Cerebral Responses to Emotional Expressions and the Development of Social Anxiety Disorder: a Preliminary Longitudinal Study

(Running: *Social phobia and emotions processing*)

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Abstract

Background: Cross-sectional studies report biased reactivity to facial expressions among shy children, anxious adolescents, and adults with social anxiety disorder. It remains unknown whether cerebral reactivity to facial expressions can predict longitudinally the development of social anxiety disorder in adolescence, and characterize the degree of social anxiety among general population adolescents.

Methods: In a longitudinal study of twenty-one general population volunteers characterized for behavioural and genetic variables, N400 event-related potentials in response to happy/neutral/angry expressions, and 3-Tesla fMRI activations were acquired at age 8-9 and 14-15, respectively.

Results: By stepwise regression, N400 amplitudes acquired at age 8-9 predicted the number of DSM-IV social anxiety disorder symptoms at age 14-15, with the sole, significant (P=0.018) contribution of the 'anger' condition. Factorial ANOVA revealed increased (Voxel-Level $P_{(FWE)}$-range:0.02-0.0001) bilateral fMRI activations of several brain areas, including the amygdala, in response to facial expressions compared to a fixation cross. The number of symptoms of DSM-IV social anxiety disorder was positively correlated with left amygdala response to angry ($P_{(FWE)}$=0.036) and neutral ($P_{(FWE)}$=0.025) facial expressions. Factorial ANOVA revealed that the 5-HTTLPR -S allele was associated with heightened left amygdala response to anger ($P_{(FWE)}$=0.05).

Conclusions: Cerebral reactivity to facial expressions -anger especially- measured at different developmental stages by different techniques is associated with adolescence social anxiety disorder. The 5-HTTLPR genotype affects the neural processing of interpersonal affective stimuli during development.

INTRODUCTION

Social anxiety disorder (SAD) is common in the population. While some childhood temperamental features -encompassing withdrawal, shyness and behavioral inhibition (BI)- are described amongst its possible behavioral antecedents,[1] the onset of full-blown SAD typically peaks around adolescence,[2] followed by considerable diagnostic stability.[3]

Some cross-sectional information is available on the endocrinological,[4] electroencephalographic,[5] and cognitive emotional correlates of some putative antecedents of SAD, including BI.[1] Likewise, the neurobiology of adult SAD has been explored by different methods[3] including brain imaging techniques.[6,7] Only few studies, however, attempted to track longitudinally the transition from childhood precursors into adolescence DSM-IV SAD in search of pathogenetic mechanisms. The few available prospective studies revealed that behavioral measures of inhibition/avoidance,[2,8-10] general demographic features such as the female sex,[9,10] the endocrinological correlate of heightened cortisol,[10] and broadly hazardous factors such as physical illness[9] or exposure to maternal stress,[10] predict SAD longitudinally. However, these predictors/risk factors are often shared with other internalizing conditions[1] and their bearing on the core cognitive-emotional features of SAD appears loose in some instances. Thus, these studies may have overlooked some salient etiopathogenetic mechanisms.

Further insights can now be added by longitudinal studies that employ contemporary neuroscience methods within a developmental psychopathology framework of reference.[11] This includes studying how developing individuals differ for neurofunctional responses that probe the cognitive-emotional processes at the basis of social interactions. If some of such neurofunctional responses are relevant to the progression of SAD, their variation among general population subjects may help predicting the illness longitudinally. Inasmuch as SAD corresponds to a condition of excessive emotionality and biased cognitions in front of social-interpersonal stimuli or contexts,[12] the investigation of neural responses to affective stimuli of social relevance such as facial expressions of emotions (EoE) fits within this framework.

Affective stimuli of interpersonal hostility -i.e., angry/contemptuous EoE- have been found to elicit relatively specific neural responses, including greater amygdala activation, altered cerebral visual event-related potentials (ERPs), and altered indexes of visual attention, in adults with SAD and in socially shy children.[6,7,13] However, it is unknown whether neurofunctional responses to EoE can help mapping the transition from earlier antecedents into adolescence SAD, and longitudinal studies of SAD based on neural reactivity to EoE are lacking. In the only investigation that neared such design, general population adults who had been categorized during childhood for having -or not- BI[14] were exposed to novel vs. familiar faces; in examining the evolution into SAD among 2 out of 13 formerly inhibited subjects,

heightened amygdala activation to novel faces (versus fixation) was not deemed specific to SAD.[14] Somehow similarly, Perez-Edgar[15] showed abnormal amygdalar responses to facial stimuli amongst formerly inhibited subjects; the implications for the development of SAD were not specified, however.

