**A neurobiological framework of separation anxiety**

**and related phenotypes**

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**ABSTRACT**

In the DSM-5, separation anxiety disorder (SAD) is newly classified in the chapter on anxiety, renewing research efforts into its etiology. In this narrative review, we summarize the current literature on the genetic, endocrine, physiological, neural and neuropsychological underpinnings of SAD *per se*, SAD in the context of panic disorder, separation anxiety symptoms, and related intermediate phenotypes.

SAD aggregates in families and has a heritability of ~43%. Variants in the oxytocin receptor, serotonin transporter, opioid receptor µ 1, dopamine D4 receptor and translocator protein genes have all been associated with SAD. Dysregulation of the hypothalamus-pituitary-adrenal axis, dysfunctional cortico-limbic interaction and biased cognitive processing seem to constitute further neurobiological markers of separation anxiety. Hypersensitivity to carbon dioxide appears to be an endophenotype shared by SAD, panic disorder and anxiety sensitivity.

The identification of biological risk markers and its multi-level integration hold great promise regarding the prediction of SAD risk, maintenance and course, and in the future may allow for the selection of indicated preventive and innovative, personalized therapeutic interventions.

**INTRODUCTION**

Separation anxiety disorder (SAD) is characterized by excessive fear or anxiety concerning separation from home or from close attachment figures (American Psychiatric Association, 2013; Baldwin et al., 2016). While previously classified in the section “Disorders Usually First Diagnosed in Infancy, Childhood, or Adolescence“, the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM 5; (American Psychiatric Association, 2013) places separation anxiety disorder in the chapter on anxiety disorders. Lifting the age restriction (< 18 years in DSM-IV) on the diagnosis of SAD reflects increasing evidence that disorder onset is not limited to childhood or adolescence, but often first manifests over the age of 18.

Data from the National Comorbidity Survey Replication (NCS-R) revealed a high lifetime prevalence of childhood-onset SAD (CSAD) of 4.1%, but a higher lifetime prevalence of 6.1% for adult-onset SAD (ASAD), with 36.1% of childhood-onset cases persisting into adulthood and as many as 77.5% of adult cases reporting first onset after the age of 18 (Shear et al., 2006). Similarly, an analysis of the World Mental Health Survey indicated a lifetime prevalence for SAD of 4.8%, with 43.1% of lifetime onsets occurring after 18 years of age. Childhood prevalence rates are higher in girls than in boys, although sex differences are less pronounced in adulthood. However, men are more likely to report disorder onset in adulthood (Shear et al., 2006; Silove et al., 2015). SAD appears to be both highly comorbid with and antecedent to a variety of other mental disorders (Silove et al., 2015), including other anxiety disorders, mood and stress-related disorders, and personality disorders. For instance, in clinical populations comprising mood and anxiety disorder patients, as many as 21.7% of patients suffered from ASAD in addition to a history of CSAD, and an additional 20.7% had ASAD without a history of the childhood disorder (Pini et al., 2010). Furthermore, a meta-analysis of 20 studies revealed a substantial sequential comorbidity for CSAD and panic disorder (OR: 3.45; (Kossowsky et al., 2013a).

The inclusion of SAD in the group of anxiety disorders and thereby lifting the age limit in the DSM-5 has renewed research efforts into its epidemiology and etiology. This may particularly pertain to the elucidation of neurobiological mechanisms, which on the one hand may constitute stable risk factors of SAD across age groups, and, on the other hand, act as neutral, adaptive or maladaptive markers depending on different time windows of age (cf. Battaglia and Khan, 2018). Thus, here we provide a comprehensive review of the current literature on the neurobiological underpinnings of SAD *per se*, SAD in the context of panic disorder, separation anxiety symptoms and related intermediate phenotypes such as heightened arousal or sensitivity to carbon dioxide. For epidemiological, nosological and neuropsychological aspects please refer to recent reviews (Baldwin et al., 2016; Battaglia, 2015; Bogels et al., 2013; Matthies et al., 2018; Milrod et al., 2014; Strawn and Dobson, 2017).

1. **EXPERIMENTAL PROCEDURES**
   1. **Search strategy and eligibility criteria**

Eligible for this narrative review were peer-reviewed studies published in English and reporting original research until June 2019 using the search term “separation anxiety”. Relevant articles were identified by searching PubMed, Web of Science and PsycINFO. Additional studies were identified manually by searching reference lists of selected articles and pertinent review articles. Results of the initial search were screened for eligibility by title and abstract by author MAS. Subsequent retrieval of full-text articles, evaluation for eligibility and data extraction was performed independently by authors MAS and KD. Disagreements were resolved by consensus.

1. **RESULTS**
   1. **Family and twin studies**

A family study of 54 children diagnosed with anxiety disorders and their parents (54 mothers and 29 fathers) found that 63% of children diagnosed with SAD had at least one parent with ASAD (OR=11.1, p<0.001) (Manicavasagar et al., 2001).

Several twin studies point to a moderate heritability of SAD and separation anxiety symptoms, with heritability estimates ranging from 0.21 to 0.74 (Battaglia et al., 2009; Bolton et al., 2006; Cronk et al., 2002; Eaves et al., 1997; Ehringer et al., 2006; Eley et al., 2003; Feigon et al., 2001; Ogliari et al., 2006; Silove et al., 1995). A meta-analysis of 18 twin cohorts including 31,859 subjects showed that genetic, shared and non-shared environmental factors contribute to the vulnerability to SAD and separation anxiety symptoms explaining 43%, 17% and 40% of the variance, respectively. A subsequent sex-specific analysis revealed a higher heritability for females (52%) than for males (26%) (Scaini et al., 2012).

