Orexin/hypocretin receptor gene (*HCRTR1*) variation is associated with aggressive behaviour

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Highlights

- Orexins are involved in the regulation of stress response

- The *HCRTR1* rs2271933 G>A genotype was associated with aggressiveness

- A/A homozygotes were more aggressive and relapsed to drunk driving

- A-allele carriers were more frequently involved in traffic accidents

- The genotype effect is sensitive to stressful life events

Abstract

Orexins, alternatively called hypocretins, are neuropeptides with crucial role in maintaining wakefulness. The orexin system is thought to mediate a coordinated defense response but thus far investigated from the flight, but never fight, response perspective. An *HCRTR1* gene variant (rs2271933 G > A) leading to amino acid substitution (Ile408Val) has been associated with migraine and mood disorders. We genotyped, and assessed aggressive behaviour in both birth cohorts (n=655 and 583) of the Estonian Children Personality Behaviour and Health Study (ECPBHS). Measures of aggressiveness were collected at age 25 or 33 and data on stressful life events (SLE-s) at age 15. Violations of traffic law were monitored in the samples of the Estonian Psychobiological Study of Traffic Behaviour. In both birth cohorts of the ECPBHS, the *HCRTR1* the A/A homozygotes reported higher aggression in both Buss-Perry Aggression Questionnaire and the Life History of Aggression Interview. With either measure of aggressiveness, the *HCRTR1* genotype effect was dependent on experience of SLE, the highest level of aggressiveness increase by environment being found in female A/A homozygotes. The *HCRTR1* A/A homozygotes scored higher in the ANGER facet of the Affective Neuroscience Personality Scale, while such an effect on FEAR was found only in females. Male *HCRTR1* A/A homozygotes were more likely to relapse into drunk driving of a

passenger car, and in two independent samples the A-allele carriers were causing traffic accidents more often. Conclusively, self-report, interview, and traffic record data converge indicating that the *HCRTR1* Ile408Val genotype is associated with aggressiveness and breach of law.

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1. Introduction

Orexins or hypocretins are hypothalamic neuropeptides derived from prepro-orexin (Sakurai et al., 1998; de Lecea et al., 1998) that have been implicated in a large variety of physiological functions, most clearly in sleep/wake regulation (Sutcliffe and de Lecea, 2000; Schwartz and Kilduff, 2015) and food intake (Sutcliffe and de Lecea, 2000; Broberger and Hökfelt, 2001; Ferrario et al., 2016). Orexin neurons are located in the dorsomedial and lateral hypothalamus, in particular in perifornical and magnocellular lateral hypothalamic nucleus (Broberger et al., 1998). More recently, orexins have been proposed to be involved in a coordinated adaptive defence response, in particular its anxiety, cardiorespiratory and endocrine aspects (Johnson et al., 2012). Orexins modulate neural circuits implicated in the expression and extinction of fear memories (Flores et al., 2015). The orexin system is activated during the acute stress response, but the effects of chronic stress on the orexin system are thought to be highly variable (Sargin, 2018). As orexin neurons are activated by a large variety of stressors, a hypothesis has been put forward that orexin neurotransmission is particularly important in behavioural regulation during aversive conditions involving high arousal (Berridge et al., 2010). Suicide attempters with major depression were found to have lower CSF levels of orexin as compared to other patient groups (Brundin et al., 2007), indirectly suggesting that orexin is important in maintaining adaptive strategies that are sensitive to mood regulation.

If the orexin peptides are crucial mediators of coordinated stress response, then the other side of the fight-flight response, the possible choice of fight and preference of confrontational and aggressive response over escape should also be examined (Dhabhar, 2018). In a unique study on epileptic patients the release of orexin A was monitored in amygdala and found to be increased while subjects reported higher levels of anger (Blouin et al., 2013). Otherwise little evidence appears to exist on the role of orexins in any aspects of aggressive behaviour. Aggressiveness is however an important component of coordinated adaptive response to challenges presented by the environment (Van Kampen, 2015) and different nuclei of the hypothalamus play a critical role in this response (Kruk, 2014). Administration of orexin A has been reported to elicit dose-dependent complex changes in mouse maternal behaviour, including, at high doses, reduction of maternal behaviour and aggression (D'Anna and Gammie, 2006). Otherwise, no evidence for a role of orexins in aggressive behaviour has previously been made available. Nevertheless, orexin neurons project to such brain regions as septum and locus coeruleus that have a wellknown role in aggressiveness, and into emotion intergrative areas such as amygdala and the bed nucleus of stria terminalis. These brain regions also express type 1 orexin receptors (Flores et al., 2015).

Polymorphism of the orexin prepropeptide gene seems rather limited in humans, but several polymorphisms have been identified in the *HCRTR1* and *HCRTR2* genes encoding their receptors (Thompson et al., 2014). One *HCRTR1* gene variant (rs2271933, G1222A) in exon 7 leads to amino acid substitution (Ile408Val) (Meerabux et al., 2005). This variant has been associated with migraine (Rainero et al., 2011b) and mood disorders (Rainero et al., 2011b). In migraine patients, aggressive behaviour is commonly observed, and their aggressiveness strongly correlates with symptoms of depression (Robbins, 2013; Park and Seo, 2018). Hence we sought to determine, by use of this marker, whether variation in the *HCRTR1* gene can be associated with aggressiveness in two independent samples. Furthermore, taking advantage of

a unique database of traffic behaviour, a common behaviour that may include breach of law that is objectively recorded, we studied the association of the *HCRTR1* genotype with drunk driving and involvement in accidents in two samples.