Amongst the biological determinants of both the processing of interpersonal-affective stimuli and the interspecific trait of anxious temperament-BI/social anxiety, the serotonin transporter (*5-HTT*) promoter polymorphism (5-HTTLPR) emerged as a promising candidate. The 5-HTTLPR has 2 common alleles -short (*S*) and long (*L*)- that influence *5-HTT* transcription activity differentially[16]. The 5-HTTLPR -*S* and -*L* alleles have been associated with greater amygdala response to angry or fearful faces in adults[17,18] and adolescents.[19] Although not all studies concord in finding the -*S* allele associated with amygdalar hyperreactivity,[19] a recent meta-analysis[20] concluded that the influence of 5-HTTLPR on both left and right amygdala activation to EoE is reliable, even though small power constitutes a caveat and the precise effect size awaits to be determined. The 5-HTTLPR -*S* allele was also associated with smaller ERP N400 amplitudes at age 8-9 in this longitudinal study group.[13]

Therefore, in a sample of general population adolescents recruited for a longitudinal psychobiological assessment of social anxiety, we addressed 3 main questions. First, can the ERP responses to EoE that were associated with shyness-BI at age 8-9, predict SAD at age 14-15? Second, can cerebral fMRI activation to expressions of happiness/neutrality/anger differentiate adolescents according to the number of SAD symptoms? Third, do these adolescents show associations between amygdalar activations and the presence of 'risk' alleles of the *5-HTT* gene?

MATERIALS AND METHODS

Subjects

Subjects were drawn from a sample of 49 normally-developing children who had participated to an ERP study of responses to EoE and shyness at age 8-9.[13] The 49 ERP study participants had been drawn from a general population cohort (N=149) assessed at age 7 for shyness and EoE categorization.[21]

In year 2007-2008 we invited all 49 children and their families to a new phase of the study, which encompassed fMRI sessions and direct psychiatric interviews: 38 (78%) accepted, 4 (8%) refused, and 7 (14%) were unavailable due to relocation. Amongst the 38 acceptant subjects, 17 withdrew for the presence of orthodontic apparels, health/family problems, or for sickness/unexpected constraints on the experiment day. This left 21 participants to this study. There were no significant differences between the 21 participants and the 28 non-participants for the key clinical, laboratory and genetic variables in the study (Table 1). Although participants were 21, fMRI analyses are shown for 19 subjects (37% girls, mean age 14.8+1.1 years), since the fMRI data of 2 girls were discarded due to movement artifacts during

MRI. The procedures were accepted by the ethical committee of the participating institutions and, after complete description of the study to the subject, parental written informed consent was obtained.

Assessment of DSM-IV SAD at Age 14-15

The presence of symptoms of DSM-IV childhood disorders was established by consensus of the first two authors via blinded reviews. K-SADS interviews were administered to parents while their children were undergoing fMRI in the experiment day. For all diagnostic categories the KSADS instructions[22] were followed and applied rigorously.

Protocol

Stimuli Selection

Children and adolescents spend most of their time with individuals of the same age, and socially anxious children fear peers' rejection.[13] Similarly to previous investigations of EoE and shyness/SAD,[13,21,24] we used a set of faces of adolescents as stimuli. These consisted of 60 black-and-white pictures of 10 boys and 10 girls standardized for size, contrast, and luminosity, displaying 3 emotions: happiness, anger, and a neutral expression. These stimuli had been developed by showing the models Ekman and Friesen's[25] prototypical pictures as a reference. The three expressions of 'happiness', 'neutrality' and 'anger' were chosen to represent non-verbal emotional-social signals of social acceptance, [26] neutrality/ambiguity[21,27] and rejection/hostility[7,27] respectively, as previously adopted by studies of social anxiety in children[13] and adults.[7] The emotional faces were presented in an oval aperture that occluded sex-specific features. Among 32 boys and girls attending the San Raffaele High School, the mean correct classification for this set of 60 pictures was 94%, well within the standards reported by other groups in the field.

Experimental Task

During fMRI scanning, stimuli were projected onto a translucid projection screen and images were viewed via a mirror attached to the head coil. The experiment reflected a 2 run block-design. Each run included 16 blocks divided into 4 quadruplets. Each quadruplet bconsisted of four randomly ordered blocks, whereby each of the first three blocks encompassed five repetitions of the same EoE (e.g., 'happiness') displayed by different subjects in a semi-randomized sex order, followed by 2 blocks of the remaining 2 EoE (e.g., 5 'anger' repetitions, followed by 5 'neutrality' repetitions), and ended by a final block of 5 repetitions of a fixation cross.

Each stimulus (face or fixation cross) was presented for 3000 ms, followed by a black screen inter-stimulus interval (2000 ms). Subjects were instructed to press the right hand side button for 'boy' faces, the left hand side for the 'girl'

faces, and a button of choice when the fixation cross appeared on the screen: by this sex discrimination task we intended to implement an implicit EoE processing task, similarly to the previous ERP study on these same children.[13] Every subject was exposed to a pre-experimental trial of 12 pictures not belonging to the same set used for the experiment to ensure comprehension of the procedures. Each participant received an educational gift of € 60 value.

Data Analyses

Longitudinal Prediction of SAD at Age 14-15 by ERP Indexes Collected at Age 8-9

In the study group of 49 children from whom we recruited the participants to the present study, the ERP waveforms' amplitudes evoked during an implicit processing task of 3 EoE at age 8-9 were predicted by children's degree of shyness/BI.[13] To assess whether the ERP activity evoked by implicit EoE processing at age 8-9 predicted SAD in adolescence, we set a stepwise regression where the predictors were the ERP N400 waveform amplitudes evoked by the angry, neutral, and happy EoE at age 8-9, and the dependent variable was the number of DSM-IV SAD symptoms collected at age 14-15 with the K-SADS interview. This regression was based on 17 subjects, since the ERP data of two girl participants to the fMRI study were discarded at the time of ERP experiment, due to artifacts.[13]

fMRI Acquisition and Data Analysis

For fMRI acquisition parameters see *Supplementary Material*. Data processing and statistical analyses were performed with Statistical Parametric Mapping (SPM5; Wellcome Department of Neurology, London) and associated toolboxes (see *Supplementary Material*).