Furthermore, several family studies found a higher frequency of SAD diagnoses among offspring of parents with panic disorder/agoraphobia, when compared to children of healthy parents (Biederman et al., 2001; Capps et al., 1996; Weissman et al., 1984). In a similar vein, 35% of the genetic variance in adult-onset panic attacks has been found to be accounted for by the genetic factor for CSAD in a sample of 1,437 twin pairs participating in the Virginia Twin Study of Adolescent Behavioral Development and those twins who later completed the Young Adult Follow-Up (YAFU) (Roberson-Nay et al., 2012). Finally, a covariation between childhood separation anxiety, hypersensitivity to carbon dioxide (CO2) and panic disorder in adulthood has been found to be largely explained by genetic factors (89%) and to a lesser degree by childhood parental loss (11%) (Battaglia et al., 2009).

* 1. **Systems and molecular genetics** 
     1. Structural genetic variation

Structural variation (duplication [dup7q11.23] or deletion [Williams syndrome]) at chromosomal region 7q11.23 can cause neurodevelopmental disorders with differential anxiety phenotypes. Williams syndrome is a rare genetic disorder characterized by pre- and post-natal growth retardation, distinctive facial features, intellectual disability, congenital heart disease and non-social anxiety (Mervis and Velleman, 2011). In a sample of 62 children with dup7q11.23, 12.9% of the children also met diagnostic criteria for SAD (Mervis et al., 2015). In a previous study, eight out of 27 children with dup7q11.23 (29.6%) were additionally diagnosed with SAD, but only 9 out of 214 children with Williams syndrome (4.2%) (Mervis et al., 2012). The authors followed subsequently developed a mouse model with decreased or increased genomic copy number of a gene from the deleted region, the general transcription factor 2I (Gtf2i [MIM 601679]): in line with the human findings, pups with additional Gtf2i copies displayed increased ultrasonic vocalizations indicating maternal separation-induced anxiety (Mervis et al., 2012).

* + 1. Oxytocin system

The oxytocin system plays a central role in prosocial behavior, bonding and attachment (Gottschalk and Domschke, 2018). In a sample of 50 adolescents with primary DSM-5 anxiety disorders, patients with SAD displayed significantly lower salivary oxytocin (OT) levels than clinically anxious youth not diagnosed with SAD, and lower salivary OT levels were associated with separation anxiety symptoms (Lebowitz et al., 2016). Greater OT response to a brief, positive youth-mother interaction was associated with categorical SAD and higher self-ratings of separation anxiety symptoms in 41 clinically anxious adolescents (Lebowitz et al., 2017). In a longitudinal sample of 127 women recruited during pregnancy and a subsample of 57 women re-assessed 3 months after delivery, lower postpartum oxytocin levels were associated with higher separation anxiety symptoms during pregnancy (Eapen et al., 2014). Reciprocally, oxytocin administration in a chicken model has been found to alleviate separation distress as measured by distress vocalizations (Panksepp, 1992).

On a genetic level (see table 1), initial mutation screening for variations in the oxytocin receptor (*OXTR*) gene in a total sample of 50 patients with major depressive disorder, bipolar I or II disorder, obsessive-compulsive disorder and panic disorder did not yield differential genotype distributions between patients additionally diagnosed with ASAD (N=36), patients without comorbid ASAD (N=14) and healthy probands (N=26) (Costa et al., 2009b). In a sample of 185 patients with unipolar or bipolar depression, the GG genotype of *OXTR* rs53576 has been linked to higher levels of adult separation anxiety (Costa et al., 2009a). In an extended sample of patients with depression (N=188), the same group found the *OXTR* rs53576 GG genotype combined with the G protein β3 subunit *Gβ3* rs5443 T allele to be associated with the presence and severity of SAD after the age of 18 years and separation anxiety in childhood (Costa et al., 2017). Another SNP in the *OXTR* gene, rs2254298, has been suggested to interact with symptoms of adult separation anxiety in the conferral of complicated grief symptoms in bereaved patients with mood and anxiety disorders (N=93) (Schiele et al., 2018). The G allele of a novel *OXTR* variant, rs968389, was associated with increased maternal separation anxiety along with decreased maternal OT response to a mother-infant interaction paradigm (Still Face Paradigm) (Mehta et al., 2016). A cumulative genetic risk score, comprising *OXTR* rs53576, rs2254298 and rs1042778, interacted with symptoms of adult separation anxiety in the moderation of maternal sensitivity in response to the Still Face Paradigm, with mothers with a higher genetic risk score and high symptoms of separation anxiety showing reduced levels of maternal sensitivity during free play with the infant, which also held true when adding in the risk information from rs968389 to the cumulative genetic risk score (Mehta et al., 2016). Finally, in a rhesus macaque model, levels of separation anxiety and arousal in response to early maternal separation were significantly modulated by a gain-of-function non-synonymous *OXTR* polymorphism (Baker et al., 2017).

