Identifying key modulators of panic attacks in panic disorder: A translational model

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Abstract

Panic disorder (PD) has a prevalence of 4% in the general population, but its pathophysiology remains elusive. This research focuses on identifying molecular and genetic modulators of unpredictable, recurrent panic attacks which characterize PD. Prior studies revealed that the serotonin transporter, 5-HTT, is involved in fear behavior and that genetic variations might affect panic-related behavior. In order to test these genetic variations on a molecular level a translational model consisting of humans and rodents was used. As follow-up of a human study, 40 male mice (background C57BL/6), wild-type and heterozygous 5-HTT knock-out, were exposed to either 9% CO_2 or room air to experimentally provoke fear-related behavior.

Furthermore, the acid-sensing ion channel ASIC1a is hypothesized to be a linking factor between fear detection and fear response. It was tested whether this ASIC1a is expressed in serotonergic neurons in the dorsal raphe nucleus, main source of serotonin, the pivotal neurotransmitter in panic responses. Localization of the ASIC1a channel was determined by use of immunofluorescence.

Analysis of the behavioral tests showed no significant difference between genotypes in both air and carbon dioxide condition. Carbon dioxide exposure however increased freezing time in both genotypes. ASIC1a has been indicated in the dorsal raphe and hippocampus in perfusion-fixated rat tissue, but could not yet be detected in the dorsal raphe of the fresh-frozen tissue of tested mice.

Introduction

Billions of years of evolution have led to complicated organisms which possess complex brains, which in turn led to adaptive behavior. Behavior is the acting and reacting of an organism to its environment, making sure the organism survives and produces offspring. Nature has created several ways to deal with harmful circumstances, of which, in case of direct life-threatening situations, panic is one. Since dealing with these circumstances is

literally of vital importance among many animals, the pivotal part that creates this panic response is located in the more preserved, ancient brain structures. Higher vertebrates like humans and rodents still possess lower structures like medulla and pons. Because of the preservation of these brain regions among species during years of evolution a translational model between humans and rodents might be reliable (1).

Neuronal signalling needs to be modulated very precisely. Hence, alterations among individuals due to genotypic differences may result in different signalling and thus behavior. In case of an insufficient stimulus detection or signalling in humans concerning panic behavior, panic disorder (PD) might develop. Sufferers from PD experience recurrent, unpredictable panic attacks (PAs) that are characterized by symptoms such as feelings of intense fear and physiological reactions like choking, dizziness and palpitations (2). Panic disorder is quite prevalent with 4% of patients in the general population (3). Panic attacks occur even in about 23% of the general population at least once in their lifetime (4).

Panic behavior occurs when being exposed to very direct threats that occur from inside the body, for example by inhalation of an elevated concentration of carbon dioxide in an experimental manner (5). Given the fact that both patients and subjects react to elevated CO_2-concentrations indicates that a similar mechanism is involved (6). Patients however might be more sensitive to stimuli at the molecular level resulting in more severe reactions.

Genetic determinant: 5-HTT

In case of panic behavior there is convincing evidence that the serotonergic (5-HT) system is involved (7). Signalling at the synaptic level is dependent on factors such as the amount of neurotransmitter, 5-HT in this case, which again is dependent on the availability of conversing enzymes, (auto)receptors and 5-HT transporters that transport the 5-HT from the synapse back into the cell. The amounts and/or efficiency of the mentioned factors determine the speed, duration and amplitude of the signal. Several experiments have been executed in which the effect of 5-HT availability and use of agonists and antagonists of the 5-HT receptor was investigated (8). These studies concluded that 5-HT signalling does modulate the response of a PA.