2. Material and methods

2.1. Subjects

2.1.1. The longitudinal Estonian Children Personality Behaviour and Health Study

Assessment of aggressiveness in this investigation has been carried out on two independent samples included in the Estonian Children Personality Behaviour and Health Study (ECPBHS). We used its original EYHS subsample that comprises two birth cohorts. At recruitment the children were at either third (younger cohort, average age 9.6 years, 52.3% female) or ninth (older cohort, average age 15.6 years, 56.2% female) grade; the participation rate was 79.1%. The rationale of sample selection and procedure has been described previously (Harro et al., 2001), and the recent data collection waves up to age 25 have been described in more detail elsewhere (e.g., Kiive et al., 2017; Laas et al., 2017). Data on aggressiveness and affective aspects of personality were collected in the most recent data collection waves, i.e., at age 25 for the younger cohort and age 33 for the older cohort. The number of participants was 441 and 504, respectively. All participants signed an informed consent form. The ECPBHS has been approved by the Tartu University Ethics Review Committee on Human Research.

2.1.2. Samples of the traffic studies

We also genotyped the subjects of two longitudinally observed samples from the Estonian Psychobiological Study on Traffic Behaviour (EPSTB) in order to examine the association of the *HCRTR1* genotype with objectively recorded antisocial behaviour. The EPSTB Sample 1 comprised subjects recruited in studies of drunk drivers and speed limit exceeders (see

Eensoo et al., 2005 and Paaver et al., 2006 for detailed description). This group of drivers included male subjects who were identified by the police driving drunk at least once during the previous year (n=203; mean age ± SD, 33 ± 11 years) or exceeding speed limits (n=292; mean age 36 ± 11 years). The control group was formed by computerised random choice of the male subjects in the driving licence database of the Estonian Motor Vehicle Registration Centre; it consisted of 509 individuals, with a mean age of 37 ± 12 years. Subjects were contacted by telephone, the description and aims of the study were provided, and they filled in self-reported questionnaires during a visit to laboratory where they donated venous blood samples. The EPSTB Sample 2 was formed during an impulsivity-focused intervention study at traffic schools (Paaver et al., 2013; Eensoo et al., 2018). Of the 1886 participants (mean age 23.0 ± 7.2 years) of the intervention study, altogether 767 subjects (41% male) had agreed to donate a blood sample. All participants signed an informed consent form. This study was approved by the Tartu University Ethics Review Committee on Human Research.

2.2. Genotyping *HCRTR1* rs2271933

Genomic DNA was extracted from venous blood samples using Qiagen QIAamp® DNA Blood Midi Kit. The real-time polymerase chain reaction (RT-PCR) for genotyping the *HCRTR1* rs2271933 polymorphism was performed using a TaqMan Pre-Designed SNP Genotyping Assay (Applied Biosystems; Foster City, CA, USA) C__15961465_10 containing primers and fluorescent probes. Genotyping reactions were performed in a total volume of 10 μl with ∼25 ng of template DNA. RT-PCR reaction components and final concentrations were as follows: 1:5 5 x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne) and 1:20 80 x TaqMan Primers Probe. Context sequence [VIC/FAM] was as follows:

CTTGTCCTTGCAGAGCCGATGCTCC[A/G]TCTCCAAAATCTCTGAGCATGTGGT.

Reactions were performed on the Applied Biosystems ViiA™ 7 Real-Time PCR System. The

amplification procedure consisted of an initial denaturation step at 95 °C for 12 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Positive and negative controls were added to each reaction plate. No inconsistencies occurred. Genotyping was performed blind to all phenotypic data. Allele frequencies agreed with National Centre for Biotechnology Information database (minor allele frequency around 0.4; https://www.ncbi.nlm.nih.gov/snp/rs2271933#frequency_tab) and published reports. All the subjects were of European origin, and genotype frequencies of all samples were in Hardy-Weinberg equilibrium (Table 1).

Table 1. *HCRTR1* rs2271933 genotype frequencies in samples included in this investigation.

	A/A	A/G	G/G	Total
ECPBHS total	185 (15%)	610 (50%)	439 (35%)	1234 (100%)
ECPBHS younger cohort	75 (13%)	309 (53%)	196 (34%)	580 (100%)
ECPBHS older cohort	110 (17%)	301 (46%)	243 (37%)	654 (100%)
EPSTB Sample 1	160 (16%)	467 (48%)	357 (36%)	984 (100%)
EPSTB Sample 2	132 (17%)	371 (48%)	273 (35%)	776 (100%)

Minor allele frequency 0.40. For comparison, around 0.40 in European samples in the NCBI database; 0.367 in the 1000G CEU population (https://www.ncbi.nlm.nih.gov/snp/rs2271933#frequency_tab).

2.3. Measures of aggressiveness

2.3.1. Self-report questionnaire

The 29-item Buss-Perry Aggression Questionnaire (BPAQ; Buss and Perry, 1992) assesses four aspects of aggressive behaviour: Physical aggression, Verbal aggression, Anger, and Hostility. Participants rated each statement on a 5-point Likert Scale (uncharacteristic = 1, characteristic = 5). Data of Buss-Perry Aggression Questionnaire were available for 427 subjects in the younger ECPBHS cohort and for 499 subjects in the older cohort.

2.3.2. Aggressiveness interview

The Life History of Aggression interview (LHA; Coccaro et al., 1997) was carried out by an experienced clinical psychologist. Items were scored only for the history of actual behaviour.

LHA has three subscales: Aggression (temper tantrums, verbal aggression, indirect aggression, non-specific fighting, and physical assault against people); Consequences/Antisocial Behaviour (school disciplinary problems, problems with supervisors, antisocial behaviour not resulting in police involvement, and antisocial behaviour involving the police); and Self-Directed Aggression (self-injurious behaviour, and suicide attempts). Each item was rated on a 5-point scale, ranging from 0 = No events to 5 = More events than can be counted. Data of LHA were available for 427 subjects in the ECPBHS younger cohort and 495 subjects in the older cohort. Overall, Aggression assessed by interview was moderately correlated to measures of BPAQ self-reported aggressiveness in both sexes while Antisocial Behaviour was associated to self-reports moderately in males and weakly in females (Supplementary Table 1). Correlation of Self-directed Aggression with other measures was weak.

