Cholecystokinin B receptor gene polymorphism (rs2941026) is associated with anxious personality and suicidal thoughts in a longitudinal study

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Abstract

Objectives: Cholecystokinin is a neuropeptide with a role in the neurobiology of adaptive behaviour that is implicated in anxiety disorders, while the underlying mechanisms currently remain insufficiently explained. The rs2941026 variation in the cholecystokinin B receptor gene has previously been associated with trait anxiety. Our aim was to investigate associations between the CCK$_B$ receptor gene polymorphism rs2941026 with anxiety, personality, depressiveness and suicidality in a longitudinal study of late adolescence and early adulthood. Methods: We used reports on trait and state anxiety, depressiveness and suicidal thoughts, as well as Affective Neuroscience Personality Scales, from the two birth cohorts of the Estonian Children Personality, Behaviour and Health Study. We measured associations between the *CCKBR* gene rs2941026 and anxiety-related phenotypes both longitudinally and cross-sectionally at ages 15, 18, 25 and 33. Results: Homozygosity for both alleles of the *CCKBR* rs2941026 was associated with higher trait and state anxiety in the longitudinal analysis. Cross-sectional comparisons were statistically significant at ages 18 and 25 for trait anxiety and at ages 25 and 33 for state anxiety. Higher depressiveness and suicidal thoughts were associated with the A/A genotype at age 18. Additionally, homozygosity for the A-allele was related to higher FEAR and SADNESS in the Affective Neuroscience Personality Scales. The genotype effects were more apparent in females, who displayed higher levels of negative affect overall. Conclusions: *CCKBR* genotype is persistently associated with negative affect in adolescence and young adulthood. The association of the CCKBR rs2941026 genotype with anxiety-related phenotypes is more pronounced in females.

Significant outcomes

• Cholecystokinin B receptor genotype presented a heterosis effect, where both homozygotes displayed higher anxiety in comparison with heterozygotes.

• In addition to anxiety, depressiveness and suicidal thoughts as well as Affective Neuroscience Personality Scales FEAR and SADNESS were associated with the cholecystokinin B receptor genotype.

Limitations

• Currently, the functional significance of the rs2941026 locus has not been described.

Introduction

Anxiety is based on a variety of chemical neuroanatomical processes (Panksepp, 1998), which currently remain insufficiently explained. All major neurotransmitter systems are involved in anxiety, and these are modulated by a number of neuropeptides that are co-released with more widespread transmitter molecules, especially at higher levels of neural activity, and with higher specificity of localisation (Hökfelt et al., 2018). Indeed several neuropeptides have been implicated in anxiety regulation (Panksepp and Harro, 2004; Griebel and Holmes, 2013; Gupta and Prabhavalkar, 2021). Amongst neuropeptides, cholecystokinin (CCK) attracted particular interest owing to findings that CCK receptor agonists elicited panic attacks in healthy volunteers (de Montigny, 1989; Bradwejn et al., 1991a) and more potently in patients with panic disorder (Bradwejn et al., 1990; Abelson et al., 1991; Bradwejn et al., 1991b). In parallel, anxiogenic effects were observed in animal models (Harro et al., 1990b). These effects were blocked by CCK_B receptor antagonists both in animals and humans (Harro et al., 1993). Several pharmaceutical companies developed selective non-peptide CCK_B receptor antagonists for the treatment of anxiety, but controlled trials failed to show any efficacy in the treatment of either panic disorder or generalised anxiety disorder (Adams et al., 1995; Kramer et al., 1995; Pande et al., 1999).

However, CCK has consistently been found to elicit anxiogenic like effects in primates and in rodents (Harro, 2006), and more recently in zebrafish (Matsuda et al., 2020), whereas neuropeptide Y-deficient zebrafish that display anxiety-like behaviour have higher levels of CCK gene expression (Shiozaki et al., 2020). Thus, CCK may have played an anxiety-regulating role already before water-to-land transition. In humans, panic attacks elicited by administration of CCK-4 are accompanied by activation of a variety of anxiety-related brain areas, most notably the rostral anterior cingulate cortex, but also medial prefrontal and dorsolateral prefrontal cortex (Leicht et al., 2013), a response also observed in terms of cingulate glutamate release as measured by magnetic resonance spectroscopy (Zwanzger et al., 2013).

CCK_B receptors have also been reported to contribute to anxiety-related behaviours beyond pharmacological challenges. CCK_B receptor levels are higher in suicide victims as shown independently by either receptor binding (Harro et al., 1992) or gene expression (Sherrin et al., 2004) studies. Rats with high anxiety levels in elevated plus-maze had higher baseline levels of CCK_B receptors (Harro et al., 1990a), and an increase in CCK_B receptor levels was observed in stressful conditions (Harro et al., 1996). Additionally, in rats, social stress elicited CCK release in the medial prefrontal cortex (Becker et al., 2008), and in mice, activation of CCK_B receptors in prefrontal cortex was found to be both necessary and sufficient for behavioural deficits induced by social defeat (Vialou et al., 2014). In another study on rats, administration of pentagastrin, a CCK_B receptor agonist, into the lateral amygdala impaired the recovery from conditioned fear (Erlich et al., 2012). Furthermore, experimental overexpression of CCK_B receptors was demonstrated to lead to a persistent increase in anxiety in mice (Chen et al., 2006).

While the mechanism of CCK_B receptor upregulation has not been clarified, several lines of evidence suggest that endogenous CCK release can be reducing anxiety or act as a safety signal (Wiertelak et al., 1992; Rovira-Esteban et al., 2019; Ballaz et al., 2020) and administration of CCK-8 can be stress-protective (Sadeghi et al., 2015). Such contradictory

results suggest that the significance of the CCK signal is qualitatively context-dependent. Indeed, in CCK$_B$ receptor-deficient mice, the behavioural phenotypes were not only strongly dependent on housing conditions but also differed by sex (Abramov et al., 2008). Administration of CCK-8 into the dorsal periaqueductal grey matter elicited opposite effects in anxiety tests if the location of injection site was ventrolateral versus dorsolateral (Vázguez-Le´on et al., 2018). CCK expression in the basket cells of the hippocampus was found downregulated after stress at an early age but recovered after further stress in adulthood (Gruber et al., 2015). Another study observed that optogenetic stimulation of basal amygdalar CCK-positive interneurons facilitated fear extinction, whereas photosilencing had an opposite effect (Rovira-Esteban et al., 2019).