One can imagine that different genotypes coding for the enzymes, receptors and transporters cause different signalling and subsequently altered responses and behavior. The effect of the serotonin transporter, 5-HTT, has been investigated in prior studies (9). This transporter causes the 5-HT signalling to be terminated by removing 5-HT from the synaptic cleft. In humans, a polymorphism has been found that determines the efficacy of the transcription of this 5-HTT-gene (10). An individual can possess either the SS, SL or LL genotype. The LL genotype is transcribed more efficiently which in turn leads to

more transporters and thus a quicker termination of the signalling compared to SS or SL genotypes. Possession of minimally one S-allele was shown to result in a decreased fear response compared to the LL-genotype (9). The polymorphism is not present in mice, but in order to create a translational model we investigated wild-type and heterozygous knock-out mice concerning the 5-HTT. Both wild-type and heterozygous knock-out conditions are comparable to the polymorphism genotypes used in human studies since heterozygous mice will possess less transporters which is similar to the SS and SL genotypes in humans. By making use of behavioral tests, wild-type and heterozygous 5-HTT mice will be investigated by scoring several fear indicating parameters. We expect that heterozygous mice will show less fear-related behavior due to the longer lasting 5-HT signalling.

Molecular modulator: ASIC1a

It is known that serotonergic signalling plays a pivotal role when it comes to fear-related behavior. The initiation of the signalling however remains quite indistinct. It has however been shown that the amygdala, involved in fear-related behavior, expresses certain ion channels, ASIC1a (11,12). ASICs, acid-sensing ion channels, are ion channels through which sodium and calcium ions flow into the cell when exposed to an acid environment. This influx may then induce neuronal firing by depolarizing the membrane potential (13-15).
The most abundant ASIC subtype, ASIC1a, (16) and its modulating effect on fear responses has been elucidated by Coryell et al. in 2007 (12). The chemoreceptive property of amygdale neurons seemed to be a linking factor of sensing stimuli and responses. Additionally, a lowered pH in midbrain 5-HT neurons leads to an increased firing rate of these neurons (17), which might be explained by pH-sensing ion channels such as ASIC1a. Raphe neurons are chemoreceptive as well and react by increasing the firing rate, thus an increased 5-HT signalling.
When taking the findings of mentioned prior studies together, it sounds plausible that 5-HT raphe neurons in the midbrain might express ASICs because of their chemosensing ability. Since raphe neurons are a pivotal source of 5-HT, which plays a major role in panic responses, we hypothesize that 5-HT neurons in the dorsal raphe nucleus express ASICs which allow them to be a linking factor between extracellular sensing and neuronal firing.

Material and Methods

Effect of 5-HTT genotype on fear-related behavior

The genetic part concerning the 5-HTT genotypes was tested in humans and mice, but only the rodent study was part of this research. By means of the 5-HTT genotype 40 male

mice (background C57BL/6), wildtype and heterozygous knock-out, kindly provided by Prof. Lesch (University of Würzburg), underwent the experiments. Mice were housed paired for 10 weeks with access to mouse chow and water *ad libitum*. Then, the animals were subjected to behavioral tests and at the age of 4 months were sacrificed by decapitation. Only male animals were used because of the influence of the estrous cycle on the response to CO_2 exposure (18).

Both genotypes were tested by making use of the open field test (OFT) and aversion assay (AA) in a Plexiglass box (50x50x40cm). In the AA a septum including a passage was placed inside the box to create two separated chambers. The amount of leaking CO_2 was negligible. During the OFT mice were exposed to either normal room air or 9% CO_2 for 20 minutes. Scored parameters included total distance moved, time spent in centre and corners and time spent freezing of which the last is a correlate of panic (19). Freezing time was manually scored being blind regarding genotype and condition whereas other parameters were scored via video tracking EthoVision XT 7.5 software.

In the AA mice were placed in randomized manner in one of the chambers. Chambers contained either both normal air or one randomized side 9% CO_2. The time spent per chamber was scored for 10 minutes, however compared to others studies we corrected for freezing time (20), since total times spent will increase by freezing even though it may not be the animal's intention.

Immunohistochemical staining of ASIC1a

Staining ASIC1a was achieved after executing several pilot stainings in which multiple parameters were altered. Brains of mice used in the behavioral tests were removed quickly after decapitation, freshly frozen and stored at -80°C according to Wemmie's paper (21).

