Rasgrf2 controls noradrenergic involvement in the acute and subchronic effects of alcohol in the brain

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Abstract

Alcohol addiction is a major psychiatric disease, and yet, the underlying molecular adaptations in the brain remain unclear. Recent evidence suggests a functional role for the *ras-specific guanine-nucleotide releasing factor 2* (*Rasgrf2*) in alcoholism. Rasgrf2$^{-/-}$ (MT) mice consume less alcohol and show entirely absent dopamine responses to an alcohol challenge compared to wild types (WT). In order to further investigate how Rasgrf2 modifies the acute and subchronic effects of alcohol in the CNS, we investigated its effects on the noradrenergic (NA) and serotonergic (5-HT) systems. In-vivo microdialysis showed significantly reduced NA and 5-HT responses in the nucleus accumbens (NAcc) and caudate putamen (CPu) after an alcohol challenge in Rasgrf2$^{-/-}$ mice. A co-expression analysis showed that there is a high correlation between Rasgrf2 and α2 adrenoceptor RNA expression in the NAcc in naïve animals. Accordingly, we further assessed the role of Rasgrf2 in the neuroadaptations of the noradrenergic system following subchronic alcohol exposure. Rasgrf2-/- mice did not show the WT observed adaptation of β1 adrenoceptor expression, but expressed β2 and α2 adrenoceptors at a reduced level. These findings suggest that adaptations in the noradrenergic system contribute to the Rasgrf2 enhanced risk of alcoholism.

Key Words: Rasgrf2, alcohol, noradrenaline, serotonin, microdialysis, α2 adrenoceptor

Introduction

Alcohol addiction is a major psychiatric disease. The World Health Organisation (WHO) estimates that 4% of deaths worldwide each year are associated with the harmful use of alcohol (WHO 2011). Alcohol use develops into addiction in a significant number of people. While significant heritability estimates of alcohol addiction have been identified (Plomin 1990), underlying specific genetic influences are emerging slowly (Schumann et al. 2011; Stacey et al. 2009). In a recent study, we found evidence for a role of the *ras-specific guanine-nucleotide releasing factor 2* (RASGRF2) gene in alcoholism (Stacey et al. 2012). The study reported a genome wide association of RASGRF2 and alcohol consumption in 28,188 individuals.

Rasgrf2 belongs to a family of calcium/calmodulin associated guanine nucleotide exchange factors (GEFs). Rasgrf2 activates the RAS protein which is known to stimulate the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) signal transduction pathway, by allowing the exchange of GDP for GTP, thereby regulating signalling pathways in cells. Although deletion of RASGRF2 has the potential to have a major impact on neuronal signalling pathways, knockout of RASGRF2 does not interfere with basal developmental in mice (Fernández-Medarde et al. 2002).

Evidence suggests that Rasgrf2 acts on pathways which are also implicated in alcoholism. The RAS-MAPK/ERK pathway has been linked to dopamine D1 receptors (Girault et al. 2007; Tian et al. 2004), and has also been isolated as a binding partner of the dopamine transporter (Maiya et al. 2007). These studies report a role for Rasgrf2 both pre- and post-synaptically. Bloch-Shilderman and colleagues (2001) reported a direct role for the MAPK/ERK pathway in neurotransmitter release. Rasgrf2, as a MAPK/ERK activating protein, may therefore be linked to

neurotransmitter release. Rasgrf2$^{-/-}$ (MT) mice were subsequently found to show entirely absent dopamine responses in the nucleus accumbens (NAcc) and caudate putamen (CPu) to an alcohol challenge. This may account for the reduced alcohol intake and alcohol preference of Rasgrf2$^{-/-}$ mice when compared to their wild-type (WT) litter mates (Stacey et al. 2012). In order to further investigate how Rasgrf2 modifies the acute and subchronic effects of alcohol in the central nervous system (CNS), we investigated its control of the noradrenergic (NA) and serotonergic (5-HT) systems. These monoaminergic systems are consistently implicated in the modulation of alcohol's effects on the reward system (Fahlke et al. 2011; Spanagel 2009; Vengeliene et al. 2008). Next to DA, NA and 5-HT are important modulatory transmitters which are involved in the acute and subchronic behavioral effects of alcohol (Fahlke et al. 2011; McBride 2010) that contribute to behavioural changes associated with binge drinking and addiction (Smith et al. 2008).