If trait-wise high anxiety were related to the CCK system, then it would be expected to find genetic variation in genes encoding CCK prepropeptide or CCK receptors. Polymorphisms in the CCK system genes have indeed been found to be associated with several anxiety-related phenotypes. Several variants, most notably the (CT)n microsatellite in the 5' regulatory region of *CCKBR* (Kennedy et al., 1999; Binkley et al., 2001; Hösing et al., 2004), −36C/T in *CCK* (Wang et al.,1998; Maron et al., 2005; Kofoed et al., 2010), and −215C/A in *CCKBR* have been associated with panic disorder. Another short tandem repeat in the *CCK* gene 5' regulatory region together with a downstream −188A/G was less frequent in panic disorder and downregulated the transcription of CCK (Ebihara et al., 2003). Additionally, functional polymorphisms in the *CCKBR* have been observed to increase the receptor-mediated second messenger signalling of anxiolytic drugs (Kopin et al., 1997). A polymorphism in the *CCK* promoter region, −196G/A, was associated with suicidal behaviour in a Japanese sample (Shindo and Yoshioka, 2005). Another group found associations between 12 single nucleotide polymorphisms (SNP) in the *CCKBR* gene and suicide attempts (Sears et al., 2013). Thus, several variants in the CCKBR have been associated with anxiety, but the results have been inconsistent, not surprisingly so owing to different sampling strategies (Zwanzger et al., 2012). A study on procedural pain and anxiety in children found the rs2941026 in the intragenic region of the *CCKBR* gene to be associated with trait anxiety (Ersig et al., 2017). Interestingly, this work suggested that the A-allele was associated with lower trait anxiety in children but transmitted more often from parents to children with higher trait anxiety in a family-based transmission disequilibrium test. We decided to investigate this *CCKBR* variation further in a broader context of negative affectivity and in a sample highly representative of the target group. We used the measures of trait and state anxiety, but additionally included measures of depressiveness, suicidal thoughts and personality.

Methods

Study sample   This investigation is based on the Estonian Children Personality, Behaviour and Health Study (ECPBHS). The ECPBHS participants were first sampled in 1998 as part of the European Youth Heart Study (EYHS) that had school as a sampling unit using probability proportional to school size (by the number of students in respective grade); the participation rate of invited students was 79.1% (Harro et al., 2001). The EYHS was later incorporated into the longitudinal ECPBHS; at age 25, 79.2% of the original sample was retained. Thus far, data from the younger birth cohort have been collected at ages 9, 15, 18 and 25. The older birth cohort was first measured at age 15 and has received follow-ups at ages 18, 25 and 33. Details concerning follow-up data collection have been described elsewhere (Tomson-Johanson et al., 2020). Written informed consent was obtained from the study participants and

in the case of subjects under 18 years of age, from their parents. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu (license numbers: 49/30, 151/11, 197T-14, and 235/M/20) and was conducted in accordance with the Declaration of Helsinki.

Anxiety measurement   Anxiety was measured with the Estonian language version of the Spielberger State-Trait Anxiety Inventory (STAI; Spielberger et al., 1983). STAI measures two aspects of anxiety: STAI-S (state) addresses how the participant is feeling while filling in the questionnaire (in our case, during the visit to the laboratory); STAIT (trait) queries how the participant usually feels, thus addressing anxiety as a personality disposition. State anxiety (STAI-S) data were available at ages 15 and 18 for the younger birth cohort, at age 25 for both birth cohorts and at age 33 for the older birth cohort. Trait anxiety (STAI-T) data were available at age 18 for the younger birth cohort, at age 25 for both birth cohorts and at age 33 for the older birth cohort.

Affective neuroscience personality scales   An adaptation (Harro et al., 2019) of the short version of the Affective Neuroscience Personality Scales (ANPS) questionnaire (Davis et al., 2003) was used to measure personality traits. The ANPS was administered at age 25 for the younger birth cohort and age 33 for the older birth cohort.

Measures of depressiveness   Depressiveness was measured with the self-report format of the Montgomery – Åsberg Depression rating scale (MÅDRS; Montgomery and Åsberg, 1979) at ages 18 and 25 for both birth cohorts and age 33 for the older birth cohort.

Measures of suicidal thoughts   For measures of suicidal thoughts, we analysed the 'Suicidal thoughts' question from the MÅDRS questionnaire, which describes to what extent the study participants consider life worth living. To measure the frequency of suicidal thoughts at ages 15 for the younger birth cohort and at 18 for both birth cohorts, we used a question in the ECPBHS lifestyle questionnaire inquiring how often the participants experienced suicidal thoughts. In this measure, lower scores indicate experiencing suicidal thoughts more often.

Genotyping of CCKBR rs2941026   Genomic DNA was extracted from venous blood samples with the Qiagen QIAmp® DNA Blood Midi Kit. Genotyping of rs2941026 was conducted by real-time polymerase chain reaction (RT-PCR) using a TaqMan® pre-designed SNP genotyping assay (Applied Biosystems; Foster City, CA, USA) C__15866557_10, that contains specific primers and fluorescent probes. Genotyping reactions were performed in a total volume of 10 μl with approximately 25 ng of template DNA. The RT-PCR components and final concentrations were as follows: 1:5 5 × HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne) and 1:20 80 × TaqMan Primers Probe. The context sequence [VIC/FAM] was as follows: CAGAAGGAAATTTTTCTAAACTCAA[A/G]AAG GGAAAGTTGGAATAAATTGTAA. Reactions were performed on the Applied Biosystems ViiA™ 7 real-time PCR system. The amplification procedure consisted of initial denaturation at 95°C for 15 s and 60°C for 1 min. Each reaction plate included a positive and negative control. There were no inconsistencies in the genotyping results. Genotyping was performed blind to all phenotypic data.

