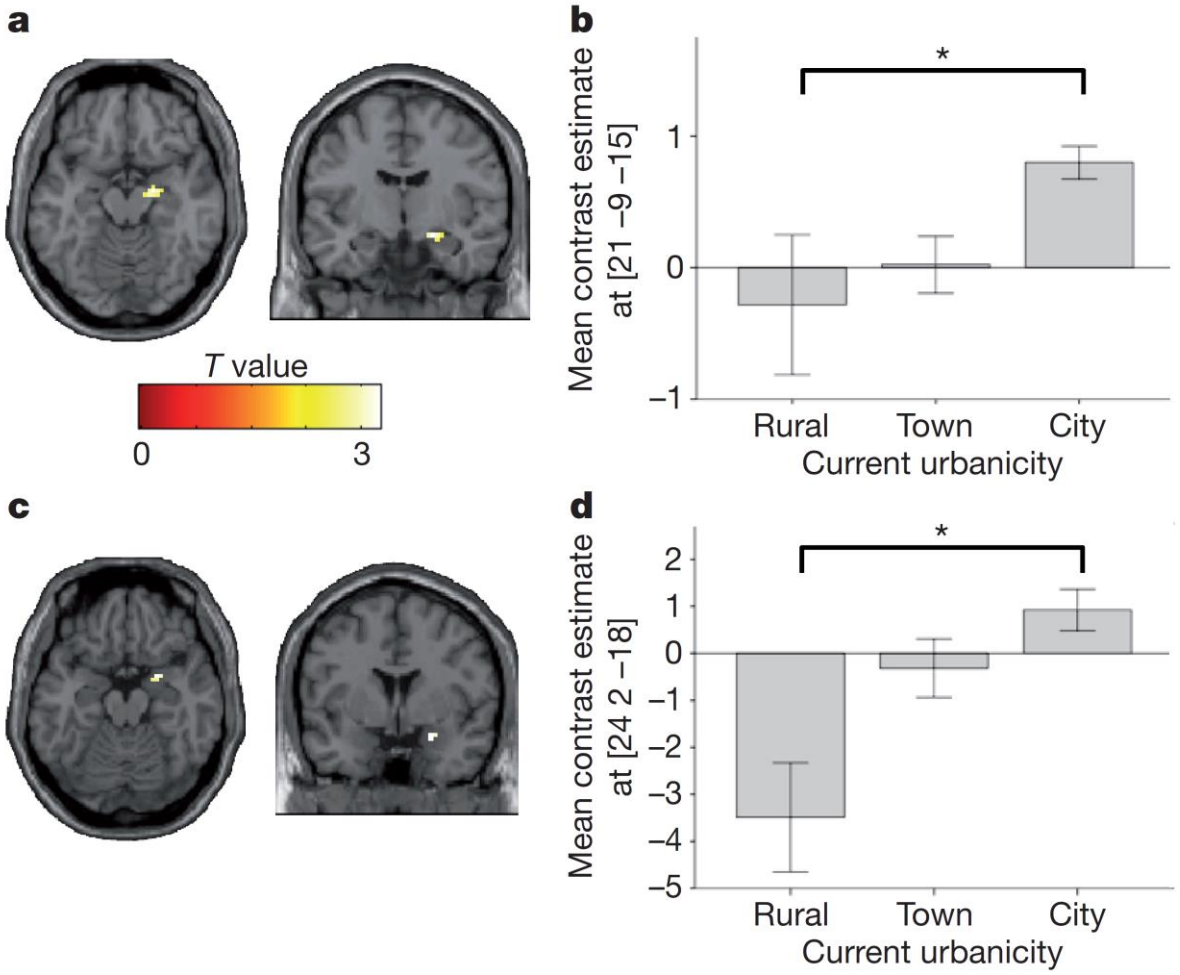

Figure II-1: Relationship between current urbanicity and amygdala activation.

a, Discovery study (N = 32): T map of significant correlations between stress-related activations (in the experimental versus control contrast) and current urbanicity scores shown at a threshold of $P < 0.005$, uncorrected. **b,** Discovery study: contrast estimates at the most significantly correlated voxel in the amygdala (located at x = 21, y = -29, z = -15) for the experimental compared to control contrast for the three current urbanicity groups (*$P < 0.05$; error bars indicate s.e.m.). **c,** Replication study (N = 23): T map of significant correlations between activations in the experimental compared to control contrast and current urbanicity scores (shown at $P < 0.05$, FEW corrected for the right amygdala as region of interest (ROI)). **d,** Replication study: contrast estimates at the most significantly correlated voxel in the amygdala (located at x = 24, y = 2, z = -18) for the experimental compared to control contrast for the three current urbanicity groups (*$P < 0.05$, error bars indicate s.e.m.).

expression (Herman, Ostrander, Mueller, & Figueiredo, 2005), modulates hypothalamic–pituitary–adrenal axis activation during stress (Diorio et al., 1993), and is implicated in processing chronic social stressors such as social defeat. In schizophrenia, reduced cingulate grey matter volume (Wright et al., 2000) has been reported in patients, emerging during adolescence (Vidal et al., 2006). Connectivity abnormalities of the pACC with the amygdala during processing of affectively negative stimuli were seen in schizophrenic patients, but not

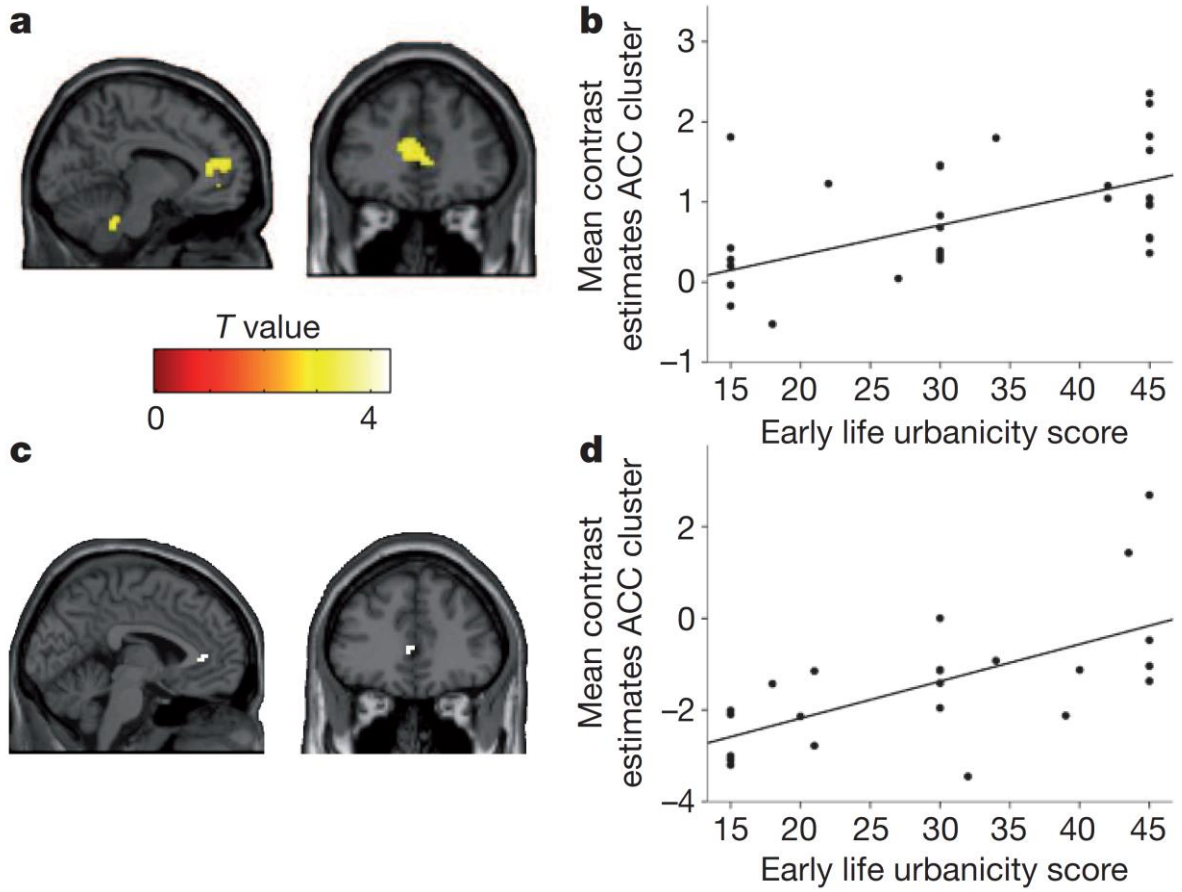

Figure II-2: Relationship between early life urbanicity scores and pACC activation.

a, Discovery study (N = 32): T map of significant correlations between stress-related activations (in the experimental versus control contrast) correlating with urbanicity scores shown at a threshold of $P < 0.005$, uncorrected. **b,** Discovery study: scatterplot of urbanicity scores and mean contrast estimates of the significantly (at $P < 0.005$) correlating voxels within the ACC in the experimental compared to control contrast. Results indicate a linear relationship between these two variables ($r = 0.56$, $P = 0.001$). **c,** Replication study (N = 23): T map of significant correlations between activations (in the experimental compared to control contrast) and urbanicity scores (shown at $P < 0.05$, FWE corrected for the rostral ACC as ROI). **d,** Replication study: scatterplot between contrast estimates for the stress compared to control contrast and the urbanicity score shown for the mean of all significantly ($P < 0.005$) correlated voxels ($r = 0.64$, $P < 0.001$).

in genetically at-risk individuals (Rasetti et al., 2009), suggesting a link to environmental factors. Therefore, the epidemiological distinction between current and early life urbanicity maps onto distinct neural regions that are associated with the disease phenotypes implicated by the environmental risk data. A direct link between psychopathology and these circuits during social evaluative stress must be established in future work, including the study of subjects with mental illness. In principle, any of the multiple factors related to urban living (Dye, 2008), such as pollution, toxins, crowding, noise, or demographic factors not captured in our analysis, could be responsible for the observed associations. However, in light of the epidemiological evidence that urbanicity is causal for mental disorders (Krabbendam & van Os, 2005; Peen et al., 2010; van Os et al., 2010), it is interesting to consider the parsimonious proposal that social stress contributes causally to the impact of urbanicity on the neural circuits identified here. Importantly, although urbanicity effects on the pACC and amygdala were dissociable, these two structures are functionally closely linked: the pACC is a key regulatory region for amygdala (LeDoux, 2000) activity in the context of negative affect, which is critical for gene–environment interactions (Pezawas et al., 2005) and extinction. Further, synaptic and neuronal remodelling of the pACC and amygdala have been described in animals exposed to social stressors (Poeggel et al., 2003), and amygdala and cingulate volume relate to social network size in humans (Bickart, Wright, Dautoff, Dickerson, & Barrett, 2011). Therefore, we investigated functional connectivity between the pACC and amygdala during the MIST stress paradigm using previously published methods (Esslinger et al., 2009). Urban upbringing was associated with reduced connectivity (Spearman's Rho = -0.39, P = 0.013; see Supplementary Figure II-3), whereas current urbanicity had no effect, supporting an effect of early urban exposure on this regulatory circuit. Because early life neurochemical alterations in the serotonin system linked to social support have enduring effects on the cingulate in animals (Spinelli et al., 2009) and humans (Cohen et al., 2006), these differential effects of early life and current urbanicity may reflect a developmental vulnerability of the cingulate. In line with this, the cortisol stress response has been found to be exaggerated in human adults who were exposed to maternal stress in utero (Entringer, Kumsta, Hellhammer, Wadhwa, & Wust, 2009). Beyond mental illness, our data are of general interest in showing a link between cities and social stress sensitivity. This indicates that an experimental approach to dissecting epidemiological associations is feasible and that it could be used to characterize further the underlying psychosocial components; for example, the effects of finer-grained quantifiers of individuals' social networks or individual social experience in urban contexts. One such potential component is unstable hierarchical position -

a social stressor related to general health that might be relevant in the context of increased socioeconomic disparities in cities- which also affects medial prefrontal cortex and amygdala function (Zink et al., 2008). Further, 'prosocial' neuropeptides, molecular mediators of social interactions, modulate the pACC-amygdala circuit (Zink, Stein, Kempf, Hakimi, & Meyer-Lindenberg, 2010), indicating that social risk and protective factors might converge on this system. This first series of studies of the neural effects of urbanicity has several limitations. First, our cross-sectional study does not prove that the observed association is causal. Second, our subjects grew up in relative safety and prosperity in Germany, a developed country, whereas greater urban–rural discrepancies are found elsewhere; however, this would have probably attenuated our findings. Third, the pronounced differences in brain processing did not correlate with cortisol levels, possibly reflecting the greater sensitivity of neural measures compared to downstream peripheral markers; nevertheless, the cortisol stress response should be studied in a larger sample. Fourth, the lack of random population sampling and the fact that our confirmation study largely used college students potentially limits the generalizability of our findings, making replication of these results in different and larger samples important. Our data reveal neural effects of urban upbringing and habitation on social stress processing in humans. These findings contribute to our understanding of urban environmental risk for mental disorders and health in general. Further, they point to a new empirical approach for integrating social sciences, neurosciences and public policy to respond to the major health challenge of urbanization.

Methods summary

The study was approved by the Ethics Committee of Heidelberg University. Three groups of healthy participants were studied after written informed consent. A thorough clinical and psychiatric examination was performed to exclude relevant illness. As in previous work, urbanicity was scored as follows4: city with more than 100,000 inhabitants (3); town with more than 10,000 inhabitants (2); and rural area (1). For urban upbringing until age 15, these numbers were multiplied by the years spent in each area and added. Blood-oxygen-level-dependent (BOLD) fMRI was performed on a 3.0 Tesla Siemens Trio scanner using an echo-planar-imaging (EPI) sequence and analysed using SPM5 (MIST study and non-stress control study) and SPM8 (stress replication study) (http://www.fil.ion.ucl.ac.uk/spm). In the discovery study, participants performed the MIST, in the replication study a variant social stress task (see Supplementary Methods), in the control study a working memory task (the n-back task) and an emotional face matching task. All imaging results were corrected for

multiple comparisons at a significance level of $P < 0.05$ via FWE. For main task effects, correction was performed over the whole brain. For hypothesis-driven analyses, bilateral a priori anatomical regions of interest (ROI) were taken from the Harvard Oxford Atlas (http://www.cma.mgh.harvard.edu). For correlations with cortisol, the amygdala, hypothalamus and ACC were specified based on previous results (Pruessner et al., 2008). For urbanicity analyses, on the basis of the correlations observed with early life and current urbanicity in the discovery study (MIST), we defined a priori anatomical ACC and amygdala ROI for the replication and control studies.

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Author notes

Author Contributions

F.L., P.K and L.H. designed and performed experiments, analysed data and wrote the paper; F.S., P.S. and S.W. designed and performed experiments, analysed data and reviewed the manuscript; H.T. analysed data and reviewed the manuscript; M.D. and M.R. designed experiments and reviewed the manuscript; J.C.P. developed the MIST paradigm and reviewed the manuscript. A.M.-L. obtained funding, designed the study and experiments and wrote the paper.

Supplementary material

METHODS

Study subjects

Participants of the discovery study were recruited via advertisements in local newspapers. Inclusion criteria were age 18-80 years, absence of present or past psychological or psychiatric therapy, absence of major or unstable general medical conditions, and ability to participate in study procedures. Exclusion criteria included history of major cerebral injury, as well as conditions prohibiting magnetic resonance imaging. Each participant underwent a thorough clinical examination including electrocardiogram and blood tests. Sample characteristics are presented in Supplementary Table II-1a. Basal hypothalamic-pituitary-adrenal (HPA)-function was assessed by four salivary cortisol concentrations collected during a regular week day at wake-up (F0) as well as ½, 8 and 14 hours (F½, F8 and F14) after wakeup. A detailed description of the sampling procedure is given elsewhere (Lederbogen et al., 2010). From these circadian samples, we computed the three cortisol secretion indices cortisol awakening reaction (CAR), slope, and area under the curve (AUC) using the trapezoidal method described by Pruessner (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The study plan had been approved by the local ethics committee. Each participant had given written informed consent prior to study inclusion. Subjects received a monetary compensation for study participation. Participants of the replication study were primarily undergraduate students who voluntarily participated in this study which was originally conducted to evaluate the new stress paradigm. Participants of the control study using the n-back and face matching task were taken from an ongoing multicenter imaging genetic study (Esslinger et al., 2009). Characteristics of the samples are presented in Supplementary Table II-1b.

Post-hoc study recruitment

Due to an unequal distribution of current urbanicity in the replication study, we collected a number of 24 additional participants, who were predominantly undergraduate students with current residency in rural areas and towns, and restudied this effect in this extended sample. In a second supplementary analysis, we increased the power of our control study controlling for non stress specific cognitive effects by including 43 additional participants and also repeated the data analysis with this extended sample. The characteristics of these combined samples are given in Supplementary Table II-1c.

Urbanicity score

Study participants provided details on their place of birth and living environment until age 15 as well as on current urban living. Following previous work, urbanization was scored in three categories (Mortensen et al., 1999): cities with more than 100,000 inhabitants (3), towns with more than 10,000 inhabitants (2) and rural areas (1). The original publication (Mortensen et al., 1999) also included the categories capital (5) and capital suburb (4) which did not occur in our sample. To calculate an urbanicity score, we multiplied the years spent in each area until age 15 by the category's score. When participants had moved from one category to another, we added up the scores from the different categories. Thus, the resulting score could range from 15 (15 years spent in rural areas) to 45 (15 years spent in a city of more than 100,000 people). Current urbanicity was defined as the category at the time of the fMRI study.

Imaging paradigms – Stress tasks

Our discovery study used the Montreal Imaging Stress Task (MIST). This paradigm poses an evaluative threat to the study subject and is described in detail elsewhere (Dedovic et al., 2005). Prior to the procedure, subjects were allowed to get acquainted to test and hardware outside the scanner. After this training session, subjects spent 30 minutes in a recumbent position in a partially darkened, quiet room to acclimatize. Inside the scanner, participants were asked to solve arithmetic tasks presented visually via goggles. They were instructed to select the correct answer by pressing different buttons on a scanner-compatible keyboard response box held in the right hand. Time pressure was created by a time bar adapting to the individual's performance in order to enforce a 25-40% success rate. The evaluative threat was established through communication with the study investigator before the start of the session, when participants were informed about the need for both speed and correctness. During the task, a mock performance indicator was presented via goggles indicating poor subject performance. After each test segment, study investigators also communicated with subjects via headphones criticizing their insufficient results in order to enhance subjects' perceived stress. The MIST used a block design consisting of three different conditions: rest, control and experimental. During rest, subjects were shown neutral surface and asked to keep their eyes open, during control, arithmetic tasks were presented as in the experimental condition, but without time pressure. The order of conditions was repeated once within one measurement sequence, resulting in a total duration of seven minutes. In order to assess cardiovascular response to the stress task, heart rate was monitored continuously during the session and blood pressure was recorded before and after sessions. Hormonal response was measured by

saliva cortisol sampling, with samples taken on participant's arrival (Cort0), after rest (Cort1), entering the scanner (Cort2), anatomical imaging (Cort3), run 1-3 (Cort4-6), and leaving the scanner (Cort7). Subjective stress was measured via visual analogue scale before and after the session, with a possible range of 0-10, "0" indicating absence of any stressor and "10" maximum stress intensity. Debriefing was scheduled after the last salivary cortisol sample. Details of the modified stress task in the replication study will be described separately. Briefly, the procedure was preceded by a training session to familiarise the subjects with the test and the hardware outside the scanner. The paradigm used a block design (two sequences of 680 seconds duration each) with repeated 60 seconds task (or control) blocks preceded by 5 seconds task announcement and followed by 20 seconds pause. In the task blocks either a mental arithmetic task or a spatial mental rotation task was presented. Responses were entered by the subjects with a response box. In the task blocks, the subject had to respond under time pressure, which was visualized by a countdown. Task speed and difficulty were adapted to the individual's performance by the software. During the scanning procedure as well between the sequences, an observer panel was presented to the subject by live video stream in order to induce social-evaluative threat. While observers remained passive in the control blocks, they gave disapproving feedback during the task blocks (visual feedback) and between the sequences (visual and verbal feedback). After the test procedure subjects received detailed debriefing.

