Amiloride-sensitive cation channel 2 genotype affects the response to a carbon dioxide panic challenge

Original Paper

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Abstract

Until recently, genetic research into panic disorder (PD) has had only limited success. Inspired by rodent research, demonstrating that the acid-sensing ion channel 1a (ASIC1a) is critically involved in the behavioral fear response to carbon dioxide (CO_2) exposure, variants in the human homologue gene amiloride-sensitive cation channel 2 (ACCN2) were shown to be associated with PD. However, the relationship between changes in brain pH and ACCN2, as done in rodents by CO_2 exposure, has not been investigated yet in humans. Here, we examined this link between the ACCN2 gene and the response to CO_2 exposure in two studies: in healthy volunteers as well as PD patients and using both behavioral and physiological outcome measures. More specifically, 107 healthy volunteers and 183 PD patients underwent a 35% CO_2 inhalation. Negative affect was assessed using visual analogue scales and the panic symptom list (PSL), and, in healthy volunteers, cardiovascular measurements. The single nucleotide polymorphism rs10875995 was significantly associated with a higher emotional response in PD patients and with an increase in systolic as well as diastolic blood pressure in healthy subjects. In all measurements, subjects homozygous for the T-allele showed a heightened reactivity to CO_2. Furthermore, a trend towards an rs685012 genotype effect on the emotional response was found in PD patients. We provide the first evidence that genetic variants in the ACCN2 are associated with differential sensitivity to CO_2 in PD patients as well as healthy volunteers, further supporting ACCN2 as a promising candidate for future research to improve current treatment options.

Keywords

Panic disorder, carbon dioxide exposure, ACCN2, ASIC1a, fear

Introduction

Panic disorder (PD) is a highly prevalent and debilitating condition characterized by recurrent, unexpected panic attacks (PAs) (American Psychiatric Association, 2013). Based on clinical and epidemiological data, a division in subtypes is made, with predominant respiratory or cardiovascular symptoms compromising a "core panic type" (Roberson-Nay and Kendler, 2011). PD is associated with high healthcare costs (Batelaan et al., 2007) and in spite of available treatments, long-term effects are modest and relapse rates are high (Yonkers et al., 2003). Insight into the molecular and genetic mechanisms of the disorder would arguably be beneficial in the search for improved and new treatments.

Until recently, genetic research into PD has been relatively scarce and less successful compared to other psychiatric disorders (Smoller, 2011). Inspired by previous work in mice, attention has been turned to the amiloride-sensitive cation channel 2 (ACCN2), the human homologue to the rodent acid-sensing ion channel 1a (ASIC1a). ASIC1a was shown to play an important role in conditioned fear (Wemmie et al., 2003, 2004), but the interest in ASIC1a in relation to PD was boosted by a seminal study (Ziemann et al., 2009) showing that ASIC1a is also essentially involved in the unconditioned behavioral fear response to carbon dioxide (CO_2) exposure by acting as a chemosensor that detects a decrease in extracellular pH.

Analogs can be drawn to humans, in whom CO_2 exposure has proven to be a reliable experimental model for panic. PD patients display a higher fear and panic response to CO_2 when compared to healthy volunteers (Griez et al., 1987, 1990). A brief inhalation of 35% CO_2 triggers short lasting, but intense symptoms resembling the ones of a naturally occurring PA in PD patients (Nardi et al., 2006; Schruers et al., 2004). We proposed that an acutely disturbed acid–base homeostasis represents the mechanism underlying the occurrence of PAs (Esquivel et al., 2009), thereby forming the molecular basis of the suffocation false alarm theory

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(Klein, 1993; Preter and Klein, 2008). CO_2 exposure was shown to cause a decrease in brain pH in rodents (Schuchmann et al., 2006; Ziemann et al., 2008, 2009). While studies investigating the direct effects of breathing CO_2 on brain pH in humans are lacking, indirect evidence for brain acidification comes from a study using intravenous infusion of bicarbonate, which caused a decrease in brain pH as shown by magnetic resonance spectroscopy (Nakashima et al., 1996). The acid–base homeostasis is tightly regulated within a narrow range and a shift out of the normal range can have fatal consequences (Rhoades and Bell, 2009). Therefore, sensing a change in pH and triggering adaptive physiological and behavioral responses are of crucial importance for an individual's survival. Hence, genes encoding for molecules involved in chemosensation such as *ACCN2* present a pivotal link between pH and panic.