We performed in-vivo microdialysis measuring NA and 5-HT levels in the NAcc and CPu after an alcohol challenge in Rasgrf2$^{-/-}$ mice. We found that a Rasgrf2 deficiency significantly reduced NA and 5-HT responses in both brain regions. In order to assess the role of Rasgrf2 in the neuroadaptations to subchronic alcohol exposure, we measured mRNA expression of noradrenergic receptors and transporter in the brain and tested whether blood mRNA adaptations would be predictive for brain adaptations.

Experimental Procedures

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals (Experimental Procedures) Act 1986. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animals

Male wild type (+/+ WT) and null mutant (-/- MT) Rasgrf2^{tm1Esn} (Fernández-Medarde et al. 2002) mice were studied. Mice were generated using a gene targeting strategy and subsequently kept on a genetic background of 129/S-J and C57BL/6. Detailed methods are described by Fernández-Medarde et al. (2002). The method inactivates the Grf2 locus by targeting its CDC25-H catalytic domain, thereby disrupting the guanine nucleotide exchange factor (GEF) activity of Rasgrf2. Animals were individually housed, provided with food and water ad libitum, and kept on a 12:12 hour light: dark cycle (lights on at 7.00 am). Experiments were performed during the light cycle between 09:00 and 16:00 h, in a pseudorandom order. Room temperature was maintained between 19°C and 22°C at a humidity of 55% (±10%). Animals were housed in Tecniplast cages (32cm x 16cm x 14cm), using Litaspen sawdust and nesting materials, (Sizzlenest, Datsand, Manchester UK).

Microdialysis surgery

Mice (WT: n=7; MT: n=6) were deeply anaesthetised with an intraperitoneal (i.p.) injection using a mixture of 4.12ml saline (NaCl), 0.38ml Ketaset (containing 100mg/ml Ketamine) and 0.5ml Domitor (containing 1mg/ml Medetomidine hydrochloride) administered at 0.1ml per 10g bogy weight. In addition 0.01ml

Rimadyl (5mg/Kg Carprofen) analgesia was given s.c. The animal was placed in a Kopf stereotaxic frame. Two guide cannulas (Microbiotech/se AB, Stockholm, Sweden) were aimed at the CPu (A: +0.5; L: ±2.3; V: -2.4 angle ±10º from midline) and the NAcc (A: +1.2; L: ±1.6; V: -4.3 angle ±10º from midline) using coordinates relative to bregma (Paxinos and Franlin 2003), and fixed in place using two anchor screws (stainless steel, d=1.4mm) and dental cement. Reverse anaesthesia was administered to the animals after approximately 45 minutes using a mixture of 3.9ml saline (NaCl) and 0.1ml Antisedan (containing 5mg/ml Atipamezole) at 0.08ml per 10g body weight (s.c.). The animals were kept warm and allowed to recover from the anaesthetic. Animals were then returned to their home cages and monitored daily, allowing at least 5 days for complete recovery.

Microdialysis procedure

One week after implantation, microdialysis probes of a concentric design (membrane lengths: 2mm for the CPu (MAB 6.14.2.); 1mm for the NAcc (MAB 6.14.1)), were inserted into the guide cannulae under a short (3-5minute) Isoflurane anaesthesia (O_2 at 1L/min, Isoflurane at 3% to induce and 2% to sustain). After probe insertion, the animal was placed into the open field (21x21x30cm) of a Truscan system (Coulbourn Instruments, Allentown, USA). Food and water were given ad libitum and room temperature maintained between 19 ºC and 22 ºC. The microdialysis probes were connected to a microinfusion pump (CMA 400, Carnegie, Sweden) via a swivel mounted on a balanced arm above the chamber, and were perfused with artificial cerebrospinal fluid (aCSF) (containing Na^+ 147 mmol, K^+ 4 mmol, Ca^{2+} 2.2 mmol, Cl^- 156 mmol, pH = 7.4) at room temperature (Müller and Huston 2006). The flow rate was set to 1.5µl/min and allowed to stabilise for at least two hours until a stable