Imaging paradigms – n-back and face matching task

During the n-back task (Esslinger et al., 2009) subjects viewed a series of digits (1-4) presented sequentially for 500 ms (interstimulus interval, 1500ms) on a screen via LCD goggles. In the 0-back control condition, subjects had to press a button corresponding to the digit presently seen. In the 2-back working memory condition they had to react to the digit seen two instances before the present digit. Four blocks of each condition were presented alternately. The task lasted 4 minutes or 124 scans.

The face matching task is used to study implicit emotion recognition (Esslinger et al., 2009). Subjects had to match two out of three pictures of angry or fearful faces (emotional condition) or geometrical shapes (control condition). The design was four blocks for each condition alternating, each consisting of six trials and lasting 30 seconds. The task lasted for 4.2 minutes or 130 scans.

Image acquisition and analysis

Blood-oxygen-level-dependent (BOLD) fMRI was performed on a 3.0 Tesla Siemens Trio scanner using an echo-planar-imaging sequence (repetition time = 2000 ms, echo time = 30 ms, flip angle = 80°, 64 x 64 matrix, 32 4mm axial slices with 1mm gap). Images were preprocessed and analysed using SPM5 (MIST study and non-stress control study) and SPM8 (stress replication study) (www.fil.ion.ucl.ac.uk/spm). Images were realigned to the first functional scan by a 6-parameter rigid body transformation, then spatially normalized to the standard Montreal Neurological Institute template including resampling into 3 x 3 x 3 mm^3 voxels and finally smoothed with a 9mm full-width at half-maximum Gaussian kernel. For each subject, one general linear model was defined containing regressors for control and experimental condition of each measurement sequence leading to a sum of 6 condition regressors. To account for motion artefacts which were not fully corrected by realignment, 6 motion regressors were also included.

Data analysis

Hormonal reactivity during the MIST (deltaCortMIST) was analyzed by subtracting saliva cortisol concentration Cort2 from Cort7. Contrast images of control versus rest, experimental versus rest and experimental versus control condition were obtained for each subject and analysed in one-sample *t*-tests to check for effects of conditions.

We used the general linear model to study the following main effects in neuroimaging: Correlational effects between stress-specific brain activations and cortisol reactivity were analysed on contrast images of the experimental > control contrast and deltaCortMIST as covariate of interest. To test for an effect of current or early-life urbanicity, experimental > control contrast images were analysed including urbanicity scores separately as covariate of interest.

Post-hoc data analyses

All post-hoc data analyses were conducted on the discovery study sample, unless explicitly stated elsewise. To address the possible effect of demographic covariates on the observed urbanicity effect, the following demographic variables were individually added to the general linear model: age, gender, years of education, currently working, living with spouse, number of children, household size, household income, subjective health (Short Form-36, 12 item version), depressive mood (Hamilton Depression Scale), social support (Berlin Social Support Scale), and personality dimensions like neuroticism, openness, conscientiousness, agreeableness and extraversion (Big Five Inventory-10) (see Table II-1a). With statistical

inference as detailed below, none of these variables had a significant effect on the reported findings.

We furthermore explored interactions of current and early-life urbanicity with age. No significant interactions were observed in the pACC or in the amygdala at a threshold of $P < 0.01$, uncorrected.

In order to account for the possible impact of potentially confounding variables such as subclinical conditions, we compared subjects of current urbanicity categories 1 and 2 with subjects of current urbanicity category 3 regarding symptoms and personality inventories via independent samples *t*-tests. There were no significant differences between groups with respect to subjective health (Short Form-36, 12 item version), depressive mood (Hamilton Depression Scale), social support (Berlin Social Support Scale), and personality dimensions like neuroticism, openness, conscientiousness, agreeableness and extraversion (Big Five Inventory-10) (all *P*-values > 0.1).

To investigate the possible impact of the current urbanicity score used in the analysis4, we reran this analysis using actual size of the city, town or region in terms of number of inhabitants. This did not materially change the findings. The resulting urbanicity score was comparably correlated to amygdala activation ($t = 3.4$, $P < 0.001$).

To validate the results of absent correlations with urbanicity scores in non-stressful cognitive tasks (control study) we repeated the analyses including only that part of the extended control study sample that did not overlap with the original sample. In the resulting sample of 43 subjects, again no significant correlations with early-life or current urbanicity were found in the ACC or the amygdala.

Statistical inference

Imaging results were corrected via FWE for multiple comparisons at a significance level of $P < 0.05$. For main task effects (stress vs. control, as reported in Supplementary Table II-3), correction was performed over the whole brain. For multiple regression analyses, we performed FWE-correction within *a-priori* anatomical regions of interest (ROI) with masks from the Harvard Oxford Atlas (http://www.cma.mgh.harvard.edu). These anatomical ROIs were chosen as follows: for correlations with cortisol, they were based on previous results (Pruessner et al., 2008) showing differential effects between stress cortisol-responders and non-responders in the hippocampus, the amygdala, the hypothalamus and the anterior cingulate cortex (ACC). For urbanicity, they were based on the regional effects of the correlations observed in the discovery study with early-life and current urbanicity during the

MIST. We used anatomical ROI of the rostral ACC (including BA 24 a-c, BA25, BA 32 and BA 33) as defined by Bush and colleagues (Bush, Luu, & Posner, 2000) and the right amygdala for our replication study in an independent sample and paradigm. For the control study we used the whole ACC and amygdala as ROI to increase the probability to identify significant correlations between activation and urbanicity in the n-back and faces paradigm.

Functional connectivity analysis

To examine pACC-amygdala functional coupling, we used a seed region approach described by Esslinger, Walter, Kirsch and colleagues (Esslinger et al., 2009). Seed time series of the pACC were extracted using all voxels within the cluster in the pACC where regional activation was associated with early-life urbanicity as displayed in Figure II-2a. Then, seed time series were temporally filtered and task related variance was removed. To account for noise, first eigenvariates from masks covering cerebrospinal fluid (CSF) and white matter were extracted for each individual and entered, together with movement covariates, into whole-brain multiple regression where seed region activity was the covariate of interest. Mean contrast estimates of the partial correlation of the pACC BOLD time series with the time series of the right amygdala were subsequently extracted, using an anatomically defined mask of the amygdala from the Harvard Oxford Atlas (http://www.cma.mgh.harvard.edu), and cross-correlated with individual urbanicity scores. Due to the presence of one substantial data outlier, significance was tested using non-parametric methods of inference, yielding a significant negative correlation between pACC-amygdala coupling and early-life urbanicity (Spearman's Rho = -0.39, $P = 0.013$, see Supplementary Figure II-3).

Supplementary methods references

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Supplementary Table II-1a: Characteristics of subjects (discovery study)

	Mean±SD / *n*	*N*
Age (years)	43.6±14.4	32
Female/male	16/16	32
Body mass index (kg/m2)	25.6±3.9	32
Smoking regularly	3	32
Currently working	14	32
Living with spouse	23	32
Number of children	0.84±1.1	32
Household size (people)	2.3±1.3	32
Household income (monthly after taxes, in Euro)	1914±823.4	32
Years of education	11.2±1.9	32
Current urbanicity categories 1/2/3	4/11/17	32
Early-life urbanicity score	31.6±11.7	32
Relocation frequency until age 15	0.4±0.8	32
Basal salivary cortisol concentrations		
F0 (nmol/L)	14.9±8.3	26
F½ (nmol/L)	22.9±11.7	30
F8 (nmol/L)	6.8±4.1	30
F14 (nmol/L)	2.1±1.3	28
Basal cortisol secretion indices		
CAR (nmol/L)	8.6±11.2	26
AUC (nmol/L)	110±42	23
Slope (nmol/L/h)	-1.0±0.6	24

F0, F½, F8 and F14 denote saliva cortisol concentration at wake-up as well as ½, 8 and 14 hours after wake-up. CAR denotes cortisol awakening reaction, AUC area under the curve. Subjects of the current urbanicity categories 1 and 2 did not differ from those of category 3 with respect to subjective health (Short Form-36, 12 item version), depressive mood (Hamilton Depression Scale), social support (Berlin Social Support Scale), and personality dimensions (Big Five Inventory-10) (independent samples *t*-test, all *P*-values > 0.1). Years of education were nominally associated with current urbanicity ($r = 0.372$, $P = 0.036$) and age with early-life urbanicity score ($r = 0.392$, $P < 0.03$). None of these associations would survive multiple comparison correction, and inclusion of these covariates in imaging analyses of urbanicity did not change the significance or specificity of the findings.

Supplementary Table II-1b: Characteristics of subjects (original replication sample [*N*=23] and original control study sample [*N*=37])

	Replication study (Mean±SD / *n*)	Control study (Mean±SD / *n*)
Age (years)	28.0±5.7	32.6±10.1
Female/male	7/16	15/22
Body mass index (kg/m²)	23.6±3.5	24.2±4.5
Smoking regularly	0	8
Currently working	2	24
Years of education	12.9±0.3	11.8±1.5
Current urbanicity categories 1/2/3	2/3/18	2/4/31
Early-life urbanicity score	29.3±11.3	35.9±10.4
Relocation frequency until age 15	1.0±1.0	0.7±0.9

No epidemiological variables were associated with current or early-life urbanicity scores.

Supplementary Table II-1c: Characteristics of subjects (extended replication sample [*N*=47] and extended control study sample [*N*=80])

	Replication study (Mean±SD / n)	Control study (Mean±SD / *n*)
Age (years)	27.0±6.0	33.1±10.4
Female/male	21/26	44/36
Body mass index (kg/m²)	23.9±3.8	23.9±3.9
Smoking regularly	0	13
Currently working	11	51
Years of education	12.8±0.7	12.0±1.4
Current urbanicity categories 1/2/3	10/12/25	6/12/62
Early-life urbanicity score	27.6±11.0	32.9±11.4
Relocation frequency until age 15	1.1±1.2	0.7±1.0

No epidemiological variables were associated with current or early-life urbanicity scores.

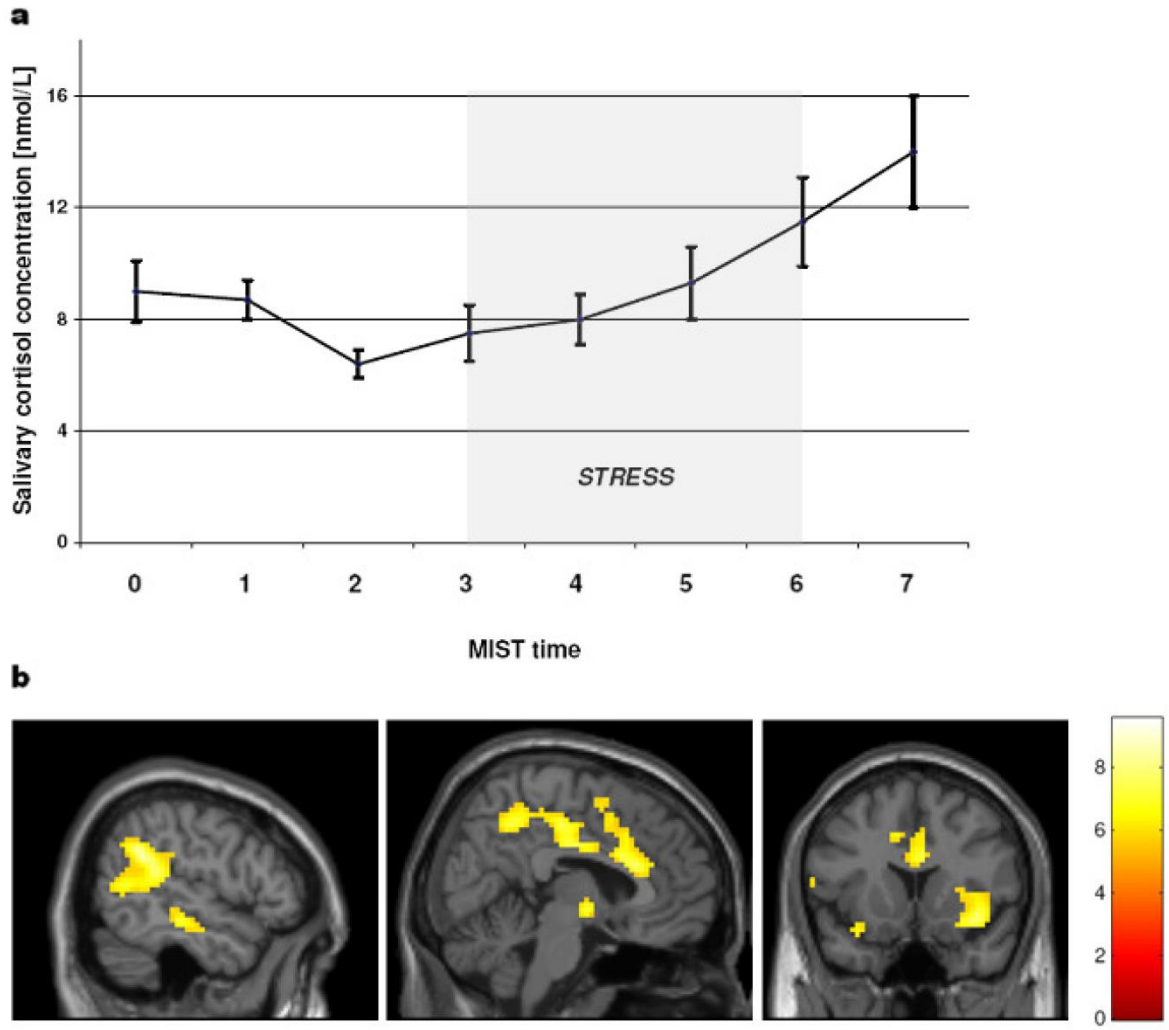
Supplementary Table II-2: Effect of stress induction in discovery study participants (*N*=32)

	Before stress*)	Stress induction*)	*P*-value †)
Heart rate (beats per minute)	68±6	73±9	< 0.01
Blood pressure (mmHg)			
systolic	124±13	136±15	< 0.001
diastolic	79±8	88±8	< 0.001
Saliva cortisol concentration (nmol/L) ‡)	6.5±3.0	14.8±10.9	< 0.001
Subjective stress intensity	2.3±1.3	7.1±1.7	< 0.001

*) Values are means±SD
†) Student's *t*-test, two-sided
‡) *N* = 28

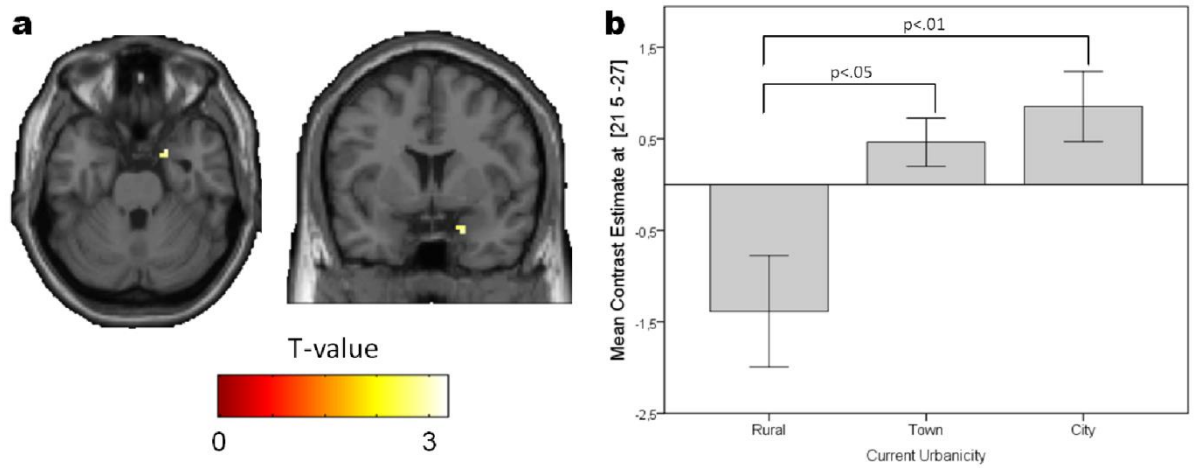
Supplementary Table II-3: Coordinates and statistics of regions significantly activated in the MIST experimental > control contrast

	Talairach coordinates			Statistics	
	x	*y*	*z*	*t*	*P*
right hypothalamus	6	-6	-6	7.12	< 0.001
left hypothalamus	-9	-9	-6	6.86	0.002
anterior cingulate cortex	3	24	21	7.91	< 0.001
posterior cingulate cortex	0	-6	30	8.09	< 0.001
precuneus	6	-51	45	7.08	< 0.001
right temporoparietal junction	60	-42	21	9.53	< 0.001
right superior temporal sulcus	57	-24	-12	7.80	< 0.001
right insular cortex	39	12	-9	8.18	< 0.001
left insular cortex	-30	12	-18	6.69	0.003
supplementary motor area	3	9	60	6.03	< 0.001
occipital cortex	18	-75	-6	9.12	< 0.001



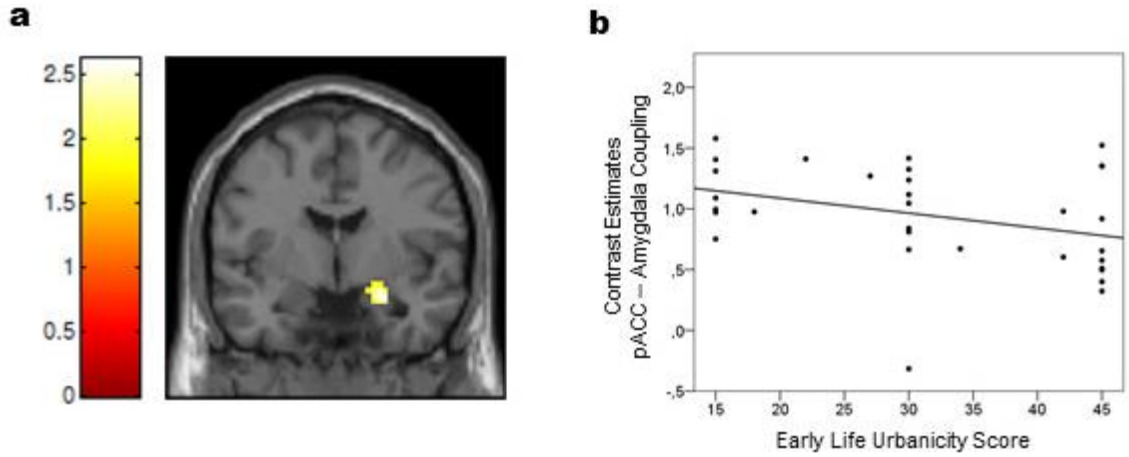
Supplementary Figure II-1: Effects of stress induction (*N* = 32).

a: Salivary cortisol concentrations before and during the stress task: Participant's arrival (0), after rest (1), entering the scanner (2), anatomical imaging (3), run 1-3 (4-6), and leaving the scanner (7). Error bars represent s.e.m. b: Brain activations during stress processing: Significantly activated regions ($P < 0.05$, FWE-corrected) in the experimental > control contrast. Slice coordinates from left to right: $x = 53$, $x = 6$, $y = 14$.



Supplementary Figure II-2: Relationship between current urbanicity and amygdala activations in the extended replication study (*N* = 47).

a: *T* map of significant correlations between activations in the experimental > control contrast and current urbanicity scores displayed at a threshold of $P < 0.005$ uncorrected ($k = 5$). **b:** Contrast estimates and significant differences at the most significantly correlated voxel in the amygdala (located at x = 21, y = 5, z = -21) for the experimental > control contrast for the three current urbanicity groups. Error bars indicate s.e.m.



Supplementary Figure II-3: Relationship between early-life urbanicity and pACC functional coupling to the right amygdala in the discovery sample (*N* = 32).

a: Voxelwise T map of the correlation of early-life urbanicity with pACC coupling to right amygdala (displayed at a threshold of $P < 0.05$ uncorrected for presentation purposes). **b:** Scatterplot of the correlation between individual mean contrast estimates of pACC – amygdala coupling and early-life urbanicity scores (Spearman's Rho = -0.39, $P = 0.013$).

Chapter III - A functional variant in the neuropeptide S receptor 1 gene moderates the influence of urban upbringing on stress processing in the amygdala

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Abstract

We have previously shown that urban upbringing and city living were associated with stress-induced activity in the amygdala and the perigenual anterior cingulate cortex (pACC). This finding might link the epidemiological risk factor "urbanicity" to neurobiological mechanisms of psychiatric disorders. However, given the heritability of stress related phenotypes, it appears likely that genetic factors can modulate the effect of urbanicity on social stress processing.