To date, only a few studies investigated the link between *ACCN2* and PD. In a first study (Hettema et al., 2008), the C-allele of the single nucleotide polymorphisms rs685012, located in the 5′ putative promoter region, was associated with anxiety disorder. The replication of this association failed, however, in a second larger sample. This is probably due to assessing a group of mixed anxiety disorder patients of whom only a subset had PD. In a large case-control study, Smoller and colleagues (2014) demonstrated that the C-allele of rs685012 and of rs10875995, located in intron 3, was associated with increased probability of having the diagnosis of PD. Similarly, a significant increase in the frequency of the rs685012 C-allele in PD patients was recently reported by Gugliandolo et al. (2016). In healthy volunteers, Smoller et al. (2014) observed that the C-allele was associated with a larger amygdala volume and heightened amygdala reactivity to visual presentation of fearful and angry faces.

The proposed mechanism linking *ACCN2* and panic in humans involves an altered threshold for sensing acidosis (Smoller et al., 2014). More specifically, variants in the *ACCN2* gene may lead to a lower threshold for detecting a decrease in pH, resulting in a heightened sensitivity to cues from within the body (caused by, e.g. CO_2 exposure). This mechanism is highly plausible, but experimental evidence linking differential CO_2 reactivity in humans to variants in the *ACCN2* gene is currently lacking. For many years it was assumed that, in contrast to PD patients, healthy volunteers are not sensitive to breathing CO_2 (Griez et al., 1987). However, by now, a dose-response relationship between CO_2 and reported fear and bodily symptoms has been repetitively shown (Griez et al., 2007; Schruers et al., 2011), whereby a double inhalation of 35% CO_2 elicits a response that closely resembles the one reported by PD patients to a single vital capacity inhalation. These observations imply the existence of a CO_2-reactivity continuum and that panic symptoms can be provoked in most individuals, depending on the concentration of CO_2 used. The findings suggest that CO_2 inhalation activates basic fear mechanisms that are present in every individual. This opens the avenue of studying panic-related fear in healthy volunteers, thus bridging the gap between animal models and research in PD patients.

The present study sought to investigate the relationship between the response to CO_2 and the *ACCN2* gene, with a focus on the two gene variants previously found to be associated with PD and amygdala reactivity. In the first study, we assessed the response to CO_2 in healthy individuals. To capture the emotional and the physiological reactivity, we examined the effects of CO_2 on self-reported emotions as well as the cardiovascular system. In the second study, in light of clinical relevance, the self-reported emotional response to CO_2 was evaluated in PD patients.

Experimental procedures

Study 1: healthy individuals

Subjects. One-hundred and seven Caucasian adult healthy volunteers participated in this study (mean age 23.76 years, SD = 6.96; 27 males; part of a previous sample (Leibold et al., 2013)). Subjects were recruited via advertisements at Maastricht University, the Netherlands. Eligibility was confirmed using a standard medical examination and a semi-structured psychiatric interview including the Mini International Neuropsychiatric Interview (MINI, Sheehan et al., 1998), performed by an experienced psychiatrist or psychologist. Exclusion criteria included current or past cardiovascular or pulmonary disease, hypertension (systolic/diastolic >170/100 mmHg), familial or personal history of cerebral aneurysm, smoking (>15 cigarettes per day), pregnancy, epilepsy, use of psychotropic medication or adrenergic receptor blockers, and a first-degree relative with PD. All participants gave written informed consent prior to the study. The study was approved by the Medical Ethics Committee of Maastricht University and the Maastricht University Hospital.

CO_2 inhalation. The standardized protocol for CO_2 inhalations in healthy volunteers has been described previously (Griez et al., 2007). In brief, subjects were informed that CO_2 might cause some effects, ranging from minor sensations up to fear. However, all effects would be short-lasting. Subjects took a double vital capacity breath of 35% CO_2. All subjects inhaled at least 80% of their vital capacity as measured using a flowmeter. Subjects were instructed to refrain from caffeine containing beverages on all inhalation days.