2.4. Affective Neuroscience Personality Scale

We used the adaptation of the short version of the ANPS (Davis et al., 2003) that is a self-report instrument constructed bottom-up to correspond to the activity in neural circuits underlying basic emotive systems as defined in animal research (Panksepp, 1998; Davis and Panksepp, 2011). It comprises scales termed ANGER, FEAR, SADNESS, SEEKING, CARE and PLAY, each measured with six items, each on a 5-point scale. Data on ANPS were available for 423 subjects in the ECPBHS younger cohort and 502 subjects in the older cohort.

2.5. Stressful life events

Stress experienced in childhood was measured at age 15 with a list of 21 adverse life experiences reported by participants or their parents, which included questions about parental death and divorce/separation, absence of both parents, unemployment in the family, financial

problems and poverty in the household, poor living conditions, poor health and chronic diseases, serious illness of a family member, death of a close relative, trauma, fear of school, bullying at school and humiliation at home (Laas et al., 2014). The events were recorded as dichotomous variables (present or not present) and were then counted to form the number of experienced stressful life events (SLE_{15}). The SLE distribution did not differ between males and females. Data were available for 478 subjects in the ECPBHS younger cohort and for 591 subjects in the older cohort.

2.6. Database search

Data on violations of traffic law and traffic accidents were obtained from databases maintained by the traffic police and the traffic insurance fund for the period of January 1, 2002 to December 31, 2011 for the EPSTB Sample 1 and for the period of January 1, 2007 to December 31, 2011 for the EPSTB Sample 2 (Eensoo et al., 2018). We focused on driving while impaired by alcohol (DWI; penalties for drunk driving with an estimated blood alcohol level of 0.2‰ or more) and traffic accidents. The accidents where the subject was at fault were classified as active accidents, and other accidents as passive.

2.7. Statistical analysis

Data were analysed using IBM SPSS (version 22.0, Chicago, IL) and SAS (version 9.4 SAS Inc., Cary, NC) software. One- and two-way analyses of variance were performed to reveal differences between *HCRTR1* genotypes with and without consideration of stressful life events (dichotomized by median split). When appropriate Tukey post-hoc tests were applied. We also used a series of multiple linear regressions for predicting the aggressiveness-related scores where we added all the simple terms and interaction terms to the models simultaneously. To increase interpretability and minimize collinearity in the models, predictors were centered according to Kraemer and Blasey (2004), and interaction terms were

residuals-centered according to Little et al. (2006). Number of stressful life events were treated as quantitative variable for regression analysis. Models were evaluated by goodness of fit statistics and analyses of residuals. Chi-square tests were conducted to assess distribution of presence of traffic violations and accidents by genotype. The conventional 5% level was applied, given that this is the first exploratory study on the association of the orexin receptor genotype and aggressiveness measures, and that the latter are significantly inter-correlated. Retrospective power calculations indicated that if the effects were small the power was low, too, starting from 0.584 in case of p-value 0.050. However, in all analyses where the p-value was ≤0.008, the power was adequate (≥0.8).

3. Results

3.1. *HCRTR1* genotype and aggressiveness in the two population-representative birth cohorts

In the ECPBHS younger cohort aggressiveness was assessed at age 25. The *HCRTR1* Ile408Val (G/A) genotype was significantly associated with total score of the Buss-Perry Aggression Questionnaire [$F_{(2,424)} = 4.36$; $p = 0.013$; $\eta^2 = 0.020$], the A/A homozygotes reporting higher hostility, anger and physical aggression (Fig. 1A and Supplementary Table 2). Similarly, the genotype was associated with the total score of the Lifetime History of Aggression interview [$F_{(2,424)} = 6.98$; $p = 0.001$; $\eta^2 = 0.032$] and its major subscale Aggression (Fig. 1B and Supplementary Table 2), the A/A homozygotes reporting higher level of aggressiveness.

Thereafter the ECPBHS older cohort was independently examined for aggressiveness at age 33. The findings were essentially similar: The total score of the BPAQ was significantly higher in the A/A homozygotes [$F_{(2496)} = 3.43$; $p = 0.033$; $\eta^2 = 0.014$], with significant differences in anger and physical aggression (Fig. 2A and Supplementary Table 2). The

HCRTR1 genotype was also significantly associated with the LHA Aggression subscale (Fig. 2B and Supplementary Table 2) as well as with self-directed aggression, while regarding total score the conventional level of statistical significance was missed (p=0.06). Given the similarity of the genotype effect on these primary outcome measures in both cohorts, further stratified analyses used the data of the birth cohorts combined.
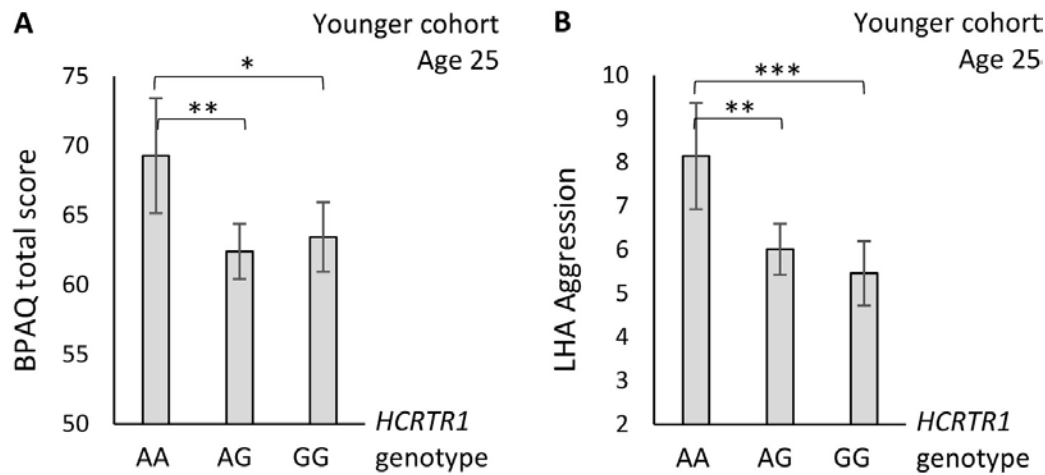