* + 1. Serotonin system

In rhesus monkeys, having been raised without the mother results in increased anxious behavior and higher vulnerability to developing behavioral pathology in the face of acute and chronic separation stress in carriers of the short allele of the rh*5-HTT*LPR variant, an orthologue of the human serotonin transporter linked polymorphic region (*5-HTT*LPR; Spinelli et al., 2007). In the rhesus monkey model, adrenocorticotropic hormone (ACTH) responses to acute experimental separation were higher in s allele carriers than in l/l genotype males, independently of rearing conditions (peer-reared vs. mother-reared): this could also be observed in females, but only against the background of prior maternal separation (peer-rearing) (Barr et al., 2004).

In humans, a gene-environment interaction (GxE) between the *5-HTT*LPR SS genotype and a higher number of separation life events was discerned in 194 panic disorder patients and, on a dimensional endophenotype level, with harm avoidance in the combined sample of healthy probands and panic disorder patients (Choe et al., 2013) (see table 1).

The first and only pharmacological randomized double-blind placebo-controlled trial (RCT) in adult separation anxiety disorder suggested efficacy of vilazodone, a selective serotonin reuptake inhibitor (SSRI) and serotonin 1a (5HT1a) receptor partial agonist, in 24 ASAD patients, leading to significant symptom improvements (Schneier et al., 2017) when compared to placebo treatment.

* + 1. Opioid system

The brain opioid system is known to be involved in mother-child bonding (Panksepp et al., 1978). Several lines of evidence suggest a deficit in endogenous opioids - possibly in interaction with the serotonin system in the dorsal periaqueductal gray (Graeff, 2017) - results in heightened CO2 sensitivity and separation anxiety in panic disorder patients, particularly against the background of actual separations and losses during childhood (see Preter and Klein, 2008, 2014). Reciprocally, exogenous opioid agonists can alleviate separation distress in socially isolated puppies (Panksepp et al., 1978).

On a genetic level (see table 1), the G allele of the functional A118G polymorphism (rs1799971) in the µ-opioid receptor gene (*OPRM1*), which interacts with parental overcontrol to predict stress reactivity in children (Partington et al., 2018), was associated with separation anxiety disorder symptoms in 44 children (Boparai et al., 2018). Furthermore, the interaction of *OPRM1* genotype and lower mother-child language style matching significantly predicted separation anxiety symptoms beyond this main effect (Boparai et al., 2018). Accordingly, *Oprm1* knock-out mice showed abnormal responses to maternal separation and deficits in infant-mother attachment (Moles et al., 2004).

* + 1. Dopamine system

Subsyndromal separation anxiety symptoms have been shown to be alleviated by 12 weeks of treatment with methylphenidate, a substance increasing synaptic availability of dopamine and norepinephrine and also binding to the serotonin 1A receptor, in a sample of 42 patients aged 8-17 years with attention-deficit/hyperactivity disorder (ADHD) and symptoms of separation anxiety (Golubchik et al., 2014).

On a genetic level (see table 1), in 66 children with autism spectrum disorders a marginally significant association of the dopamine D4 receptor gene (*DRD4*) exon 3 48-bp VNTR 7-repeat allele and maternal ratings of separation anxiety was reported (Gadow et al., 2010). Also, non-clinical 1-year-old children (total N=95) carrying the *DRD4* 7-repeat allele have been observed to be at greater risk for disorganized attachment as assessed in the ‘Strange Situation Experiment’, which tests infants’ ability to cope with separation anxiety (Gervai et al., 2005; Lakatos et al., 2000). In the same sample, this effect was further enhanced by the presence of the *DRD4* rs1800955 −521T allele (Lakatos et al., 2002).

* + 1. GABA system

The mitochondrial translocator protein 18 kDA (TSPO), formerly named the peripheral-type benzodiazepine receptor (PBR), governs cholesterol translocation into mitochondria and thereby mediates the first rate-limiting step in the biosynthesis of neurosteroids, which are allosteric modulators of GABA(A) receptor function (Nothdurfter et al., 2012). Using the specific radioligand [3H] PK 11195 in 27 adult outpatients with panic disorder and 18 healthy controls, lower PBR density on platelet membranes was observed only in the subgroup of panic disorder patients which also fulfilled diagnostic criteria for comorbid SAD. Accordingly, PBR density correlated negatively with separation anxiety severity scores (Pini et al., 2005). This finding was replicated in 40 adult outpatients with major depression and 20 healthy controls, where again a reduction of platelet TSPO density mean values was found in depressed patients with comorbid ASAD symptoms only, and PBR density correlated negatively with separation anxiety severity (Chelli et al., 2008). Also in a sample of 24 outpatients with bipolar disorder compared to 14 healthy controls, only the patients with comorbid ASAD displayed lower TSPO density on platelet membranes, with separation anxiety scores again correlating negatively with individual TSPO density values (Abelli et al., 2010).

On a genetic level (see table 1), in a sample of 182 patients with unipolar or bipolar depression (80 with comorbid ASAD) and 190 healthy controls, the functional *TSPO* rs6971 A allele was found to be associated with comorbid ASAD diagnosis. Also, higher separation anxiety scores were discerned in AG heterozygotes as compared to GG genotype carriers, independent of depression diagnosis (Costa et al., 2009c).