As specified in the Introduction, the fMRI analyses were principally aimed at assessing whether DSM-IV SAD symptoms were associated with specific patterns of brain activation in response to EoE, and whether the participants' 5-HTTLPR genotype could be associated with different degrees of brain activation in the experiments. Preliminarily, we compared the activation associated with EoE viewing to the activation associated with the fixation cross viewing by voxel-wise within-subjects factorial ANOVA (see Figure 1). We adopted a voxel-level significance of $P \leq 0.05$, with a whole brain family-wise error (FWE) correction for multiple comparisons. FWE correction is based on Gaussian Random Field Theory and on the expected Euler characteristics of the contrast images[29].

Then, based upon the factorial ANOVA results (*vide infra*) and upon previously published studies,[28] we focused on the amygdalae as ROIs that become activated in response to EoE. We estimated the correlation between the degree of amygdalar activation in response to EoE and the number of DSM-IV SAD symptoms at the K-SADS in 3 separate linear regression models (one for each EoE), while the degree of amygdalar responses in relation to the 5-HTTLPR was

analyzed by ANOVA. Participants were subdivided into two genotypic groups (respectively: *S*-carriers/*L*-homozygotes), based on neurofunctional data[20] that showed the viability of this option, and on the limited sample size. The Main Effect of Group (*S*-carriers/*L*-homozygotes) was analyzed by a massed univariate F-test, and the post-hoc between-group paired comparisons by one-tailed t-Student contrasts.

For the ROI analyses we used a Small Volume Correction (voxel-level $P<0.05$, FWE correction for multiple comparisons) to test the *a priori* hypothesis of the influence of DSM-IV SAD symptoms and of the 5-HTTLPR genotype on the degree of amygdalar activation.

To this purpose, a spherical volume of interest of radius 8 mm was defined around the stereotaxic coordinates [left amygdala: x = -24, y = -6, z = -17; right amygdala: x = 19, y = -8, z = -16] reported by a meta-analysis study.[28] By using the SPM Marsbar toolbox (http://marsbar.sourceforge.net), we also extracted the mean percent BOLD signal changes from the amygdalar ROI regions defined on spherical volumes of radius 6 mm around the peaks of activation yielded by the correlations with DSM-IV SAD symptoms and the effect of 5-HTTLPR genotype (see also captions to Figures 2 and 3 for further details).

RESULTS

Prevalence of DSM-IV Diagnoses at Age 14-15

According to the interviews of participants carried out at age 14-15 with the K-SADS, we found that 5 out of 19 participants (26%, of whom 40% were girls) had DSM-IV SAD. Compared to the K-SADS interviews undertaken when these same subjects were 8-9 years old, there were four new cases of DSM-IV SAD, and one subject who remained stable with the SAD diagnosis received at age 8. According to the K-SADS evaluations, there were two boys who met a full DSM-IV diagnosis of Generalized Anxiety Disorder in addition to a SAD diagnosis. None of these subjects was receiving a treatment. There were no other current DSM-IV disorders amongst participants to the fMRI study, as two subjects who had qualified for Separation Anxiety Disorder at age 8-9 were found in remission at age 14-15.

Genotypes and Allelic Frequencies

The 5-HTTLPR allelic and genotypic frequencies among the participants to the fMRI study are provided in Table 2.

Relationship Between ERP Responses to Facial Expressions at Age 8-9 and DSM-IV SAD at Age 14-15

By stepwise regression, we found that the ERP N400 amplitudes obtained by implicit processing of three EoE (anger/neutrality/happiness) at age 8-9[13] predicted the number of DSM-IV SAD symptoms at age 14-15 ($F_{(1,16)}=7.035$,

adjusted R^2=0.27, P=0.018), with the anger expression as the only significant predictor retained by the final equation yielding β=0.57, $t_{(16)}$=2.65, P=0.018, while both the neutrality and the happiness expression conditions were removed from the final regression solution, due to non-significant contribution.

A polytomous regression of the number of SAD symptoms collected with the K-SADS at age 8-9 upon the DSM-IV SAD diagnosis at age 14-15 yielded no significant prediction ($F_{(1,17)}$= 0.22, adjusted R^2=0.00, P=0.64). Similarly, a polytomous regression of the score of a shyness-BI index at age 8-9 upon DSM-IV SAD symptoms at age 14-15 yielded no significant prediction ($F_{(1,17)}$= 2.18, adjusted R^2=0.06, P=0.16).