baseline was obtained. Samples were collected every 20 minutes into vials containing 2.73µl of antioxidant (0.1 M perchloric acid and 500 pg dihydroxybenzylamine (DHBA) as internal standard). Three samples taken during the first testing hour of the experiment provide baseline quantities of NA and 5-HT. An injection of alcohol was then administered at 2g/Kg, v_{inj}= 10 ml/kg (i.p.). A further ten samples were collected simultaneously to behavioural data collection.

HPLC-ED analysis

All samples were analysed using High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) to measure NA and 5-HT levels in the CPu and NAcc. The column used was an ET 125/2, Nucleosil 120-5, C-18 reversed phase column (Macherey–Nagel, Germany) perfused with a mobile phase composed of 75 mM NaH_2PO_4, 4 mM KCl, 20µM ethylenediamine tetraacetic acid (EDTA), 1.5 mM sodium dodecyl sulfate, 100 µl/l diethylamine, 12% methanol, and 12% acetonitrile adjusted to pH 6.0 using phosphoric acid (Amato et al. 2011; Pum et al. 2008). The electrochemical detector (Intro, Antec, The Netherlands) was set at 500 mV vs an in situ Ag/AgCl (ISAAC) reference electrode (Antec, Leyden, Netherlands) at 30°C. The detection limit of the assay was 0.1pg with a signal–noise ratio of 2:1.

Behavioural analysis

Locomotor activity was measured simultaneously to in-vivo microdialysis experiments. Horizontal locomotor activity was automatically calculated by the Truscan system in 20 min intervals.

Histological analysis

Once microdialysis experiments were complete, animals were sacrificed by cervical dislocation and the localization of microdialysis probes was verified. Only animals with probe placement within the CPu and the NAcc were considered for data analysis.

Subchronic alcohol treatment

In order to investigate the link between Rasgrf2 and the noradrenergic and serotonergic systems in the brain and the neuroadaptations following subchronic alcohol treatment, we tested mRNA expression in the brain and blood. Single housed Rasgrf2$^{-/-}$ (n=8-9/group) and WT mice (n=8-9/group) received a daily i.p. injection with either alcohol (3.5 g/kg) or saline for 7 days. After each injection animals were placed back to their home cages. 120 minutes after the last injection, animals were sacrificed by cervical dislocation and the brain and spleen were removed and immediately cooled on dry ice. Tissue was kept at -80 °C until further analysis.

Tissue preparation

The frozen brains were placed on a cold dissecting surface and hemispheres were separated by cutting in the sagittal plane. After removing the cerebellum and frontal cortex, the dorsal and ventral striatum and the hippocampus were dissected. Total RNA was extracted using a modified Qiagen-protocol: A phenol-extraction in Qiazol (Qiagen) was followed by column-purification with Rneasy Mini Kit (Qiagen), including DNase digestion according to manufacture's protocol. cDNA was synthesized using iScript cDNA Synthesis Kit (Biorad, Munich/Germany) following manufacturer's instructions.

RNA amplification and microarray hybridisation

We generated biotinylated, amplified RNA from total RNA by using the Illumina® TotalPrep™ RNA Amplification kit (Applied Biosystems/Ambion, Austin, TX, USA) following the manufacturer's instructions. Briefly, 500ng of total RNA was converted into first strand cDNA. After second strand cDNA synthesis and cDNA purification, *in vitro* transcription overnight generated biotinylated amplified RNA, which was purified and quantified using a Qubit® 2.0 Fluorometer (Invitrogen Ltd., Paisley, UK). The size distributions of amplified RNA were made by running each sample on the Bioanalyzer (Agilent Technologies UK Ltd., Wokingham, UK) using the Eukaryotic mRNA Assay with smear analysis.