* 1. **Neuroendocrinology**

In a sample of 56 persons having experienced acute bereavement or impending loss, Jacobs et al. reported increased urinary free cortisol output in the subgroup of probands with worsening separation anxiety within one month of the event (Jacobs et al., 1987). In female healthy probands (N=22), separation sensitivity correlated negatively with morning cortisol blood levels (Dell'Osso et al., 2012). In a sample of 57 healthy adult men, parental death during childhood was associated with higher cortisol levels as compared to those having never experienced loss or separation for more than 2 weeks from their parents, while cortisol patterns in probands who had temporarily been separated from their parents were inconclusive (Nicolson, 2004). Exposure to the ‘strange situation procedure’ (including two brief separations from the mother) elicited a significantly increased cortisol response in 152 children at 12 months of age (Tollenaar et al., 2011).

Analyzing diurnal cortisol profiles, van Hulle et al. discerned a significant and positive correlation of separation anxiety symptoms with the afternoon-evening cortisol slope in 129 8-year old children (Van Hulle et al., 2017). Furthermore, separation anxiety symptoms were observed to trend-wise predict cortisol concentrations at noon in a sample of 99 8- to 16-year-old children or adolescents with mixed anxiety disorders (Kallen et al., 2008). In contrast, in a sample of 152 children aged 8-12 years with highly comorbid mixed anxiety disorders including SAD, a low diurnal cortisol profile at noon and in the evening was discerned when compared to 200 same-aged non-clinical children (Dieleman et al., 2015).

In a psychophysiological challenge paradigm, 31 10-year-old children with SAD displayed significantly increased hypothalamic pituitary adrenal (HPA) axis activity with increased cortisol secretion in anticipation of (as well as during) a separation exposure paradigm with the mother leaving the laboratory room to another building for an uncertain period of time followed by a social exposure paradigm (the Trier Social Stress Test) (Brand et al., 2011). By contrast, Gerra et al. (2000) did not discern significant changes in ACTH or cortisol levels after psychological stress (Mental Arithmetic, Stroop Color Word Interference Task, Trier Social Stress Test) in 20 male peripubertal patients with anxiety disorders including SAD, when compared to matched healthy controls.

* 1. **Neurophysiology**

In 49 children with SAD, elevated reactivity in respiratory variability, heart rate, and *M*. corrugator supercilii activity when compared to clinical control children with anxiety disorders other than SAD and to healthy control children was observed in response to a voluntary hyperventilation task, possibly indicating difficulty with breathing regulation. However, self-report measures of anxiety and panic symptoms did not differ between groups (Kossowsky et al., 2013b). In 30 children with non-comorbid (‘pure’) anxiety disorders including SAD, generalized anxiety disorder and social phobia, only the diagnosis of SAD (N=10) significantly predicted panic symptoms in response to a 5% CO2 inhalation (Pine et al., 2000), and childhood separation anxiety has been shown to predict CO2 reactivity in adulthood (Ogliari et al., 2010). CO2 hypersensitivity has been closely linked to panic disorder vulnerability (for review see Battaglia, 2017; Leibold and Schruers, 2018), and SAD and panic disorder have been shown to share increased emotional and respiratory reactivity to CO2 as a related intermediate phenotype (Klein, 1993). For instance, in a total sample of 212 children, those with CSAD and at least one parent with panic disorder (N=13) had a three-fold higher rate of experiencing a panic attack in response to 5% CO2 inhalation, when compared to children with or without CSAD without a family history of panic disorder (Roberson-Nay et al., 2010). Applying a 35% CO2 challenge paradigm in 38 adult patients with panic disorder and 31 with ASAD without a history of panic attacks, CO2 induced panic attacks occurred more frequently in both clinical groups as compared to 40 healthy controls (Atli et al., 2012). Finally, the covariation between childhood separation anxiety, CO2 hypersensitivity and adult panic disorder has been related to a shared genetic diathesis and to childhood parental loss as a common environmental risk factor (Battaglia et al., 2009). Altered CO2 reactivity has also been observed in animal models of separation anxiety, for instance applying protocols such as repeated neonatal maternal separation or repeated cross-fostering has been shown to affect respiratory regulation and reactivity to CO2 exposure in rodents (cf. (Battaglia and Khan, 2018; Battaglia et al., 2014). Furthermore, ‘anxiety sensitivity’, that is the cognitive inclination to interpret bodily symptoms as dangerous – which has also been found to be predictive of symptomatological responses to CO2 inhalation in panic disorder (Perna et al., 2003) – was significantly elevated in patients with panic disorder and ASAD, but did not predict the occurrence of panic attacks (Atli et al., 2012). Similarly, anxiety sensitivity was found to be most strongly correlated with both panic and separation anxiety subtypes in 300 twin pairs at around 8 years of age (Waszczuk et al., 2013). In 37 patients with ASAD, separation anxiety severity was found to be inversely associated with low frequency heart rate variability at a trend-wise significance level (Milrod et al., 2016). Taken together, separation anxiety/SAD, panic disorder, CO2 sensitivity and anxiety sensitivity may thus constitute spectrum disorders or spectrum dimensions, respectively (see (Battaglia et al., 2014).

* 1. **Brain imaging**

Voxel-based morphometry in 38 medication-free adolescent patients with anxiety disorders (including SAD) and 27 healthy controls found that the patients displayed increased gray matter volumes in the dorsal anterior cingulate and decreased gray matter volumes in the inferior frontal gyrus (ventrolateral prefrontal cortex), postcentral gyrus, and cuneus/precuneus (Strawn et al., 2015).