In the present exploratory study, we investigated if a functional sequence variation in the neuropeptide S receptor gene (*NPSR1* rs324981) is associated with brain activation patterns under acute psychosocial stress and if it modulates the link between urbanicity and central stress processing. In animals, neuropeptide S has strong anxiolytic effects and it induces hypothalamus-pituitary-adrenal (HPA) axis activation. In humans, rs324981 was found to be associated with anxiety and stress related phenotypes.

Forty-two subjects were exposed to a psychosocial stress task for scanner environments (Scan*STRESS*). While no main effect of rs324981 on amygdala and pACC activity was detected, we found a distinct interaction between rs324981 and urban upbringing modulating right amygdala responses (t = 6.10, P = 0.004, whole-brain FWE corrected). Moreover, right amygdala responses were significantly higher in subjects who also showed a salivary cortisol response to the stress exposure.

The present finding of a gene x environment interaction further supports the view that the brain NPS system is involved in central stress regulation. This study provides first evidence for the assumption that a *NPSR1* variant modulates brain activation under stress, interacting with the environmental risk factor urban upbringing.

Introduction

Individuals born and raised in an urban environment suffer more often from schizophrenia (Pedersen & Mortensen, 2001, van Os et al., 2004), anxiety disorders and depression (Peen et al., 2010) than people living in the countryside. Previously, we found an association of urbanicity with neural stress processing (Lederbogen et al., 2011). Current urban environment exposure was associated with increased amygdala activity under acute stress while urban upbringing was associated with increased activity in the perigenual anterior cingulate cortex. These structures are involved in stress regulation and social cue processing and show alterations in subjects with psychiatric disorders (Monk, 2008) as schizophrenia (Radua et al., 2012) and anxiety disorders (Holzschneider & Mulert, 2011).

Stress is a well-known risk factor for psychopathology (Chrousos, 2009). Genetic factors do also influence the risk for psychopathology and they influence stress reactivity as revealed in family studies on major depression (Sullivan et al., 2000), anxiety (Kendler et al., 2008), schizophrenia (Cardno & Gottesman, 2000) and HPA axis regulation (Federenko et al., 2004, Wüst et al., 2000). A molecule recently implied in anxiety and stress regulation is Neuropeptide S (NPS). NPS acts via the G-protein-coupled NPS receptor 1 (NPSR1) (Xu et al., 2004), which is expressed throughout the brain including limbic structures (Clark et al., 2011, Leonard & Ring, 2011). A promising NPS receptor gene (*NPSR1*) variant is rs324981, a single nucleotide polymorphism encoding an amino acid change (A>T Asn107Ile). The minor Ile107 variant shows increased surface receptor expression and a five- to tenfold higher NPS-induced signaling response than the Asn107 variant (Reinscheid et al., 2005). First evidence suggests associations between rs324981 and anxiety related phenotypes. While in one study genotype A/A was underrepresented only in male panic disorder patients (Okamura et al., 2007), in another sample the T allele was associated with panic disorder only in females (Domschke et al., 2011). In panic disorder and agoraphobia patients the T allele was associated with higher anxiety sensitivity, heart rate and symptom reports during a behavioral avoidance test (Domschke et al., 2011). Healthy subjects with genotype T/T showed increased anxiety sensitivity. In the same study, also an influence of rs324981 on the effect of childhood maltreatment and recent life events on anxiety sensitivity was found (Klauke et al., 2014). Studies that used anxiety related fMRI paradigms showed an association between rs324981 and brain activation changes in the rostral dorsomedial prefrontal cortex (Raczka et al., 2010), the dorsolateral prefrontal, lateral orbitofrontal and anterior cingulate cortex (Domschke et al., 2011) and the right amygdala (Dannlowski et al., 2011). Recently, Kumsta and colleagues

(2013) found an association between rs329481 T and increased salivary cortisol as well as with subjective stress responses to the Trier Social Stress Test for Groups.

In the present study we investigated the association between rs324981 and neural responses to acute stress. Moreover, we analyzed the gene x environment interaction of rs324981 with current and early urbanicity on neural responses. Heart rate, plasma adrenocorticotropic hormone (ACTH) and salivary cortisol were measured to assess peripheral stress responses.

Methods

Sample

Participants were recruited via flyers and the email system of the universities of Mannheim and Heidelberg as part of a study investigating gene-environment interactions in schizophrenia (EU-GEI). On the first contact, a screening interview was carried out on the phone. Exclusion criteria comprised current presence or lifetime history of significant general medical, psychiatric, or neurological illness, psychotropic pharmacological treatment, head trauma, incompatibility with fMRI scanning (metal parts or other health risks) and left-handedness. To ensure a genetically homogeneous sample, only participants with German grandparents were included. We only recruited participants that previously underwent MR scanning to reduce the risk of a stress reaction caused by the novelty of the MR environment. The study was approved by the ethics committee of the University of Heidelberg and written informed consent was obtained from all participants. The volunteers received a small monetary compensation for study participation. The present sample is a subgroup of a previous cohort (Lederbogen et al., 2011) and we included all subjects who from the outset were collected for genetic analyses. Only from these subjects DNA samples were available. In total, 22 men and 20 women of German descent between 20 and 43 years (mean = 28.0) were included.

Neuroimaging stress paradigm

The Scan*STRESS* paradigm was developed by our group to induce social stress in the fMRI environment. The social stress induction includes several crucial components such as pressure to perform, time pressure, forced failure, social-evaluative threat, uncontrollability and unpredictability. Subjects receive the instruction that the aim of the study is the investigation of brain activation correlates of maximal individual mental performance and that therefore it is of crucial importance that they show maximal effort.

The Scan*STRESS* paradigm was implemented in Presentation® software (Version 12.9, www.neurobs.com). It consists of two different tasks presented to the participant via a monitor. In the first task participants have to match a three-dimensional figure to its rotated form from three options presented below the target figure (source of stimulus material: Peters & Battista, 2008). In the second task, participants have to continuously subtract the number 13 from a 4 digit number, analogous to the subtraction task implemented in the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993). They have to choose the correct answer out of four presented numbers (Figure III-1b). In case of an error, participants have to start again from the beginning. In both tasks answers are given with a 4 button response box (Current Design, Philadelphia, PA), with the layout of the answer options corresponding to the diamond shaped layout of the keys.

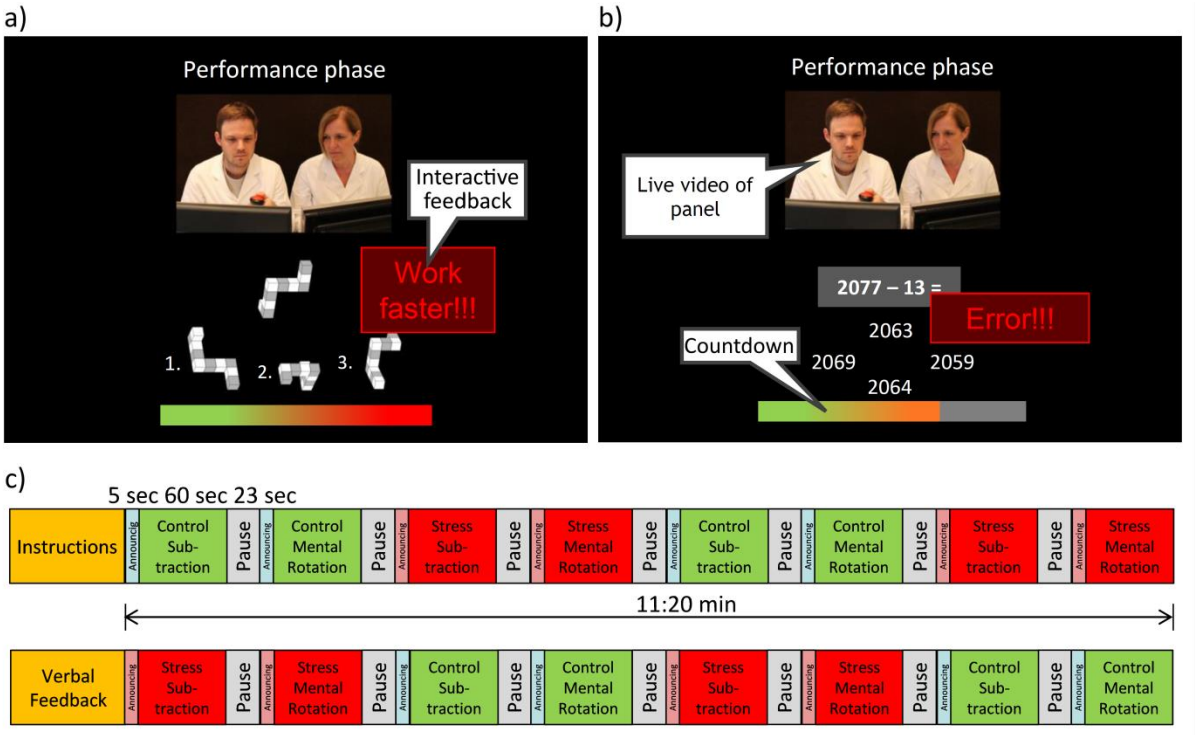

Figure III-1: Screenshot of the two different tasks, mental rotation (a) and subtraction task (b), presented in the performance phase of the stress paradigm and (c) the design of the Scan*STRESS* paradigm with two runs, preceded by an instruction phase and interrupted by critical verbal feedback given by one panel member to the participant.

The paradigm uses a block design (two runs of 680 seconds duration each) with repeated 60 seconds task (or control) blocks preceded by 5 seconds task announcement and followed by 20 seconds rest period. The paradigm consists of 16 epochs of 60 seconds each with alternating stress performance and control blocks presented in two runs (see Figure III-1c). In

the task blocks, the subject has to respond under time pressure, which is visualized by a countdown timer, signaling the remaining time. Both task speed and difficulty are adapted to the individual's performance by the software, thus ensuring frequent failure. In the control conditions, participants performed a less demanding task without time pressure (number matching without subtraction, figure matching without rotation). The number of trials in control and stress blocks is matched.

Before entering the scanner room, subjects are introduced by the experimenter to an observer panel consisting of one female and one male researcher in professional attire, sitting in the control room. The participants are informed that their behavior, mimics and answers will be monitored by the panel, which will be able to see a live video transmission of their faces. A mock-camera is installed on the head-coil for that purpose. A live video transmission of the panel is presented to the participant during the scanning procedure to induce social-evaluative threat (Figure III-1a & b).

After immersion into the scanner tube and subsequent pre-measurements (e.g. localizer) and directly before starting the paradigm, the tasks and the role of the panel are introduced to the participant (via the scanner's audio system and video transmission) by one member of the panel. During the task blocks they explicitly watch the monitors and give disapproving visual feedback after wrong or slow answers of the subject. This is done via button presses on a "buzzer" visible to the subject on the video transmission. Depending on the button pressed, either a message indicating an error ("Error!") or a message asking the subject to work faster ("Work faster!") is displayed on top of the answer options. In the control blocks, the panel members remain passive and do not look into the camera. The video picture is overlaid by a grey diagonal cross to signal that the subject is not monitored. No feedback is given to the subject in control blocks. Between the two runs one of the panel members provides the subjects with the information that her/his performance is below average and that the fMRI data will be useless, if she/he doesn't try harder in the next half of the experiment.

After completion of the stress induction, the study was explained in detail to the subjects in a comprehensive debriefing. Amongst others, they were informed that the disapproving feedback was part of the standard procedure and independent of individual performance. The Scan*STRESS* paradigm is available on request from the corresponding author.

Experimental protocol

Participants reported to the laboratory twice, first for an appointment of 45 minutes (detailed information on study, exclusion criteria check) and then for a second session of 2.5 hours, which included the Scan*STRESS* paradigm.

Heart rate

During fMRI scanning, heart rate recordings were obtained with a MRI compatible finger oxiometer with a sampling rate of 50 Hz. For each subject, the average heart rate was computed for the control blocks and the stress blocks for each run. The difference between both values was used to quantify stress induced heart rate increase.

Saliva and blood sampling

In all 42 participants, eight saliva samples were obtained using Salivette® sampling devices (Sarstedt, Nuembrecht, Germany): 45, 22 and 10 minutes before and 35, 45, 60, 75 and 90 minutes after onset of the stress induction (see supplementary Figure III-1). Time point 0 was defined as the beginning of the introduction given by the expert panel directly prior to the first run. The sample at time point -10 was collected immediately before immersion of the participant into the scanner tube (around time point -7), while the sample at time point +35 was collected immediately after leaving the scanner tube. Those samples were placed in the participants' mouth and taken after two minutes by the experimenter with a glove, to minimize head movement. Saliva was not sampled between the two runs to avoid excessive head movement that could severely reduce the quality of fMRI data. In addition, nine blood samples were collected before (time points -45 and -22), during (time points -1.5, +13.5 and +27.5) and after the scanning procedure (time points +45, +60, +75 and +90; see supplementary Figure III-1). For that purpose a catheter was placed in a forearm vein of the left non-dominant arm (at minute -48). To allow blood drawing in the scanner without moving the bench, all blood samples were drawn through a 3-way stopcock connected to a 100 cm long intravenous line (volume 4 ml). For every blood drawing, the first 10 ml were discarded before a 2.7ml EDTA blood vial was filled. To keep the line and the catheter patent, the 3-way stopcock was connected to a slow drip of physiological saline solution. At the end of each blood draw, a bolus of 10 ml was slowly flushed through the tube to clear the line. We waived all blood samples that could not be drawn within 90 seconds in order to adhere to our strict time schedule. For that reason, we stopped sampling in 7 subjects. In another 13 of the 42 subjects we failed to collect blood samples for logistical reasons. Therefore, complete

blood sample data were available in a subsample of 22 participants (10 females). The total test duration was not different in subjects with and without complete blood sample data.

Blood samples were immediately stored on ice and centrifuged within 10 minutes at 200 g and 4°C for 10 minutes. EDTA plasma was divided into aliquots and stored at -80°C until analysis. Saliva samples were kept at room temperature throughout the test session and then stored at -20°C. After thawing for biochemical analysis, samples were centrifuged at 2000 g at 10°C for 10 minutes. Plasma ACTH concentrations were determined by an ELISA assay according to the manufacturer's protocol (Biomerica Inc., Irvine, California, USA). The assay sensitivity was 0.22 pg/ml. Intra- and inter-assay variabilities were below 6.0 % and 7.0 %, respectively. Salivary cortisol concentrations were determined by a time resolved immunoassay with fluorescence detection (DELFIA) described elsewhere (Dressendörfer et al., 1992). The assay sensitivity was 0.173 nmol/l. Intra- and inter-assay variabilities were less than 6.7 % and 9.0 %, respectively.

DNA-extraction and genotyping

Genomic DNA was extracted from EDTA blood according to standard procedures. The single nucleotide polymorphism rs324981 was genotyped on an Applied Biosystems 7900HT Fast Real-Time PCR System, using a TaqMan 5' nuclease assay (TaqMan® SNP Genotyping Assay ID C___2959781_10; Applied Biosystems). Alleles designated A (detected by the VIC labelled probe; AAU = Asparagine/Asn according to the standard RNA genetic code) and T (detected by the FAM labelled probe; AUU = Isoleucine/ Ile according to the standard RNA genetic code) were detected in the sequence context of CTGGTCAACATCTTGACAGATATTA[A/T]TTGGCGATTCACTGGAGACTTCACG.

Genotyping accuracy was assessed by running 15 % of the sample in duplicates. Reproducibility was 100 %.

Urbanicity score and socioeconomic variables

Participants provided detailed information on their current place of living, place of birth and living environment of the first 15 years of their lives. Urbanicity for the respective time points was scored by classifying the municipality the subject was living in at the respective time point into 3 different categories: city (3 = more than 100,000 inhabitants), town (2 = between 10,000 and 100,000 inhabitants) and rural area (1 = less than 10,000 inhabitants). Early urbanicity scores were calculated as described previously (Lederbogen et al., 2011, Pedersen & Mortensen, 2001) by multiplying the years spent in each area until age 15 by the category's

score. When participants had moved from one category to another, we added up the scores from the different categories. This resulted in scores between 15 (15 years upbringing in rural areas) and 45 (15 years upbringing in a city with a population bigger than 100,000). For repeated measures models investigating the effects of urban upbringing, we categorized the participants into three groups representing the environment they mainly grew up in (urbanicity score 15 - 22 = low urbanicity, 23 - 37 = middle urbanicity, 38 - 45 = high urbanicity). In addition, the participants' years of school education and the formal school education of their parents (no degree, secondary general school certificate ("Hauptschulabschluss"), secondary modern school certificate ("Realschulabschluss"), university-entrance diploma ("Abitur")) have been assessed.

Image acquisition and analysis

The experiment was carried out on a 3.0 Tesla MRI scanner (Siemens Trio, Erlangen, Germany) scanner. Blood-oxygen-level-dependent (BOLD) fMRI was performed using a gradient-echo echo planar imaging (EPI) sequence (repetition time (TR) = 2000 ms, echo time (TE) = 30 ms, flip angle = 80°, 64 x 64 matrix, 192 mm field of view, 32 3mm axial slices with 1 mm gap). To minimize T1 equilibration effects the first 4 scans were discarded. Images were preprocessed and analyzed using SPM8 (www.fil.ion.ucl.ac.uk/spm). Images were realigned to the first functional scan by a 6-parameter rigid body transformation, then spatially normalized to the standard Montreal Neurological Institute (MNI) template including resampling into 3 x 3 x 3 mm^3 voxels and smoothed with a 9 mm full-width at half-maximum (FWHM) Gaussian filter. For each subject, one general linear model was defined containing regressors for control and social stress conditions and the respective announcement phases for each measurement sequence leading to a sum of 12 condition repressors. Six motion regressors were included to account for motion artifacts which were not fully corrected by realignment. Contrast images of social stress versus control condition were computed for each subject and analyzed in one-sample t-tests to check for effects of conditions. To study the effects in neuroimaging we used the general linear model. We carried out multiple regression analyses with the contrast images of the social stress > control contrast and urbanicity scores or number of alleles score as covariate of interest. The interaction between genotype and early or current urbanicity was calculated by adding the product of the centralized variables as a covariate of interest while retaining urbanicity and genotype as covariates of no interest in the model. The participants' years of education were included as a nuisance covariate in the neuroimaging regression models.

Imaging results were corrected via family-wise error (FWE) for multiple comparisons at a significance level of $P < 0.05$. For the main task effects (stress > control, control > stress) correction was performed over the whole brain and peak voxels are reported separately for anatomical volumes of interest (AVOI) as defined by Tzourio-Mazoyer et al. (2002) (see Supplementary Table III-2 a & b). Effects of rs324981 and the interaction of early and current urbanicity effects with rs324981 were FWE-corrected for multiple comparisons within two a priori defined anatomical regions of interest (ROI) using masks from the Harvard Oxford Atlas (http://www.cma.mgh.harvard.edu) as reported previously (Lederbogen et al., 2011): the rostral ACC (including BA 24 a-c, BA25, BA 32 and BA 33) as defined by Bush and colleagues (2000) and the right amygdala. Effects outside of these ROIs are reported if they reached significance after FWE whole-brain correction.

Analysis of demographic, heart rate and endocrine data

Descriptive and endocrine data analysis was performed using the SPSS software (version 20, IBM). Chi-square tests and analyses of variance were performed to test differences in scores and frequencies between genotype groups. Independent t-tests were applied to for posthoc comparisons. Group differences in ACTH and salivary cortisol outcome measures were analyzed with ANOVA models for repeated measurements. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported.

Results

Manipulation check: heart rate, ACTH and cortisol stress responses

In the first part of the statistical analysis we tested whether the Scan*STRESS* paradigm induced significant cardiovascular and endocrine stress responses. As shown in Figure III-2, mean heart rates were significantly higher in stress blocks than in control blocks ($F_{1, 31} = 78.17$, $P < 0.001$). The average heart rate difference between conditions was 8.98 bpm. Posthoc comparisons confirmed that the stress vs. control block difference was significant for both runs (run1: $t_{31} = 7.559$, $P < 0.001$; run 2: $t_{31} = 8.650$, $P < 0.001$). All 32 participants with complete heart rate data showed increased heart rates during the stress conditions compared to control blocks. This effect did not differ between the two runs (main effect run: $F_{1, 31} = 0.22$, $P = 0.64$; interaction stress x run: $F_{1, 31} = 0.822$, $P = 0.37$).