Statistical analysis   Linear mixed-effects regression models were used to evaluate the association between the CCKBR rs2941026 genotype and anxiety, depressiveness and suicidal thoughts longitudinally. For state anxiety, the baseline was 15 years of age, and follow-up

points were at ages 18, 25 and 33. For trait anxiety, depressiveness and suicidal thoughts, the baseline was 18 years of age, and follow-up points were at ages 25 and 33. In these models, *CCKBR* rs2941026 genotype (A/A, A/G, G/G) and sex were defined as independent variables and time was treated as a continuous variable. For trait anxiety, the STAI-T score, and for state anxiety, the STAI-S score, were used as the outcome variables, respectively, and A/G heterozygotes were defined as the reference group. For depressiveness and suicidal thoughts, the MÅDRS items and item scores were used as outcome variables, and A/A homozygotes were defined as the reference group. We used a random intercept model that allows individual variations at baseline values. Unstructured variance– covariance structure and the restricted maximum likelihood method were used for model estimation. Mixed model coefficients of categorical variables can be interpreted as the conditional mean difference in outcome variable scores with respect to the reference group. The linear mixed model for age accounts for intra-individual changes in time as random effects by drawing the intercepts and slopes of individual trajectories distribution. Maximum likelihood estimation in a mixed model uses all available observations and is, therefore, more efficient than repeated-measures ANOVA, which requires an equal number of observations at all time points and a constant covariance between the time points. The coefficient of age represents the linear slope of change in the outcome variable with each additional year of age. Linear mixed model analyses were conducted in R 4.0.3. Cross-sectional analyses were carried out by Factorial Analysis of Variance (ANOVA), with *CCKBR* rs2941026 genotype and sex as independent variables. ANOVA with LSD post hoc analyses was carried out to compare the mean levels of measures on anxiety, ANPS, depressiveness and suicidal thoughts between the CCKBR genotype groups and to estimate the respective effect sizes. The statistical significance p-value cut-off for analysis was set at 0.05. Analysis of Variance was conducted on IBM SPSS Statistics 27. Data that have been used in these analyses are available from the authors upon reasonable request.

Results

CCKBR rs2941026 genotype distribution

We were able to successfully genotype 1232 subjects. The distribution of the A/A, A/G and G/G genotypes of the *CCKBR* rs2941026 were 22.5%, 50.4% and 27.1%, respectively. Genotype frequencies were in Hardy–Weinberg equilibrium. The A-allele frequency was 0.477, and the allele frequencies were in accordance with the National Centre for Biotechnology Information database (A-allele frequency 0.474 for Estonia). There was no significant difference in the distribution of genotype frequencies between male (A/A = 122, 21.6%; AG = 281, 49.6%; GG = 163, 28.8%) and female (A/A = 155, 23.3%; A/G = 340, 51.1%; G/G = 171, 25.7%) subjects (χ^2 test, p = 0.44). Trait and state anxiety Longitudinally, trait anxiety decreased at a rate of 0.33 points (95% CI [0.26, 0.40]) and state anxiety at a rate of 0.45 points (95% CI [0.37, 0.51]) per year. Females had higher scores for trait and state anxiety (Fig. 1). Linear mixed-effects regression models revealed that both A/A and G/G homozygotes had higher anxiety as compared to heterozygotes (Table 1).

Table 1. Estimated main effects (mean and 95% CI) of the ECPBHS sample in anxiety measurements between *CCKBR* rs2041026 genotype from 18 to 33 years of age for trait anxiety and 15 to 33 years of age for state anxiety according to the linear mixed effects regression model.

	Coefficient	95% CI	*p*-value
Trait anxiety			
A/A	2.33	1.07; 3.60	<0.001
G/G	1.51	0.31; 2.71	<0.05
State anxiety			
A/A	1.83	0.81; 2.84	<0.001
G/G	1.10	0.14; 2.06	<0.05

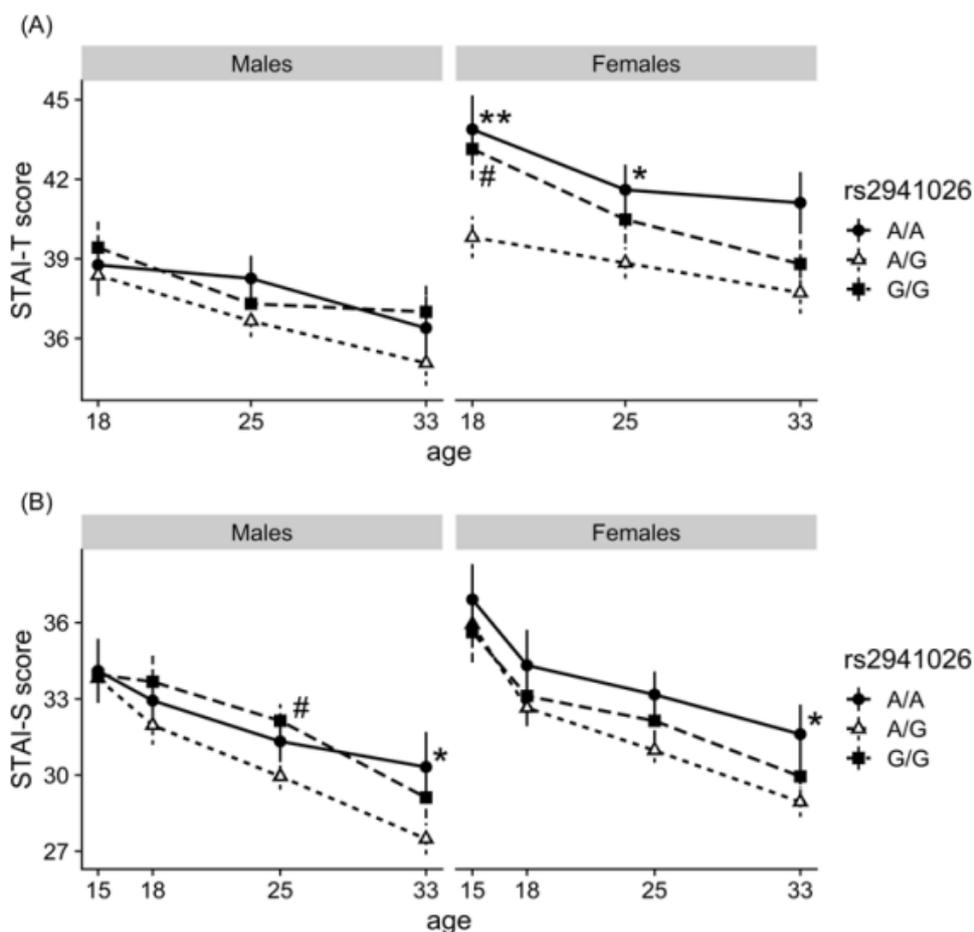Here and elsewhere, A/G genotype was used as the reference group.