Separation anxiety symptoms were linked to increased amygdala responses to negative emotional faces as well as, on a structural level, increased amygdala gray matter volumes in a large fMRI sample of 320 healthy adults from the Münster Neuroimaging Cohort (Redlich et al., 2015). In a longitudinal assessment of 45 children, a history of pre-school separation anxiety predicted less functional connectivity between the amygdala and the ventral prefrontal cortices and greater connectivity between the amygdala and dorsal prefrontal cortices in response to angry faces at school-age (Carpenter et al., 2015). Differential amygdala response to social-affective cues of trustworthiness predicted the severity of separation anxiety symptoms over a 2-year period in 74 youths with early deprivation experiences (Green et al., 2016), while in a sample of 41 children and adolescents with generalized anxiety disorder, social anxiety disorder and/or SAD (17.1% of the sample) greater activation of the dorsolateral prefrontal cortex and ventrolateral prefrontal cortex as well as precentral/postcentral gyri during processing of threatening faces was associated with better response to SSRI treatment and cognitive-behavioral psychotherapy (Kujawa et al., 2016).

Functional MRI of the Multisource Interference Task measuring conflict- and error-related activations in 23 adolescents with anxiety disorders including SAD (N=3) and healthy controls (N=25) revealed decreased activation of the dorsolateral prefrontal cortex in patients (Fitzgerald et al., 2013). An fMRI study on neural correlates of attachment security in 21 children discerned greater activation of the dorsolateral prefrontal cortex, the amygdala, the cingulate cortex and the striatum in securely attached children as opposed to children with lower quality of attachment (Choi et al., 2018).

In a rhesus monkey model, maternal separation was associated with activation in the right dorsolateral prefrontal cortex and ventral temporal/occipital lobes and decreased activity in the left dorsolateral prefrontal cortex (Rilling et al., 2001).

* 1. **Neuropsychology**

Anxiety disorders are characterized by cognitive biases, which may affect symptom maintenance and treatment success. With regard to SAD, cognitive biases have mostly been addressed in children with mixed results. In an eye-tracking study probing visual attention to separation anxiety-related images in 23 children with SAD and 17 non-anxious comparison children, children with SAD displayed a gaze pattern characterized by enhanced early vigilance followed by stimulus avoidance (In-Albon et al., 2010). In a follow-up study by the same group, this pattern was shown to be reversible by cognitive-behavioral therapy (In-Albon and Schneider, 2012). In response to picture stimuli depicting separation and social scenes in a forced-choice reaction time task in 72 children with SAD, 31 children with social anxiety disorder, and 42 healthy control children, children with SAD and social anxiety disorder did not interpret ambiguous pictures more negatively than non-anxious children, and no group differences emerged regarding reaction time: however, children with SAD perceived separation images as less pleasant and more arousing than did control children (In-Albon et al., 2009). In 101 children and adolescents with mixed anxiety disorders including generalized anxiety disorder, social phobia and SAD compared to 51 control children, a greater attentional bias to threatening stimuli in a visual dot-probe task was observed in clinically anxious children, though no differences between the diagnostic groups were observed (Roy et al., 2008). By contrast, in a large sample comprising 1,291 children and adolescents, attentional bias to threat as assessed using a visual dot-probe task was positively and specifically associated with symptoms of social anxiety and school phobia, but not with symptoms of separation anxiety, panic, or generalized anxiety (Abend et al., 2018). In an EEG study employing a Go/No-Go task in 139 children aged 5-8 years, parent-reported symptoms of separation anxiety were related to more No-Go errors and a smaller error-related negativity, suggesting impaired error-monitoring function in SAD (Lo et al., 2017).

1. **DISCUSSION**

Separation anxiety disorder aggregates in families and has an overall heritability of ~43%, which is higher in females than in males. A substantial proportion of SAD heritability is shared with panic disorder and sensitivity to carbon dioxide inhalation (CO2). On a molecular genetic, expression/protein and pharmacological level, the oxytocin *(OXTR* rs53576, rs2254298, rs968389, rs1042778), serotonin (*5-HTT*LPR), opioid(*OPRM1* rs1799971), dopamine (*DRD4* exon 3 48-bp VNTR; rs1800955) and GABA-related (*TSPO* rs6971) systems have been proposed to be involved in the pathogenesis of SAD. Structural genetic studies point to a potential role of the dup7q11.23 chromosomal region. A suggested dysregulation of the hypothalamus-pituitary-adrenal axis in separation anxiety seems to depend on the environmental context, developmental stage and diurnal variation. Hypersensitivity to CO2 appears to be an endophenotype common to SAD, panic disorder, and anxiety sensitivity, and is partly shared across species. On a neural network level, a dysfunctional cortico-limbic interaction has been observed to be associated with separation anxiety, with the direction of activation varying greatly on task and phenotype. Symptoms of separation anxiety may be accompanied by biased cognitive processing, however, specificity to SAD is unclear. Future studies – considering several aspects as detailed below – should aim at further disentangling the neurobiological underpinnings of separation anxiety and establishing the potential for specific diagnosis and treatment (also see figure 1).