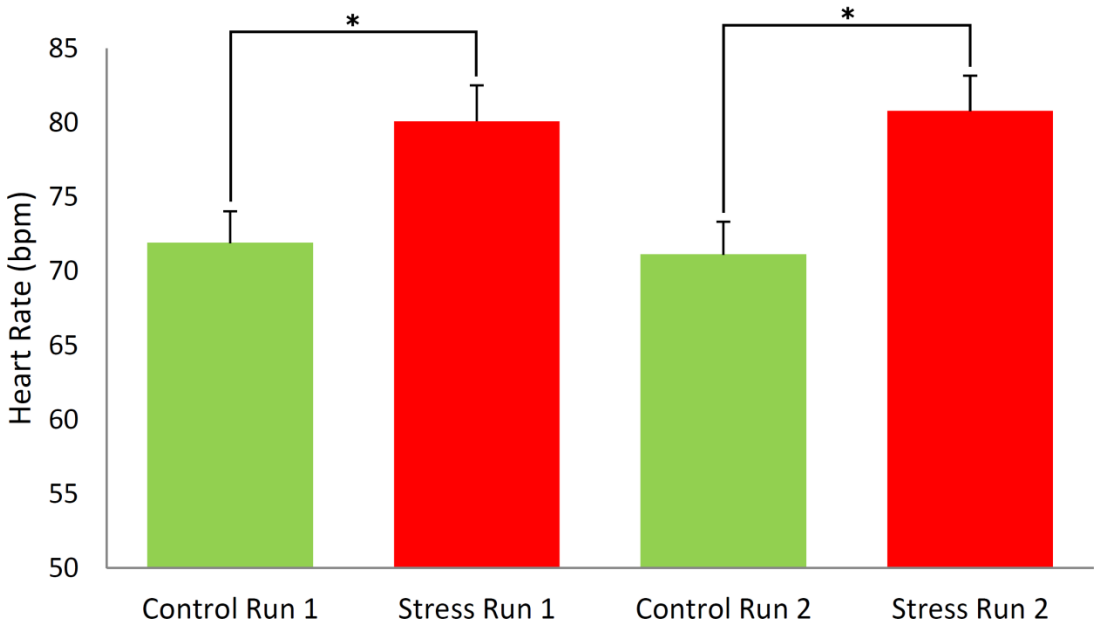

Figure III-2: Mean (±SEM) heart rate (in beats per minute) responses to the Scan*STRESS* paradigm. Asterisks indicate significant differences between two conditions ($P < 0.001$, independent t-tests for posthoc comparison)

The majority of subjects showed, in part rather pronounced, decreases of ACTH and salivary cortisol levels over the three pre-stress measurements (main effect time: $F_{1.57, 39.30} = 6.79$, $P = 0.005$ for ACTH and $F_{1.61, 65.85} = 5.07$, $P = 0.014$ for cortisol), suggesting a considerable HPA axis anticipation response in several subjects. When the entire sample was included, neither for ACTH nor for salivary cortisol a significant mean increase was observed (main effect time: $F_{3.03, 63.61} = 1.14$, $P = 0.34$ (ACTH); $F_{1.58, 64.84} = 1.98$, $P = 0.16$ (cortisol)). Nevertheless, 15 of 22 subjects with complete blood sample data showed an ACTH rise after stress (Figure III-3a) and for 22 of 42 subjects a salivary cortisol increase was detected (Figure III-3b). Participants were categorized as responders when they showed an absolute hormone level increase (>0) between the last pre-stress measurement and any of the following three measurements (ACTH: +13.5, +27.5, +45; Cortisol +35, +45, +60). As expected, this classification resulted in significant group differences: ACTH responders showed a significantly higher rise than non-responders (responder x time effect: $F_{3.16, 63.20} = 4.34$, $P < 0.01$, Figure III-3a) and cortisol responders showed a significantly higher response than cortisol non-responders (responder x time effect: $F_{1.83, 73.51} = 16.46$, $P < 0.001$, Figure III-3b). Moreover, 12 out of 15 ACTH responders were also cortisol responders and cortisol responders showed significantly higher mean heart rate differences between control and stress

blocks (mean delta = 11.51 bpm) than non-responders (mean delta = 6.47 bpm) (responder x time effect: $F_{1,30} = 7.45$, $P = 0.011$).

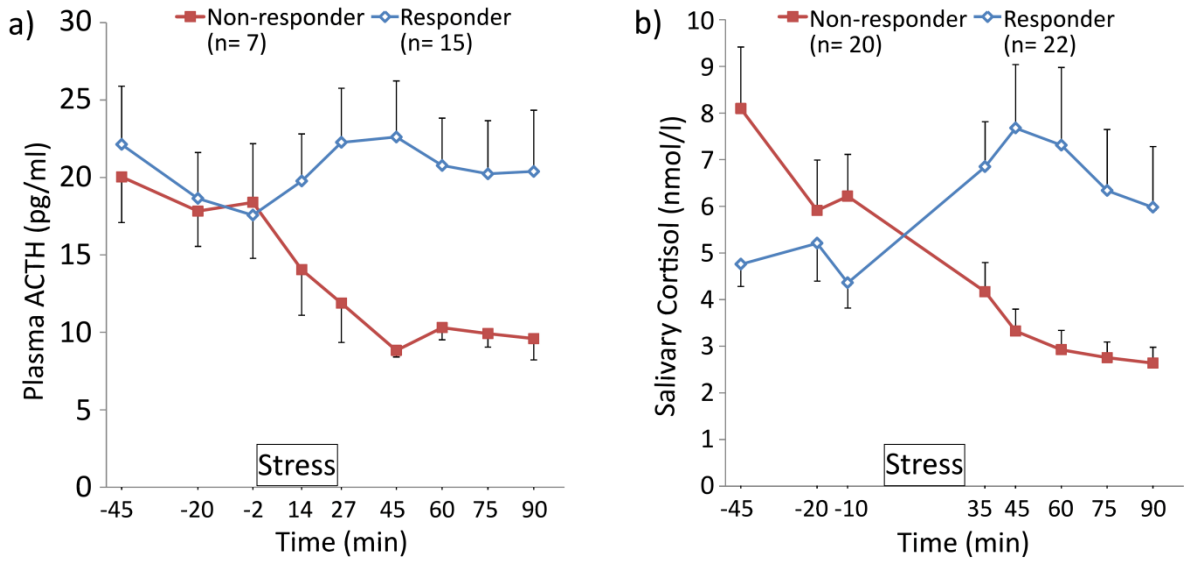

Figure III-3: Mean (±SEM) plasma ACTH (a) and salivary cortisol (b) responses to the Scan*STRESS* paradigm in responders and non-responders.

Genotypes

Of the 42 participants, 9 were homozygous for the A allele, 21 were heterozygous and 12 homozygous for the T allele. The obtained allele frequencies did not deviate from Hardy-Weinberg equilibrium (HWE; $P = 0.97$). There was no difference between the three genotype groups with respect to age ($P = 0.79$), sex ($P = 0.11$), current urbanicity ($P = 0.31$), urban upbringing ($P = 0.62$) and years of education ($P = 0.22$).

Urbanicity and socioeconomic background

Early urbanicity scores ranged from 15 (first 15 years spent in rural environment) to 45 (first 15 years spent in urban environment; Mean of 29.25). Early urbanicity scores did not correlate significantly with age ($P = 0.95$) or years of education of the participant ($P = 0.30$) and were not significantly associated with the education of the mother ($P = 0.31$) or the father ($P = 0.20$).

The analysis of the current urbanicity status showed that 6 subjects currently lived in a rural environment, 12 subjects lived in a small city, and 24 subjects lived in a bigger city. Current urbanicity groups did not differ significantly in age ($P = 0.90$), early urbanicity score ($P = 0.81$), numbers of relocations in the first 15 life years, and education of the mother ($P = 0.77$) or the father ($P = 0.97$). There was a trend for a difference in years of education ($P = 0.078$)

with participants currently living in a town (10,000 - 100,000 inhabitants) showing marginally less years of education (M = 12.42) than both the urban (M = 12.96) and the rural group (M = 12.67).

Neural substrates of the stress response and associations with NPSR1 rs324981

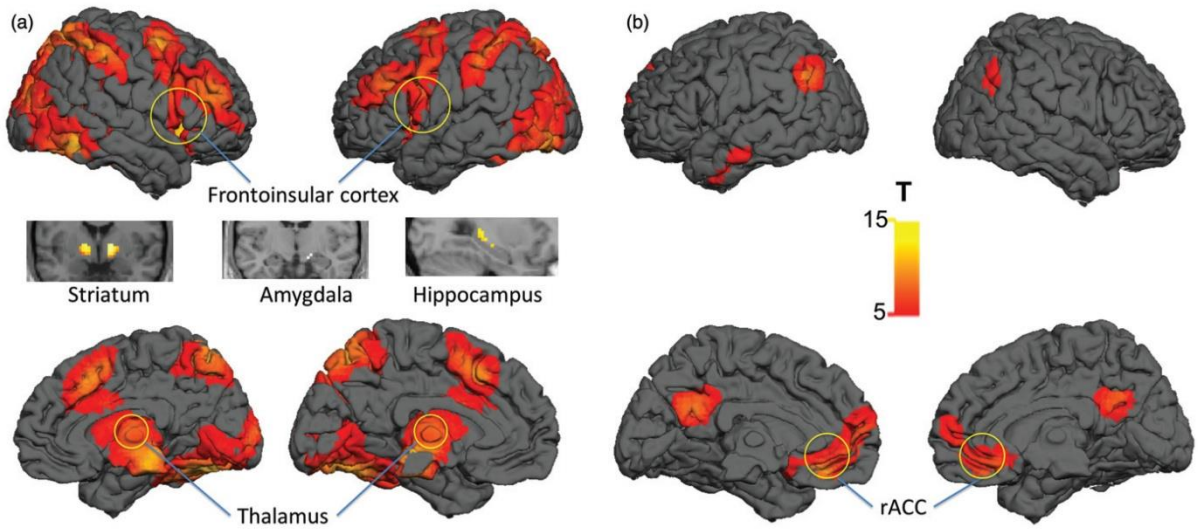

Figure III-4: Main effect of social stress induction: activation (a) and deactivation (b).
The functional maps are thresholded at a significance threshold of $P < 0.05$ FWE corrected for multiple comparisons across the whole brain. Neural response included activations in ventral striatum, thalamus, frontoinsular cortex, hippocampus and amygdala and deactivations in rACC (all $P < 0.05$, whole-brain FWE corrected); FWE = family-wise error corrected for multiple comparisons.

Under psychosocial stress a distributed network of activations and deactivations was observed including activations in ventral striatum, thalamus, frontoinsular cortex, hippocampus and amygdala and deactivations in rACC (all $P < 0.05$, whole-brain FWE corrected, see Figure III-4a & b; a detailed list can be found in Supplementary Table III-1 & III-2).

Early urbanicity was significantly correlated with ACC activity (r = 0.34, P = 0.034) and current urbanicity with right amygdala activity (r = 0.35, P = 0.022), as reported previously (Lederbogen et al., 2011).

There was no significant main effect of *NPSR1* rs324981 on activation patterns in the ROIs defined for the amygdala or the ACC. However, rs324981 modulated the stress response in a cluster in the right cerebellum (x = 42, y = -49, z = -39, P = 0.004; t = 6.1, whole-brain FWE corrected). Subjects homozygous for the T allele showed the strongest activation while subjects homozygous for the A allele showed the lowest activation.

While we did not detect a genetic main effect in our predefined ROIs, we did find evidence for a gene x environment interaction in our sample. Rs324891 significantly modulated the

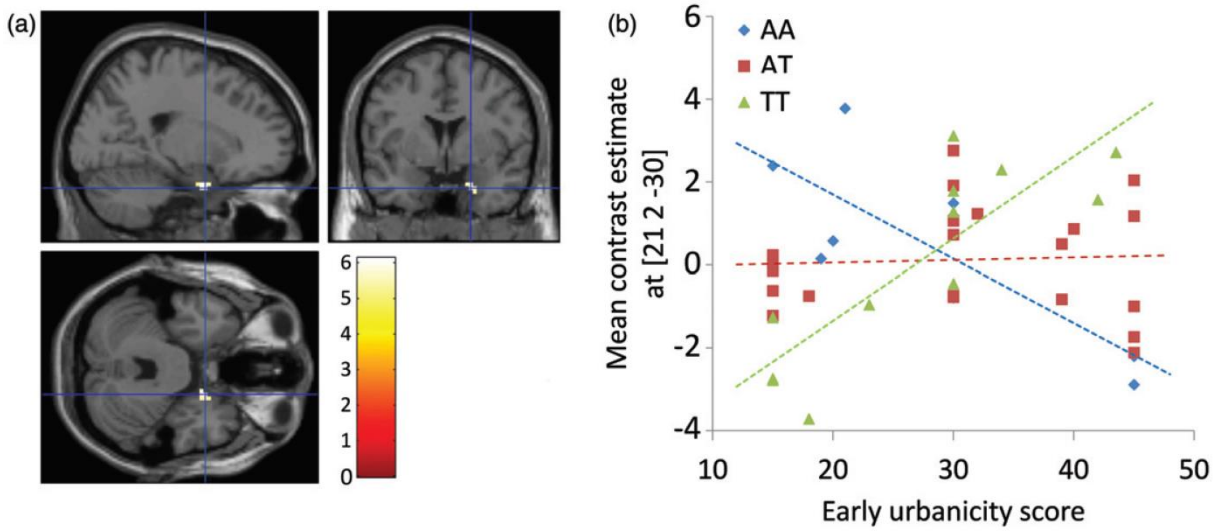

Figure III-5: Interaction of early life (birth until age 15) urbanicity scores and genotype of *NPSR1* polymorphism rs32489 1 on amygdala activation under acute psychosocial stress (a & b).

a) T-map of significant correlations between activations in the stress > control contrast with the interaction term for the urban upbringing score and the genotype for rs324981 displayed at a threshold of $P < 0.05$ FWE whole-brain corrected. b) Scatterplot of the correlation between the urban upbringing score and the most significant correlated voxel in the right amygdala (located at $x = 21$, $y = 2$, $z = -30$) for the interaction term in the stress > control contrast depicted separately for the three genotype groups of rs324981; FWE = family-wise error corrected for multiple comparisons

effect of urban upbringing on activation within the ROI amygdala mask ($x = 21$, $y = 2$, $z = -30$, $P < 0.001$, $t = 6.03$, FWE corrected within ROI; Figure III-5a), which even yielded a significant result on the whole-brain level ($P = 0.005$, whole-brain FWE corrected). While in subjects with the genotype rs324891 TT, amygdala activity under stress was positively associated with the early urbanicity score, a negative association between neural activity and early urbanicity was observed in subjects with the genotype AA (Figure III-5b). In subjects with the heterozygous genotype AT, no substantial association between early urbanicity and amygdala activity could be detected. To control for a possible influence of sex we added sex as a covariate of no interest to our model. This did not substantially alter the interaction results ($P < 0.001$, $t = 6.19$, FWE corrected within ROI). In a supplementary analysis, we investigated if the observed gene x environment interaction is substantially driven by outliers. Therefore, two subjects whose activation in the peak voxel slightly exceeded two standard deviations from the group average were excluded. The effect remained significant after this step ($P < 0.001$, $t = 5.48$, whole-brain FWE corrected).

In the peak voxel for this gene x environment interaction, mean activations were significantly higher in cortisol responders compared to non-responders ($t_{40} = 2.20$, $P = 0.033$). We did not

observe any significant gene x environment interaction effects in the defined ROIs when we analyzed the interaction of current urbanicity with rs324981 on stress activation.

In an exploratory analysis, neither cortisol nor ACTH responses were significantly associated with rs324981 (main effects genotype, interactions genotype x time: all $F < 0.82$, all $P > 0.47$). We did not find evidence for a gene x environment interaction effect on endocrine responses in our sample (interactions genotype x early / current urbanicity, interactions genotype x early / current urbanicity x time: all $F < 0.80$, all $P > 0.59$). Consistently, genotype frequencies did not differ significantly between responders and non-responders both for ACTH and salivary cortisol (all $X^2_{(2)} < .45$, $P > .80$).

Discussion

This study provides first evidence for an influence of *NPSR1* variant rs324981 on brain activation during acute stress. Previously, we have linked early and current urbanicity to ACC and amygdala stress processing (Lederbogen et al., 2011). Now, we found that the environmental risk factor "early urbanicity" interacts with rs324981 constituting a gene x environment interaction effect on the amygdala stress response. It has repeatedly been shown that variation in genes influencing physiological systems involved in stress related disorders, as the HPA axis (Polanczyk et al., 2009) or the serotonergic system (Caspi et al., 2003), can modulate the impact of environmental risk factors. In animals, NPS shows strong anxiolytic effects and it increases arousal at the same time (Xu et al., 2004). NPS induced HPA axis activation in rats (Reinscheid, 2008, Smith et al., 2006) and in turn, brainstem NPS neurons were triggered by CRH, resulting in NPS release in areas including the amygdala (Jüngling et al., 2012). NPS injected into the amygdala prevented both anxiety-like behaviour and enhanced conditioned fear responses after stress (Chauveau et al., 2012).

The psychological components of a stressful situation, including novelty, uncontrollability, unpredictability and ego-involvement (Dickerson & Kemeny, 2004) are closely related to anxiety. The main finding of our study is an interaction of rs324981 with early urbanicity on right amygdala activation under stress. In subjects homozygous for the T allele, higher early urbanicity scores were associated with increased amygdala activity, while A/A subjects showed an association in the opposite direction. While we previously found an effect of current urbanicity on amygdala activity under stress (Lederbogen et al., 2011), the present finding suggests that amygdala stress responses, via a gene x environment interaction, are also influenced by urban upbringing. The absence of a main effect of urban upbringing on amygdala activity is not inconsistent with this observation. As genetic variation is already

operative at the early life phase that urban upbringing indexes, it appears plausible that an effect of an environmental factor on amygdala stress processing can depend on the genetic background. Furthermore, NPS expression profiles predict effects in subcortical structures, notably in the amygdala. Assuming that growing up in a city constitutes on average a more stressful environment, our results are consistent with the results of Klauke and colleagues (2014) who reported highest anxiety sensitivity in subjects homozygous for the T allele, who were exposed to childhood maltreatment or recent stressful life events. Anxiety sensitivity is considered an intermediate phenotype of anxiety disorders, which are also associated with amygdala reactivity (Holzschneider & Mulert, 2011). A stress-induced NPS release within the amygdala was shown in animals, identifying the amygdala as a target of the NPS pathway (Ebner et al., 2011). Moreover, amygdala functioning was shown to be modulated by genetic variation (Munafo et al., 2008) as well as early and current environment (Taylor et al., 2006, Zink et al., 2008). In healthy subjects, rs324981 T was positively associated with harm avoidance and right amygdala responses to fear-relevant faces (Dannlowski et al., 2011). In anxiety disorder and major depression patients, amygdala hyperactivity was reported.

The more active T allele that mediates enhanced NPS signaling was previously associated with increased amygdala activity (Dannlowski et al., 2011) and a higher cortisol stress response (Kumsta et al., 2013) as well as with panic disorder and anxiety (Domschke et al., 2011). Consistently, in our study right amygdala responses were increased in subjects who also showed cortisol stress responses. It can be speculated that the arousal-increasing effects of NPS observed in animals might be more relevant for the reported phenotypes in humans than its anxiolytic effects. Furthermore, genetic variations exert their effects during different stages of neurodevelopment. Regarding the current study, the operationalization of early urbanicity comprised the first 15 years of life. The individual *NPSR1* genotype might interact with environmental factors during sensible phases in childhood, shaping later neurophysiological stress reactivity.

We also observed genotype-dependent activation differences in the cerebellum. Independent of genotype and consistent with previous reports (Gianaros et al., 2007), the cerebellum was activated under stress (see Supplementary Table III-1) possibly due to its role in cognitive and affective processing (Strick et al., 2009). Therefore, the modulation of cerebellum activity by *NPSR1* genotype should be followed in future studies.