Fig. 1. An association between *CCKBR* rs2941026 genotype and trait anxiety scores (A) from 18 to 33 years of age in male and female subjects and an association between *CCKBR* rs2941026 genotype and state anxiety (B) scores from 15 to 33 years of age in male and female subjects. * - Statistically significant differences of the A/A genotype group from the A/G genotype group cross-sectionally at the corresponding age. # - Statistically significant differences of the G/G genotype group from the A/G genotype group cross-sectionally at the corresponding age.

Cross-sectionally at ages 18, 25 and 33, associations between trait anxiety and the *CCKBR* genotype were statistically significant at ages 18 and 25, and there was a trend of association at age 33 (Table 2). At age 18, both homozygotes had higher scores for trait anxiety as compared to heterozygotes. At age 25, only the difference between A/A homozygotes and A/G heterozygotes remained statistically significant. The effects of *CCKBR* on trait anxiety derived from females, but the interaction between the genotype and sex was not statistically significant.

Table 2. Anxiety scores (mean and SD) at the ages of 15, 18, 25 and 33 of the ECPBHS sample by *CCKBR* rs2941026 genotype and sex (n).

	Genotype effects		Genotype by sex effects		
	Statistics		Statistics	Males	Females
Trait anxiety					
18[a]	$F_{(2, 441)} = 3.68$	A/A = 41.7 ± 9.1 (100)*	$F_{(2, 441)} = 1.77$	A/A = 38.8 ± 7.3 (43)	A/A = 43.9 ± 9.7 (57)**
	$p = 0.03$	A/G = 39.2 ± 8.4 (225)	$p = 0.17$	A/G = 38.4 ± 7.6 (96)	A/G = 39.8 ± 9.0 (129)
	$\eta^2 = 0.016$	G/G = 41.4 ± 8.7 (122)*	$\eta^2 = 0.008$	G/G = 39.4 ± 7.4 (57)	G/G = 43.1 ± 9.4 (65)*
25	$F_{(2, 956)} = 4.09$	A/A = 40.3 ± 10.0 (222)*	$F_{(2, 956)} = 0.37$	A/A = 38.3 ± 8.1 (89)	A/A = 41.6 ± 11.0 (133)*
	$p = 0.02$	A/G = 37.9 ± 9.4 (482)	$p = 0.69$	A/G = 36.7 ± 8.7 (205)	A/G = 38.8 ± 9.8 (277)
	$\eta^2 = 0.008$	G/G = 39.0 ± 9.6 (258)	$\eta^2 = 0.001$	G/G = 37.3 ± 8.0 (118)	G/G = 40.5 ± 10.7 (140)
33[b]	$F_{(2, 496)} = 2.87$	A/A = 39.4 ± 9.6 (118)	$F_{(2, 496)} = 0.81$	A/A = 36.4 ± 8.0 (44)	A/A = 41.1 ± 10.0 (74)
	$p = 0.06$	A/G = 36.6 ± 9.3 (248)	$p = 0.44$	A/G = 35.1 ± 8.8 (107)	A/G = 37.7 ± 9.4 (141)
	$\eta^2 = 0.011$	G/G = 38.0 ± 8.9 (136)	$\eta^2 = 0.003$	G/G = 37.0 ± 7.9 (60)	G/G = 38.8 ± 9.5 (76)
State anxiety					
15[a]	$F_{(2, 456)} = 0.23$	A/A = 35.8 ± 10.0 (103)	$F_{(2, 456)} = 0.12$	A/A = 34.1 ± 8.0 (40)	A/A = 36.9 ± 11.0 (63)
	$p = 0.80$	A/G = 35.0 ± 8.2 (232)	$p = 0.89$	A/G = 33.8 ± 7.3 (103)	A/G = 35.9 ± 8.8 (129)
	$\eta^2 = 0.001$	G/G = 34.8 ± 8.5 (127)	$\eta^2 = 0.001$	G/G = 34.0 ± 7.6 (66)	G/G = 35.6 ± 0.3 (61)
18[a]	$F_{(2, 441)} = 1.14$	A/A = 33.7 ± 9.3 (101)	$F_{(2, 441)} = 0.39$	A/A = 32.9 ± 7.5 (44)	A/A = 34.3 ± 10.6 (57)
	$p = 0.32$	A/G = 32.4 ± 7.9 (225)	$p = 0.68$	A/G = 32.0 ± 7.6 (96)	A/G = 32.6 ± 8.2 (129)
	$\eta^2 = 0.005$	G/G = 33.4 ± 8.3 (121)	$\eta^2 = 0.002$	G/G = 33.7 ± 7.8 (58)	G/G = 33.1 ± 8.8 (63)
25	$F_{(2, 956)} = 5.08$	A/A = 32.4 ± 9.4 (221)*	$F_{(2, 956)} = 0.73$	A/A = 31.3 ± 7.7 (89)	A/A = 33.2 ± 10.4 (132)
	$p = 0.006$	A/G = 30.5 ± 7.9 (482)	$p = 0.48$	A/G = 30.0 ± 7.5 (205)	A/G = 31.0 ± 8.1 (277)
	$\eta^2 = 0.011$	G/G = 32.1 ± 8.1 (259)*	$\eta^2 = 0.002$	G/G = 32.1 ± 7.1 (119)*	G/G = 32.1 ± 8.9 (140)
33[b]	$F_{(2, 495)} = 5.02$	A/A = 31.1 ± 9.7 (118)*	$F_{(2, 495)} = 0.07$	A/A = 30.3 ± 9.2 (44)*	A/A = 31.6 ± 10.0 (74)*
	$p = 0.007$	A/G = 28.3 ± 6.8 (247)	$p = 0.93$	A/G = 27.5 ± 6.4 (106)	A/G = 28.9 ± 7.1 (141)
	$\eta^2 = 0.020$	G/G = 29.6 ± 7.2 (136)	$\eta^2 = 0.000$	G/G = 29.1 ± 6.4 (60)	G/G = 30.0 ± 7.8 (76)

*p < 0.05, **p < 0.01 significant difference as compared to the mean value of the CCKBR rs2941026 A/G genotype by the LSD method.
a Data available for the younger cohort only.
b Data available for the older cohort only.