Fig. 1. *HCRTR1* genotype and aggressiveness in the younger ECPBHS birth cohort at age 25. A - Buss-Perry Aggression Questionnaire (BPAQ) total score; number of subjects A/A = 53, A/G = 230, G/G = 144; B - Life History of Aggression (LHA) interview Aggression subscale; number of subjects A/A = 53, A/G = 230, G/G = 144. * - p < 0.05, ** - p < 0.01, *** - p < 0.001 difference between genotypes. Whiskers indicate 95% confidence intervals.
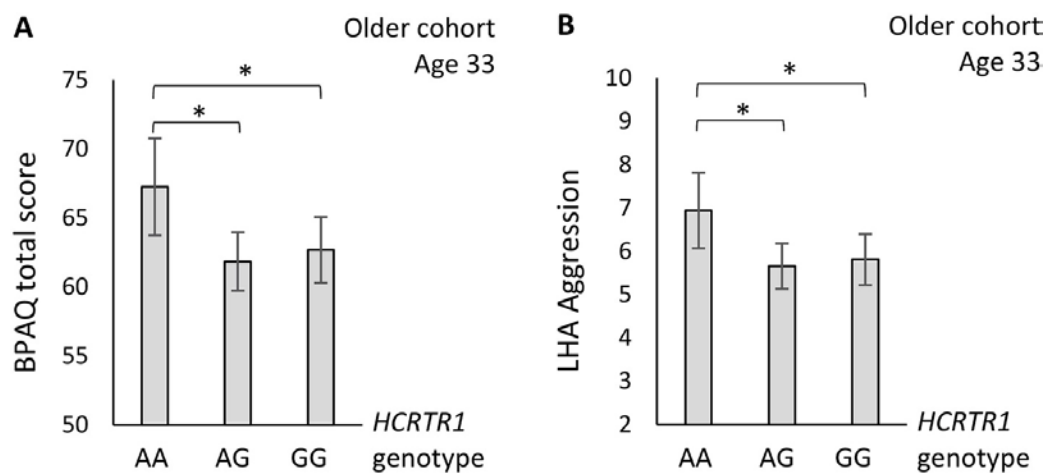

Fig. 2. *HCRTR1* genotype and aggressiveness in the older ECPBHS birth cohort at age 33. A - Buss-Perry Aggression Questionnaire (BPAQ) total score; number of subjects A/A = 84, A/G = 232, G/G = 183; B - Life History of Aggression (LHA) interview Aggression subscale; number of subjects A/A = 83, A/G = 230, G/G = 182. * - p < 0.05 difference between genotypes.

Analysis of both cohorts together revealed highly significant genotype effects on the BPAQ and LHA total scores and most aspects of aggressive behaviour (Supplementary Table 2). No genotype by sex interaction was statistically significant (data not shown).

3.2. *HCRTR1* genotype and the Affective Neuroscience Personality Scale

Based on the findings above we developed an a priori hypothesis that the *HCRTR1* genotype should be associated with scores of the ANGER scale, and given the literature on stress reactivity and orexin, possibly with the FEAR scale, as a large share of overall aggressiveness is of defensive type. Indeed we found that ANGER was significantly higher in the A/A homozygotes [$F (2, 927) = 6.39$; $p = 0.002$; $\eta^2 = 0.014$]; this was the case in both males and females (Table 2). As to FEAR, the genotype main effect missed the conventional level of significance [$F (2, 927) = 2.44$; $p = 0.088$; $\eta^2 = 0.005$] and no statistically significant genotype $\times$ sex interaction emerged [$F (2,924)=2.36$; $p=0.095$; $\eta2=0.005$], but the scores were much higher in females, so we analysed the sexes separately. While in males the genotypes were identical [$F (2, 396) < 0.01$; $p = 0.997$; $\eta^2 < 0.001$], a variation appeared among females [$F (2, 396) = 5.09$; $p = 0.006$; $\eta^2 = 0.019$]. The female A/A homozygotes had the highest levels of FEAR scores. Exploratory analysis of other affect domains (Table 2)

Table 2. *HCRTR1* genotype and the Affective Neuroscience Personality Scale scores in both ECPBHS birth cohorts combined, by sex.

	Males			Females		
	AA	AG	GG	AA	AG	GG
ANGER	17.5±4.4	15.9±4.4*	16.0±4.5*	18.2±4.5	16.6±4.4*	17.1±5.0
FEAR	15.7±3.3	15.7±3.1	15.8±3.0	18.2±3.5	16.8±3.3**	17.4±3.3
SADNESS	13.5±4.0	13.1±4.1	13.0±4.2	16.2±4.8	15.0±4.7	15.6±5.0
SEEKING	23.5±2.9	23.0±3.7	22.7±3.6	22.2±3.4#	23.2±3.5	23.2±3.6
CARE	20.6±4.2	20.0±3.9	20.5±3.8	23.1±3.0	22.7±3.6	23.2±3.5
PLAY	22.5±4.6	22.1±3.9	22.1±3.9	21.9±4.0	22.7±3.8	22.6±4.5

Mean ± S.D. * - $p < 0.05$, ** - $p < 0.01$, difference from A/A genotype in the same sex. # - $p < 0.05$, difference from the A/A male genotype. Genotype frequencies: Males, A/A = 61, A/G = 193, G/G = 145; Females, A/A = 74, A/G = 273, G/G = 184.

revealed no main effect of genotype, but a genotype × sex interaction was revealed for SEEKING [$F_{(2, 924)} = 3.25$; $p = 0.040$; $\eta^2 = 0.016$]. This was owing to the relatively higher SEEKING score of the A/A males contrasted to the relatively low SEEKING in A/A females (Table 2).