Gene expression profiling

In order to investigate how expression of noradrenergic and serotonergic genes depends on Rasgrf2, we measured expression profiles in the NAcc of Rasgrf2$^{-/-}$ and WT mice after saline treatment. Gene expression profiling was performed using the Illumina Mouse WG-6 v2.0 Expression BeadChip that contains 45,281 probes (Illumina Inc., San Diego, CA, USA). 1.5µg of amplified RNA was used for hybridization of each array. The hybridization, washing and scanning were performed according to the manufacturer's instructions.

RT-PCR analysis

Gene expression analysis in the NAcc showed a relationship of Rasgrf2 with α2 adrenoceptor mRNA expression, but not with serotonergic gene expression. Accordingly, we focused the analysis of alcohol-induced brain-wide gene expression on noradrenergic genes only. Quantitative RT-PCR was performed using SYBR

Green I Master Mix buffer (Applied Biosystems), and reactions were run on an iCycler (Roche) using a three-step standard protocol. The annealing temperature was optimized for all primer pairs and ranged from 55 to 65 °C. PCR products were visualized on standard 2% agarose gels with ethidium bromide to eliminate the possibility of having amplified genomic DNA. β-Actin was used as internal standard, and CT values were calculated from differences between β-actin and the target genes. All experiments were repeated at least three times and the mean value used for further analysis.

The following primer pairs were used:

B-Actin-F: CTGTATTCCCCTCCATCGTG

B-Actin-R: GAAGATCTGGCACCACACCT

NAT-F: TCAACTTCAAGCCGCTTACC

NAT-R: GGTTCAGATAGCCAGCCAGT

Adra2a-F: GTTCTGGCTGAGAGGG

Adra2a-R: AAGGAAGGGGGTGTGGAG

Adra2b-F: GCAGAGGTCTCGGAGCTAA

Adra2b-R: GCCTCTCCGACAGAAGATA

Adra1b-F: GAAAAGAAAGCAGCCAAA

Adra1b-R: CTGGAGCACGGGTAGATGAT

Statistical Analysis

Behavior and neurochemistry: All graphical output data were expressed as a mean ± SEM. Baseline behavioural and neurochemical data were analysed using pre-planned t-test comparisons to determine the differences seen between genotype

groups prior to alcohol administration. To compare the effect size of alcohol affected by genotype, the area under the curve (AUC) was established for locomotor activation. Data was analysed using planned t-test comparisons. Alcohol induced neurochemical data was expressed as a percentage of the mean of the three baseline samples which were taken as 100%. Data were compared using two-way ANOVAs for factor genotype (3) and time (13). To compare alcohol effects of certain time points, pre-planned Bonferroni-corrected t-test comparisons were performed versus average baseline value.

Gene expression: The mRNA expression was calculated as deltaCT (mean$\pm$SEM). For statistical analysis, two-way ANOVAs with factors genotype and treatment were used. To evaluate genotype effects on single brain area and blood level, pre-planned comparisons using Bonferroni-corrected Fisher's LSD test were calculated. A correlation analysis of brain and blood mRNA expression (Pearson correlation) was performed for all target mRNA with pooled genotypes. The software used was Statistica 7.0. A significance level of $p \leq 0.05$ was used to test for statistical significance.

Results

Rasgrf2 deficiency is associated with increased locomotion and basal noradrenaline activity

Basal activity of Rasgrf2$^{-/-}$ and WT mice was significantly different (Fig.1A). Rasgrf2$^{-/-}$ mice were found to have a higher level of locomotor activity than WT mice ($t=2.71$, $df=34$, $p=0.01$). Rasgrf2$^{-/-}$ mice were also found to have increased basal NA levels in the NAcc ($t=4.32$, $df=37$, $p<0.001$) and the CPu ($t= 5.56$, $df=37$, $p<0.0001$), compared to WT mice (Fig. 2A). Rasgrf2$^{-/-}$ mice showed enhanced basal 5-HT levels in the NAcc ($t=-4.87$, $df=37$, $p<0.0001$) but decreased levels the CPu ($t= 3.78$, $df=38$, $p<0.0006$), compared to WT mice (Fig. 3A).