State anxiety was cross-sectionally analysed at ages 15, 18, 25 and 33 (Table 2). Genotype effects on state anxiety emerged at ages 25 and 33. At age 25, both homozygotes had higher state anxiety as compared to heterozygotes. By age 33, only the difference between A/A homozygotes and heterozygotes remained statistically significant. For state anxiety, genotype effects are derived from males as well as females.

The affective neuroscience personality scales

The *CCKBR* genotype was associated with FEAR and SADNESS, and there was a trend of association with ANGER in the ANPS (Table 3). Genotype-wise, A/A homozygotes had statistically significantly higher scores as compared to heterozygotes and G/G homozygotes.

As with trait anxiety, the genotype effects derived from females, yet there was no statistically significant interaction effect between genotype and sex.

Table 3. The effect of *CCKBR* genotype on the affective Neuroscience personality scales

	Genotype effects		Genotype by sex effects		
	Statistics		Statistics	Males	Females
ANGER	$F(2, 927) = 2.63$	A/A = 17.3 ± 4.6	$F(2, 927) = 0.19$	A/A = 16.7 ± 4.1	A/A = 17.7 ± 4.9
	$p = 0.07$	A/G = 16.4 ± 4.5	$p = 0.82$	A/G = 15.9 ± 4.5	A/G = 16.8 ± 4.5
	$\eta^2 = 0.006$	G/G = 16.4 ± 4.6	$\eta^2 = 0.000$	G/G = 16.2 ± 4.7	G/G = 16.7 ± 4.6
FEAR	$F(2, 922) = 3.45$	A/A = 17.2 ± 3.4	$F(2, 922) = 0.78$	A/A = 16.0 ± 3.0	A/A = 17.9 ± 3.4
	$p = 0.03$	A/G = 16.3 ± 3.3**	$p = 0.46$	A/G = 15.6 ± 3.1	A/G = 16.9 ± 3.3**
	$\eta^2 = 0.007$	G/G = 16.5 ± 3.3*	$\eta^2 = 0.002$	G/G = 15.7 ± 3.1	G/G = 17.1 ± 3.3*
SADNESS	$F(2, 922) = 3.85$	A/A = 15.2 ± 4.8	$F(2, 922) = 0.54$	A/A = 13.7 ± 4.0	A/A = 16.2 ± 5.0
	$p = 0.02$	A/G = 14.0 ± 4.6**	$p = 0.58$	A/G = 12.9 ± 4.3	A/G = 14.9 ± 4.6**
	$\eta^2 = 0.008$	G/G = 14.4 ± 4.7*	$\eta^2 = 0.001$	G/G = 12.9 ± 3.8	G/G = 15.6 ± 4.9
SEEKING	$F(2, 923) = 0.14$	A/A = 23.1 ± 3.4	$F(2, 923) = 0.08$	A/A = 23.1 ± 3.3	A/A = 23.1 ± 3.4
	$p = 0.87$	A/G = 23.0 ± 3.6	$p = 0.93$	A/G = 22.9 ± 3.6	A/G = 23.1 ± 3.6
	$\eta^2 = 0.000$	G/G = 22.9 ± 3.6	$\eta^2 = 0.000$	G/G = 22.8 ± 3.6	G/G = 23.0 ± 3.6
CARE	$F(2, 926) = 0.58$	A/A = 21.84 ± 3.8	$F(2, 926) = 1.74$	A/A = 20.4 ± 3.7	A/A = 22.8 ± 3.7
	$p = 0.56$	A/G = 21.9 ± 3.9	$p = 0.18$	A/G = 20.5 ± 4.1	A/G = 22.9 ± 3.5
	$\eta^2 = 0.001$	G/G = 21.6 ± 4.0	$\eta^2 = 0.004$	G/G = 19.7 ± 3.7	G/G = 23.1 ± 3.5
PLAY	$F(2, 930) = 0.61$	A/A = 22.2 ± 3.8	$F(2, 930) = 0.15$	A/A = 21.8 ± 3.6	A/A = 22.4 ± 4.0
	$p = 0.54$	A/G = 22.5 ± 4.1	$p = 0.87$	A/G = 22.4 ± 4.0	A/G = 22.6 ± 4.1
	$\eta^2 = 0.001$	G/G = 22.4 ± 4.1	$\eta^2 = 0.000$	G/G = 22.1 ± 3.9	G/G = 22.6 ± 4.2

Mean ± SD * p < 0.05, **p < 0.01 difference from A/A genotype in the same sex.
Genotype frequencies: Males, A/A = 84, A/G = 202, G/G = 114; Females, A/A = 133, A/G = 262, G/G = 139; Total, A/A = 217, A/G = 463, G/G = 253.

Table 4. Estimated main effects (mean and 95% CI) of the ECPBHS sample in depressiveness and suicidal thoughts measurements between *CCKBR* rs2041026 genotype from 18 to 33 years of age according to the linear mixed effects regression model

	Coefficient	95% CI	*p*-value
Depressiveness			
A/G	−1.03	−1.76; −0.30	<0.01
G/G	−0.84	−1.66; −0.01	<0.05
Suicidal thoughts			
A/G	−0.15	−0.26; −0.04	<0.05
G/G	−0.16	−0.29; −0.03	<0.05