The observed associations between *NPSR1* genotype and neural stress responses were not paralleled by associations between genotype and endocrine responses in our sample, possibly reflecting greater sensitivity of neural measures compared to peripheral markers. The

association with HPA axis measures can better be studied in larger samples exposed to stress protocols applicable outside the scanner, e.g. the TSST. The moderate sample size and its limited statistical power are the major limitations of the present study. Consequently, we decided to test only a single sequence variant and regions of interest defined a priori. Due to the small sample size, all findings on genotype-phenotype associations can only be regarded as preliminary. Still, it appears remarkable that the significant effects are rather pronounced and reach significance on the whole-brain level. In a recent review paper (Duncan & Keller, 2011) the robustness of the majority of gene x environment findings in psychiatry has been challenged. Although this fundamental scepticism is not shared by all authors (Caspi et al., 2010, Dick, 2011) a replication of our finding in a larger sample would be valuable. Nevertheless, some features of our study may at least partly counterbalance these limitations. First, we used an experimental design and a promising intermediate phenotype approach. Central stress regulation represents a phenotype sensitive to genetic and environmental factors that might increase disease vulnerability. Intermediate phenotypes were shown to be a valuable tool to target brain mechanisms of psychopathology even in limited samples (Mier et al., 2010). Moreover, our main effect was not driven by statistical outliers. The exclusion of two participants with the most deviating neural activation in the peak voxel did not significantly alter our results. As only one variant was studied we are not able to estimate the overall size of the genetic effect of *NPSR1* on our phenotypes. Still, considering the functional characteristics of rs324981 we speculate that differences in NPS-induced signaling modulate the link between environmental factors and stress processing in the amygdala.

Urbanicity is a broad concept that is influenced by several factors, particularly by variables related to socioeconomic status. However, urbanicity was shown to predict the risk for stress related psychopathology, especially schizophrenia (Pedersen & Mortensen, 2001, van Os et al., 2004). Epidemiological studies reported urbanicity effects independent of socioeconomic factors and selective migration (overview see Krabbendam & van Os, 2005). In accordance with these findings, our results maintained stable when the educational level of our participants was controlled for.

The newly developed Scan*STRESS* paradigm proved to be a useful tool. Previous findings on neural correlates of urbanicity with this paradigm were consistent with those obtained with the established Montreal Imaging Stress (MIST, Dedovic et al., 2005) in an independent sample (Lederbogen et al., 2011). Scan*STRESS* elicited solid effects on brain activity and heart rate, with all subjects showing heart rate responses. Although the majority of the participants showed ACTH and cortisol rises, mean responses across all subjects were not

statistically significant in the present sample. On the one hand this reflects the well-known interindividual variability in HPA axis stress responses (Kudielka et al., 2009). Moreover, a weak or absent cortisol response does not preclude a preceding robust central stress response. On the other hand, HPA axis responses to a stress paradigm are an important and conservative validation criterion and we thus consider the modest endocrine responses another limitation of our study. We cannot rule out that the more distant subject-panel-interaction in the scanner reduced stress intensity to a certain degree. However, one could also assume that some components of the situation are more stressful than in protocols performed outside the scanner, e.g. the scanning procedure itself, the (mock) camera, etc. We speculate that the modest endocrine responses -that also occurred in fMRI studies which used the MIST (Pruessner et al., 2008)- can at least partly be explained by HPA axis anticipation effects (and consequently lower responses to the paradigm) related to the upcoming MRI procedure and the necessary technical preparations prior to scanning. To minimize this expected effect we only studied scanner-experienced subjects but still relatively high endocrine pre-stress levels were observed in several subjects, consistent with previous reports (Muehlhan et al., 2011). Moreover, we most likely missed cortisol response peaks in some subjects due to a 45-minutes interval in which no saliva samples were collected to prevent head movements during scanning.

In summary, our findings suggest a modulation of the neural stress response in the right amygdala by genetic variation in *NPSR1* in interaction with early urbanicity. This result is consistent with earlier findings indicating a gene x environment interaction of the same variant with early adversity on anxiety sensitivity. Our results support the view that the NPS system modulates stress responses and contributes to individual risk susceptibility for psychiatric disorders.

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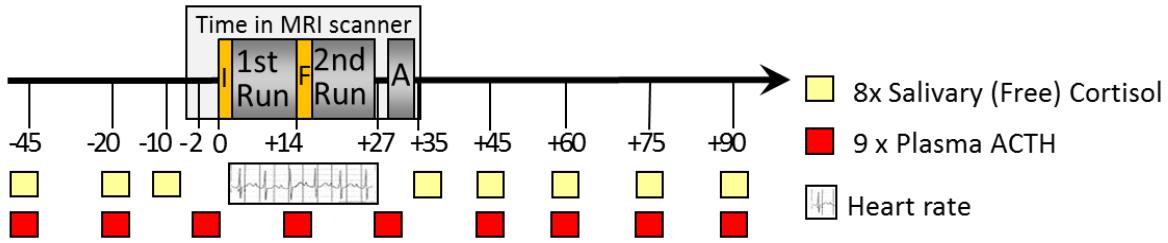
Supplementary material



Supplementary Figure III-1: Collection time points for saliva and blood samples.

Time points are given relative to stress onset, which started with the verbal introduction ("I") of the tasks and the presentation of the panel to the participant (time point 0).

Supplementary Table III-1: Structures activated under social stress (FWE-corrected for the whole brain with a threshold of 0.05) with the T-values, p-values and the localization of the peak voxel within each structure.

Structure			**Statistics**		**MNI coordinates**		
		k	*P*	*t*	*x*	*y*	*z*
Precentral gyrus	left	543	<0.001	12.61	-45	5	30
	right	265	<0.001	12.04	45	8	30
Postcentral gyrus	left	202	<0.001	10.75	-42	-37	45
	right	20	<0.001	9.79	42	-37	54
Frontal lateral lobe	left	1386	<0.001	12.30	-42	8	27
	right	1894	<0.001	12.43	30	2	51
Frontal medial lobe	left	328	<0.001	10.80	-3	20	48
	right	265	<0.001	10.92	9	20	45
Frontal orbital lobe	left	27	<0.001	6.19	-36	23	-6
	right	136	<0.001	13.22	33	23	-9
Temporal lateral lobe	left	324	<0.001	11.86	-42	-64	-9
	right	753	<0.001	12.67	57	-55	-12
Parietal lateral lobe	left	1050	<0.001	15.56	-24	-67	42
	right	1074	<0.001	13.96	24	61	48
Parietal medial lobe	left	382	<0.001	14.07	-15	-70	60
	right	342	<0.001	13.54	12	-64	54
Occipital lateral lobe	left	1347	<0.001	14.68	-24	-70	36
	right	1041	<0.001	14.36	27	-67	42
Occipital medial and inferior lobe	left	1083	<0.001	12.36	-30	-67	-15
	right	1093	<0.001	13.27	33	-61	-15
Limbic lobe	left	150	<0.001	8.21	0	5	30
	right	238	<0.001	10.00	9	20	42
Insula	left	198	<0.001	13.63	-33	20	3
	right	190	<0.001	14.08	36	20	-3
Caudate nucleus	left	43	<0.001	8.13	-21	-19	24
Lenticular nucleus	left	44	<0.001	9.72	-12	-1	0
	right	103	<0.001	10.34	15	-1	-3
Thalamus	left	327	<0.001	12.50	-6	-19	-3
	right	288	<0.001	12.36	6	-19	-3
Cerebellum	left	1566	<0.001	12.03	-6	-76	-36
	right	1375	<0.001	12.04	27	-40	-18
Vermis		603	<0.001	11.57	-3	-73	-33

Supplementary Table III-2: Structures deactivated under social stress (FWE-corrected for the whole brain with a threshold of 0.05) with the T-values, p-values and the localization of the peak voxel within each structure.

Structure		**Statistics**			**MNI coordinates**		
		k	*P*	*t*	*x*	*y*	*z*
Frontal medial lobe	left	161	<0.001	10.07	-6	53	3
	right	70	<0.001	8.10	3	53	0
Frontal orbital lobe	left	111	<0.001	11.22	-6	35	-15
	right	103	<0.001	8.89	0	32	-12
Temporal lateral lobe	left	43	0.002	6.24	-63	-19	-21
Parietal lateral lobe	left	170	<0.001	11.30	-54	-64	36
	right	62	<0.001	8.26	54	-67	36
Parietal medial lobe	left	122	<0.001	9.76	-6	-55	27
	right	62	<0.001	9.18	3	-52	27
Limbic lobe	left	223	<0.001	10.15	-3	-55	27
	right	81	<0.001	9.05	3	-55	30

Chapter IV - Sex-specific association between functional neuropeptide S receptor gene (*NPSR1*) variants and cortisol and central stress responses

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Abstract

The brain neuropeptide S (NPS) system has recently generated substantial interest and may be of major relevance for central stress regulation. The NPS receptor (NPSR1) is highly expressed in the limbic system, exogenous NPS exerts pronounced anxiolytic and fear-attenuating effects in rodents and extensive close crosstalk between the NPS system and the hypothalamic-pituitary-adrenal (HPA) axis has been demonstrated. In humans, associations between *NPSR1* variants and anxiety and panic disorder, as well as amygdala responsiveness to fear-relevant faces and prefrontal cortex activity in a fear conditioning paradigm have been reported. Moreover, a *NPSR1* sequence variant was found to be associated with cortisol stress responses in males.

Here, we performed a haplotype-based analysis covering three functional *NPSR1* single nucleotide polymorphisms in the promoter (rs2530547), in exon 3 (rs324981) and exon 6 (rs727162) in 277 healthy subjects who were exposed to the Trier Social Stress Test (TSST). A significant sex-specific association with salivary cortisol responses to acute psychosocial stress was detected for the common TTC haplotype 2 (frequency of about 20%). In an additional study using an imaging genetics approach, 65 healthy subjects were exposed to a stress paradigm for scanner environments ("Scan*STRESS"*). We found a significant and, again, sex-specific interaction between rs324981 (whose minor T-allele is harbored by haplotype 2) and the neural stress response in a cluster close to the parahippocampal gyrus (whole-brain corrected). Moreover, as in the TSST sample, *NPSR1* variation was associated with salivary cortisol responses (on a trend level) in a sex-specific way.

In summary, our preliminary findings in two independent cohorts exposed to different stress paradigms suggest that the NPS system significantly influences acute stress responses and that sequence variation in *NPSR1* may contribute to sex differences in stress regulation.

Introduction

Chronic stress is a significant risk factor for several disorders, including highly prevalent psychiatric diseases such as depression and anxiety disorders (Chrousos, 2009; de Kloet et al., 2005; Grant et al., 2005). Stress also increases the risk for primarily somatic disorders including cardiovascular diseases (Cohen et al., 2007; Kivimäki et al., 2006; Rosengren et al., 2004) and the metabolic syndrome (Chandola et al., 2006). Additionally, a core component of the endocrine stress response, the hypothalamic-pituitary-adrenal (HPA) axis, has been shown to be dysregulated in those disorders (Chrousos, 2009; de Kloet et al., 2005). While the association between stress and disease is well established, the mechanisms mediating this link are still poorly understood.

A molecule that has recently been implicated in psychiatric disorders and stress regulation is neuropeptide S (NPS). NPS is a highly conserved 20 amino-acid peptide (Xu et al., 2004) and in the brain it is synthesized in clusters of the brain stem close to the locus coeruleus and the Barrington's nucleus, as well as in neurons in the medial amygdala and dorsomedial hypothalamus. NPS selectively binds and activates its receptor NPSR1 (Xu et al., 2004), which is widely expressed throughout the brain including the thalamus, hypothalamus, hippocampus, parahippocampal regions and the amygdala (Clark et al., 2011; Leonard and Ring, 2011; Xu et al., 2007). In animal models the administration of NPS exerts strong anxiolytic effects (Jüngling et al., 2008; Xu et al., 2004) and at the same time increases arousal (Xu et al., 2004). There is evidence that the NPS system interacts with stress regulating processes. The administration of NPS results in HPA axis activation including the release of corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and corticosterone (Smith et al., 2006). Conversely, CRH triggers NPS release in different brain areas including the amygdala, and stress leads to increased NPS levels in the amygdala (Ebner et al., 2011). Recently, the significant influence of genetic variability in the NPS system on stress and anxiety regulation was demonstrated in rodents selectively bred for 'High vs. Low Anxiety-Related Behavior' (Slattery et al., 2015).

As to date no NPSR1 agonist or antagonist for the application in human pharmacological studies is available, no direct effects of NPS have been studied. However, genetic studies on the gene coding for the neuropeptide S receptor 1 (*NPSR1*) have implicated the NPS system in stress regulation and the etiology of psychiatric disorders as well as disorders related to the immune system. Regarding psychiatric phenotypes, the most studied variant is rs324981 in exon 3. It leads to an amino acid change (A>T Asn107Ile) in the first extracellular loop of the receptor protein (Reinscheid et al., 2005). The minor Ile^{107} variant shows increased surface receptor expression and a five- to tenfold higher NPS-induced signaling response than the major Asn^{107} receptor variant (Reinscheid et al., 2005). This variant has been associated with psychiatric phenotypes including anxiety, panic disorder and schizophrenia (e.g. Domschke et al., 2011; Klauke et al., 2014; Lennertz et al., 2012). Results suggest that these associations may in part be sex-specific (Domschke et al., 2011; Laas et al., 2014a; Okamura et al., 2007) and interact with environmental risk factors such as early trauma or stressful life events (Klauke et al., 2014; Laas et al., 2014a). *NPSR1* rs324981 was also targeted in studies that assessed intermediate phenotypes for psychiatric disorders. In a fear conditioning paradigm T allele carriers exhibited stronger conditioned stimulus (CS)-evoked brain activity in the rostral dorsomedial prefrontal cortex (dmPFC) (Raczka et al., 2010). In panic disorder patients, T allele carriers showed increased anxiety sensitivity, heart rate and fear symptoms in a behavioral avoidance test as well as decreased activity in the dorsolateral prefrontal, lateral orbitofrontal and anterior cingulate cortex during presentation of fearful faces (Domschke et al., 2011). In healthy subjects T allele carriage was associated with increased right amygdala responsiveness to fear-relevant faces and self-reported harm avoidance (Dannlowski et al., 2011). Kumsta et al. (2013) showed that in healthy males, the rs329481 modulated the subjective and endocrine response to the Trier Social Stress Test for Groups (von Dawans et al., 2011), with T carriers exhibiting increased stress reactions. Previously, we showed that the rs324981 genotype is related to amygdala activity under stress in interaction with early urbanicity, a risk factor for schizophrenia (Streit et al., 2014).

However, it appears unlikely that the proposed genetic effects of *NPSR1* are solely based on rs329481 and this view is supported by a genetic case-control study on panic disorder that comprehensively analyzed *NPSR1* variability (Donner et al., 2010). Based on these findings the present study aimed at investigating the role of genetic variability in *NPSR1* for human stress regulation. In a functional characterization of all common *NPSR1* promoter and coding SNPs, Anedda et al. (2011) recently identified three common functional variants, one promoter SNP (rs2530547) affecting gene expression levels and two non-synonymous SNPs

associated with altered NPS signaling (rs324981 and rs72716) and showed that specific allele combinations (haplotypes) of these SNPs were associated with inflammatory bowel disease. Therefore, we studied the association between these three functional and common *NPSR1* gene variants and salivary cortisol responses to the TSST in a sample of healthy females and males in a haplotype-based approach. Additionally, in a smaller sample that was exposed to an fMRI stress paradigm, we assessed cortisol as well as neural stress responses and we limited our association analysis on rs329841. Genetic variation in *NPSR1* has been linked to psychiatric disorders such as depression and anxiety disorders (e.g. Domschke et al., 2011; Donner et al., 2010; Klauke et al., 2014; Laas et al., 2014a). These disorders show a higher prevalence in females (Altemus, 2006). Furthermore, *NPSR1* SNPs have shown associations with anxiety and panic disorder in a sex-specific way (e.g. Domschke et al., 2011; Laas et al., 2014a). Additionally, sex differences in HPA axis regulation are well established (Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005) and gene x sex interactions on HPA axis responses have been reported before (e.g. Kumsta et al., 2007). Therefore, we explicitly explored the sex-specificity of genotype-phenotype associations.

Methods

TSST study

Participants

The TSST data set comprised initially data of a total of 337 healthy young participants (126 females, all taking oral contraceptives) who underwent the TSST. It consisted of two previously described subsamples (Federenko et al., 2004; Kumsta et al., 2007). As the study by Federenko et al. (2004) included 58 male twin pairs (33 MZ, 25 DZ), one sibling of each twin pair was randomly chosen and entered into the present analysis. This resulted in a final sample of 277 participants (126 females, mean age = 23.74 years, range 16 - 41 years).

Stress paradigm and physiological measures

For a detailed description of the protocol see Kumsta et al. (2007) and Federenko et al. (2004). In brief, participants underwent the TSST (Kirschbaum et al., 1993), which consists of a short preparation period, an oral presentation and a mental arithmetic task of 15 min duration, performed in front of an expert panel and a camera. Participants were tested between

15:00 and 17:00 h. Saliva samples were collected 2 min before and 1, 10, 20, 30, 45, 60 and 90 min after the onset of the TSST. EDTA blood samples were collected for genetic analyses.

ScanSTRESS study

Participants

The Scan*STRESS* data set consisted of 65 healthy young participants (32 females; mean age = 26.46 and range 19 - 42 years), selected from previously described study samples (Akdeniz et al., 2014; Streit et al., 2014). The inclusion criterion for the present analysis was the availability of a DNA sample of the respective subject.

Stress paradigm and physiological measures

Participants underwent the Scan*STRESS* paradigm, a protocol for the induction of social stress in the fMRI environment (Streit et al., 2014). Subjects had to perform mental arithmetic and mental rotation tasks under time pressure in the MRI scanner, while being monitored by an investigator panel.

The paradigm was implemented with Presentation® software (Version 12.9, www.neurobs.com). It uses a block design (two runs of 11.3 minutes duration each) with repeated 60 seconds task (or control) blocks preceded by 5 seconds task announcement and followed by 20 seconds rest period. In the task blocks, the subject has to respond under time pressure. Speed and difficulty are adapted to the individual's performance. In the control conditions, the participant performs a less demanding task without time pressure, with the number of trials being matched to the stress condition. In both tasks, answers are given using a four-button response box (Current Design, Philadelphia, PA). An investigator panel, consisting of one female and one male, is visible via live video stream to the subject immersed in the scanner. During the stress blocks the panel gives disapproving feedback via button presses on a buzzer which is displayed visually ("Error!", "Work faster"!). In between the runs the panel gives disapproving verbal feedback on the subject's performance.

In total, eight saliva samples were collected per participant (45, 22 and 10 min before and 35, 45, 60, 75 and 90 min after begin of stress induction; for details see Streit et al., 2014).

Imaging data acquisition and preprocessing

As previously described (Akdeniz et al., 2014; Streit et al., 2014), data was acquired on a 3.0 Tesla MRI scanner (Siemens Trio, Erlangen, Germany) using a gradient-echo echo planar

imaging (EPI) sequence (TR = 2000 ms, TE = 30 ms, 80° flip angle, 192 mm field of view, 64 x 64 matrix, 32 3mm axial slices with 1 mm gap). Images were preprocessed and analyzed using standard routines in SPM8 (www.fil.ion.ucl.ac.uk/spm). All images were realigned to the first functional image, then spatially normalized into a standard stereotactic space (MNI template), resampled into 3 x 3 x 3 mm^3 voxels and smoothed with a 9 mm full-width at half-maximum (FWHM) Gaussian filter.

DNA-extraction and genotyping

Genomic DNA was extracted from EDTA blood samples following standard procedures. In the TSST sample *NPSR1* SNPs rs2530547, rs324981 and rs727162 were genotyped. Due to the lower statistical power of the haplotype-based approach we did not perform this analysis in the smaller Scan*STRESS* sample. Instead, only rs324981 was analyzed as this variant shows the best empirical support for an association with stress-related phenotypes. SNPs were genotyped using an Applied Biosystems 7900HT Fast Real-Time PCR System, applying a TaqMan 5' nuclease assay. To determine genotyping accuracy, 15 % of the sample was run in duplicates showing a reproducibility of 100 %.

Statistical analysis

TSST study

Descriptive and endocrine data analysis was performed using the SPSS software (version 20, IBM). In the larger TSST data set a haplotype-based analysis was performed. Haplotypes are sets of (SNP) alleles on one chromosome that tend to be inherited together. The likelihood that an individual is a carrier of a specific haplotype can be estimated based on the known association (linkage) structure of the respective SNPs in the population. The association between haplotype structure and cortisol responses was tested in a two-level analysis. First, a likelihood based association analysis incorporating haplotype estimation uncertainty was carried out in UNPHASED (Dudbridge, 2008) with the area under the curve (AUC) of salivary cortisol responses as the dependent variable. This approach determines the global association of the haplotype structure as well as the association of each haplotype with the dependent variable, while correcting for multiple comparisons.