Phenotype

Research on neurobiological mechanisms in separation anxiety greatly depends on the quality of the investigatedphenotype. Separation anxiety is mostly ascertained via self-report questionnaires. For childhood and adolescence, several general anxiety self-report measures such as the Screen for Child Anxiety-Related Emotional Disorders questionnaire (SCARED,; Birmaher et al., 1997) or the Spence Children’s Anxiety Scale (SCAS; Spence, 1997) comprise subscales addressing separation anxiety. For the specific assessment of separation anxiety symptoms, the Separation Anxiety Assessment Scale (SAAS; Eisen and Schaefer, 2005), the Separation Anxiety Avoidance Inventory (SAAI; In-Albon et al., 2013), and the Separation Anxiety Scale for Children (SASC; Mendez et al., 2008) are available. The Separation Anxiety Daily Diary (SDCC-C; Allen et al., 2010) constitutes a daily self-monitoring tool that can be used to record the daily number of anxious and non-anxious separations over an eight-day assessment period. In adult populations, the Separation Anxiety Symptom Inventory (SASI; Silove et al., 1993) allows for the retrospective assessment of separation anxiety symptoms during childhood and adolescence. Adult separation anxiety can be evaluated via the Adult Separation Anxiety Questionnaire (ASA-27; Manicavasagar et al., 2003), a 27-item self-report questionnaire that purports to examine symptoms of separation anxiety experienced after 18 years of age with a proposed cut-off total score of 22 for ASAD caseness. The Screen for Adult Anxiety Related Disorders (SCAARED; Angulo et al., 2017), an adapted version of the SCARED to assess DSM-5 anxiety disorders in adulthood, contains seven items loading on the factor “separation anxiety”. Additionally, SAD symptom severity can be assessed and monitored using the self-administered 10-item Severity Measure for Separation Anxiety Disorder according to DSM-5 criteria offered by the American Psychiatric Association (Craske et al., 2013a; Craske et al., 2013b).

With the Structured Clinical Interview for Separation Anxiety Symptoms (SCI-SAS; Cyranowski et al., 2002), a structured clinician-administered instrument assessing separation anxiety according to DSM-IV criteria is also available for administration in adult populations. The SCI-SAS comprises two subscales, one retrospectively assessing separation anxiety symptoms in childhood, and the other assessing presence of these same separation anxiety symptoms in adulthood. For each subscale, the SCI-SAS can be scored categorically according to DSM-IV-defined thresholds, or dimensionally as a measure of separation anxiety symptoms. There is, however, a pressing need for validated translations of instruments to assess dimensional and categorical separation anxiety in order to ensure comparability across study populations and the advancement of assessment instruments according to current diagnostic criteria with high discriminatory power especially given the high comorbidity and/or symptom overlap between SAD and a host of other mental disorders (see for example Baldwin et al., 2016; Bogels et al., 2013; Matthies et al., 2018).

Comorbidities

The high levels of concurrent or sequential comorbidity between SAD and panic disorder reported in the literature further complicate definition of the phenotype and raise the question whether the reported neurobiological markers reflect a symptom overlap between spectrum disorders or rather represent pathogenetic patterns underlying both SAD and panic disorder as distinct categories with, however, high levels of genuine comorbidity. In addition, the clinical distinction of SAD and separation anxiety symptoms from generalized anxiety disorder and dependent personality disorder is not a trivial process (cf. Baldwin et al., 2016), which might introduce a further phenotypical confounder to studies on the neurobiological underpinnings of separation anxiety. Future neurobiological studies employing longitudinal approaches may elucidate the common and diverging central causal mechanisms and trajectories of SAD and panic disorder.

Environmental and mediating factors

Longitudinal epidemiological studies accompanied by research focusing on biological markers, studies on gene-environment interactions (GxE),and models extending this stress-vulnerability model through the additional dimension of resilience-increasing factors (such as coping strategies or high self-efficacy, cf. Schiele et al., 2016) - are urgently warranted in order to disentangle biological and environmental factors as well as state vs. trait markers in the trajectory of separation anxiety etiology across the life span. Also, the link between potentially mediating factors such as attachment styles – particularly insecure attachment – and separation anxiety/SAD remains to be elucidated empirically. Available studies to date have associated symptoms of separation anxiety with anxious attachment in clinical populations (Manicavasagar et al., 2009; Pini et al., 2014) and in first time mothers (Kohlhoff et al., 2015), however, separation anxiety/SAD can also occur in patients with a secure attachment style (Pini et al., 2014). Parental intrusiveness (Wood, 2006), indifference, abuse and over- control (Kohlhoff et al., 2015) might be related to SAD, while evidence for actual separation experiences such as parental loss or absence is inconclusive (Bandelow et al., 2001; Battaglia et al., 2009; Battaglia et al., 2016; Cronk et al., 2004).

Genetics and gene expression

Future efforts to further unravel the genetic underpinnings of separation anxiety/SAD will have to explore further candidates such as brain derived neurotrophic factor (BDNF) (cf. Dalle Molle et al., 2012) or molecules involved in cytokinesis (cf. Mohan et al., 2012), and expand to hypothesis-free approaches such as genome-wide association studies (GWAS) in sufficiently powered samples. Also, mRNA expression studies in humans – comparable to an examination of mRNA content in amygdala tissue collected from rhesus monkeys having been separated from their mothers (Sabatini et al., 2007) – might reveal yet undiscovered mechanisms of separation anxiety.