Behavioural Results

The mean reaction time (in milliseconds ±SD) during the sex discrimination task was 1224.5 (±223.6), ranging from 306 to 2981 milliseconds. The mean accuracy (expressed in percent of correct answers ±SD) was 75 % (±12%), ranging from: 33% to 90%. Neither accuracy nor reaction time differed significantly between boys and girls (respectively, t_{18}= -0.82; P=0.42 and t_{18}=-1.25; P=0.23). Reaction times did not differ significantly across the three emotion conditions, while the percent of correct gender classifications was significantly reduced when subjects were looking at the anger expression, compared to neutral and happy expressions (Main Effect of the experimental condition: F(2,30)=14.92; P<0.001; Post-hoc Tukey HSD test of anger vs. happiness: P<0.001; anger vs. neutral: P=0.004).

fMRI results

Cerebral Activation in Response to Facial Expressions vs. a Fixation Cross

The within-subjects factorial ANOVA showed that the EoE experimental conditions were associated with increased bilateral activation of amygdalae, hippocampus, fusiform gyrus, and inferior occipital pole cortex, compared to the fixation basal condition (see Figure1 and *Supplemental Table 1*). The right inferior frontal gyrus and cerebellar vermis were also activated. In the light of these results and available experimental evidence on the amygdalar reactivity to EoE, [17,26] we focused the remaining analyses on amygdalar responses to EoE.

Relationship between Adolescence DSM-IV SAD and Amygdala Activation

We found a significant positive correlation between left amygdala response to the neutral and the angry EoE and the number of DSM-IV SAD symptoms (see Figure 2). No significant results were found for the right amygdala (Figure 2).

Influence of the 5-HTTLPR Genotype on Amygdala Responses to Facial Expressions and ERP Responses

The left amygdala ROI activation across the three EoE was significantly associated with the 5-HTTLPR genotype, in that the 5-HTTLPR *–S* allele was associated with heightened amygdala response to facial expressions (see Figure 3); further analyses showed that this effect was associated with the angry, but not the neutral or happy EoE.

Similarly, a multiple regression showed that the 5-HTTLPR genotype predicted the ERP N400 amplitudes obtained at age 8-9 by implicit processing of anger (β=0.64, $t_{(15)}$=3.20, P=0.005) but not of happiness, (β=-0.07, $t_{(15)}$=-0.26, P=0.79) or of the neutral (β=0.17, $t_{(15)}$=0.66, P=0.52) EoE at age 8-9.

The amygdala ROI activation at age 14-15 and the ERP N400 amplitudes obtained by implicit EoE processing at age 8-9, were not significantly correlated across the three EoE, however (for anger, $P_{(FWE)}$=0.36).

DISCUSSION

Our data show that individual variation at a neurobiological readout –namely cerebral reactivity to EoE- measured at different developmental stages with different techniques, can map individual dispositions towards social anxiety and predicts transition from childhood precursors into symptoms of DSM-IV SAD in adolescence.

Among the three basic EoE (anger/neutrality/happiness) presented in implicit processing tasks, anger –a prototypical signal of social rejection- proved effective in eliciting cerebral responses that distinguished individuals on the basis of their symptoms of SAD. This was true longitudinally (as the ERP N400 amplitudes in response to angry expressions at age 8-9 predicted DSM-IV SAD symptoms at age 14-15) and cross-sectionally (as the amygdala activation in response to angry –as well as to neutral- expressions differentiated adolescents according to their SAD symptoms). In our study group the ERP response, rather than the number of symptoms of SAD collected at age 8-9 were predictive of DSM-IV SAD in adolescence. While methodological issues and limitations related to the sample size apply (see limitations section), this finding supports the adoption of ERP to predict SAD longitudinally.

A relatively specific pattern of cerebral activity in response to peers' rejection signals is in accordance with current cognitive, behavioural and evolutionary theories that emphasize social/evaluative information biases in SAD,[12] and with empirical evidence of selective bias towards signals of anger/rejection among socially anxious children and adults.[15,21,30,31] The finding of a relative inaccuracy in gender classification in front of the angry expressions further supports the theory that 'anger' is perceptually more challenging to process, compared to other EoE [32,33].

Neurofunctional continuity between the ERP N400 childhood data and the adolescence fMRI data (including our focus on amygdala activation) is supported by the fact that the N400 waveform is thought to reflect a temporal correlate of a

cortico-amygdala pathway of emotional processing,[34] while the centroparietal regions involved in N400 production are interconnected with emotion processing networks that encompass the amygdala and the prefrontal cortex.[26,35,36] However, we did not find a significant association between the ERP N400 waveforms collected at age 8-9 and the fMRI responses at age 14-15, possibly due to the small sample size.

The finding of greater left amygdala activation to EoE that correlates with SAD symptoms is similar to that of Lau et al, [19] who studied adolescent patients with various anxiety disorders. Our finding that amygdalar activation in response to neutral and angry EoE correlates with SAD symptoms among adolescents, is germane to the finding of greater amygdala activation during anticipated peer evaluation reported by Guyer et al[24] in their case-control study of anxious adolescents, 57% of whom had SAD.

The greater activation in response to angry and neutral EoE fits well with the notion that the amygdala is sensitive to both hostile and ambiguous stimuli,[26] while the association with 1-2 copies of the 5-HTTLPR –*S* allele replicates a finding that is common in adult studies,[20] but may still be controversial in developmental samples.[19] Overall, our data of greater amygdala response to rejection signals in adolescent SAD and in carriers of the –*S* allele of the 5-HTTLPR is in agreement with brain imaging studies of adult SAD patients[18] exposed to social anxiogenic stimuli/contexts, and of adolescents followed up for BI.[15] There is also recent evidence that the –*S* allele of 5-HTTLPR is associated with attention biases to angry faces.[37]

At least 3 potential limitations apply. First, the sample size is small, implicating reduced power and the need for replication in larger longitudinal samples, as warranted for any preliminary study. However, we found no clear indications of participation bias (Table 1): the main constraints to participation were relocation, and orthodontic apparels. Still, subjects with SAD were overrepresented (26%) among those who finally underwent fMRI. While all efforts were undertaken in order to recruit the whole original sample,[13] a tendency towards positive self-selection for the most socially anxious appears plausible here.