In a second step, a haplotype for which a significant association with the dependent variable has been detected can be followed up with a *post hoc* general linear model using the full information of the repeated cortisol measures. For this model haplotype dosage scores (range 0 – 2, incorporating estimation uncertainty) were used as between-subject factor. Greenhouse-

Geisser corrections were applied where indicated and adjusted results are reported. For graphical presentation of haplotype effects, the best estimate haplotypes were used to contrast carriers and non-carriers of the respective haplotype.

ScanSTRESS study

For fMRI data analysis, one general linear model was defined for each participant containing regressors for control and social stress conditions and the respective announcement phases for each measurement sequence leading to a sum of 12 condition regressors. To account for motion artifacts six motion regressors were included. To check for effects of conditions, contrast images of social stress versus control condition were computed for each subject and incorporated into second-level one-sample t-tests. In addition, multiple regression analyses were carried out with the contrast images of the social stress > control contrast and number of minor alleles score and sex as covariate of interest. The interaction between sex and genotype was assessed by adding an interaction term as a covariate of interest to the model. To calculate the interaction term, the variables were centralized by subtracting the respective mean (resulting in a variable with mean = 0) and subsequently they were multiplied. Imaging results were corrected via family wise error (FWE) for multiple comparisons at a significance level of $p < 0.05$. Effects of rs324981 and of the interaction of sex with this gene variant were FWE corrected for multiple comparisons within a priori defined anatomical regions of interest (ROI) of the rostral ACC (including BA 24 a-c, BA25, BA 32 and BA 33) as defined by Bush and colleagues (2000) and of the right amygdala as reported previously (Lederbogen et al., 2011). These ROIs were chosen, as we could previously show that neural activation in the ScanSTRESS paradigm and another fMRI social stress paradigm was modulated by the environmental risk factor urbanicity in these ROIs (Lederbogen et al., 2011). Additionally, these regions have been associated with anxiety and stress processing (Holzschneider and Mulert, 2011) and previous studies on *NPSR1* could show a modulation of neural activity in the ACC (Domschke et al., 2011) and the amygdala (Dannlowski et al., 2011; Streit et al., 2014) related to rs324918 genotype. Outside of these ROIs, effects were reported if they remained significant after FWE whole-brain correction ($P_{FWE} < 0.05$).

To analyze endocrine responses, general linear models were computed, assessing effects of genotype on hormonal levels as well as the interaction between sex and genotype. Greenhouse-Geisser corrections were applied were indicated and adjusted results are reported.

Results

Genotype distribution

Genotype frequencies were corresponding to data reported previously (see Table IV-1; Consortium, 2012). The distribution of the three SNPS did not deviate significantly from Hardy-Weinberg Equilibrium (all $p > 0.57$).

Table IV-1: Genotype frequencies for the investigated *NPSR1* single nucleotide polymorphisms (SNPs) in the two subsamples

SNP	**rs2530547**	**rs324981**	**rs727162**
Location	Promoter	Exon 3	Exon 6
Major allele	C	A	C
Minor allele	T	T	G
MAF data set TSST	0.40	0.48	0.25
MAF data set ScanSTRESS		0.51	

MAF = minor allele frequency; designation as major / minor allele according to the dbSNP database

TSST study - endocrine reaction and association with NPSR1 haplotypes

The *NPSR1* haplotype structure analysis revealed eight haplotypes, with four of them showing a frequency higher than 10% (haplotype 1, 2, 3 and 4; Table IV-2). Globally, the likelihood association analysis showed a significant association between *NPSR1* haplotype structure and salivary cortisol stress responses to the TSST as indicated by the AUC ($\chi^2 = 16.32$, df = 7, $p = 0.022$). Moreover, the single haplotype analysis revealed a significant effect of haplotype 2 (allele combination TTC for rs2530547, rs324981 and rs727162; $\chi^2 = 4.92$, $p = 0.026$; see Table IV-2). To follow up the haplotype 2 effect, a general linear model was computed, taking the repeated cortisol measurements into account and adding sex as a predictor. This analysis showed a significant haplotype 2 x sex interaction on cortisol responses ($F_{1,266} = 5.90$, $p = 0.016$, $eta^2 = 0.022$). Consistent trends for a haplotype 2 main effect ($F_{1,266} = 2.92$, $p = 0.089$, $eta^2 = 0.011$) and a haplotype 2 x sex x time interaction ($F_{3.20,850.59} = 2.258$, $p = 0.076$, $eta^2 = 0.008$) were also detected. As depicted in Figure IV-1, male carriers of haplotype 2 showed higher cortisol levels than male non-carriers, while in females no such effect of haplotype 2 carriage was observed. It should be noted that overall males showed on average larger cortisol responses than females ($F_{1,266} = 17.06$, $p < 0.001$, $eta^2 = 0.060$). As mentioned

earlier, the TSST data set was a combination of two subsamples with one subsample consisting only of male subjects (Federenko et al., 2004; Kumsta et al., 2007). To rule out that the observed haplotype 2 x sex interaction was biased by this uneven distribution of sex across subsamples we repeated the GLM in the mixed-sex subsample and found virtually the same effect (haplotype 2 x sex: $F_{1,210} = 4.903$, p = 0.028, eta² = 0.023).

Table IV-2: Estimated haplotypes in the TSST data set and likelihood based association analysis results

	***NPSR1* SNPS**				
Haplotype	**rs2530547**	**rs324981**	**rs727162**	**Frequency**	**P**
HAP1	C	A	C	0.32	0.33
HAP2	**T**	**T**	**C**	**0.19**	**0.026**
HAP3	C	T	C	0.14	0.53
HAP4	C	T	G	0.13	0.11
HAP5	T	A	C	0.099	0.32
HAP6	T	A	G	0.081	0.063
HAP7	T	T	G	0.023	0.082
HAP8	C	A	G	0.015	0.35
Overall likelihood ratio: $\chi^2 = 16.32$; df = 7; P = 0.022					

Allele combinations, frequencies and associations with salivary cortisol AUC are shown for each haplotype. Haplotypes are sorted by their observed frequency.

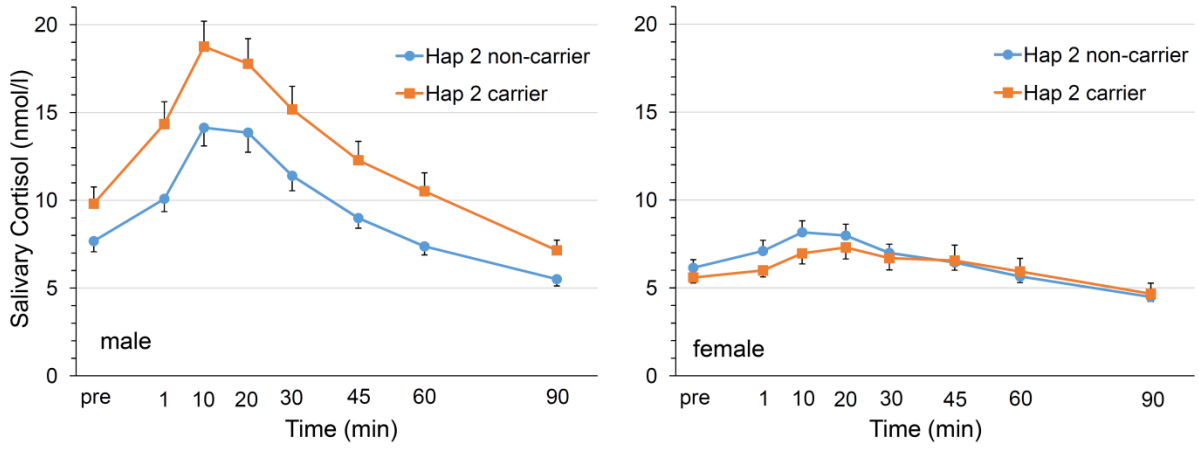

Figure IV-1: Graphical illustration of the sex-specific modulation of salivary cortisol levels (mean ± SEM) in response to the TSST by haplotype 2 in male and female participants.

For graphical presentation of haplotype effects, the best estimate haplotypes were used to contrast carriers and non-carriers.

ScanSTRESS study - cortisol responses and association with NPSR1 rs324981

Due to the lower statistical power of the haplotype-based approach only rs324981 was analyzed in the smaller Scan*STRESS* sample. However, it should be noted that the rs324981 T allele is harbored by haplotype 2 (see Table IV-2). Analysis of genotype effects on cortisol levels revealed a trend for a rs324981 x sex interaction that was consistent with the haplotype 2 x sex interaction in the TSST sample (rs324981 x sex: $F_{1,61} = 2.94$, $p = 0.092$, $eta^2 = 0.046$). On a descriptive level, the T allele was associated with higher salivary cortisol levels in males but with lower levels in females. When the first two pre-stress samples at -45 and -20 minutes were excluded from the analysis to enhance the comparability with the sampling scheme of the TSST, this trend was no longer detectable (rs324981 x sex: $F_{1,61} = 1.38$, $p = 0.245$, $eta^2 = 0.022$). When analyzed separately in males and females, the main effects of rs324981 as well as the rs324981 x time interactions failed to reach statistical significance (all $p > 0.1$).

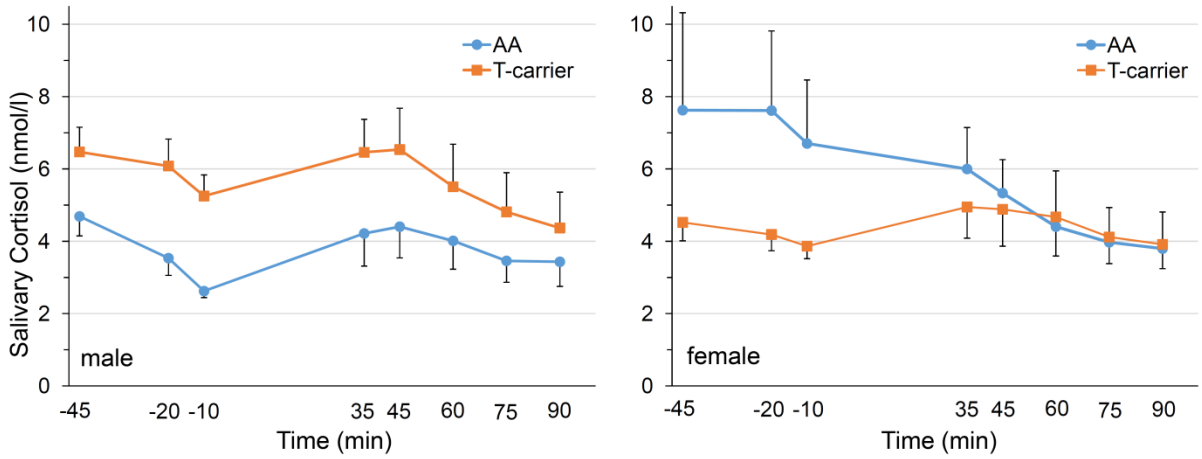

Figure IV-2: Salivary cortisol levels (mean ± SEM) by *NPSR1* rs324981 genotype in the ScanSTRESS paradigm in male and female participants.

ScanSTRESS study – neural stress response and association with NPSR1 rs324981

No main effect of genotype and no genotype by sex effect on the neural stress response were observed in the amygdala or ACC ROIs. However, in the explorative analysis a cluster close to the hippocampal gyrus reached whole-brain significance ($P_{FWE} = 0.024$; T = 5.08; -33, -33, -27, k = 2). The rs324981 T allele, also harbored by haplotype 2, was associated with an increased neural activation in males, while it was associated with a reduced activation in females (Figure IV-3).

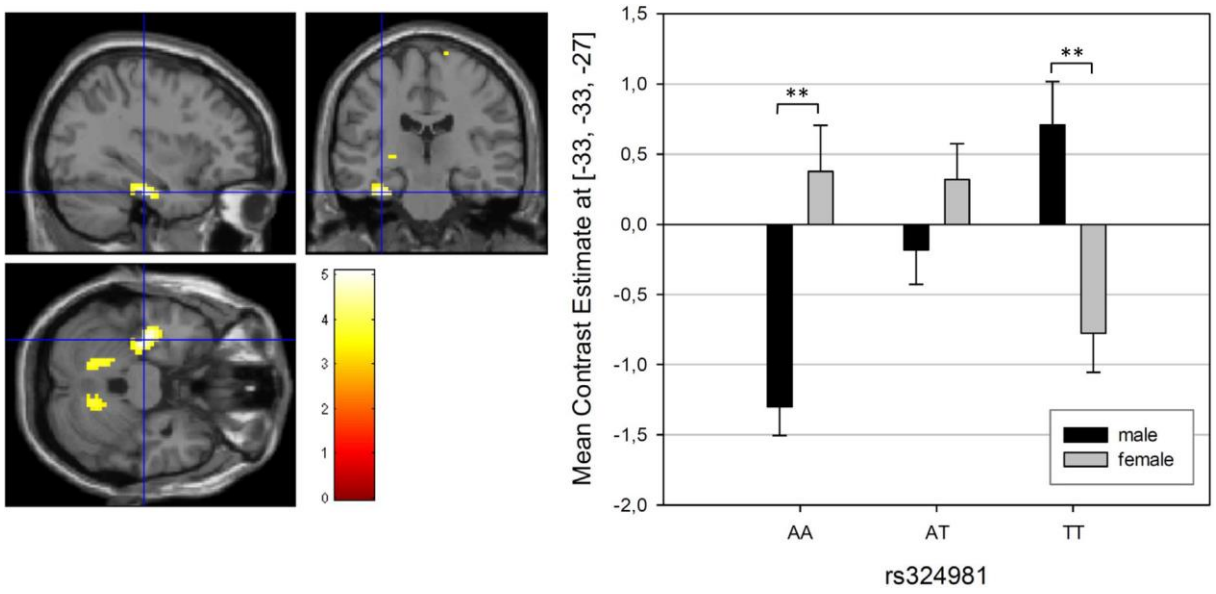

Figure IV-3: Significant interaction effect of rs324981 x sex on the neural stress response in a cluster close to the parahippocampal gyrus (BA36) (whole-brain corrected; P_{FWE} = 0.024; T = 5.08; -33, -33, -27).
The T allele was related to increased activity in males and decreased activity in females. A functional map is shown at a threshold of P = 0.001 for presentation purposes. Error bars represent 1 ± standard error of the mean (SEM). Significant sex differences in the genotype groups are indicated. ** = p < 0.01.

Discussion

In the present study we report preliminary evidence for a sex-specific association between functional sequence variants in the neuropeptide S receptor gene and acute stress responses. Using different social stress induction paradigms, we found consistent effects in independent cohorts at different levels of analyses, i.e. the adrenocortical stress response and the neural stress processing systems.

In the TSST study, the overall haplotype structure as well as the common *NPSR1* TTC haplotype comprising the minor alleles of rs2530547 and rs324981 and the major allele of rs727162, were significantly associated with the area under the salivary cortisol response curve as a measure of overall cortisol output during stress exposure. As shown in Table IV-2 also for haplotypes 6 and 7 a trend for an association was found but the low frequency of these haplotypes prohibits any interpretation of these statistical trends. Furthermore, the analysis of stress response patterns showed a more pronounced endocrine stress reaction in male carriers of haplotype 2. Figure IV-1 illustrates that the interpretation of the significant haplotype 2 x sex interaction is not entirely straightforward. This finding could be explained by a true impact of sex on the relation between *NPSR1* haplotype and cortisol responses or,

alternatively, by a pure genetic main effect that was only detected in males due to relatively low mean cortisol responses and response variability in females. This substantially lower cortisol response to psychosocial stressors in woman is a consistently replicated finding (review see Kudielka et al., 2009). However, support for a true sex-specific association between genetic variability in *NPSR1* and stress responses was found in our ScanSTRESS study. In this data set a trend for a sex-specific association between rs324981 and salivary cortisol was detected, with higher cortisol levels in male T allele carriers and lower levels in female T allele carriers. Although this effect just failed to reach statistical significance we consider it relevant as it is consistent with the finding in the TSST study, it was observed in an independent sample and with a different stress paradigm. Moreover, from a more general perspective, the idea of a sex-specific relation between *NPSR1* variability and stress regulation is also validated by our functional imaging data (see below for discussion).

In humans, variation in *NPSR1* has been shown to modulate both anxiety (Domschke et al., 2011; Laas et al., 2014a) and stress regulation (Kumsta et al., 2013; Streit et al., 2014). An influence of genetic variation in the NPS system on those phenotypes is strongly supported by rodent models selectively bread for ”High vs. Low Anxiety-Related Behavior“ (Slattery et al., 2015). In humans, Kumsta and colleagues (2013) showed in a sample of healthy males an increased cortisol response to the TSST in T-allele carriers. This is in line with the present findings, as in our TSST sample male carriers of haplotype 2, harboring the T allele of rs324981, showed increased cortisol responses as well. While a *NPRS1* x sex interaction effect on endocrine stress responses has not been reported before, general sex differences in HPA axis regulation are consistently observed (Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005). Weiss and colleagues (Weiss et al., 2006) noted that the cellular environment in women and men differs substantially regarding the hormonal milieu and they highlighted the impact of sex on penetrance and expressivity of a wide variety of traits. Differences in hormonal levels might lead to differential expressivity of underlying genetic networks and thus, gene by ‘cellular environment’ interactions can result in differential effects of the same variation in men and women. As NPS activates the HPA axis and as *NPSR1* is widely expressed in brain areas that are closely involved in HPA axis regulation, sex-specific effects of *NPSR1* variants on cortisol responses appear mechanistically plausible. Moreover, sex-specific effects of genetic variants on HPA axis activity have been described for other genes as the glucocorticoid receptor gene (e.g. Kumsta et al., 2007). Besides associations with the HPA axis, sex-specific associations of *NPSR1* sequence variants have already been reported, namely between rs324981 and psychiatric phenotypes (Laas et al., 2014a, 2015).

Previous findings suggest that *NPSR1* rs324981 is associated with activity of brain areas involved in stress and anxiety regulation (Dannlowski et al., 2011; Domschke et al., 2015; Domschke et al., 2011; Guhn et al., 2015; Neufang et al., 2015; Raczka et al., 2010; Streit et al., 2014; Tupak et al., 2013). In our study, subjects were exposed to acute psychosocial stress in the scanner but we failed to find evidence for a modulatory influence of this genotype on brain activation changes in the amygdala or the ACC ROI. We did, however, find a significant (whole-brain corrected) and again sex-specific effect in a cluster close to the parahippocampal gyrus. While the rs324981 T allele was associated with increased neural stress-related activation in in males, it was associated with reduced activation in females. Even though most rs324981 x sex interactions seem to be driven by genotype effects observed exclusively in one sex, opposing effects of rs324981 genotype in males and females have been reported, e.g. on ADHD-related symptomatology by Laas et al. (2014b).

A modulation of the parahippocampal region by NPS signaling is consistent with the observation that in animals, *NPSR1* mRNA is expressed in parahippocampal regions (Xu et al., 2007). In humans, a modulation of this region by endocrine stress signaling has been suggested. After a single stress-level equivalent dose of cortisone (25 mg), decreased cerebral blood flow in the parahippocampus was observed during a declarative memory retrieval task (de Quervain et al., 2003). Additionally, parahippocampal activation has been linked to stress-related processes. In untreated patients with major depressive disorder, a PET study reported glucose hypermetabolism in the parahippocampus that normalized along with HPA axis dysregulation after successful antidepressant treatment (Aihara et al., 2007). In anxiety patients a hyperactivation of this region was observed during anticipatory anxiety (Boshuisen et al., 2002). In light of this empirical support, our finding suggesting an influence of NPS signaling on the neural stress response in the parahippocampus appears plausible. However, it has to be noted that the sex interaction with *NPSR1* genotype did not only affect the cortisol reaction to the stressor. In male rs324981 T-allele (Scan*STRESS* study) and in male haplotype 2 carriers (TSST study), increased cortisol levels prior to stress onset were observed at least on a descriptive level. In light of the involvement of NPS signaling in anxiety regulation, it can be speculated that this observation reflects anticipatory anxiety processes.