Rasgrf2 contributes to noradrenergic and serotonergic activation and behavioral activity after acute alcohol treatment

Rasgrf2 WT mice showed a tendency towards an increased level of locomotor activation after an acute alcohol injection compared to Rasgrf2$^{-/-}$ mice ($t=-2.06$, $df=10$, $p=0.06$; Fig 1B).

Noradrenaline levels in the NAcc steadily increased after an alcohol challenge (Fig. 2B). Analysis showed a significant time effect ($F_{12,120}=7.38$, $p<0.001$), but no genotype, or genotype x time interaction ($p>0.05$). A within group analysis using pre-planned pairwise comparisons suggested that, compared to basal levels, WT mice show a significantly increased level of noradrenaline at 100 min ($p<0.03$), 120 min ($p=0.05$) and 200 min ($p=0.05$) after an acute injection of alcohol. Rasgrf2$^{-/-}$ mice show no significant differences in NA levels after the same alcohol treatment ($p>0.05$).

The NA response to alcohol in the CPu shows a similar reactivity to that seen in the NAcc. Two-way ANOVA revealed a significant time effect ($F_{12,120}$=5.03, p<0.001; Fig. 2C) effect, but no genotype effect or genotype x time interaction (p>0.05). Within group analysis confirmed significantly increased NA levels in the WT mice at 100 min (p=0.01), 120 min (p<0.001), after acute alcohol administration. Rasgrf2$^{-/-}$ did not significantly differ from basal transmitter levels after the same treatment (p>0.05).

Alcohol treatment increased extracellular 5-HT levels in the NAcc of WT mice, but had no effect in Rasgrf2$^{-/-}$ mice (Fig. 3B). Analysis showed a significant effect of genotype ($F_{1,9}$=17.41, p=0.002), and time ($F_{12,108}$=2.02, p=0.03), but no genotype x time interaction (p>0.05). A within group analysis using pre-planned pairwise comparisons suggested that, compared to basal levels, WT mice show a significantly increased 5-HT level 20 min (p=0.03) after an acute alcohol injection. Rasgrf2$^{-/-}$ did not significantly differ from basal 5-HT levels in the Nacc after the same treatment (p>0.05). A similar 5-HT response was observed in the CPu. Alcohol treatment increased extracellular 5-HT levels in WT, but not in Rasgrf2$^{-/-}$ mice (Fig. 3C). Analysis showed a significant genotype effect ($F_{1,10}$=11.81, p=0.006), but no time effect (p>0.05). However, there was a significant genotype x time interaction ($F_{12,120}$=1.91, p=0.04). A within group analysis using pre-planned pairwise comparisons suggested that, compared to basal levels, WT mice showed a significantly increased 5-HT level 20 min (p=0.02), 60 min (p=0.05) and 120 min (p=0.02) after an acute injection of alcohol. Rasgrf2$^{-/-}$ did not significantly differ from basal 5-HT levels in the CPu after the same treatment (p>0.05).

Rasgrf2 and *α*2 adrenoceptor co-expression in the ventral striatum

A co-expression analysis showed that there was a high correlation between Rasgrf2 and *α*2 adrenoceptor (Adr2a) mRNA expression in the ventral striatum in WT animals (R=0.9277, p=0.0125). No other correlation of Rasgrf2 with noradrenergic or serotonergic gene expression reached significance (Suppl. Tab. 1 and 2). These findings suggest a link between Rasgrf2 and noradrenergic, but not serotonergic systems at the level of the ventral striatum.