The mice brains were cut in 16 µm thick slices by using a Leica CM3050 cryostat and put on microscope glasses. Samples thawed followed by post-fixation in a 4% formaldehyde and 4% sucrose solution for 10 minutes at 4°C. Thereafter, the coupes were kept at room temperature (RT) and dried for 30 minutes, followed by three times 5 minutes washing steps in consecutive two times Tris-buffered saline 1x (TBS) and one time 0.3% TBS-T. Then the glasses incubated 30 min. in citrate buffer (pH=6.0) followed by 30 min. 5% donkey serum. Subsequently the primary antibody against ASIC1a (Santa Cruz goat-α-mouse 1:100 in 0.03% donkey serum in TBS-T) was added. Samples incubated overnight at 4°C.

The protocol's second day was executed at RT and started off by two times washing in TBS for 5 minutes. The 5% donkey serum incubated for 30 min followed by the application of the Alexa488 secondary antibody (Invitrogen 1:100 in 2% donkey serum in TBS-T) which incubated for 2 hours in the dark. The last described TBS washing steps were

then repeated, this time in the dark. Samples were then placed conversely on a plastic plate before pipetting 300 µl Hoechst (1:500 in TBS) to determine cell positions per glass between the plate and glass. Hoechst incubated for half an hour in the dark after which the two washing steps in TBS were executed again. Thereafter the glasses were covered by mounting solution, 80% glycerol and 20% TBS, and a coverslip was mounted and secured on the glass. By means of an Olympus BX-51 microscope and cellSens imaging software stainings were examined.

Statistical analysis

Freezing times scored during the OFT and AA were statistically analyzed. Mice were grouped per genotype and condition. In case of the AA starting side and CO_2-side were used as differentiating parameters also. The mean values of freezing times of the four groups (n=10) in both tests were compared by means of a one- and two-way ANOVA-test and LSD post-hoc test in the IBM SPSS Statistics 21-software, using a significance level of $p<0.05$.

Results

Behavioral test analysis

As can be seen in Figure 1, different conditions affected the amount of time freezing significantly independent of genotype ($p<0.001$) in OFT and AA. However, between genotypes, no significant difference in freezing times was found per condition.

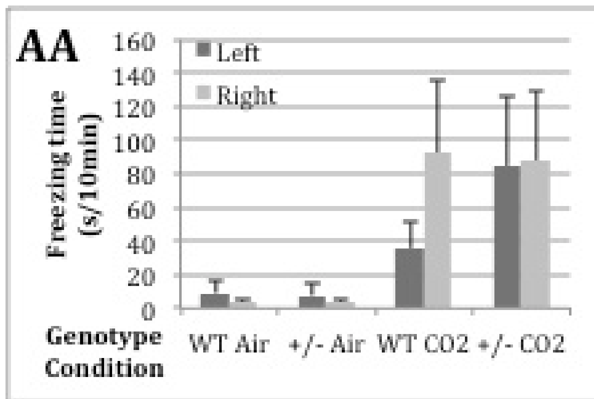

Figure 1. Freezing time in OFT per genotype and condition. Means + SEM. Significant differences (* $p<0.001$) among groups.

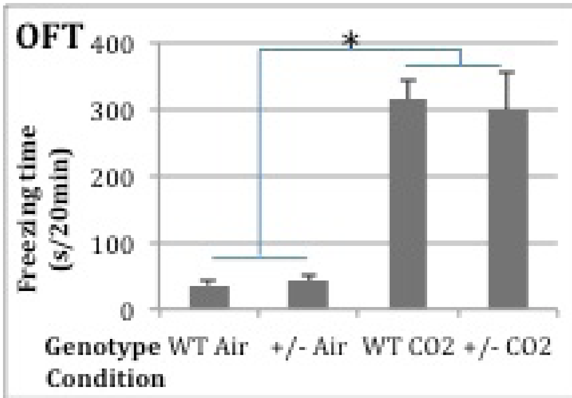

Figure 2. Freezing time in AA per genotype and condition in both left and right chamber. Means + SEM, corrected for starting side. Significant differences ($p<0.05$) among groups, but no effect of genotype and CO_2- side.