3.3. *HCRTR1* genotype and stressful life events (SLE): interaction effect on aggressiveness

Three-way ANOVA-s as well as multidimensional linear regression analysis suggested the presence of *HCRTR1* genotype × SLE × sex interactions for the BPAQ and LHA measures (Supplementary Tables 3 and 4). Higher level of experience of stressful life events by age 15 had a general tendency, in some genotype-wise comparisons statistically significant, to increase aggressiveness, except in the group with highest aggressiveness, the male A/A homozygotes (Fig. 3). In females, higher exposure to SLE-s was in A/A homozygotes associated with a dramatic increase. This was the case for both questionnaire (BPAQ) and interview (LHA) measures.
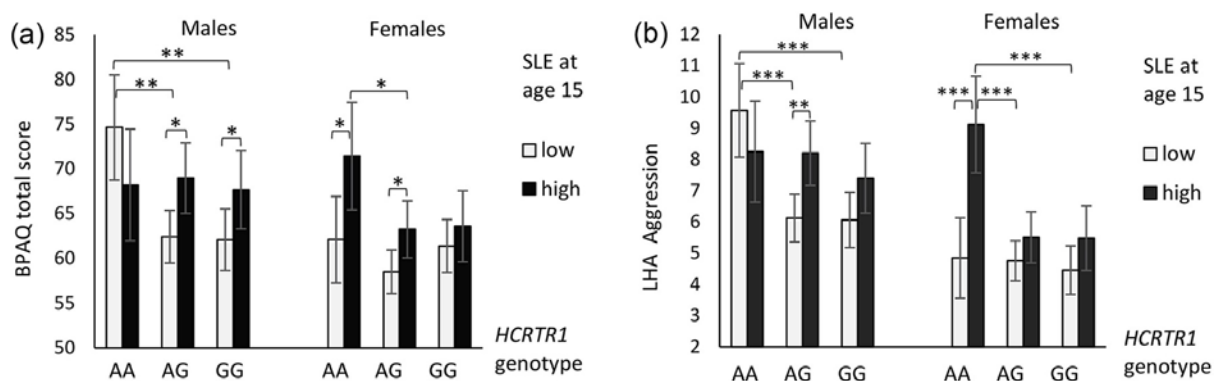

Fig. 3. Aggressiveness by *HCRTR1* genotype, level of exposure to stressful life events and sex. Panel A: Buss-Perry Aggression Questionnaire. Number of males: Low SLE, A/A= 27, A/G = 107, G/G =79; High SLE, A/A = 24, A/G = 60, G/G =49. Number of females: Low SLE, A/A= 40, A/G = 157, G/G =106; High SLE, A/A=26, A/G=92, G/G=59. Panel B: Lifetime History of Aggression. Number of males: Low SLE, A/A=28, A/G=108, G/G=80; High SLE, AA=24, AG=59, GG =50. Number of females: Low SLE, A/A=38, A/G=154, G/G=103; High SLE, A/A=26, A/G=93, G/G=59. * - $p<0.05$, ** - $p <0.01$, *** - $p<0.001$ difference between genotypes. Whiskers indicate 95% confidence intervals.

3.4. HCRTR1 genotype and re-occurrence of driving while impaired

Fig. 4 presents the occurrence of drunk driving during the observation period by year. While some cases of DWI appeared over the years in the random control group and among the speed limit exceeders, the subjects recruited owing to a recorded episode of drunk driving had the levels of relapse that far exceeded the prevalence of DWI in other groups. This re-occurrence of DWI was however related to the *HCRTR1* genotype (Supplementary Table 5). Higher re-occurrence of DWI in the *HCRTR1* A/A homozygotes appeared soon after initial recruitment, became statistically significant at the sixth year of follow-up (DF = 2, $\chi^2 = 7.4$, p= 0.025) and remained so until the last year of follow-up (DF = 2, $\chi^2 = 7.7$, p=0.022). At the recruitment, the drunk drivers, speed limit exceeders and controls were not different in terms of genotype distribution (data not shown).