Depressiveness

Longitudinally, depressiveness scores decreased by 0.11 points (95% CI [0.08, 0.15]) per year. Female participants had higher depressiveness (Fig. 2). Genotype-wise, according to the mixedeffects regression model, A-allele homozygotes had higher depressiveness as compared to heterozygotes and G-allele homozygotes (Table 4). Cross-sectionally at ages 18, 25 and 33, the associations between depressiveness and the *CCKBR* genotype were statistically significant at age 18, where A/A homozygotes had the highest depressiveness (Table 5). This
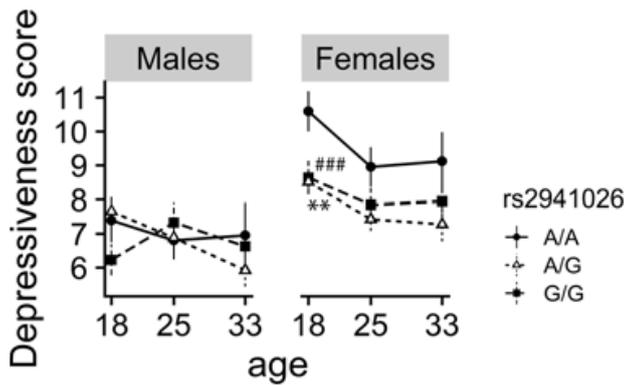
Fig. 2. An association between *CCKBR* rs2941026 genotype and depressiveness score from 18 to 33 years of age in male and female subjects. * - Statistically significant difference of the A/G genotype group from the A/A genotype group cross-sectionally at the corresponding age. # - Statistically significant difference of the G/G genotype group from the A/A genotype group cross-sectionally at the corresponding age.

genotype effect was derived from females, and for this outcome, an interaction effect between genotype and sex was statistically significant. Suicidal thoughts Longitudinally, the extent of suicidal thoughts decreased at a rate of 0.03 points (95% CI [0.03, 0.04]) per year. There was a tendency for females to report a greater extent of suicidal thoughts (Fig. 3). We could see in the linear mixed-effects regression models, that heterozygotes and G-allele homozygotes experienced suicidal thoughts to a lesser extent as compared to A-allele homozygotes (Table 4). Cross-sectional analysis revealed a significant association with the prevalence of suicidal thoughts at age 18, where A/A homozygotes had the highest extent of suicidal ideation as compared to the other two *CCKBR* genotypes (Table 5). There was no association at ages 25 or 33. This effect emerged from females, and even though not statistically significant, a trend of association can be seen for the interaction between genotype and sex. Cross-sectional analysis on the frequency of suicidal thoughts at ages 15 and 18 revealed statistical significance for association with the genotype at age 18, where A/A homozygotes reported having suicidal thoughts more often as compared to heterozygotes and G/G homozygotes (Table 5). This effect derived from females, and there was an interaction effect between genotype and sex.
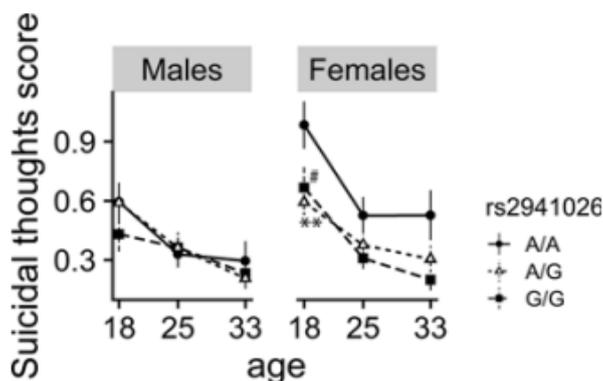

Fig. 3. An association between *CCKBR* rs2941026 genotype and frequency of suicidal thoughts from 18 to 33 years of age in male and female subjects. * - Statistically significant difference of the A/G genotype group from the A/A genotype group cross-sectionally at the corresponding age. # - Statistically significant difference of the G/G genotype group from the A/A genotype group cross-sectionally at the corresponding age.

Table 5. Depressiveness scores (mean and SD) at ages 18, 25 and 33 of the ECPBHS sample by *CCKBR* rs2941026 genotype and sex (n)