Self-reports. The affective response to CO_2 was evaluated in accordance with the DSM-IV criteria for a PA (American Psychiatric Association, 2000). Fear and discomfort were assessed using the Visual Analogue Scale (VAS-F and VAS-D, respectively), consisting of a horizontal line of 100 mm length and ranging from 0 (not at all) to 100 (the worst imaginable). Panic symptomatology was evaluated using the panic symptom list (PSL), a questionnaire listing the 13 DSM PA symptoms such as palpitations, sweating, shortness of breath, dizziness, and choking, and ranging from 0 (absent) to 4 (very intense). Self-reports were obtained directly before and immediately after the inhalation.

Cardiovascular recordings. Blood pressure and heart rate were measured continuously throughout the entire procedure in healthy volunteers using a computerized system (Carbon Dioxide Tolerance Tester, CTT, Maastricht Instruments BV, Maastricht, the Netherlands). Both parameters were measured by means of a finger cuff that was connected to a cardiovascular monitor (Nexfin, BMEYE BV, Amsterdam, the Netherlands). All measurements were acquired with custom-made software (IDEEQ, Maastricht Instruments BV, Maastricht, the Netherlands).

Study 2: PD patients

Subjects. One-hundred and eighty-three Caucasian PD patients (mean age 36.14 years, SD = 10.94; 71 men) participated in this

study. Patients were recruited from the outpatient setting of the Academic Anxiety Centre, Maastricht, the Netherlands. PD (with or without agoraphobia) as main diagnosis was established via a semi-structured psychiatric interview by an experienced psychiatrist, including the MINI (Sheehan et al., 1998) (see assessment scores shown in Supplementary table 1 online). Patients underwent a standard medical examination to confirm eligibility. Exclusion criteria were current or past cardiovascular or pulmonary disease, hypertension (systolic/diastolic >170/100 mmHg), familial or personal history of cerebral aneurysm, pregnancy, and epilepsy. In total, 106 patients had one or more comorbid disorders. Patients were free of antidepressant and benzodiazepine treatment for at least 4 or 2 weeks, respectively, before taking part in the experiment. Written informed consent was obtained from each subject before participating. The study was approved by the Medical Ethics Committee of Maastricht University and the Maastricht University Hospital.

CO_2 inhalation. In PD patients, CO_2 inhalations took place according to a standardized protocol as described previously (Klaassen et al., 1998). Subjects were informed that the subsequent inhalation might cause some effects, ranging from minor sensations up to fear. However, all effects would be short-lasting. Subjects took a single vital capacity breath of 35% CO_2. Using a flowmeter it was ensured that at least 80% of their previously measured vital capacity was inhaled.

Self-reports. In line with the DSM-IV criteria for a PA (American Psychiatric Association, 2000), experienced fear was evaluated using the Visual Analogue Scale for fear (VAS-F), consisting of a horizontal line of 100 mm length and ranging from 0 (not at all) to 100 (the worst imaginable). Panic symptoms were assessed by means of the PSL, a questionnaire listing the 13 DSM PA symptoms, and ranging from 0 (absent) to 4 (very intense). Self-reports were obtained directly before and immediately after the inhalation.

Genotyping

Saliva was collected from all subjects using Oragene® saliva self-collecting kits (DNA Genotek Inc, Ontario, Canada). Genomic DNA was isolated using the AutoGenFlex DNA Isolation system (Autgen, Hilliston, MA, USA) according to manufacturer's instructions and subsequently genotyped using Taqman SNP Genotyping Assays for rs10875995 and rs685012 (Life Technologies). The selection of these particular polymorphisms was based on the results of the recent study by Smoller et al. (2014), in which an association with PD and amygdala reactivity was found. Results were independently scored by two experienced technicians.

Data and statistical analysis

Primary analyses. Differences in the change of self-reported emotions and symptoms depending on the genotype were analyzed using univariate analysis of variance (ANOVA). Six PD patients were excluded from the rs685012 analysis due to genotyping failure (final sample mean age 36.14 years, SD = 10.94; 71 men).

To analyze physiological measurements in healthy subjects, locally weighted polynomial regression (Cleveland and Devlin, 1988) was used to avoid strong effects of outliers. Based on smoothed data, the maximum, the area under the curve (AUC), and the standard deviation (log transformed) were calculated within the first 60 s after inhaling CO_2. The maximum represents the highest value/peak in this period, which might reflect a few seconds of the response only. To capture the response in more detail, the AUC was calculated. The standard deviation serves as index for fluctuations caused by CO_2. Due to a technical failure, the final sample for physiological analyses consisted of 92 subjects (mean age 23.56 years, SD = 0.44; 24 men).