Regarding the sex-specificity of our imaging finding it is of note that previous studies did not investigate (Domschke et al., 2015; Domschke et al., 2011; Neufang et al., 2015) or did not find substantial (Dannlowski et al., 2011) sex-specific differences in brain activation modulated by rs324981. However, various reasons may account for this difference including the most obvious one that these previous studies were not focused on neural responses to

acute stress and thus did not use a robust stress induction protocol like the Scan*STRESS* paradigm.

A limitation of the present study is the fairly small number of participants in the Scan*STRESS* sample. Even though the assessment of neural structure and activity with (f)MRI is considered to be a most promising intermediate phenotype in genetic psychiatry (Meyer-Lindenberg and Weinberger, 2006), phenotypic effects of single genetic sequence variants are usually small. Thus, although our sample size was comparable to previous samples investigating rs324981, an increased sample size would enhance the reliability of detected effects. Moreover, it would allow investigating more genetic variants in the NPS system in one analysis and increase the statistical power to integrate endocrine with fMRI data. A larger sample size would also allow to further investigate effects of the rare haplotypes 6 and 7 (frequency < 10%), which showed a statistical trend for association with salivary cortisol AUC in the TSST sample. Another limitation is that in the Scan*STRESS* sample the reported genetic effect was not found in *a priori* defined ROIs but in a cluster close to the hippocampal gyrus in the explorative analysis. From a more general methodological perspective it should also be noted as a limitation that in both subsamples (TSST and Scan*STRESS*) genetic association analyses have been performed and published before. Thus, a principle cross-study alpha error accumulation cannot be ruled out. These limitations emphasize the exploratory nature of our findings and interpretations.

In summary, the present findings provide exploratory evidence for the view that genetic variation in the neuropeptide S receptor gene modulates both cortisol responses to acute stress as well as neural stress processing in a sex-specific manner. A particular strength of our study is that consistent effects have been found for complementary stress-related biological systems, in two independent cohorts and with two different stress induction paradigms. Notably, pronounced sex differences are consistently observed in both HPA axis regulation (Kudielka and Kirschbaum, 2005) as well as in the prevalence of stress-related psychiatric disorders, including depression (Ferrari et al., 2013) and anxiety disorders (Baxter et al., 2014). It appears most likely that these two phenomena have overlapping causes as a dysregulation of the HPA axis is a significant risk factor for these disorders (Chrousos, 2009; de Kloet et al., 2005). However, the underlying psychobiological mechanisms including the genetic architecture are only poorly understood. The potential overall effect size of *NPSR1* (or *NPS*) X sex interaction effects on stress regulation and psychopathology is currently not known. Nevertheless, based on the present findings it can be assumed that the NPS system modulates stress regulation and disease risk and that this modulatory influence can differ between females and males. Therefore, to further explore this finding and its potential clinical

relevance we propose that future studies on the phenotypic effects of genetic variability in the NPS system should take gene by sex interactions into account.

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Chapter V - Concordance of phantom- and residual limb pain phenotypes in double amputees: evidence for the contribution of distinct and common individual factors

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Abstract

Most, but not all, limb amputees develop phantom limb pain (PLP) and/or residual limb pain (RLP), and large inter-individual differences in pain intensity and course are apparent. The present cross-sectional study of 122 double amputees investigated the possible role of genetic factors in PLP and RLP, assuming that strong individual predisposition will result in high intra-individual concordance in pain phenotype. Intra-individual concordance was observed in 116 (95%) subjects for development of PLP; and in 110 subjects (90%) for development of RLP. For both pain types, high intra-individual concordance was also observed for remission and current intensity. Moderate association for lifetime history and current intensity of PLP and RLP was observed both within and between limbs. The high intra-individual concordance in pain phenotypes suggests strong individual predisposition for PLP and RLP development. However, the finding of only moderate association between PLP and RLP suggests that susceptibility to these pain phenomena involves distinct, as well as common, risk factors. Genome-wide studies in large samples of single amputees may facilitate the dissection of these phenotypes and their underlying mechanisms.

Perspective

The observation of high intra-individual concordance for PLP and RLP in 122 double amputees suggests that individual factors contribute to post-amputation pain. The relatively low intra-individual association between PLP and RLP suggests that these factors are at least partially specific for each pain type.

Introduction

Neuropathic pain is related to lesions, disease, toxins, or medications that disrupt the structure/function of the somatosensory nervous system (Treede et al., 2008). This type of pain affects up to 18% of the population, is difficult to treat, and causes long-term suffering, depression, and disruption of function (Smith & Torrance, 2012). Surgical or traumatic limb amputation is associated with two major types of neuropathic pain (Borsook, Kussman, George, Becerra, & Burke, 2013; Kehlet, Jensen, & Woolf, 2006): pain referred to the missing limb (phantom limb pain, 'PLP'), and residual limb pain (pain in the remaining portion of the limb, RLP) (Ephraim, Wegener, MacKenzie, Dillingham, & Pezzin, 2005; Sherman, Sherman, & Parker, 1984). PLP occurs in about 75% of the limb amputees, while about 60% suffer from RLP (Foell, Bekrater-Bodmann, Flor, & Cole, 2011). Both types do not necessarily co-occur although they are correlated (Foell et al., 2011). Sensory-discriminative characteristics, such as pain intensity, frequency, and duration, remain stable for many years after amputation, with a low rate of spontaneous remission but high inter-individual variability (Hunter, Katz, & Davis, 2008; Sherman et al., 1984).

A considerable proportion of the variability observed in these pain phenotypes may be driven by the individual genetic background (Belfer, 2013; Mogil, 2012). Twin and family studies of other chronic pain conditions have revealed heritabilities of 20 to 70% (Nielsen, Knudsen, & Steingrimsdottir, 2012). Corresponding heritability estimates for postamputation pain are unavailable, as for obvious reasons the required twin samples do not exist. However, animal models of neuropathic pain have yielded heritability estimates similar to those seen in human chronic pain conditions (Devor, del Canho, & Raber, 2005; Mogil et al., 1999; Young, Costigan, Herbert, & Lariviere, 2014). For example, a study in rats based on autotomy behavior following paw denervation as a model of neuropathic pain, compared autotomy levels expressed after sequential denervation of two limbs spaced weeks apart. The investigators reported that autotomy levels after the first denervation were highly predictive of the levels of the same behavior following the second denervation. The authors proposed that this finding may indicate a strong individual predisposition for certain levels of autotomy behavior (Rabin & Anderson, 1985).

In humans, two studies have reported a high concordance in pain phenotype between injured body sites following surgery (Bruce et al., 2003) or amputation (Lacoux, Crombie, & Macrae, 2002), and their results have been interpreted as "tentative evidence of heritability" (Devor, 2004). Bruce et al. (Bruce et al., 2003) compared postsurgical chronic pain in 1080 patients

who underwent coronary bypass surgery that necessitated grafting the saphenous vein in the leg. Those who developed chronic postsurgical pain in the chest also tended to develop chronic pain in the leg. In a sample of 11 bilateral upper limb amputees, Lacoux et al. (2002) reported concordance for the presence of PLP and non-painful phantom sensation in all subjects, while 10 of the 11 subjects were concordant for RLP. However, the small sample size clearly limits the generalizability of this study.

The aims of the present study were to: (i) investigate the hypothesis that PLP and RLP are each determined by a strong individual predisposition that will result in high concordance in pain phenotype between limbs; and (ii) determine the association between PLP and RLP, both between and within limbs, in order to examine whether common or distinct risk factors are implicated in their development. This was investigated in a sample of 122 double amputees. A precise phenotype characterization of PLP and RLP was performed for each limb.

To investigate the individual predisposition for each type of pain, we tested between-limb concordance of the lifetime history and the current intensity of both PLP and RLP. For the relationship between PLP and RLP, we tested the association between PLP and RLP both between and within limbs in terms of life time history and current intensity.

Methods

Sample

Between August 2009 and November 2013, a total of 31,887 questionnaires were sent to cooperating organizations in Germany for distribution to individuals with a major amputation of one or more limbs (in the context of the PHANTOMMIND project) (e.g. Bekrater-Bodmann et al., 2015). In total, 3,862 questionnaires were returned together with a signed/dated informed consent document (response rate, 12%). Returned but incomplete questionnaires were completed via telephone interviews. A total of 361 questionnaires were excluded due to missing information. Of the remaining 3,501 questionnaires, 122 questionnaires were completed by individuals who had undergone a double amputation. The study was approved by the ethics review board of the Medical Faculty Mannheim, University of Heidelberg, and was conducted in accordance with the Declaration of Helsinki (Association, 2001).

Questionnaire

The questionnaire was based on the Phantom and Stump Phenomena Interview (Winter et al., 2001), with the addition of items concerning demographic variables, other physical diseases,

and general pain experiences. For all questions about the amputation procedure and subsequent pain experiences, the subjects were asked to provide separate responses for each limb. For the purposes of the present analyses, the following items were evaluated:

a) The subjects were asked to indicate the site of the amputation, the length of the residual limb, the reason for the amputation, when it was performed (year and month), and whether they had experienced pain in the affected limb prior to the amputation.
b) Subjects were asked whether they had experienced PLP and/or RLP during the following two time-frames: "ever, i.e., since the loss of the limb" and "within the last three months". PLP and RLP were categorized using both: (i) two categories (no lifetime history of pain vs. lifetime history of pain); and (ii) three categories (no lifetime history, symptom free at least the last three months ("remission"), or lifetime history of pain with symptoms within the last 3 months ("current")).
c) Subjects asked to rate the average intensity of PLP and RLP experienced during the four weeks preceding the survey on a scale of 0 ("no pain") to 10 ("intolerable pain"). These intensity ratings were used to quantify the current intensity of PLP and RLP.

Statistics

The data analysis was performed with the SPSS software (version 20, IBM Corp., Armonk, NY, USA). Concordance in PLP and RLP between different amputation sites, and intra-individual association between PLP and RLP both within and between limbs, were assessed using the χ^2 test for categorical variables and correlational analysis for continuous variables. The analyses were carried out separately in three groups (leg-leg, arm-arm, leg-arm). All data were complete except three intensity ratings of current RLP, which were excluded from correlational analysis. To investigate whether the time points of the two amputations (i.e., sequential or simultaneous) had an influence on concordance patterns, the largest group, i.e., the leg-leg group, was divided into: (i) subjects whose limbs had been amputated at the same time point (i.e., within the same month; n = 61); and (ii) subjects whose limbs had been amputated at different time points (n = 28; median difference = 2.38 years).

For categorical variables with two levels (lifetime vs. no lifetime history of pain), association effect sizes were calculated as the phi coefficient (φ). This index ranges from -1 in the case of perfect negative association to+1 in the case of perfect positive association. For categorical variables with three levels (no lifetime history, remission, or current pain), Cramér's-V (V_c) was calculated. This index ranges from 0 in the case of no association to +1 in the case of

perfect association. Associations with a lifetime history of pain were assessed using χ^2 statistics in the leg-leg group. Exact tests were applied in the smaller arm-arm and leg-arm groups. Exact tests were also applied in the analysis that included the category "remission". Since the PLP and RLP ratings showed deviations from the normal distribution, Spearman correlation coefficients (ρ) were used to assess the correlation between parameters and to assess the respective associations.

To account for age, sex, length of the residual limb, and time since amputation -all known contributors to PLP and RLP (e.g. (Bosmans, Geertzen, Post, van der Schans, & Dijkstra, 2010; Dijkstra, Geertzen, Stewart, & van der Schans, 2002))- separate generalized linear mixed models were computed for PLP and RLP in the largest group (i.e., "leg-leg"; 73% of the total sample). A binary distribution and a logit link were used, an approach which can be conceptualized as an analogue to a logistic regression but which allows for correlated data. For both limbs, lifetime history of PLP or lifetime history of RLP was modeled as the respective outcome variable (1 = yes, 0 = no). Age, sex, length of the residual limb, and time since amputation were included in a stepwise manner into the model as fixed effects together with a random intercept effect. Intraclass correlations (ICC) were determined using the intercept-only model (null model) in order to assess the degree of dependence between the two limbs and to quantify the proportion of variability which is accounted for by the individuals.

Results

Characteristics of the study sample

The present analyses included data from 122 double amputees (93 males and 29 females; mean age = 64.55 years: standard deviation (SD) = 15.44 years; range 20 - 95 years). The sample comprised subjects with: (i) bilateral foot/leg amputations (n = 89; "leg-leg" group); (ii) bilateral hand/arm amputations (n = 16; "arm-arm" group); and (iii) amputations of one hand/arm and one foot/leg (n = 17; "leg-arm" group).

The majority of subjects reported a lifetime history of PLP (74%) and RLP (75%) in at least one limb. The average time since amputation was 31.21 years (SD = 23.22; range 5 months - 80 years). In the majority (70%) of subjects, the two amputations had been performed at the same time point. Preamputation pain was reported for 25% of the amputations (for details see Supplementary Table V-1). Only a small number of subjects reported complete pain remission (16% for PLP and 18% for RLP; for an overview see Table V-1). In limbs for which subjects reported current pain, the average intensity was 6.14 (range 0 – 10; SD = 2.65) for PLP and

5.87 (range 0 – 10; SD = 2.40) for RLP (Table V-1). A traumatic event was the most frequent reason for amputation (66% of the amputations), followed by vascular disease (24%) (for details see Supplementary Table V-1). In the leg-leg group, a traumatic event was the most common cause of simultaneous amputation (78% of amputations). In contrast, traumatic events accounted for only 11 % of amputations performed at different time points. Here, cardiovascular disease was the most common reason for amputation (80% of amputations).

Table V-1: Prevalence and intensity of PLP and RLP after double amputation

Prevalence of PLP and RLP for each limb ("No history of pain", "Remission", "Current") in the 3 subgroups (Leg-leg, Arm-arm and Leg-arm) in absolute numbers and in percentages.

Pain type	Group	Amputated limb	No history of pain, N (%)	Remission, N (%)	Current, N (%)	Intensity of current pain (SD)
PLP	Leg-leg (n = 89)	Left leg	24 (27)	13 (15)	52 (58)	6.73 (2.41)
		Right leg	24 (27)	14 (16)	51 (57)	6.23 (2.68)
	Arm-arm (n =16)	Left arm	7 (44)	2 (13)	7 (44)	4.14 (1.77)
		Right arm	8 (50)	1 (6.3)	7 (44)	3.43 (1.99)
	Leg-arm (n =17)	Arm	4 (24)	5 (29)	8 (47)	6.63 (2.50)
		Leg	3 (18)	2 (12)	12 (71)	5.62 (3.10)
RLP	Leg-leg (n = 89)	Left leg	35 (39)	9 (10)	45 (51)	5.95 (2.43)
		Right leg	35 (39)	10 (11)	44 (49)	6.03 (2.34)
	Arm-arm (n = 16)	Left arm	6 (38)	3 (19)	7 (44)	3.86 (2.12)
		Right arm	9 (56)	2 (13)	5 (31)	4.40 (1.82)
	Leg-arm (n = 17)	Arm	7 (41)	5 (29)	5 (29)	7.00 (3.00)
		Leg	6 (35)	3 (18)	8 (47)	6.50 (2.07)

Note: For subjects reporting pain in the past 3 months, current pain intensity was rated as the average pain intensity experienced during the past 4 weeks using a scale of 0 (no pain) to 10 (intolerable pain).

Concordance of phantom limb pain

Lifetime presence of PLP (lifetime history vs. no lifetime history) was concordant in 116 of the 122 subjects (concordance rate: 95%): 84 subjects developed PLP in both limbs, and 32 subjects developed PLP in neither limb. Only 6 subjects developed PLP in one limb but not in the other (5% discordance).

The φ coefficients in the three groups were: (i) leg-leg: φ = .87, P < .001; (ii) arm-arm: φ = .88, P < .01; and (iii) leg-arm: φ = .84, P < .01 (see Figure V-1). After additional categorizing of the subjects into the categories "no lifetime history of pain", "remission", and "current", the Cramér's-V coefficients were: (i) leg-leg: V_c = .83, P < .001; (ii) arm-arm: V_c = .85, P < .001; and (iii) leg-arm: V_c = .60, P < .01; exact tests).

A high between-limb correlation was observed for current PLP intensity in the three groups 'leg-leg', 'arm-arm' and 'leg-arm' (range: ρ = .70 - .95; all P < .01; see Figures V-1b, d & f).

Overall, the lifetime history, course, and current intensity of PLP were highly concordant between limbs.

Concordance of residual limb pain

Lifetime presence of RLP (lifetime history vs. no lifetime history) was concordant in 110 of the 122 subjects (concordance rate: of 90%): 67 subjects developed RLP in both limbs, and 43 subjects developed RLP in neither limb. Only 12 subjects developed RLP in one limb but not in the other (10% discordance).

High φ coefficients were obtained for the lifetime presence of RLP (lifetime history vs. no lifetime history), irrespective of the combination of limbs affected (leg-leg: φ = .86 and P < .01, arm-arm: φ = .68, P < .01, leg-arm: φ = .63, P < .01; Figure V-1).

Following categorization of the subjects into the categories "no lifetime history of pain", "remission", and "current", high concordance for RLP was observed (leg-leg: V_c = .77, P < .001; arm-arm: V_c = .76, P < .001; leg-arm: V_c = .61; P < .01; exact tests).

A high between-limb correlation was observed for current RLP intensity in the three groups 'leg-leg', 'arm-arm' and 'leg-arm' (range: ρ = .67 - .70; all P < .05; see Figures V-1b, d & f).

As with PLP, the lifetime history, course and current intensity of RLP were highly concordant between limbs.

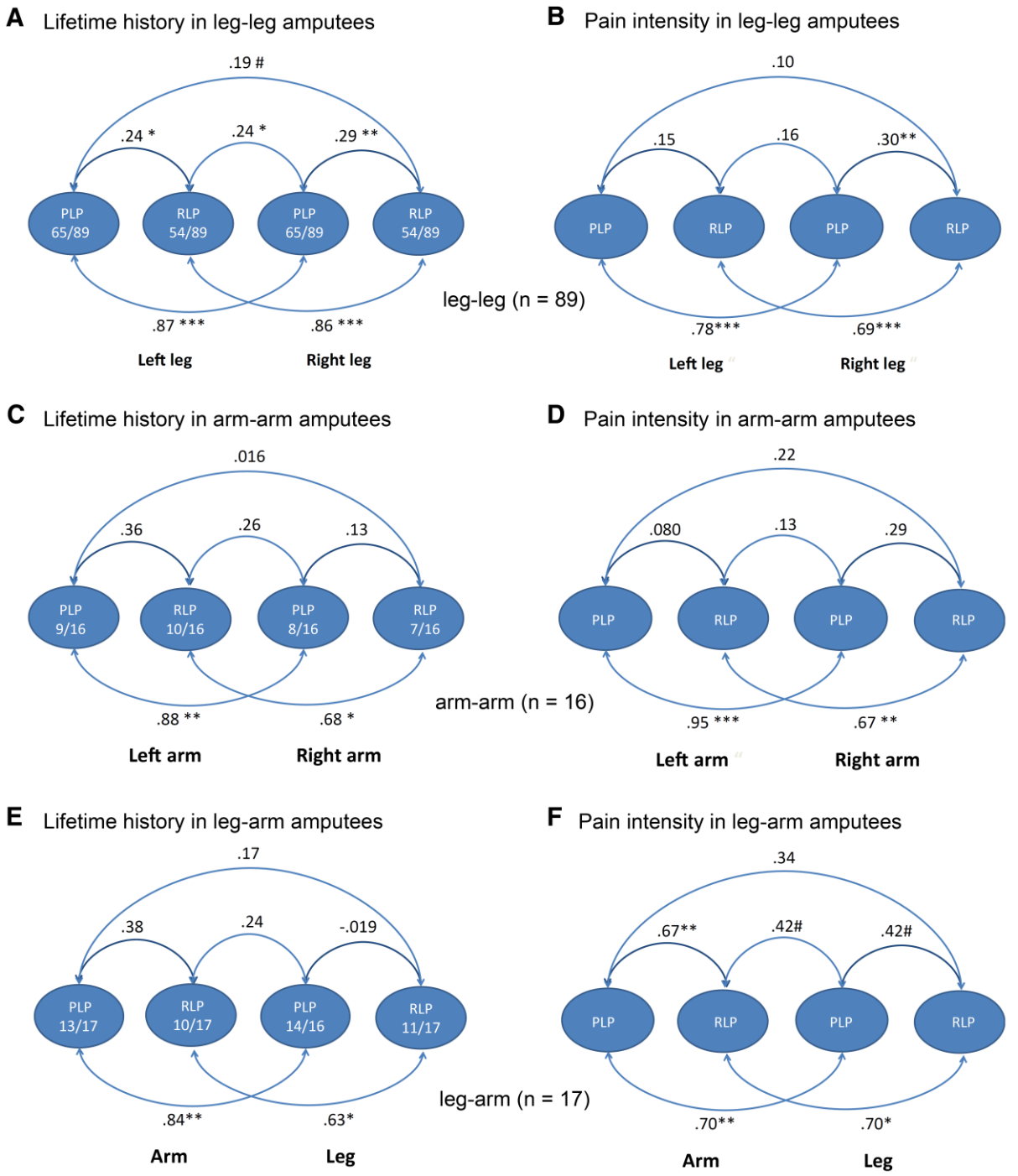

Figure V-1: Association between lifetime history and current intensity of PLP and RLP both within and between limbs.