		Genotype effects		Genotype by sex effects		
		Statistics		Statistics	Males	Females
Depressiveness						
18	$F(2, 872) = 3.98$	A/A = 9.29 ± 6.50 (207)	$F(2, 872) = 3.28$	A/A = 7.39 ± 5.94 (84)	A/A = 10.59 ± 6.56 (123)
	$p = 0.02$	A/G = 8.15 ± 5.72 (440)*	$p = 0.04$	A/G = 7.66 ± 5.73 (190)	A/G = 8.52 ± 5.69 (250)**
	$\eta^2 = 0.009$	G/G = 7.50 ± 5.29 (231)**	$\eta^2 = 0.007$	G/G = 6.23 ± 4.80 (109)	G/G = 8.64 ± 5.46 (122)***
25	$F(2, 963) = 1.25$	A/A = 8.10 ± 6.26 (220)	$F(2, 963) = 1.56$	A/A = 6.81 ± 5.31 (88)	A/A = 8.95 ± 6.71 (132)
	$P = 0.29$	A/G = 7.19 ± 5.58 (486)	$P = 0.21$	A/G = 6.88 ± 5.39 (207)	A/G = 7.43 ± 5.71 (279)
	$\eta^2 = 0.003$	G/G = 7.61 ± 6.07 (263)	$\eta^2 = 0.003$	G/G = 7.33 ± 6.32 (121)	G/G = 7.85 ± 5.86 (142)
33[b]	$F(2, 495) = 2.37$	A/A = 8.31 ± 7.05 (118)	$F(2, 495) = 0.21$	A/A = 6.95 ± 6.33 (44)	A/A = 9.12 ± 7.37 (74)
	$p = 0.10$	A/G = 6.70 ± 5.60 (248)	$p = 0.81$	A/G = 5.92 ± 4.97 (106)	A/G = 7.28 ± 5.98 (142)
	$\eta^2 = 0.009$	G/G = 7.37 ± 5.28 (135)	$\eta^2 = 0.001$	G/G = 6.63 ± 5.20 (60)	G/G = 7.95 ± 5.30 (75)
Severity of suicidal thoughts						
18	$F(2, 872) = 3.12$	A/A = 0.83 ± 1.18 (207)	$F(2, 872) = 2.41$	A/A = 0.60 ± 0.88 (84)	A/A = 0.98 ± 1.32 (123)
	$p = 0.045$	A/G = 0.59 ± 1.01 (439)*	$p = 0.09$	A/G = 0.59 ± 1.05 (251)	A/G = 0.59 ± 0.96 (188)**
	$\eta^2 = 0.007$	G/G = 0.56 ± 1.11 (232)**	$\eta^2 = 0.006$	G/G = 0.43 ± 0.93 (109)	G/G = 0.67 ± 1.24 (123)*
25	$F(2, 964) = 0.76$	A/A = 0.45 ± 0.93 (221)	$F(2, 964) = 1.49$	A/A = 0.33 ± 0.64 (88)	A/A = 0.53 ± 1.07 (133)
	$P = 0.47$	A/G = 0.37 ± 0.79 (486)	$p = 0.23$	A/G = 0.36 ± 0.76 (207)	A/G = 0.38 ± 0.80 (279)
	$\eta^2 = 0.002$	G/G = 0.33 ± 0.74 (263)	$\eta^2 = 0.003$	GG = 0.36 ± 0.84 (121)	G/G = 0.31 ± 0.65 (142)
33[b]	$F(2, 494) = 2.49$	A/A = 0.44 ± 0.96 (118)	$F(2, 494) = 1.03$	A/A = 0.30 ± 0.67 (44)	A/A = 0.53 ± 1.09 (74)
	$P = 0.08$	A/G = 0.26 ± 0.70 (247)	$P = 0.36$	A/G = 0.21 ± 0.55 (10)6	A/G = 0.30 ± 0.79 (141)
	$\eta^2 = 0.010$	G/G = 0.21 ± 0.48 (135)	$\eta^2 = 0.004$	G/G = 0.23 ± 0.50 (60)	G/G = 0.20 ± 0.45 (75)
Frequency of suicidal thoughts						
15[a]	$F(2, 469) = 1.75$	A/A = 3.47 ± 0.91 (103)	$F(2, 469) = 0.87$	A/A = 3.85 ± 0.59 (39)	A/A = 3.23 ± 0.99 (64)
	$p = 0.18$	A/G = 3.54 ± 0.77 (241)	$p = 0.42$	A/G = 3.73 ± 0.56 (109)	A/G = 3.37 ± 0.88 (132)
	$\eta^2 = 0.007$	G/G = 3.40 ± 0.94 (131)	$\eta^2 = 0.004$	G/G = 3.64 ± 0.75 (67)	G/G = 3.14 ± 1.05 (64)
18	$F(2, 887) = 2.70$	A/A = 3.33 ± 0.89 (209)	$F(2, 887) = 4.33$	A/A = 3.65 ± 0.67 (86)	A/A = 3.11 ± 0.97 (123)
	$P = 0.07$	A/G = 3.52 ± 0.76 (447)	$p = 0.01$	A/G = 3.61 ± 0.67 (189)	A/G = 3.46 ± 0.82 (258)**
	$\eta^2 = 0.006$	G/G = 3.50 ± 0.82 (237)	$\eta^2 = 0.010$	G/G = 3.66 ± 0.63 (112)	G/G = 3.35 ± 0.91 (125)*

Mean ± SD * p < 0.05; **p < 0.01; ***p < 0.001 significant difference as compared to the mean value of the CCKBR rs2941026 A/A genotype by the LSD method.
a Data available only for the younger birth cohort.
b Data available only for the older birth cohort.

Discussion

This study demonstrates a consistent association of a variation in the *CCKBR* gene (rs2941026) with anxiety, anxiety-related aspects of personality, depressiveness and suicidal thoughts. These associations were similarly persistent in young adulthood and more robust in females. A previous study (Ersig et al., 2017) has reported that the A-allele of the CCKBR rs2941026 was associated with trait anxiety, and indeed we have found that A/A homozygotes had significantly higher trait anxiety levels, while the A/G heterozygotes had the lowest trait anxiety. In this birth cohort representative longitudinal sample, we also observed that A-allele homozygosity led to higher and heterozygosity to lower levels of anxiety-related personality traits, depressiveness and suicidal thoughts. Longitudinally, the levels of trait and state anxiety, as well as depressiveness and suicidality, decreased in both males and females. There is currently limited knowledge on the longitudinal nature of anxiety-related traits, but some evidence does exist for anxiety and depression to decrease with age (Jorm, 2000).

In this study, females reported higher levels of negative affectivity-related traits. Both population-based and clinical evidence shows that women report higher anxiety and depressiveness (McLean and Anderson, 2009; Parker and Brotchie, 2010). As for ANPS, females have previously been shown to score higher on the anxiety-related FEAR and SADNESS scales (Pingault et al., 2012) and while the rate of completed suicide is higher in males (Canetto and Sakinofsky, 1998), a meta-analysis conducted in 2019 on adolescents and young adults concluded that females present a higher risk for suicide attempt (Miranda-Mendizabal et al., 2019). It has been proposed that female vulnerability for the onset of anxiety and depression is regulated by sex-specific genetic risk factors (Altemus et al., 2014). In this regard, it is noteworthy that the associations observed between *CCKBR* rs2941026 and anxiety-related traits were more pronounced in females. Indeed, both age and sex are factors associated with the state of the CCK system. For example, differences between the sexes in anxiety development have been described in mice, where females had stronger fear conditioning and more generalisation of context (Keiser et al., 2017), whereas males may have higher CCK upregulation in response to stress (Karisetty et al., 2017). Also in studies on rats, CCK concentrations were significantly higher in males and increased with age in both sexes, while the levels of mRNA showed a decrease in older male rats (Miyasaka et al., 1995), as does the CCK-B receptor binding (Harro and Oreland, 1992). Findings in humans have been similar (Flint et al., 2002). These age-related alterations are unlikely to be related to neurodegeneration as neither CCK levels nor CCK-B receptor binding was found to be changed in Alzheimer's disease (Löfberg et al., 1996).