Rasgrf2 controls noradrenergic gene expression in the brain after alcohol treatment

In order to further explore the role of Rasgrf2 in the establishment of alcoholism, we investigated the expression of noradrenergic genes in response to alcohol in Rasgrf2$^{-/-}$ and WT mice.

β1-adrenoceptor: There was no difference in β1-adrenoceptor mRNA between Rasgrf2$^{-/-}$ and WT after saline treatment (Fig. 2; ANOVA; genotype: $F_{1,166}$=47.37, p<0.0001; treatment: $F_{1,166}$=20.72, p=0.001; interaction: $F_{1,166}$=24.67, p<0.0001). Subchronic alcohol treatment reduced mRNA expression significantly in the WT (p<0.0001), but not in the Rasgrf2$^{-/-}$ mice (p>0.05). Pre-planned comparisons showed a significant difference between Rasgrf2$^{-/-}$ and WT after alcohol (p<0.001), but not after saline treatment (p>0.05). This difference was found in each brain area tested after alcohol treatment (frontal cortex: p=0.036; ventral striatum: p=0.012; dorsal striatum: p=0.008; hippocampus: 0.014; cerebellum: p=0.0008).

β2-adrenoceptor: There was no difference in β2-adrenoceptor mRNA expression between Rasgrf2$^{-/-}$ and WT mice after saline treatment (Fig. 2; ANOVA; genotype:

$F_{1,166}$=30.53, p<0.0001; treatment: $F_{1,166}$=13.90; p=0.0003; interaction: $F_{1,166}$=7.09, p=0.006). Subchronic alcohol treatment had no effect on β2-adrenoceptor mRNA expression in the WT (p>0.05), but reduced mRNA expression in the Rasgrf2$^{-/-}$ mice (p<0.0001). Pre-planned comparisons showed a significant difference between Rasgrf2$^{-/-}$ and WT after alcohol (p<0.0001), but not after saline treatment (p>0.05). This effect was found at single brain area level in the ventral striatum (p=0.003), hippocampus (p=0.022), and cerebellum: p<0.0001), but not in the frontal cortex (p>0.05), and only as a tendency in the dorsal striatum (p=0.08).

α2-adrenoceptor: There was no significant difference in α2-adrenoceptor mRNA expression between Rasgrf2$^{-/-}$ and WT mice after saline treatment (Fig. 2). Subchronic alcohol treatment reduced mRNA expression significantly in the Rasgrf2$^{-/-}$, but not in the WT mice (ANOVA; genotype: $F_{1,166}$=47.37, p<0.0001; treatment: $F_{1,166}$=20.72, p=0.001; interaction: $F_{1,166}$=24.67, p<0.0001). Pre-planned comparisons showed a significant difference between Rasgrf2$^{-/-}$ and WT mice after alcohol (p<0.0001), but not after saline treatment (p>0.05). The effect of Rasgrf2 was visible in all brain areas investigated after the alcohol treatment (frontal cortex: p=0.0004; ventral striatum: p=0.0013; dorsal striatum: p=0.011; hippocampus: p=0.0006; cerebellum: p=0.0002).

Noradrenaline transporter: NAT mRNA expression did not differ between Rasgrf2$^{-/-}$ and WT mice, neither after saline nor after subchronic alcohol administration (ANOVA; genotype: $F_{1,166}$=0.014, p=0.90; treatment: $F_{1,166}$=2.38, p=0.12; interaction: $F_{1,166}$=1.60, p=0.21). Pre-planned comparisons at single brain area level showed no difference in all brain areas tested (p>0.05).