3.5. HCRTR1 genotype and involvement in traffic accidents

We examined in this sample whether or not the *HCRTR1* genotype could be related to involvement of the participants in traffic accidents. It was revealed that the accumulation of active traffic accidents in the DWI group was higher in the A-allele carriers (Fig. 5 and Supplementary Table 5). A tendency for this was present early in the observation period but as the cases accumulated this trend became statistically significant by the seventh year of observation ($\chi^2 = 4.2$, p = 0.041) and remained so until the last year examined ($\chi^2 = 5.0$, p=0.025). Similar findings were present in an independent sample of passenger car drivers observed since they entered the study (Fig. 6 and Supplementary Table 5). In this sample the number of the *HCRTR1* A-alleles seemed associated with the involvement of traffic accidents, and in particular with active accidents, but statistical significance was found in comparison of either A/A homozygotes or A-allele carriers with G/G homozygotes.
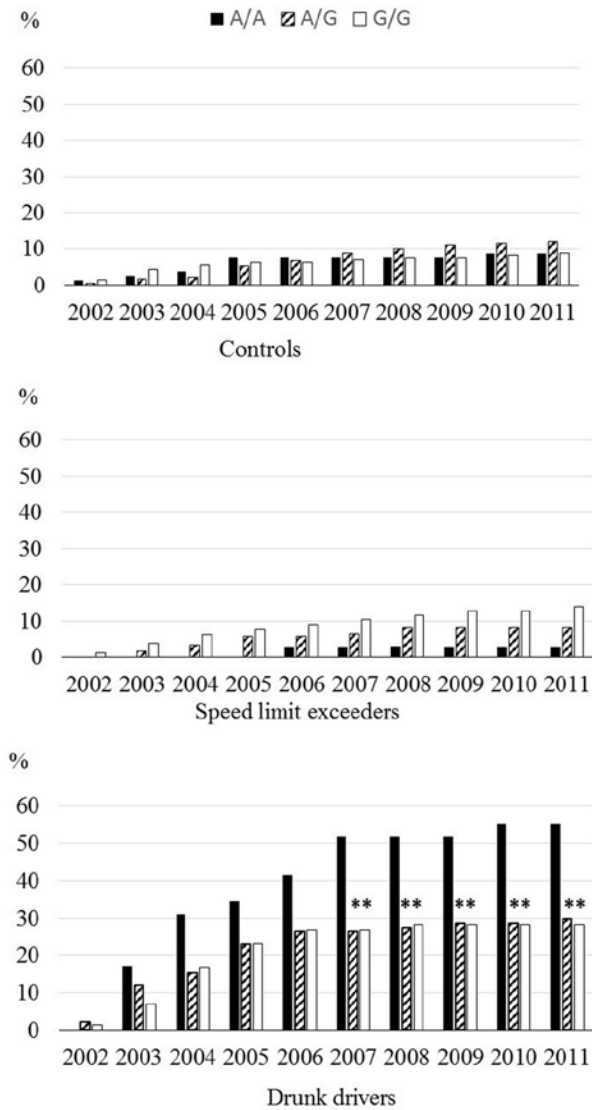
Fig. 4. Cumulative occurrence of driving while impaired in 2002–2011 by year in subjects recruited in 2001–2003, by the *HCRTR1* genotype. The Y-axis represents the proportion of subjects with the given genotype who had by that year driven vehicle while intoxicated. For the drunk drivers group this refers to re-occurrence. * - p < 0.05, difference from the A/A genotype. Genotype frequency: Controls, A/A = 80, A/G = 190, G/G = 160; Speed limit exceeders, A/A = 34, A/G = 121, G/G = 78; Drunk drivers A/A = 29, A/G = 91, G/G = 71.
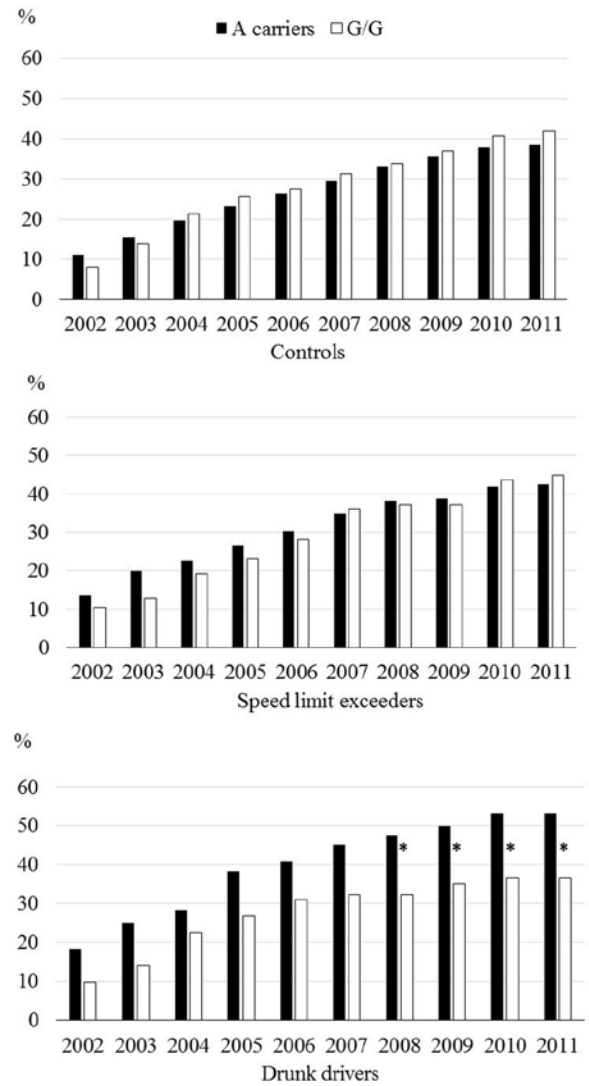
Fig. 5. Cumulative occurrence of active traffic accidents in 2002–2011 by year in subjects recruited in 2001–2003, by the *HCRTR1* genotype. The Y-axis represents the proportion of subjects with the given genotype who had by that year caused a traffic accident. * - p<0.05, difference from the A-allele carriers. Genotype frequency: Controls, A-allele carriers = 270, G/G = 160; Speed limit exceeders, A-allele carriers = 151, G/G = 78; Drunk drivers, A-allele carriers = 120 G/G = 71.
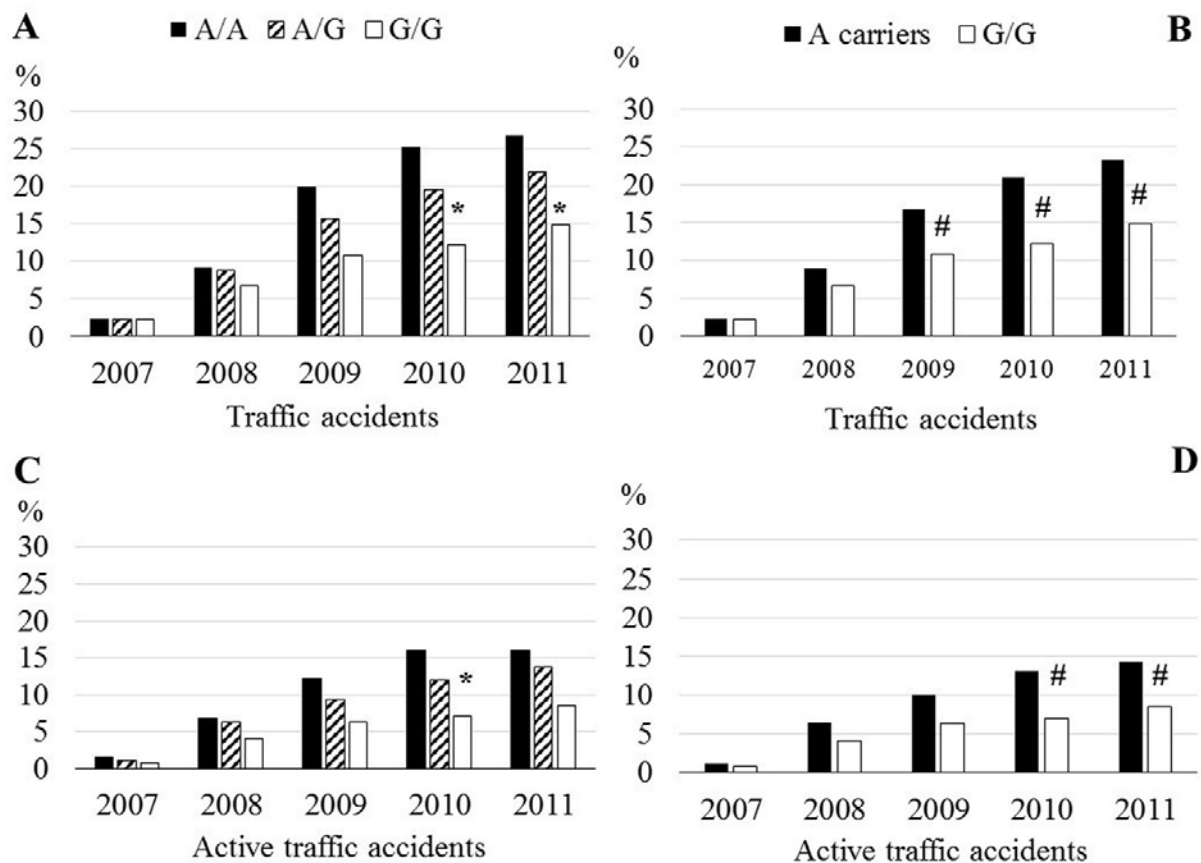
Fig. 6. Cumulative occurrence of total number (A and B) and active (C and D) traffic accidents in 2007–2011 by year in subjects recruited in 2007 at traffic schools, by the *HCRTR1* genotype. The Y-axis represents the proportion of subjects with the given genotype who had by that year been involved in a traffic accident. * - $p < 0.05$, difference from the A/A genotype; # - difference from the A-allele carriers. Genotype frequency: A/A=131, A/G=365, G/G=269.