Second, the genetic analyses were based upon dichotomous t-contrasts: while simplistic, this approach is often adopted in small-to-medium samples size studies,[17,18] as is based on evidence that the presence of the 'risk' alleles imply functional differences, including reduced transcription and diminished serotonin uptake for the 5-HTTLPR –*S* allele.[16]

Third, we found significant effects of SAD and genotype on left amygdala responses. While the importance of the right hemisphere for the neural representation of emotions is clear,[38] a meta-analysis of 106 imaging studies reported an equivalent number of left- and right-side effects for emotion processing.[28] Specific left amygdala activation in response to EoE is documented among adult SAD patients,[7] and left amygdala activation by angry EoE has been found

associated with SAD and the 5-HTTLPR –*S* allele in adults.[18] Still, we agree with Stein[7] that such findings do not necessarily reflect true laterality of the amygdala response.

CONCLUSION

Biased processing of social-emotional stimuli during development can be characterized by ERP in childhood and confirmed by fMRI in adolescence, when it marks DSM-IV SAD symptoms at their peak of incidence. By the study of functional traits en route between multiple elements of liability, neurofunctional systems, and the related developmental phenotypes, dimensionally-distributed functions of risk can be approximated in the general population and promote a characterization of vulnerability that is more relevant to core developmental processes.

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TABLES

Table 1. Comparisons of Demographic, Psychometric and Genetic Features of Participants vs. non-Participants in the fMRI Experiment

	Participants (n=21)	**Nonparticipants (n=28)**	***t* Value/χ^2**	**df**	**P Value**
Age at fMRI time, mean ± SD	15 (±0.7)	14.7 (±0.7)	-1.39	47	NS*
Symptoms of SAD by K-SADS interview, mean no ± SD	2.67 (±2.99)	1.96 (±2.52)	-0.89	47	*NS**
Shyness-BI index, mean ± SD	9.38 (±7.28)	11.96 (±5.98)	1.36	47	*NS* *
N400 ERP Amplitude, mean ± SD	-17.33 (±4.63)	-18.57 (±5.83)	-0.75	40	*NS* *
CBCL Withdrawn scale, mean ± SD	3.66 (±3.31)	2.54 (±2.32)	-1.41	47	*NS* *
5-HTTLPR, No. (%) *S*-carriers	14 (66)	19 (68)	0.01	1	*NS* †
Sex, No. (%) male	12 (52)	14 (50)	0.25	1	*NS* †
SES, No.					
Lower	3	3	1.46	2	*NS* †
Middle	8	15			
Upper	10	9			

The comparisons in Table 1, except for mean age, are based on data collected at age 8-9 in a study group of 49 children, from whom participants to the present fMRI study were enrolled.

Two girls among the 21 participants produced movement artefacts during fMRI, and were thus removed from the final sample.

Abbreviations: **fMRI**, functional magnetic resonance imaging; **SAD**, DSM-IV social anxiety disorder; **K-SADS**, Schedule for Affective Disorders and Schizophrenia for School-age Children; **BI**, Behavioral Inhibition; **ERP**, event related potentials; **CBCL**, Child Behavior Checklist;[39] **5-HTTLPR**, serotonin transporter promoter polymorphism; **SES**, socioeconomic status calculated on the basis of the Hollingshead scale (lower range, score of 1-3; middle range, score of 4-6; upper range, score of 7-9).

*By *t* test.

†By χ^2 test.

Table 2: Allelic and Genotypic Frequencies of 5HTTLPR polymorphism among the 19 participants

	Allelic frequencies		Genotypic frequencies		
	***S*-allele**	***L*-allele**	***SS***	***SL***	***LL***
5-HTTLPR	N=16 (42,1%)	N=22 (57,9%)	N=3 (16%)	N=10 (53%)	N=6 (31%)

The 5-HTTLPR allelic and genotypic frequencies were in Hardy-Weinberg equilibrium, without sex-related differences in distribution (respectively $\chi 2 = 0.371$, P=0.54 and $\chi 2 = 0.663$, P=0.72 for 5-HTTLPR)

Abbreviations: 5-HTTLPR, serotonin transporter promoter polymorphism

FIGURE LEGENDS

Figure 1. Pattern of Cerebral Activation in Response to Facial Expressions of Emotions vs. A Fixation Cross among 19 General Population Adolescents

Results from within-subject factorial ANOVA (Factor: Experimental Condition; 4 levels: happiness/neutrality/anger/fixation; equal variance; levels not independent) at P<0.05 (voxel-level, whole brain FWE correction for multiple comparisons).

Activations are superimposed on axial slices of the normalized mean anatomical image of participants. The fixation cross baseline condition was excluded from all successive analyses because this factorial ANOVA had shown significant and marked differences in amygdalar activation between the fixation point and the EoE viewing, and given our interest in studying the brain differential activations in response to EoE of distinct social valence.