Secondary analysis. Patients with predominantly respiratory symptoms during their PAs have been proposed as forming a "core" panic subtype (Roberson-Nay and Kendler, 2011). Smoller et al. (2014) showed a particularly strong relationship between *ACCN2* genotype and such a respiratory panic subtype. We have previously demonstrated the existence of a similar respiratory subtype in response to a CO_2 challenge (Colasanti et al., 2008). Analysis of the respiratory cluster was based on this previous cluster analysis study (Colasanti et al., 2008) and consisted of the symptoms shortness of breath, feeling of choking, dizziness, sweating, palpitations, and chest pain. The effect of genotype on the sum of the change scores of these six symptoms was analyzed by means of univariate ANOVA.

Based on Smoller et al. (2014), C-allele carriers were compared to T/T homozygotes in all analyses for both polymorphisms. Significance was set at $p < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 20.0.0.1 for Mac; SPSS Inc., Chicago, IL) or the software R (version 3.1.1, 2014, R Development Core Team, Vienna/Austria).

Results

Demographical data of healthy volunteers and PD patients are presented in Table 1. The distribution of *ACCN2* genotypes was in Hardy–Weinberg equilibrium for both samples. Genotypes did not differ with respect to sex and age and the duration of PD.

Study 1: Healthy volunteers

Self-reports to CO_2 inhalation. In healthy volunteers, no effect of the rs10875995 genotype on the change score regarding VAS-F ($p = 0.264$, Figure 1(a)), VAS-D ($p = 0.208$), and PSL ($p = 0.196$, means shown in Supplementary table 2) was present. No rs685012 genotype effect was found on VAS-F ($p = 0.646$, Figure 2(a)), VAS-D ($p = 0.131$), and PSL ratings ($p = 0.453$, means shown in Supplementary table 3).

Cardiovascular response to CO_2 inhalation. Healthy subjects homozygous for the T-allele of rs10875995 showed a significantly higher AUC ($p = 0.043$, means shown in Supplementary table 4) and a tendency towards a higher peak ($p = 0.089$) of the systolic blood pressure after the CO_2 inhalation. Similarly, also the AUC ($p = 0.049$, Figure 3(a)) and the peak ($p = 0.030$, Figure 3(b)) of the diastolic blood pressure were elevated after inhaling CO_2 compared to C-allele carriers. No effect was found on any

Table 1. Demographics of healthy volunteers and PD patients.

		rs10875995		rs685012	
		C/C and C/T	T/T	C/C and C/T	T/T
Healthy volunteers	***N***	55	52	56	51
	Sex (% male)	21	29	20	30
	Age (SD)	23.70 (7.02)	23.83 (7.22)	24.04 (7.17)	23.45 (6.78)
PD patients	***N***	90	93	91	86
	Sex (% male)	40	38	36	42
	Age (SD)	36.06 (11.55)	36.22 (10.37)	36.18 (11.63)	36.31 (10.36)
	Duration PD (years (SD))	11.08 (11.30)	8.82 (9.84)	11.12 (11.31)	9.05 (10.07)