4. Discussion

In this study we have demonstrated that the orexin system is associated with aggressiveness and accident-prone behaviour in humans. Specifically, homozygocity for the minor variant of the *HCRTR1* gene (rs2271933, G1222A) that causes amino acid substitution (Ile408Val) was associated with aggressiveness measured with either self-reports or clinical interviews. While the paper-and-pencil and interview measures of aggressiveness are in positive correlation (Kiive et al., 2017 and this study), these correlations in birth cohort representative samples are not particularly high. Nonetheless, the genotype effect was apparently very similar with both types of measurement, suggesting that the *HCRTR1* genotype is related to aspects of

aggressiveness that are represented equally in these two instruments. Importantly, the findings in the younger ECPBHS birth cohort, assessed for aggressiveness at age 25 in 2014/2015 were replicated in the older ECPBHS birth cohort assessed at age 33 in 2016/2017. It is of notice that genotype associations with behavioural measures in these two birth cohorts have not been similar in a variety of previous analyses. Indeed, for behaviours with strong secular changes such as age of alcohol use debut, striking genotype by birth cohort interactions have been revealed (Vaht et al., 2014, 2016). These may be related to changes in attitudes toward alcohol use in adolescents and alcohol availability during the societal changes that were very rapid in some of the countries of Central and Eastern Europe during the period when the two birth cohorts were in critical age (Harro and Vaht, 2019). The high similarity of the association of the *HCRTR1* genotype with two different measures of aggressive behaviour in these two cohorts not only provides a necessary replication of the original observation but also suggests that the *HCRTR1* genotype has an effect on aggressiveness that transcends such gene-environment effects that stem from societal changes.

Nevertheless, in females the *HCRTR1* genotype effect was sensitive to stressful life events at the personal level. Again, the findings were similar with questionnaire and interview measures. Subjects with higher exposure to SLE in childhood had higher level of aggressiveness. This increase was small and not significant in female G/G homozygotes, and in the A/A males who had the highest levels of aggressiveness the stress exposure had not further effect, rather a nonsignificant trend towards reduction was observed. The interaction between genotype, SLE and sex was largely driven by the very large increase of aggressiveness in female A/A homozygotes with higher exposure to stress, while the female A/A homozygotes with low stress exposure did not differ from other genotypes.

An initial attempt to understand the mediating mechanisms of the sex differences was made by examining the facets of the Affective Neuroscience Personality Scale in males and females. The ANPS (Davis and Panksepp, 2011) has been constructed bottom-up to measure the aspects of personality arising from persistent individual differences in the emotive phylogenetically old brain circuits and has been advocated for use in biologically oriented psychological studies (Montag and Reuter, 2014). Facets of the ANPS have been linked to characteristic patterns of resting state functional connectivity in the human brain (Deris et al., 2017). We expected to observe higher ANGER in both male and female *HCRTR1* A/A homozygotes, and this was found indeed. Scores of FEAR were higher in females than males, and in particular in female A/A homozygotes. Female A/A homozygotes also had the lowest scores of SEEKING of all sex by genotype groups, while the A/A males had the highest. Altogether these findings lead to speculation that in male A/A homozygotes the relatively high aggressiveness levels that are not dependent of the experience of life adversities originate from higher activities of neural systems for ANGER and SEEKING, the latter promoting resource orientated behaviour. In female A/A homozygotes the high sensitivity to stressful life events may emerge from higher FEAR and low SEEKING. However, as aggressiveness and the personality facets were assessed cross-sectionally in adulthood, this hypothesis requires examination in further longitudinal studies.