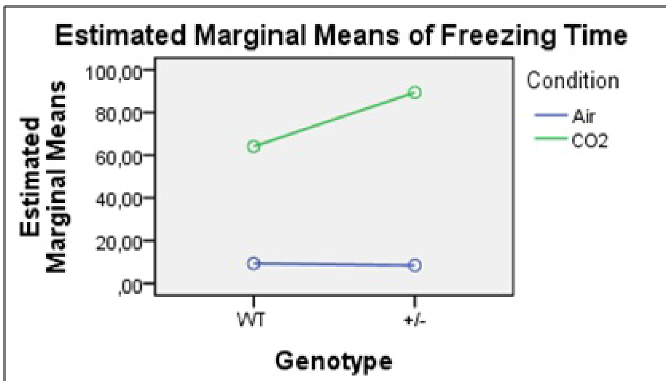

Figure 3. Chart of the Genotype*Condition interaction effect in AA. Genotype*Condition: $p<0.475$.

Genotype and CO_2-side do not show significant differences in freezing time in the AA (Fig. 2-3). Testing the effect of starting side as a covariate in the two-way ANOVA turned out to be insignificant ($p=0.705$). As Figure 3 shows, an significant interaction between genotype and condition could not be determined. Both tests resulted in significantly different freezing times between normal air and carbon dioxide conditions, regardless of genotype and side of $CO_{2.}$

Immunofluorescent staining

Concerning the immunofluorescent staining promising results were achieved. Specific signals on cell bodies and dendrites in the hippocampus were found in fresh-frozen tissue as can be seen in Figure 4, which corresponds to a prior study (16).

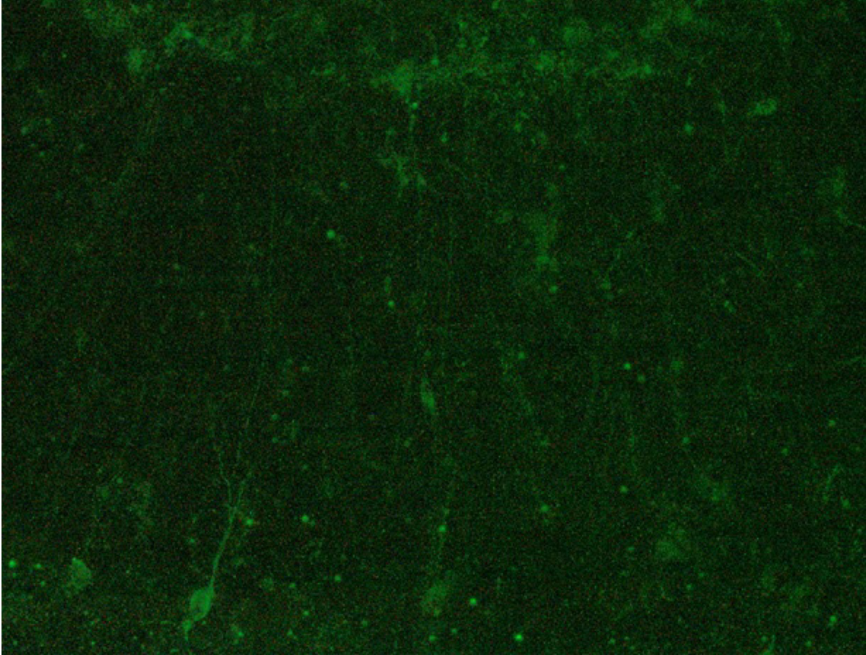

Figure 4. Signal from cell bodies and sprouting fibers in the CA2 region of the hippocampus. Condition: Santa Cruz goat-α-mouse, blocking buffer, citrate buffer incubation and Alexa488. Magnification 20x, exposure time: 50ms, FITC-filter.

However, these results were not found in the dorsal raphe nucleus. Since we hypothesized the presence of ASIC1a in this structure, we used another approach by performing the same protocol on perfusion-fixated rat tissue in contrast to Wemmie's statement (21) . Testing this material led to good results in both hippocampus and dorsal raphe, respectively in Figures 5 and 6. The right images represent the negative control conditions in which no specific signals can be seen. Between structures the kind of cells seem to be different, but this was not examined precisely.