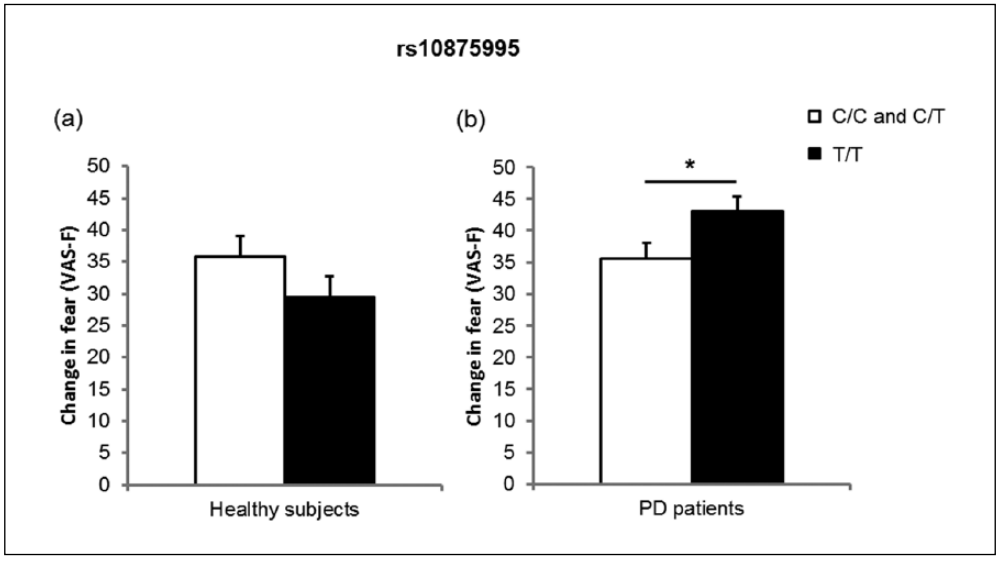

Figure 1. Effects of CO_2 on the VAS-F ratings depending on the rs10875995 genotype. (a) No effect of genotype on the VAS-F was found in healthy subjects. (b) In PD patients, homozygous T-allele carriers scored significantly higher than C-allele carriers. *$p < 0.05$. Data represent mean + SEM.

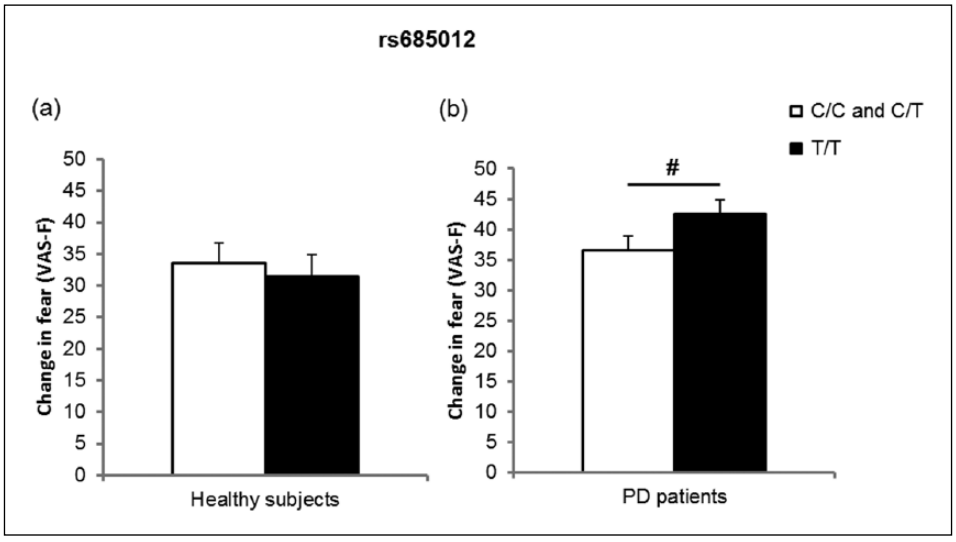

Figure 2. Effects of CO_2 on the VAS-F ratings depending on the rs685012 genotype. (a) No effect of genotype was present in healthy subjects. (b) In PD patients, a trend towards a higher change score was found in carriers homozygous for the T-allele in comparison with C-allele carriers. #$0.05 < p < 0.1$. Data represent mean + SEM.