Epigenetics

Studies on epigenetic mechanisms at the intersection between genetic vulnerability and adverse environment (Schiele and Domschke, 2018) will be instrumental in elucidating the complex-genetic pathomechanism of separation anxiety. In this regard, a first study in mice showing heightened separation anxiety and hyperventilation to 6% CO2-enriched air after repeated cross-fostering (RCF) identified differential H3Ac and H3K4me3 histone marks inter alia in the acid sensing channel 1 (*Asic1*) gene (Cittaro et al., 2016). In a translational, cross-species approach, differential DNA methylation in genes involved in chemoreception (e.g., *Asic2*/*ASIC2*) has been discerned both in a mouse model of RCF-associated intergenerational transmission of CO2 sensitivity and in monozygotic human twins discordant for emotional reactivity to CO2 challenge (Giannese et al., 2018).

Brain imaging

On a neural network level, beyond the known fear circuit revolving around the amygdala, the bed nucleus of the stria terminalis (BNST), proposed to play a pivotal role in the mediation of particularly sustained anxiety (Lebow and Chen, 2016), might constitute a promising target of investigation with respect to separation anxiety. Also, given the above mentioned prominent mediating role of CO2 sensitivity in SAD, magnetic resonance spectroscopic lactate measurement or pH-sensitive MR imaging (T1 relaxation in the rotating frame; T1ρ) – as already probed in patients with panic disorder (Maddock et al., 2013; Magnotta et al., 2014) – might reveal an altered function of acid-sensitive fear circuits also in the separation anxiety/SAD spectrum. Furthermore, neuropsychological, personality and temperamental dimensions such as attentional control, harm avoidance, intolerance of uncertainty, or behavioral inhibition related to separation anxiety (Boelen et al., 2014; Mertol and Alkin, 2012; Paulus et al., 2015; Sportel et al., 2011) might constitute promising intermediate phenotypes for the investigation into the neurobiology of separation anxiety.

Impact of lifting the age limit on neurobiological research

Lifting the age limit for the diagnosis of SAD in the DSM-5 as compared to previous nosology will help guide novel directions in neurobiological research towards a better understanding of disorder pathogenesis and course. For instance, it has to be taken into account that a dynamic role of genetic factors has been suggested in anxiety and depression phenotypes (Waszczuk et al., 2014), with a relatively high heritability in childhood, but a decreased importance of genetic factors during adulthood due to an increase in environmental variance (Nivard et al., 2015). Neuroimaging research in patients with SAD might want to explore a potentially insufficient maturation of fronto-limbic connectivity from childhood to adolescence and early adulthood (Gabard-Durnam et al. 2014), which has previously been implied in anxiety-related phenotypes (cf. Domschke et al., 2017). On a hormonal level, research into the neurobiological underpinnings of SAD in adults as opposed to children will have to take into account the influence of gonadal hormones, which might play a crucial mediating role in the interaction of developmental risk factors and biological mechanisms towards sex differences in anxiety phenotypes (cf. Jaric et al., 2019). Finally, given that adult-onset SAD prevalence peaks at childbearing and early child-rearing age (Silove et al., 2017), the role of oxytocin – pivotally involved in pregnancy, breast feeding and mother-infant bonding – warrants further investigation in ASAD (cf. 2.2.2; Eapen et al., 2014).

In conclusion, the only recently burgeoning research into its neurobiological substrates as reviewed above has opened up promising new avenues to advance the understanding of the causes of separation anxiety disorder. The identification of biological risk markers holds great promise for predicting disorder risk, maintenance and course, and in the future may permit the selection of indicated preventive and innovative, personalized therapeutic interventions.

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**Contributors**

MAS and KD managed the literature search and wrote the first version of the manuscript. All authors contributed to and have approved the final version of the manuscript.

**Conflict of Interest**

DSB is a Medical Patron and Anxiety UK and Clinical Advisor to the UK National Clinical Audit of Anxiety and Depression. All other authors declare that they have no potential conflicts of interest.

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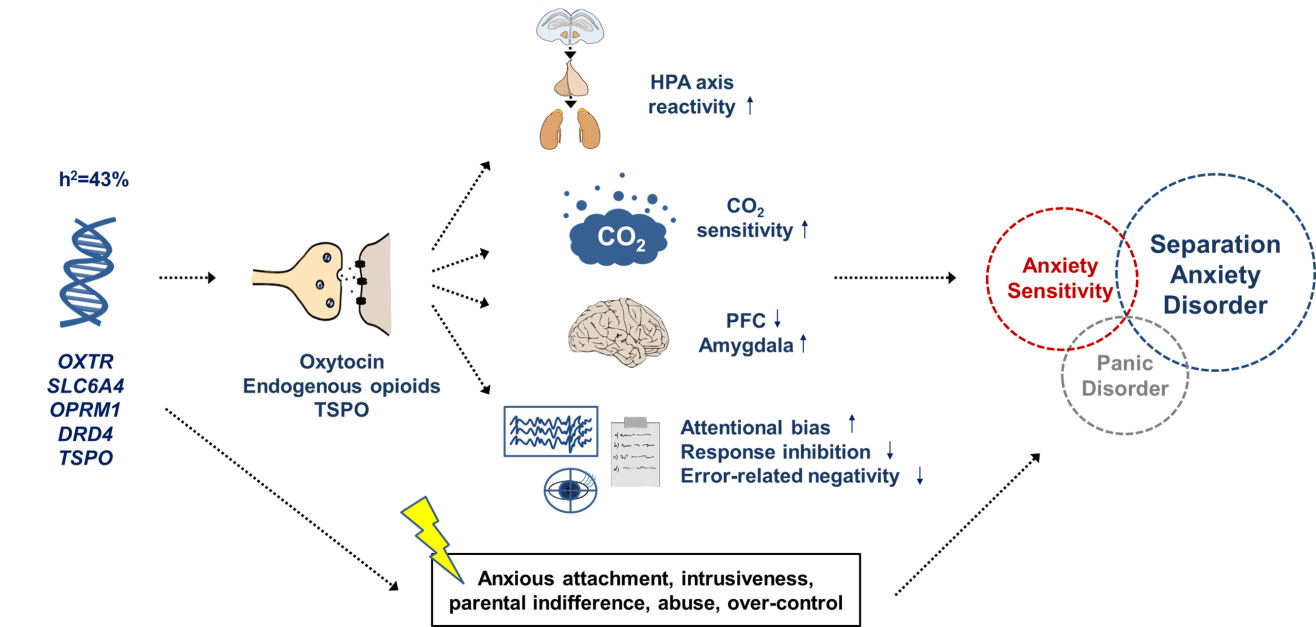
**TABLE 1**