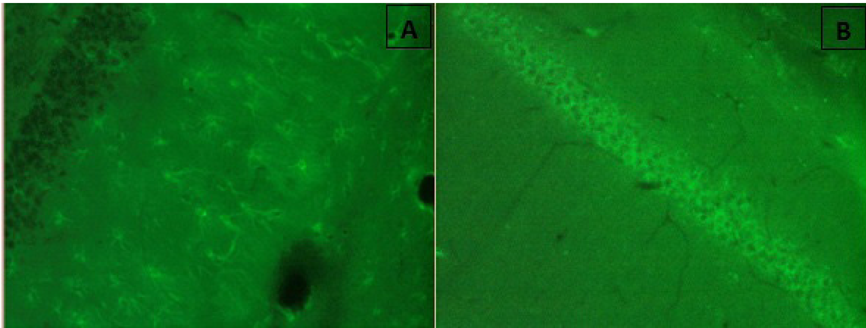

Figure 5. A Image of fluorescent signal Alexa 488 in hippocampus. Magnification 20x. Exposure time 50ms, FITC-filter. **B** Negative control. Exposure time 150 ms.

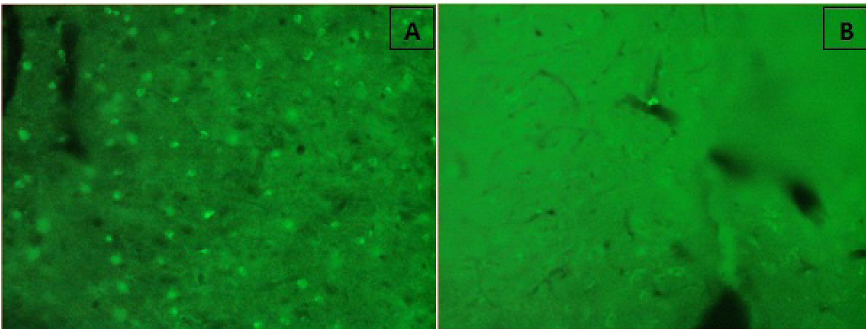

Figure 6. A Image of fluorescent signal Alexa 488 in dorsal raphe nucleus. Magnification 20x. Exposure time 100ms, FITC-filter. **B** Neg. Exposure time 150ms. Blur due to folded tissue.

Discussion

Concerning the 5-HTT the genotypic effect seemed to not have an significant influence on freezing times scored during the behavioral tests, although different genotypes among human subjects resulted in differing fear responses when being experimentally exposed to the elevated carbon dioxide concentration (9). Other scored parameters indicated that knock-out mice showed less fear-related behavior, but these results are yet to be analyzed. The immunofluorescent staining of the ASIC1a-channel showed differing results between perfusion-sacrificed and fresh-frozen tissue, but according to Wemmie, ASIC1a can exclusively be stained in fresh-frozen tissue (21). In contrast, we showed that by making use of a citrate buffer incubation (pH=6.0) in perfusion-fixated tissue serious signals were found in hippocampus and dorsal raphe. Addition of the pH-buffer might have caused an

altered conformation of the channel in such a way that the antibody's epitope becomes more approachable since the channels will open at a lowered pH (20). However, because mice subjected to the behavioral tests were sacrificed by decapitation and their brains were freshly frozen the protocol should be adjusted to produce similar results in fresh frozen tissue as has been seen in perfusion-fixated tissue.
Since the brains of the animals used in the behavioral part are fixated by freezing them freshly, the protocol has to be adjusted to this kind of fixation method. Future protocols will be based on trying to mimic the mechanisms of perfusion-fixation. When the protocol has led to specific staining of cells in the dorsal raphe nucleus, it additionally has to be replicated in a double-staining protocol including tryptophan hydroxylase, TPH2, antibodies in order to determine whether the stained cells are 5-HT neurons (22). This extra staining might lead to altered results or additional reactions to which the protocol might be adapted again.

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