Blood mRNA expression of noradrenergic genes and brain neuroadaptations to subchronic alcohol

In order to test whether mRNA expression in peripheral blood could predict genotype- and/or alcohol-induced expression differences in the brain, we measured the expression of α2-, β1-, β2- and NAT mRNA in blood cells. There was no difference between genotypes in mRNA expression of all targets after saline treatment ($p>0.05$, Fig. 5). Visual inspection of the data suggested an alcohol-induced decrease in β1 expression in WT animals, and a decrease of α2- and β2 expression in Rasgrf2$^{-/-}$ animals. These effects, however, did not reach statistical significance ($p>0.05$). A correlation analysis for single brain areas, suggest that blood mRNA expression may predict β1 expression in the dorsal striatum and β2 expression in the ventral striatum after saline treatment in WT animals. It may also predict NAT mRNA expression after alcohol in the WT animals in the frontal cortex. However, for the other investigated brain areas and treatment conditions there was no significant correlation between blood and brain mRNA expression ($p>0.05$; Tab. 1). These findings suggest the use of blood mRNA as a marker for selective brain area expression of β1 and β2 adrenoceptor mRNA under basal conditions, and NAT adaptations in the frontal cortex after sub-chronic alcohol treatment.

Discussion

The monoaminergic systems have consistently been implicated in the modulation of alcohols effects in the reward system (Fahlke et al. 2011; Spanagel 2009; Vengeliene et al. 2008). Noradrenaline and serotonin are involved in the modulation of alcohol's behavioural and neurochemical effects (Fahlke et al. 2011; McBride 2010). Both systems have been implicated in long term adaptive changes induced by alcohol consumption (Berggren et al. 2002; Fahlke et al. 1999; Nutt and Glue 1988). Rasgrf2 has recently been shown to mediate the dopaminergic responses to alcohol in the NAcc and CPu by controlling sensitivity of dopaminergic neurons in the ventral tegmental area (Stacey et al. 2012). The aim of the current study was to further characterize how Rasgrf2 contributes to acute and subchronic alcohol effects in the noradrenergic and serotonergic systems of the CNS. In this study, WT animals showed an alcohol-induced increase of extracellular NA and 5-HT levels in both the NAcc and the CPu. A corresponding increase in locomotor activity was also apparent. Mice lacking Rasgrf2 showed an enhanced baseline locomotor activity and enhanced NA levels in the NAcc and CPu. Serotonin levels were enhanced in the NAcc, but attenuated in the CPu. These findings suggest that a lack of Rasgrf2 leaves mice in a hyperarousal state. Interestingly, an alcohol challenge induced less locomotor activation and NA responses in the Rasgrf2$^{-/-}$ mice compared to WT. The 5-HT response, in addition, was completely lacking in Rasgrf2$^{-/-}$ mice. These findings may suggest that the locomotor stimulating effects of a medium dose of alcohol depend on Rasgrf2 integrity and on downstream NA and 5-HT activation. Given that basal locomotion and monoamine levels were already enhanced during baseline, the lack of an additional activation may be due to a ceiling effect in systems activity. Present data also suggest that Rasgrf2 controls basal NA and 5-HT activity. These

findings are largely in line with previously reported role of Rasgrf2 in the control of basal and alcohol-induced dopaminergic activity (Stacey et al. 2012).

An analysis of mRNA co-expression of Rasgrf2 and important components of the noradrenergic and serotonergic systems showed that in the ventral striatum only the α2 adrenoceptor mRNA expression correlated highly with Rasgrf2. This suggests a strong link between Rasgrf2 and the NA system in an alcohol-reinforcement related brain area. Subsequent analysis, which sought to compare expression between different brain areas, focused on noradrenergic receptors and the NAT. After subchronic alcohol treatment, β1 adrenoceptor levels were elevated in WT mice in all brain areas tested, but there were no changes in β2 and α2 adrenoceptor levels, or in expression levels of NAT. Rasgrf2$^{-/-}$ mice after subchronic alcohol showed no adaptation of β1 adrenoceptors, but did differentially express β2 adrenoceptors in the ventral striatum, hippocampus and cerebellum, and α2 adrenoceptors in all brain areas tested at a reduced level. Like WT mice, Rasgrf2$^{-/-}$ mice showed no change in NAT expression. For both, Rasgrf2$^{-/-}$ and WT mice, blood levels showed a tendency towards the effects seen in brain tissue, but the findings could not be statistically verified. Although partial correlations between blood and brain tissue were obtained, they rather applied for saline treated animals and did not predict alcohol-induced adaptations.