It would be most important to know whether the higher aggressiveness in the *HCRTR1* A/A homozygotes can lead to consequences in terms of real life behaviour. For this reason we genotyped the participants of a longitudinal study of traffic behaviour. Risky driving and traffic accidents are in strong relationship with aggressive and anxious states while driving, and share such affective and cognitive characteristics as emotional reactivity and threat appraisal (Zinzow and Jeffirs, 2018). We have previously shown in the EPSTB Sample 1 of this study that driving while impaired is highly recurrent, and that even in terms of other,

additional traffic violations the repeat DWI drivers represent the most hazardous group of car drivers (Tokko et al., 2019). Driving while impaired is a high-risk behaviour that derives not just from the drinking problems but also temperament, personality, and development of basic behavioural tendencies (Stacy et al., 1991). The present study has found that recurrent DWI is more frequent in the *HCRTR1* A/A homozygotes. Drunk driving was not more prevalent with this genotype in subjects with no record of major traffic violations, and if anything it was lower in drivers who had to a very significant extent exceeded speed limits. This suggests a rather specific relationship of *HCRTR1* A/A homozygocity with drunk driving: It appears to aggravate problems with behavioural regulation in subjects who use alcohol irresponsibly. In the drunk drivers group the cumulative causing of traffic accidents was lower in *HCRTR1* G/G homozygotes than in the A-allele carriers. Among the speed limit exceeders a rather different trend of the A/A homozygotes to cause less traffic accidents was revealed, but indeed the speed limit exceeders and drunk drivers have very different personality profiles (Paaver et al., 2006). We next examined the association of the *HCRTR1* genotype with traffic accidents in a different sample of younger passenger car drivers that was recruited directly in traffic schools and consisted of both males and females. It was observed again that the genotype was associated with the active traffic accidents. The *HCRTR1* A/A homozygotes had the highest cumulative accident rate of accidents, while the G/G homozygotes had the lowest. Given that the findings in these younger and less experienced car drivers resembled those obtained only in drunk drivers if the sample had longer driving records, it could be hypothetized that the *HCRTR1* genotype relates to accident proneness in conditions of low behavioural control.

Previously, orexins have been implicated in coordinating defense response but mostly without focus on aggressiveness. However, one highly relevant study on epileptic patients monitored the release of orexin A in amygdala and found it to be increased while subjects

reported higher levels of anger, but also more universally during social interactions; particularly high levels were observed during experiencing positive emotions (Blouin et al., 2013). Orexin A overflow was not related to arousal unspecifically, as it did not occur if arousal was related to postoperative pain. These findings in humans are consistent with data showing higher orexin neuron firing during operant reinforcement (Borgland et al., 2009; McGregor et al., 2011; Sharf et al., 2010). It is however not clear what is the respective contribution of ORX_1 and ORX_2 receptors in these responses. The ORX_2 receptors were recently shown to contribute to emotion-related adaptive strategies dependent on environmental conditions (Staton et al., 2018).

To understand fully the neurobiological mechanisms behind the findings presented herewith, further information would be needed on the functional consequences of the *HCRTR1* rs2271933 G > A variation. The G1222A variation leads to amino acid substitution in the region of receptor that may be interacting with G proteins and other proteins (Thompson et al., 2014), but direct in vitro evidence of functionality is missing, while the valine in the respective position could promote the phosphorylation of the immediately adjacent serine and alter signal transduction upon activation of the receptor (Meerabux et al., 2005). The A-allele was found to be associated with higher prevalence of major depressive disorder (Rainero et al., 2011a), with a tendency for a larger difference from control with the A/A homozygotes than with A/G heterozygotes; however, the association of migraine with the *HCRTR1* genotype rather rested on heterozygotes. These studies were not sufficiently large to suggest clearly the mode of inheritance. A recent study that separated migraine patients to those with aura and those without replicated the overrepresentation of the *HCRTR1* A-allele in migraine patients without aura (Kowalska et al., 2018). The *HCRTR1* genotype was not associated with narcolepsy in a small sample (Peyron et al., 2000) or with panic disorder (Annerbrink et al., 2011). Nevertheless, the presented findings should increase interest in elucidating the

possible functional role of the *HCRTR1* rs2271933 G > A polymorphism and other gene variations in the orexin signalling system to reveal the genetic mechanisms of the role of orexins in the coordinated defense response.

As a limitation of the presented study, the reader should be aware of the uncorrected p-values used throughout the text and illustrations. Given that the fair level of correction is not simple to select for the intercorrelated measures, and that it would put limits to relevant analyses given the fixed sample sizes of longitudinal studies, it was preferred to present all analyses as exploratory and leave the decision of the appropriate significance level with the reader. The strength of the study is inclusion of multiple cohorts and convergence of findings with related yet independent constructs.

Conclusively, we have found a consistent association of the *HCRTR1* rs2271933 G > A variation with aggressiveness in two birth cohort samples. This gene polymorphism was also associated with high relapsing to drunk driving and with higher rate of causing traffic accidents among drivers with limited experience or hazardous driving behaviour. This evidence suggests on important role of orexins in the formation of aggressive responses and their control.

Declarations of interest

None.

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Appendix A.

Supplementary data Supplementary data to this article can be found online

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