Figure 2. Relationship Between DSM-IV Social Anxiety Disorder Symptoms and Amygdala Activation among 19 General Population Adolescents

Results from the correlation analyses in SPM5 revealed a significant positive correlation between the number of DSM-IV SAD symptoms and amygdala activation in response to anger (P(FWE)=0,036) and neutral (P(FWE)=0,025) EoE, but not with amygdala activation in response to happiness (P(FWE)= 0,072).

The scatterplot shows amygdalar BOLD responses to three Expressions of Emotions in participants divided into 3 ranks of SAD symptoms (0=no symptoms, 1= 1 to 3 SAD symptoms, 2= more than 3 SAD symptoms).

All data refer to left amygdala activation. We report the percentage of BOLD signal change relative to the signal global mean. All significant effects corresponded to an activation peak at x=-20; y=-10; z=-20, within the corrected small volume defined for the left amygdala.

According to the Anatomy toolbox of SPM (www.fz-juelich.de/inm/inm-1/DE/Forschung/_docs/SPMAnantomyToolbox/SPMAnantomyToolbox_node.html) this activation coordinate corresponds to a cytoarchitectonic probability of 60% and 50%, respectively, for the Latero-Basal and for the Superficial subregions of the amygdala. The percentages (+SD) of left amygdala BOLD signal changes in response to neutral, happy and angry EoE among the symptoms rank groups were respectively: a) No symptoms: -0,86 ± 0,62; -0,62 ± 0,66; -0,75 ± 0,63;

b) 1 to 3 SAD symptoms: 0,7 ± 0,99; 0,42 ± 0,82; 0,60 ± 0,73; c) more than 3 SAD symptoms: 0,86 ± 0,76; 0,70 ± 0,77; 0,76 ± 0,86.

Figure 3. Influence of the 5-HTTLPR Genotype on Amygdala Responses to Facial Expressions of Emotions among 19 General Population Adolescents

Results from a group-level factorial ANOVA in SPM5 ('flexible' option) with factors: Subject (n= 19; equal variance; levels independent), Group (2 levels: *S*-carriers, *L*-homozygotes; unequal variance; levels independent) and Experimental Condition (3 levels: happiness/neutrality/anger; equal variance; levels not independent).

Analyses across the three EoE yielded evidence in favor of differential amygdala response to facial expressions between the two genotypic groups (Z score=2.99; $P_{(FWE)}$= 0.05). Further analyses showed that this effect was sustained by the augmented amygdalar activity displayed by 5-HTTLPR *S*-carriers compared to *L*-homozygotes in response to angry- (Z score=2.99; $P_{(FWE)}$= 0.05), but not to neutral-(Z score=2.82; $P_{(FWE)}$= 0.07), or happy (Z score=2.90; $P_{(FWE)}$= 0.06) expressions.

All significant effects (percent BOLD signal change relative to the signal global mean) corresponded to an activation peak at x=-30; y=-4; z=-16, within the corrected small volume defined for the left amygdala (corresponding to 40% cytoarchitectonic probability for the Latero-Basal subregion of the amygdala, according to the Anatomy toolbox of SPM). The percentage (+SD) of left amygdala BOLD signal changes in response to neutral, happy and angry EoE among the genotypic groups were respectively: a) L-homozygotes: -0,37 ± 0,44; -0,25 ± 0,54; -0,26 ± 0,55;

b) S-carriers: -0,35 ±0,76 ; -0,16 ±0,70 ; -0,14 ± 0,74.

Figure 1

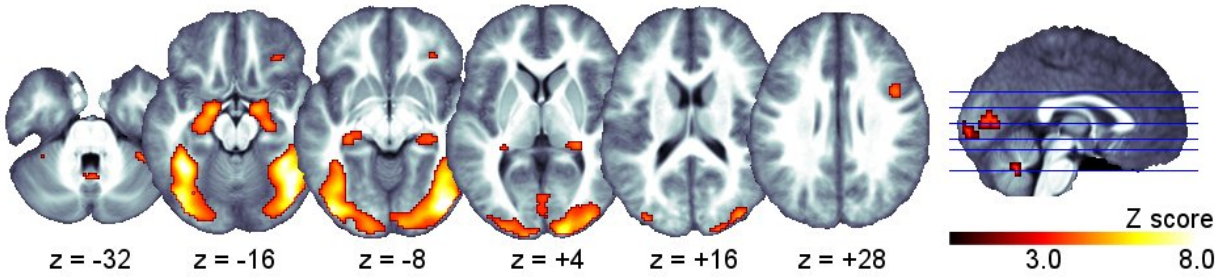
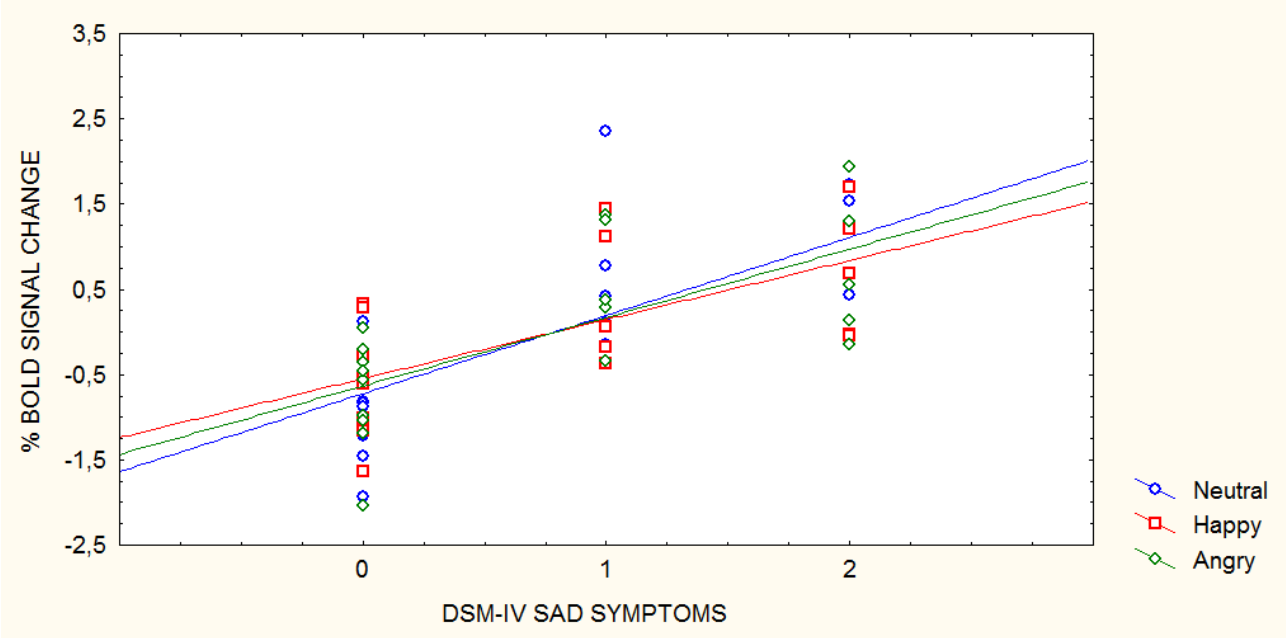