**Human genetic association studies in separation anxiety**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Genes** | **Polymorphisms** | **Samples** | **Results** | **Studies** |
| *OXTR* | rs17339677 (3’ UTR), rs34097556 (intron 2) | 50 adult patients with MDD, bipolar I or II disorder, OCD or PD with (N=36) or without (N=14) comorbid SAD; 26 healthy probands | No association with SAD | Costa et al., 2009b |
| *OXTR* | rs53576 (6930A/G),  rs2254298 (9073A/G) | 185 adult patients with unipolar or bipolar depression; 192 healthy probands | rs53576 GG genotype associated with high separation anxiety (ASA-27) in patients with unipolar depression (N=93) | Costa et al., 2009a |
| *OXTR* | rs53576 | 188 adult patients with depression; 225 healthy probands | Combined GG genotype and G protein β3 subunit (*Gβ3*)rs5443 T allele associated with childhood and adult SAD | Costa et al.,  2017 |
| *OXTR* | rs2254298 | 93 bereaved adult patients with affective and anxiety disorders | In GG genotype carriers, higher separation anxiety scores (ASA-27) were related to increased complicated grief (ICG) | Schiele et al., 2018 |
| *OXTR* | rs968389, rs53576, rs2254298, rs1042778 | 96 women pre- and post-partum | rs968389 GG genotype associated with increased maternal separation anxiety post-partum (MSAS);  combined rs968389, rs53576, rs2254298 and rs1042778 higher cumulative genetic risk score and high separation anxiety pre-partum (ASA-27) associated with reduced maternal sensitivity post-partum | Mehta et al.,  2016 |
| *SLC6A4* | *5-HTT*LPR | 194 adult PD patients, 172 healthy probands | Interactive effect of SS genotype and a higher number of separation life events on PD diagnosis and on increased harm avoidance (HA) in the combined sample | Choe et al., 2013 |
| *OPRM1* | rs1799971 (A118G) | 44 non-clinical children | G allele associated with separation anxiety disorder symptoms in interaction with low mother-child language style matching | Boparai et al., 2018 |
| *DRD4* | Exon 3 48-bp VNTR | 66 children with autism spectrum disorders | Association of 7-repeat allele with maternal ratings of separation anxiety | Gadow et al., 2010 |
| *DRD4* | Exon 3 48-bp VNTR; rs1800955 (−521C/T) | 90/95 non-clinical 1-year-old children | VNTR 7-repeat allele associated with disorganized attachment reflecting infants’ ability to cope with separation anxiety; effect further enhanced by −521 T allele | Gervai et al., 2005; Lakatos et al., 2000; Lakatos et al., 2002 |
| *TSPO* | rs6971 (439A/G, Ala147Thr); 485A/G | 182 adult patients with unipolar or bipolar depression (80 with comorbid SAD); 190 healthy probands | 439 A allele was associated with comorbid ASAD diagnosis; 439 A/G genotype associated with higher separation anxiety scores | Costa et al., 2009c |

Legend to table 1: 3’ UTR = 3’ untranslated region; *SLC6A4* = serotonin transporter gene; 5-HTTLPR = serotonin transporter-linked polymorphic region; ASA-27 = Adult Separation Anxiety Checklist; ASAD = adult separation anxiety disorder; *DRD4 =* dopamine D4 receptor; HA = harm avoidance in Temperament and Character Inventory; ICG = Inventory of Complicated Grief; MDD = major depressive disorder; MSAS = Maternal Separation Anxiety Scale; OCD = obsessive compulsive disorder; *OPRM1 =* µ-opioid receptor gene; *OXTR* = oxytocin receptor; PD = panic disorder; SAD = separation anxiety disorder; *TSPO* = translocator protein 18 kDA.

**FIGURE 1**



Legend to figure 1: Proposed risk factor model of separation anxiety disorder. h2: heritability estimate; *OXTR*: oxytocin receptor gene; *SLC6A4*: serotonin transporter gene; *OPRM1*: µ-opioid receptor gene; *DRD4*: dopamine D4 receptor gene; *TSPO*: translocator protein; HPA axis: hypothalamic-pituitary adrenal axis; CO2: carbon dioxide; PFC: prefrontal cortex.