Concordance between PLP and RLP within and between limbs is displayed for the following groups: leg-leg (*n* = 89), arm-arm (*n* = 16), and leg-arm (*n* = 17). Lifetime history of PLP and RLP: number of patients with pain in the respective limb, φ coefficients of the associations and their significance (A) between and within right and left leg, (C) between and within right and left arm, and (E) between and within arm and leg. Current pain intensity of PLP and RLP: Spearman r of the correlations for pain intensity and their significance (B) between and within right and left leg, (D) between and within right and left arm, and (F) between and within arm and leg. ***P < .001; **P < .01; *P < .05; #P < .1.

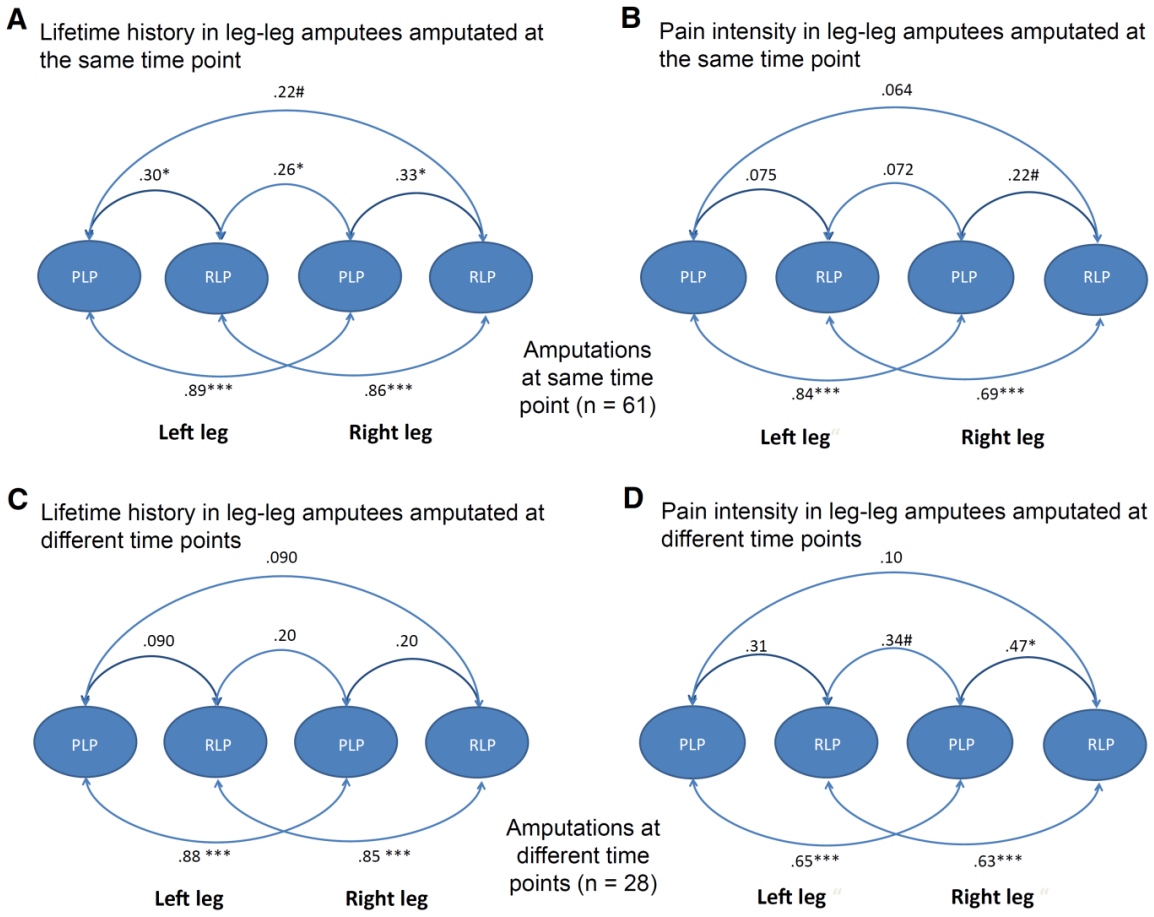

Figure V-2: Association between lifetime history and current intensity of PLP and RLP both within and between limbs for amputations performed at the same time or at different time points.

Concordance between PLP and RLP within and between legs is displayed for leg-leg amputees whose limbs were amputated at the same time point (n = 61) and leg-leg amputees whose limbs were amputated at different time points (n = 28). Lifetime history of PLP and RLP: φ coefficients of the associations and their significance between and within right and left leg in leg-leg amputees whose limbs were (A) amputated at the same time point and (C) at different time points. Current pain intensity of PLP and RLP: Spearman r of the correlations for pain intensity and their significance between and within right and left leg in leg-leg amputees whose limbs were (B) amputated at the same time point and (D) at different time points. ***P < .001; **P < .01; *P < .05; #P < .1.

Influence of covariates on lifetime history of phantom limb pain and residual limb pain

In the largest subgroup, the leg-leg group, the influence of covariates on the concordance of PLP and RLP was explored.

Time point:

In the leg-leg group similar high levels of concordance were observed in both the subgroup of subjects whose two legs had been amputated at the same time point (n = 61), and in the subgroup of subjects whose legs had been amputated at different time points (n = 28; Figure V-2).

Reason of amputation:

While lifetime history of PLP was more frequently reported for amputations caused by vascular disorder, lifetime history of RLP was not (for details see Supplementary Table V-2). Both PLP and RLP were highly concordant in the analysis of 1) patients reporting vascular disorders as the reason of amputation; and 2) patients reporting traumatic events as the reason of amputation (for details see Supplementary Table V-3).

Preamputation pain:

Preamputation pain was more prevalent in amputations with a lifetime history of PLP, but not with a lifetime history of RLP (for details see Supplementary Table V-2). Preamputation pain did not impact the high correlation of pain phenotypes across limbs. PLP and RLP were highly concordant 1) in patients reporting preamputation pain; and 2) in patients reporting no preamputation pain (for details see Supplementary Table V-3).

Age, sex, time since amputation and length of residual limb:

A generalized linear mixed model for PLP revealed no significant effects for age ($P = .27$), or sex ($P = .35$). Refitting the model without age and sex revealed significant effects for both time since amputation ($F_{1,87} = 9.93$; $P < .01$), and length of the residual limb ($F_{1,87} = 7.54$; $P < .01$). Here, recent amputation and short length of residual limb were associated with a higher probability of PLP. The ICC resulting from the null model was .50. Following the addition of the predictors “time since amputation” and “length of residual limb”, the ICC showed only a marginal change, i.e., an increase to .51.

Slightly different results were obtained after the same multilevel model was fitted to lifetime history of RLP. Age, sex, and time since amputation were not significant predictors of RLP (all $P > .5$). After refitting of the model, a trend towards a significant association was found for the length of the residual limb ($F_{1,88} = 3.82$; $P = .054$). Again, the ICC of .49 for the null model was quite high, and increased to .50 in the final model.

Concordance was observed in the subgroups established according to reason of amputation and presence of preamputation pain. Furthermore, the inclusion of the variables age, sex, time since amputation, and length of residual limb in the general mixed models resulted in only small changes in the ICCs. These findings indicate that the strong intra-individual concordance for both PLP and RLP was independent of these potential contributors.

Association between phantom limb pain and residual limb pain both within and between limbs

In the leg-leg group, the association between a lifetime history of PLP and a lifetime history of RLP was low to moderate both within each leg (left φ = .24, P < .05; right φ = .29, P < .001), and between PLP in the right leg and RLP in the left leg (φ = .24, P < .05). The association between lifetime history of PLP in the left leg and RLP in the right leg showed only a trend towards significance (φ = .19, P < .1; see Figure V-1a).

In this group, a significant association was observed between current intensity of PLP and current intensity of RLP in the right leg (ρ = .30, P < .01). For other limb combinations, PLP and RLP intensity showed as well a tendency for positive association. However, these associations failed to reach significance (ρ ranges between .10 and .16, all P > .1; see Figure V-1b).

In the two smaller groups, no significant association between lifetime history of PLP and lifetime history of RLP was found, either between or within limbs (range: φ = -.019 - .38; all P > .1, see Figures V-1c & e). Only one significant association was found between current intensity of PLP and current intensity of RLP. This was observed for the arm in the leg-arm group (ρ = .67, P < .01; all other ρ range between .080 and .42, all P > .05; see Figures V-1d & f).

A high between-limb concordance was found for each pain type. In contrast, apart from one correlation of pain intensity in the leg-arm group (r = .67), significant associations between PLP and RLP either within or between limbs were only found in the biggest group (leg-leg) and the strengths of those associations were only low to medium (all ρ and $\varphi \leq .30$).

Discussion

The present study investigated whether there is an individual predisposition for the development of PLP and RLP, along with intensity and remission by analyzing phenotypic concordance in 122 double amputees. The frequency and intensity of PLP and RLP reported in the present sample are comparable to those shown in single limb amputees (Clark, Bowling, Jepson, & Rajbhandari, 2013; Ephraim et al., 2005; Sherman et al., 1984). The present study reports high intra-individual concordance in lifetime history (ranging in the three groups from V_c = .84 to .88 for PLP, and .63 - .86 for RLP). High intra-individual concordance was also observed for current pain intensity (ρ: .70 - .95 for PLP, and .67 - .70 for RLP) and remission (V_c ranged between .60 and .85 for PLP, and between. .61 and .76 for RLP).

The high intra-individual concordances for lifetime history, current intensity, and remission observed for both PLP and RLP suggest that the development and maintenance of these neuropathic pain conditions (as well as the recovery from these conditions) do not occur at random, but are strongly determined by individual susceptibilities. Individual differences in pain-perception and the susceptibility to develop chronic pain have been shown to be influenced by demographic factors such as sex (Bosmans et al., 2010) and age (Dijkstra et al., 2002), as well as behavioral, cognitive, affective and further underlying neurobiological factors (Flor, Nikolajsen, & Jensen, 2006; Turk, 1999). For example, chronic pain has been shown to be associated with individual differences in depression, anxiety (Gorczyca, Filip, & Walczak, 2013), neural plasticity and pain memory (Flor et al., 2006), as well as processing of painful stimuli (Nielsen et al., 2008). Many of these factors are shaped by the environment, such as early adversity or previous pain experiences (Beggs, Currie, Salter, Fitzgerald, & Walker, 2012; Gorczyca et al., 2013; Joseph, 1999; Low & Schweinhardt, 2012). Therefore, we cannot exclude the possibility that the high intra-individual concordance rates observed in the present sample may be attributable, at least in part, to environmental factors shaping individual predisposition to develop chronic pain. Furthermore, postamputation pain can be influenced by limb specific pre-, peri- and postamputational factors, such as preamputation pain (Jensen, Krebs, Nielsen, & Rasmussen, 1983; Nikolajsen, Ilkjaer, Kroner, Christensen, & Jensen, 1997); the amputation procedure (Kehlet et al., 2006); conditions following amputation (e.g. infections, neuroma, insufficient blood circulation) (Bourke, Yelden, Robinson, Sooriakumaran, & Ward, 2011; Katz, 1992); related secondary surgeries (Bourke et al., 2011); the time period since amputation (Bosmans et al., 2010; Jensen et al., 1983); as well as the length of the residual limb, and the use of a prosthesis (Kern, Busch, Rockland, Kohl, & Birklein, 2009). It is a limitation of this study that not all relevant factors could be assessed in the questionnaire. It is therefore impossible to determine whether the high concordance rates observed in the present sample are due to similarities within individuals in terms of factors common to both amputations, or whether they are due to individual predisposition, which might in turn be either genetically or environmentally determined.

To account for some of the variables that might enhance the concordance of PLP and RLP independently of the assumed individual predisposition, we computed generalized linear mixed models, which included age, sex, time since amputation, and the length of residual limb as predictors. Short length of the residual limb and a recent amputation were associated with an increased risk of PLP. For RLP, only a trend towards an effect of the length of the residual limb was observed. Inclusion of these predictors did not reduce the strength of the

association of RLP or PLP between limbs. Additionally, analyses in subsamples of the leg-leg group revealed concordance of PLP and RLP independent of the patients' reported preamputation pain, vascular disorder as reason of amputation or simultaneous or non-simultaneous amputations. Interestingly, we observed significant associations between lifetime history of PLP and RLP in the subjects amputated at the same time point, but not in the subjects amputated at different time points. Such a pattern was not observed for pain intensity.

As in previous reports of single amputees (Kooijman, Dijkstra, Geertzen, Elzinga, & van der Schans, 2000; Sherman et al., 1984), a moderate association between PLP and RLP was observed in the present sample of double amputees. However, this association was much weaker than the intra-individual concordance observed for either PLP or RLP between the two amputations. This supports the hypothesis that while these phenotypes have shared causal factors, they also are influenced by distinct, non-shared factors. This is consistent with the hypothesis that the different aspects of postamputation pain vary in terms of individual susceptibility. Furthermore, the low association observed intra-individually between PLP and RLP, as assessed on the basis of self-reported lifetime history and intensity, cannot be explained by an individual predisposition to the reporting of pain in general. It is therefore unlikely that the high concordances observed for each pain phenotype were driven by individual differences in response tendency. However, the inclusion of physiological and behavioral measures of pain relevant phenotypes would have been desirable. Although no new treatment methods can be derived from our results, the present data indicate that PLP and RLP should be seen as distinct phenotypes during the evaluation of therapy outcomes in patients with postamputation pain, since specific interventions might benefit one type of pain but not the other.

The fact that PLP and RLP following the two amputations remained highly concordant despite correction for certain confounding factors is consistent with the hypothesis that individual predisposition plays a major role in the development of postamputation PLP and RLP. Previous authors have proposed that genetic factors influence the susceptibility to neuropathic pain (e.g. Belfer, 2013; Seltzer, 2014). This hypothesis is supported by genetic findings in animal models of neuropathic pain (e.g. Devor, Gilad, et al., 2005; Nissenbaum et al., 2008; Seltzer, Wu, Max, & Diehl, 2001). Genetic effects with an influence on susceptibility to postamputation pain may be mediated through various pathways. A genetic component has been demonstrated for several factors associated with pain perception or pain disorders, such as personality (Vassend, Roysamb, & Nielsen, 2013), coping styles (Busjahn,

Faulhaber, Freier, & Luft, 1999), brain plasticity (Brans et al., 2010; Missitzi et al., 2011), memory processes (Kremen, Koenen, Afari, & Lyons, 2012), and processing of painful stimuli (Nielsen et al., 2008). Numerous genetic mechanisms may underlie PLP and RLP, some of which may be shared and some unique to each phenotype. This hypothesis is supported by empirical data both in animal and human models, which suggest that individual differences in pain sensitivity are distinct for different modalities of pain (Young et al., 2014). In twin studies, for example, only a small proportion of genetically mediated variance could be explained by genetic factors common to pain induced by cold-pressor test and by heat, suggesting that the genetic basis of these traits is mostly unique per trait (Nielsen et al., 2008; Vassend et al., 2013).

As for other complex phenotypes, the investigation of genetic risk factors is a promising approach for the identification of mechanisms underlying chronic pain, which may facilitate the development of new treatment approaches. Animal models of postamputation pain suggest that a moderate-to-high portion of the inter-strain variability is explained by heritable risk, which maps to quantitative trait loci on chromosome 15 in the mouse (Devor, Gilad, et al., 2005; Seltzer et al., 2001), and chromosome 2 in the rat (Nissenbaum et al., 2008). In humans, a small number of successful genome-wide association studies (GWAS) of pain have been reported, including investigations of diffuse musculoskeletal pain, migraine, and the analgesic efficacy of early postoperative morphine (e.g. Esserlind et al., 2013; Nishizawa et al., 2014; Peters et al., 2013). To date, only one GWAS has investigated chronic neuropathic pain (Meng et al., 2014). This yielded suggestive evidence for an association on chromosome 8 (at band p21.3).

The present study generated two findings of relevance to future pain genetics research: First, our findings suggest that a systematic search for genetic factors underlying postamputation pain would be a promising approach for the identification of underlying biological mechanisms. Second, our data suggest that PLP and RLP are different phenomena, and are caused by distinct as well as shared factors. Analysis of genome-wide data of large cohorts of amputees would facilitate the classification of pain phenotypes on a genetic level. This could be achieved through the application of methods such as polygenic score analyses (Purcell et al., 2009), or Genome-wide Complex Trait Analysis (GCTA) (Lee, Yang, Goddard, Visscher, & Wray, 2012), methods which allow estimation of the heritable components that are specific for, and shared by, complex traits on the basis of genome variation data. These methods could also be used to investigate genetic overlap between PLP and RLP and risk factors such as depression or anxiety.

In summary, the results of the present study provide strong support for the hypothesis of an individual predisposition for the development of PLP and RLP following limb amputation. This predisposition appears to be partially distinct for each type of pain, and is very likely to be influenced by both distinct as well as overlapping genetic factors. These findings may be of value in selecting promising phenotypes for studies of postamputation related pain types. The present data suggest that PLP and RLP should be considered separate phenomena in GWAS of genetic risk factors for postamputation pain.

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Supplementary Material

Supplementary Table V-1: Cause of amputation and prevalence of pre amputation pain

Group	Age	Sex m/f	Limb	Reason of amputation					Pre amputation pain			
				Traumatic[a,b]	Infection[a]	Tumor[a]	Vascular disease[a]	Other[a]	Yes	No	Not known	missing
leg-leg	63.63	65/24	Left leg	50	9	-	27	7	28	56	4	1
			Right leg	51	8	1	27	9	29	55	4	1
arm-arm	68.06	15/1	Left arm	16	-	-	-	-	-	16	-	-
			Right arm	16	-	-	-	-	-	16	-	-
arm-leg	66.06	13/4	Arm	14	-	-	1	1	1	15	-	1
			Leg	14	1	-	3	1	4	12	-	1

Cause of amputation and prevalence of pre amputation pain by limb in absolute numbers. a: multiple answers were possible for reason of amputation. b: the category "traumatic" comprises the answer options "accident" and "injury". m = male; f = female.

Supplementary Table V-2: Association of preamputation pain and reason of amputation with phantom limb pain and residual limb pain

	Phantom limb pain		Residual limb pain	
	Left leg	**Right leg**	**Left leg**	**Right leg**
Vascular disorder	.29**	.18#	-.069	-.069
Preamputation pain	.19#	.26*	.069	.02

Phi coefficients and their significance of the association of lifetime history of phantom limb pain (PLP) and residual limb pain (RLP) with vascular disorder as reason of amputation and preamputation pain for each limb in the leg-leg group. ** $P < .01$; * $P < .05$; # $P < .1$

Supplementary Table V-3: Concordance of phantom limb pain and residual limb pain in subgroups divided for preamputation pain and reason of amputation

	Concordance of PLP between left leg and right leg	Concordance of RLP between left leg and right leg
Vascular disorder	.85***	.85***
Traumatic events	.87***	.83***
Preamputation pain	.88**	.93***
No Preamputation pain	.87***	.85***

Phi coefficients and their significance of the concordance of lifetime history of phantom limb pain (PLP) and residual limb pain (RLP) in subgroups of the leg-leg group reporting vascular disorder or a traumatic event as reason of the amputation as well as for subjects reporting preamputation pain or no preamputation pain. *** $P < .001$

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

1. .....

2. .....

3. .....

Weitere Personen waren an der inhaltlich-materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich versichere die Richtigkeit der vorangegangenen Erklärung und bin mir der strafrechtlichen Folgen einer Falschaussage bewusst.

.....	.....
Ort, Datum	Unterschrift