Figure 2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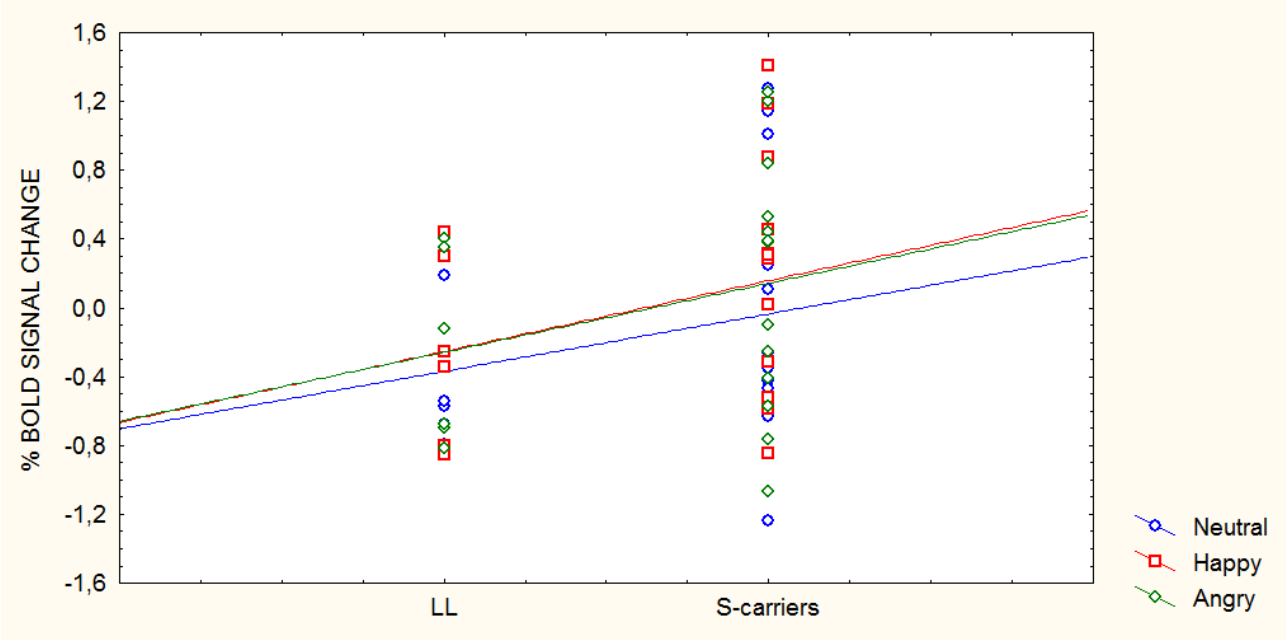
2,5
1,5
0,5
-0,5
-1,5
-2,5
% BOLD SIGNAL CHANGE
0
1
2
DSM-IV SAD SYMPTOMS
Neutral
Happy
Angry

Figure 3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1,2
0,8
0,4
0,0
-0,4
-0,8
-1,2
-1,6
% BOLD SIGNAL CHANGE
LL
S-carriers
Neutral
Happy
Angry

Supplementary Material

DNA Extraction and Genotyping

Genomic DNA was extracted from mouthwash samples collected in 4% sucrose by means of a reagent for isolation of genomic DNA (DNAzol Genomic DNA Isolation Reagent; Molecular Research Center Inc, Cincinnati, Ohio). The polymorphism in the transcriptional control region upstream of the *5-HTT* coding sequence (5-HTTLPR) was analyzed by polymerase chain reaction.[1,2] Two fragments were generated: a short variant (*S*) of 484 base pairs and a long variant (*L*) of 528 base pairs.