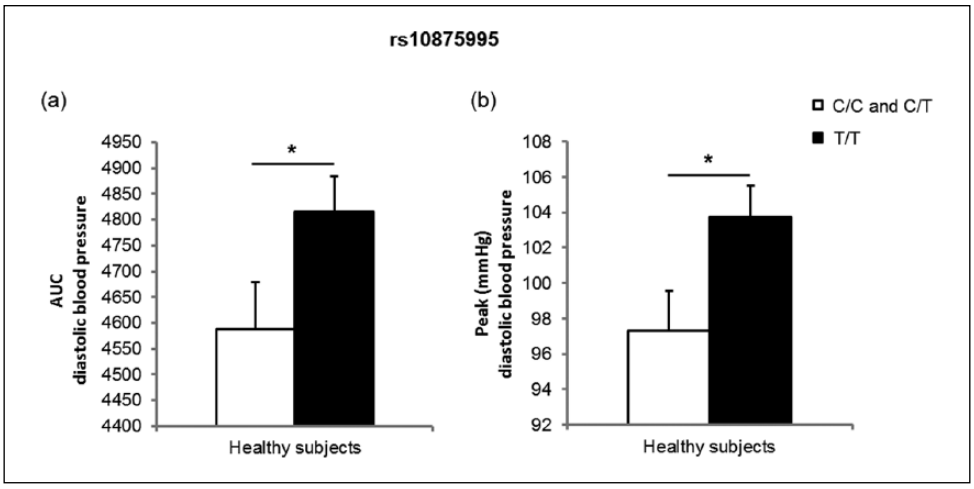

Figure 3. Effects of the rs10875995 genotype on the diastolic blood pressure in response to CO_2 in healthy subjects. (a) Homozygous T-allele carriers had a significantly higher AUC after inhaling CO_2 compared to subjects of the C/C and C/T genotype. (b) The same effect was observed with regard to the peak after inhaling CO_2. *$p < 0.05$. Data represent mean + SEM.

outcome of heart rate (AUC $p = 0.357$, peak $p = 0.698$). With regard to rs685012, no effect was present on any outcome (Supplementary table 5).

Study 2: PD patients

Self-reports to CO_2 inhalation in PD patients. In PD patients, subjects homozygous for the rs10875995 T-allele scored significantly higher on the VAS-F in comparison with C-allele carriers ($p = 0.032$, Figure 1(b), means shown in Supplementary table 2), whilst no effect of genotype on reported PSL scores was found ($p = 0.388$). With regard to rs685012, there was a trend towards higher VAS-F ratings in subjects with the T/T genotype in comparison with C-allele carriers ($p = 0.061$, Figure 2(b)). No significant effect of *ACCN2* genotype on PSL scores was observed ($p = 0.519$, means shown in Supplementary table 3).

Secondary analysis. *Respiratory cluster analysis.* We next analyzed the effect of genotype on the respiratory symptom cluster in the response to the CO_2 challenge, as defined in a previous study. There was no main effect of rs10875995 ($p = 0.659$) or rs685012 genotype ($p = 0.889$) (means shown in Supplementary tables 2 and 3).

Discussion

In this study, we show an association between two genetic variants in the *ACCN2* gene, selected based on prior data (Smoller et al., 2014), and CO_2 sensitivity in PD patients and healthy individuals. More specifically, the rs10875995 T/T genotype was associated with higher fear scores in patients, and with a higher blood pressure response to a CO_2 panic challenge in healthy subjects compared to C-allele carriers.

The *ACCN2* gene is the human orthologue of the rodent gene encoding the pH-sensitive ion channel ASIC1a, which was shown to be critically involved in conditioned (Wemmie et al., 2003, 2004) as well as unconditioned CO_2-induced fear behavior in mice (Ziemann et al., 2009). Therefore, ASIC1a is a prime candidate to be involved in the molecular pathophysiology of panic in humans. To date, the few previous human studies investigating an association between the *ACCN2* gene and PD reported an association of the rs685012 and/or rs10875995 C-allele with PD or anxiety disorders (Gugliandolo et al., 2016; Hettema et al., 2008; Smoller et al., 2014). Smoller and colleagues also found a larger amygdala volume and heightened functional amygdala reactivity in a sample of healthy C-allele carriers of the rs10875995 genotype. The authors suggested that this observed amygdala-association might reflect an increased sensitivity to a decrease in pH, secondary to neuronal activity to process the visual emotional stimulus. The elevated sensitivity would be associated with *ACCN2* gene variants that contribute to a decreased threshold for sensing acidosis. Furthermore, they speculated that "such activation would clearly be less direct (and less intense) than that induced by a direct CO_2 inhalation."