The measure of trait anxiety is part of a broader concept of negative affectivity, which includes anxiety- and depression-related internalising phenotypes (Knowles and Olatunji, 2020). In addition to trait anxiety, we observed an association with state anxiety, which was not found in the previous study concerning *CCKBR* rs2941026. One study in mice has observed that a CCKB antagonist reduced state but not trait anxiety (Belzung et al., 1994); however, the differential involvement of the CCK system in trait and state anxiety has overall not been extensively explored. The general interconnections between anxiety, depression and the ANPS have however been described previously (Montag et al, 2017; Fuchshuber et al., 2019). Trait anxiety is in strong association with both SADNESS and FEAR scales of the ANPS (Davis et al., 2003). Given the known association of the CCK system with panic disorder, it may be worth pointing out that the original denomination for the underlying neural circuit of the SADNESS scale was PANIC, referring to the neurophysiology of fear of social loss (Panksepp and Watt, 2011). To the best of our knowledge, no research has explicitly studied the association between CCK system gene polymorphisms and personality dimensions. Personality has however been observed to impact CCK receptor agonist sensitivity. Social introversion has been shown as a predictor for CCK-4 induced panic in patients with panic disorder (Koszycki et al., 1996), and aggression and suspicion were found to predict discomfort as a response to a low dose of CCK-4 administration (Radu et al., 2003). Lack of assertiveness, detachment, embitterment, verbal aggression, physical aggression, irritability, somatic anxiety and stress susceptibility has also been found to predict CCK-4 induced panic response in healthy volunteers (Tõru et al., 2010).

Interestingly, the CCK system gene variants have not been identified by any anxiety-related genome-wide association studies (GWAS) despite several anxiety-related GWAS having reached sample sizes of up to hundreds of thousands of individuals (Liet al. 2018; Levey et al. 2020; Hill et al., 2020; Wendt et al., 2021). In the birth cohort representative samples

investigated herewith, the *CCKBR* rs2941026 heterozygotes had the lowest level of anxiety. The heterosis effect observed herein proposes certain variations in the CCK system as possibly anxiety-protective. The dichotomous nature of the CCK system in the emergence of anxiety has been suggested previously. CCK_B receptor blockade in itself is rather behaviourally activating than anxiolytic (Harro, 2006), and can alter adaptation to novelty in a manner that is distinct in subjects with different baseline expressions of novelty-related traits (Ballaz et al., 2007). While several recent studies have confirmed that higher CCK-ergic activity can be related to higher anxiety, other experiments have continued to provide evidence of CCK as a safety signal. For example, recently, the anxiety-like behaviour in Otsuka Long-Evans Tokushima fatty rats was linked to higher densities of CCK-positive neurons in several brain areas (Ochi et al., 2020), and in a study of stress resilience, sensitive animals in a model of post-traumatic stress disorder developed higher immediate-early gene expression in CCK-positive interneurons in hippocampus and basolateral amygdala (Regev-Tsur et al., 2020). In contrast, in experiments with rats, freezing behaviour on exposure to a cat was reversed by a CCK_B receptor agonist (Farook et al., 2004), and in another study, CCK-4 administration had an anxiolytic-like effect specifically in extensively handled animals (Greisen et al., 2005). The role of CCK in anxiety states may depend on the context and previous experiences. Interestingly, CCK mRNA expression was shown to emerge in many nerve cells following auditory stimulation with conspecific song in zebra finches (Lovell and Mello, 2011). In rats, learned fear (but not confrontation with the resident in learned safety) increased CCK release in the prefrontal cortex (Becker et al., 2001), yet friendly social interactions in the resident-intruder tests led to relatively lower CCK levels in the brain of the intruders (Panksepp et al., 2004). Selectively in mice susceptible to social stress, social defeat caused ΔFosB induction in the medial prefrontal cortex, and viral overexpression of ΔFosB in the prelimbic area rendered mice more susceptible to social defeat (Vialou et al., 2014). In resilient mice, social defeat downregulated CCK_B receptor mRNA, while overexpression of ΔFosB upregulated CCK_B receptor mRNA expression. Further experiments with local infusion of CCK-8 or a CCK_B receptor antagonist and optogenetic manipulation of specific prelimbic projection neurons have suggested that CCK_B receptors mediate an anxiogenic-like and depressogenic-like effect via separate projections, to basolateral amygdala and nucleus accumbens, respectively (Vialou et al., 2014). CCK, via CCK_B receptors, has a direct excitatory effect on GABA-ergic interneurons in the basolateral amygdala (Chung and Moore, 2007). More recent studies suggest that in this brain region, neurons that express CCK_B receptors comprise a molecularly heterogenous population of excitatory and inhibitory neurons (Bowers and Ressler, 2015), and further research should establish whether and how CCK_B receptor-mediated regulation of the balance between excitation and inhibition in the amygdala is driven by context.

The sample of the ECPBHS consists of individuals of European descent, hence the described associations might not be found in other ethnicities. The previously reported association of *CCKBR* rs2941026 with trait anxiety was found in a study that was based on White/non-Hispanic individuals in Midwestern USA (Ersig et al., 2017). Another factor in the interpretation of the present findings is that the functional meaning of the intronic *CCKBR* rs2941026 variant has not been described, yet intronic region polymorphisms are known for having an effect on gene expression or being associated with other regulatory polymorphisms (Cooper, 2010). Indeed the fine tuning of CCK_B receptor expression related to anxiety can be speculated as regulated by non-coding RNAs in the intronic region (e.g. Rearick et al., 2011;

Mills et al., 2016) that could explain the heterosis effect. The size of observed effects is modest and bears no immediate consequence for clinical practice. Yet, for a single SNP, these effects are substantial and highlight the significance of CCK neurotransmission in anxiety in this welldefined population. The GTEx Portal does not report the *CCKBR* rs2941026 polymorphism as resulting in a significant difference in gene expression in the brain (https://gtexportal.org/ home/snp/rs2941026). This indeed would not be expected to be found with a polymorphism for which a heterosis effect is predominant. However, the *CCKBR* rs2941026 A-allele that was somewhat more anxiety-related in this study was found related to higher expression of *CCKBR* in a few tissues, most significantly in the skin. This suggests a functionality of this variation, but its nature remains to be clarified.

Conclusively, we report that a variation in the CCK_B receptor gene is persistently associated with trait as well as state anxiety, anxious personality, depressiveness and suicidal thoughts in young adults, these associations being more prominent in females. These results further support a role of the neuronal CCK system in anxiety regulation.

Author contributions.

AL designed and wrote the manuscript and along with DM analysed and interpreted the data. DM, TK and TV critically reviewed and edited the manuscript. JH designed, wrote and edited the manuscript. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest.

None.

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