fMRI Data Acquisition

MRI scans were acquired on a 3T Achieva Philips body scanner (Philips Medical Systems, Best, NL) using an 8 channels-sense head coil (sense reduction factor=2). Whole-brain functional images were obtained with a T2*-weighted gradient-echo, echo-planar sequence, using blood-oxygenation-level-dependent contrast. Each functional image comprised 30 contiguous axial slices (4 mm thick), acquired in interleaved mode, and with a repetition time of 2000 ms (echo time: 30 ms; field of view: 240 mm x 240 mm; matrix size: 128 x 128). Each participant underwent 2 functional scanning sessions. The duration of each session was 225 scans, preceded by 5 dummy scans that were discarded prior to data analysis. A fieldmap was acquired for unwarping of the echo-planar images. We also acquired a high-resolution whole-brain structural T1 weighted scan (150 axial slices, resolution 1mm x 1mm x 1mm) of each participant for brain tissue segmentation, anatomical localization and visualization of brain activations.

fMRI Data processing

Data processing and statistical analyses were performed with Statistical Parametric Mapping (SPM5; Wellcome Department of Neurology, London) and associated toolboxes. First, we used the TOM toolbox (http://dbm.neuro.uni-jena.de/software/tom) to create age-matched brain tissue maps (grey and white matter, CSF) based on the objective 1 NIH data (n = 404), in the age range of 5-18 years.[3] We then segmented and normalized the T1 image of each subject to the age-matched brain tissue maps. Subsequently, the realigned and unwarped echo-planar functional images of each subject were segmented by using the VBM 5.1 toolbox (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5) with the 'no Bayesian tissue priors' option and the normalized individual T1 tissue maps as tissue probability maps. Finally, the normalized images were smoothed by a 8 mm FWHM Gaussian isotropic kernel and submitted to the GLM statistical analysis.

We adopted a two-stage random-effects approach to ensure generalizability of the results at the population level. At the first stage, the time series of each participant were high-pass filtered at 128 s and pre-whitened by means of an autoregressive model AR(1). No global scaling was performed. Hemodynamic evoked responses for all experimental conditions were modeled as canonical hemodynamic response functions. For each participant, we modeled the factor experimental conditions with 2 separate sessions (4 levels: joy/neutrality/anger/fixation). First-level t-Student contrasts were specified, with each contrast including a weight of one for a particular set of regressors of interest and a weight of zero for all the other regressors. This resulted in 1 contrast per experimental condition for each participant. At the second stage of analysis, the one-sample t-Student contrasts defined at the single-subject level were used to compute a set of voxel-wise and region-of-interest (ROI)-based mixed effects models assessing their significance at the group-level.

Table 1. Cerebral Activation in Response to Facial Expressions vs. a Fixation Cross among 19 General Population Adolescents (Voxel-Level P<0.05, FWE corrected at Whole Brain Level)

	Cluster size	Voxel Level		MNI coordinates		
Brain region (cytoarchitectonic probability[§])	***K (voxels)***	***Z-score***	***P-value***	***x***	***y***	***z***
Right amygdala (area SF 40%)	266	6.75	< 0.0001	16	-6	-16
Right hippocampus	"	6.27	< 0.0001	26	-20	-12
Left amygdala (area SF 70%)	254	7.21	< 0.0001	-18	-8	-16
Left hippocampus (area CA 60%)	"	6.41	< 0.0001	-24	-14	-16
Left hippocampus (subiculum 80%)	5	4.89	0.021	-18	-34	-4
Left fusiform gyrus	3680	Inf	< 0.0001	-40	-46	-20
Right fusiform gyrus	"	Inf	< 0.0001	38	-46	-20
Left inferior occipital gyrus	"	Inf	< 0.0001	-42	-82	-8
Right inferior occipital gyrus	"	Inf	< 0.0001	46	-78	-8
Right calcarine gyrus (BA 17 70%)	"	Inf	< 0.0001	18	-100	4
Right inferior frontal gyrus,m pars opercularis (BA 44 40%)	29	5.73	< 0.0001	44	8	28
Right inferior frontal gyrus, pars triangularis	8	5.00	0.013	46	24	24
Right inferior frontal gyrus, pars orbitalis	25	5.40	0.002	32	34	-12
Cerebellum, vermis	20	5.53	0.001	2	-58	-32

[§]According to the Anatomy toolbox of SPM (www.fz-juelich.de/inm/inm-1/DE/Forschung/_docs/SPMAnantomyToolbox/SPMAnantomyToolbox_node.html).

All reported effects on this whole-brain analysis are voxel-level related statistics with a $P<0.05$ family wise error (FWE) type, whole-brain correction for multiple comparisons and a cluster extent threshold greater than 5 voxels.

Abbreviations: **FWE**, Family Wise Error; **SF**, superficial amygdala (includes the anterior amygdaloid area, the amygdalopyriform transition area, the amygdaloid-hippocampal area and the ventral (intermediate, dorsal, ventral) and posterior cortical nuclei); **CA**, Cornu Ammonis; **BA**, Brodmann Area.

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