The present studies show an rs10875995 genotype effect on the emotional as well as cardiovascular reactivity following such a direct 35% CO_2 inhalation. In PD patients, VAS-F ratings were higher in carriers homozygous for the rs10875995 T-allele compared to C-allele carriers and consistent with this we also found a heightened cardiovascular response in healthy T/T carriers. This is in contrast to the study by Smoller et al. (2014), in which the C-allele was considered as risk allele. This might be explained by focusing on different phenotypes. In Smoller's study, the C-allele was shown to be associated with the *diagnosis of PD* and with *amygdala volume and function*, i.e. *largely anxiety-related endophenotypes*, whereas the present study showed a relationship between the T-allele and *experimentally provoked fear/panic sensations*, i.e. *specific fear-related endophenotypes*. The amygdala has been implicated in rodent (Ziemann et al., 2009) and human (Holmes et al., 2012) fear behavior as well as in enhanced reactivity to emotional threats in humans (Shin and Liberzon, 2010). Furthermore, altered amygdala structure and functioning has been reported in PD patients (Kim et al., 2012). However, PD is a complex disorder and consists of different aspects (PAs, anticipatory anxiety, and avoidance behavior), which are likely

subserved by different parts of the brain and not solely by the amygdala. While the amygdala may be most important in the conditioning process leading to avoidance behavior, the nature of PAs might be more related to other structures such as the brainstem (Goossens et al., 2014). Therefore, focusing on a complex anxiety disorder (diagnosis of PD) or a fear-specific phenotype (response to CO_2) might lead to different results.

Moreover, observing opposite alleles associated with risk disease between studies is a known phenomenon in association studies and is referred to as "flip-flop phenomenon" (Lin et al., 2007). Although the exact cause is unknown it is assumed that it occurs when the focus is on one genetic locus while in fact many genetic variants or environmental factors, and their mutual interaction, contribute to complex phenotypes such as neuropsychiatric disorders. In addition, in spite of similar ethnicities, two populations can differ in terms of linkage disequilibrium between the two genetic loci, leading to differences in the direction of the association between studies (Lin et al., 2007). Moreover, Clarke and Cardon (2010) have calculated that the probability of finding at random flip-flop associations is negligible. Therefore, this phenomenon may have biological implications.

Given that PAs are accompanied by profound physiological symptoms, we also included cardiovascular measurements in healthy volunteers to complement self-reports. Consistent with an elevated emotional response in patients of the rs10875995 T/T genotype, we found a heightened cardiovascular response in healthy T/T carriers. It has been shown that ASIC1a interacts with ASIC2, a channel implicated in baroreceptor sensitivity and thus blood pressure regulation (Lu et al., 2009). In addition, interaction of the two channels can shift the pH sensitivity (Wemmie et al., 2013). Currently, the functional consequences of variants in the *ACCN2* gene are not known. It might be speculated that the T-variant increases the pH sensitivity, associated with an elevated autonomic response. In addition to cardiovascular symptoms, respiratory ones are common during PAs. PAs with prominent respiratory symptoms have been proposed as a core panic subtype (Roberson-Nay and Kendler, 2011) and Smoller et al. (2014) showed a particularly strong association between the *ACCN2* genotype and the respiratory subtype in a secondary analysis. We could not confirm this in the present study, possibly due to methodological differences between the studies. We based our definition of a respiratory subtype on a cluster of six PSL sub-items in response to the CO_2 inhalation, that we previously identified using a principal component factor analysis (Colasanti et al., 2008), whilst Smoller et al. (2014) grouped patients depending on the predominance of certain symptoms during real-life PAs, assessed via diagnostic interview. On the molecular level, the role of ASIC1a in respiration is still under investigation, but ASIC1a knock-out mice that do not express the protein were shown to have normal increases in minute ventilation to CO_2 (Ziemann et al., 2009).

It has to be noted that we did not observe all effects consistently in both human samples. While patients having the T-allele of rs10875995 showed an increased fear response to CO_2, no effect was found in healthy volunteers. We have previously reported that a double vital capacity inhalation of 35% CO_2 in healthy volunteers induced a condition complying with the formal criteria of panic in the current psychiatric nosology (Griez et al., 2007). However, this constitutes a qualitative comparison; recently we provided a direct quantitative comparison showing that PD patients on average still show a higher sensitivity to CO_2 (Leibold et al., 2016). This might explain, together with the smaller sample size in the healthy group, why the effect of genetic variation can be detected more easily in patients. Further, in patients, a moderating effect of the *ACCN2* genotype on VAS scores, but not on reported bodily symptoms, may be explained by the finding that the VAS is a more specific instrument than the PSL to measure reactivity to a 35% CO_2 challenge (Battaglia and Perna, 1995; Verburg et al., 1998). Similar effects were found for rs685012.

In light of the current theories about the pathophysiology of PD, we have proposed that PAs are caused by an acute metabolic disturbance of the acid–base homeostasis (Esquivel et al., 2009). Such a metabolic disturbance might form the molecular basis of Klein's suffocation false alarm theory (Klein, 1993; Preter and Klein, 2008) and can be induced by inhaling CO_2. In rodents, it has been shown that ASICs are important in detecting and triggering behavioral responses to CO_2 (Ziemann et al., 2009). The present study and the ones in the past few years that show an association between ASIC and PD (phenotypes) suggest that these channels might be one of the neural substrates in the alarm system in humans.

ASIC1a is expressed throughout the entire central nervous system including the brainstem (Price et al., 2014) and is a likely molecular candidate to act as chemosensor in a variety of neurons. Neurons intrinsically sensitive to CO_2/pH are located within many brain structures (Biancardi et al., 2008; da Silva et al., 2011; Dean et al., 1990; Mulkey et al., 2004; Putnam et al., 2004; Ziemann et al., 2009). Some of those structures most likely serve different functions such as breathing as well as defensive behavior (Esquivel et al., 2009). The exact contribution of these brain structures in CO_2 sensitivity, sensing changes in pH and eliciting a fear and panic-related behavioral response, is still elusive. In rodents, the amygdala has been identified as an essential brain structure in this process (Ziemann et al., 2009), while research in Urbach–Wiethe disease patients having a bilaterally dysfunctional amygdala could not confirm this (Feinstein et al., 2013). A recent functional magnetic resonance imaging study by our group (Goossens et al., 2014) suggests that, at least in humans, the brainstem is the principal brain area in sensing changes in CO_2/pH.

Future studies should address the relationship between variants in the *ACCN2* gene and the locus of brain activity in response to CO_2 in a large sample. In addition, the functional consequences of those variants should be investigated. This is also interesting in relationship to cardiovascular and respiratory measures in patients, which we did not assess in the present study. Further, the exact effects of inhaling CO_2 on brain pH in humans remain to be elucidated. In rodents, it was shown that exposure to CO_2 decreases brain pH (Ziemann et al., 2009). In humans however, direct evidence for such a CO_2-induced brain acidosis is lacking. Indirect evidence is available, provided by MRI studies showing that PD patients have a larger brain lactate response to visual stimuli (Maddock et al., 2009) and after lactate infusion (Dager et al., 1997, 1999), compared to healthy controls. Work in rodents showed that an increase in lactate causes a decrease in brain pH (Erlichman et al., 2008), suggesting that a similar effect may occur in humans. Combining CO_2 administration and MR spectroscopy, able to non-invasively detect brain pH changes, may provide the lacking direct evidence.

Limitations

The results of this study should be interpreted keeping in mind some potential limitations. First, the sample size of the present study is relatively small for genetic analyses. Due to the low number of subjects homozygous for C, we clustered C-allele carriers into one group to allow meaningful statistical analyses instead of a possibly more sensitive quantitative (0-1-2 alleles) approach. Second, we did not correct for multiple testing, given our confirmatory and clear hypothesis-driven approach of two highly correlated variants. Third, family history of PD was an exclusion criterion in the healthy volunteers group. With hindsight, this may have led to the inclusion of a "hyper-normal" group, thereby increasing the possibility of a type II error. This might explain the less homogeneous results in the healthy group. To increase the sensitivity on outcome measurements, future studies should include samples with a larger variation in anxiety, such as individuals with differential anxiety sensitivity or first-degree relatives of PD patients.

Conclusion

The results of the present study extend on those of previous studies into the mechanisms of panic and provide the first evidence for a relationship between the *ACCN2* gene and the reactivity to a CO_2 panic challenge in humans. Thereby, the association between the *ACCN2* gene and PD is strengthened and the importance of investigating both the role of variants in the *ACCN2* gene in humans as well as ASIC1a in rodents in the pathophysiology of panic is further supported. In the long-term, *ACCN2* may become a potential therapeutic target in the treatment of PD, a suggestion that is also supported by rodent research in which pharmacological inhibition of ASIC1a reduced fear-behavior (Ziemann et al., 2009).

Declaration of conflicting interests

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