Present data suggest that Rasgrf2 regulates the adaptation of adrenoceptors to subchronic alcohol administration. Rasgrf2 appears to be essential for β1 adaptation, whilst normally preventing β2 and α2 adaptations. It appears that, only if Rasgrf2 is absent, can the adrenoceptor system adapt and possibly make the animal less vulnerable to alcohols rewarding effects. This would account for the diminished alcohol preference seen in the Rasgrf2$^{-/-}$ mice (Stacey et al. 2012). Increases in NA

have been shown to result in the down regulation of the β1 adrenoceptor in cardiac tissue (Dong et al. 1999; Thomas and Marks 1978). This may account for the WT adaptations we have observed in response to subchronic alcohol exposure. Since the Rasgrf2$^{-/-}$ NA response is reduced acutely, this may explain the absence of β1 adaptation in these mice. In response to repeated alcohol treatments, WT mice do not typically alter their β2 and α2 adrenoceptor levels. However, Rasgrf2$^{-/-}$ mice show a distinct down regulation of these receptors. NA signalling through the α2 adrenoceptor is known to be involved in the negative feedback control mechanism in neuronal synapses, thus, regulating NA release from the presynaptic nerve terminals of the CNS (Schwartz 1997; Starke 2001). Present data suggest that Rasgrf2 is capable of controlling the amount of NA released to the synaptic cleft. Rasgrf2 activates the Ras protein and thereby stimulates the MAPK/ERK signaling pathway. Adrenoceptors have been shown to differentially alter the activation of the MAPK/ERK pathway (Williams et al. 1998). Williams and colleagues demonstrated that stimulation of the β1 adrenoceptors results in ERK activation, while stimulation of α2 adrenoceptors does not. Although this contravenes earlier studies, suggesting that α2 adrenoceptors also activate the MAPK/ERK pathway (Alblas et al. 1993; Howe and Marshall 1993; Winitz et al. 1993), the present study suggests Rasgrf2 may play a role in this process.

Since microdialysis was performed after acute treatment only, we cannot rule out the possibility of chronic adaptations of the neurochemical response (Berggren et al. 2002), which may result from changes in adrenoceptor expression levels. In addition to this, not all adrenoceptors were measured in the present study, thus, one cannot say to what extent they might play a role in the NA response to subchronic alcohol exposure. Nor do our results rule out NAT expression changes in general, but only in

target area neurons. For example, there is a chance that NAT expression might be affected in the locus coeruleus neurons by Rasgrf2 (Zippel et al. 1997).

Altogether, the current study reveals an important role of Rasgrf2 in the noradrenergic system's response to alcohol. Rasgrf2 appears to mediate the presence of NA and 5-HT in the synaptic cleft at both, basal level and after acute alcohol stimulation. Rasgrf2 and the NA system are specifically linked by co-expression of Rasgrf2 with α2 adrenoceptor mRNA at basal conditions. After repeated alcohol exposure, the NA system is modified, and Rasgrf2 regulates alterations in expression levels of adrenoceptor mRNA. Expression levels in blood/spleen samples may serve only with a very restricted focus as a predictor for noradrenergic adaptations in the brain after subchronic alcohol exposure. A lack of Rasgrf2 results in the failure of the noradrenergic system to adapt in the normal way to subchronic alcohol, which coincides with animals being less vulnerable to the incentive effects of alcohol.

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Figure Legends:

Figure 1: The role of Rasgrf2 in acute alcohol effects on locomotor activity. **(A).** Baseline locomotor activity and **(B).** Alcohol (2 g/kg, i.p.) induced locomotor activation as area under the curve (AUC) for 200 min after injection (**p<0.01, t-test).

Figure 2: The role of Rasgrf2 in alcohol effects on noradrenaline activity. **(A).** Noradrenaline basal levels in the nucleus accumbens (NAcc) and caudate putamen (CPu) prior to alcohol treatment (***p<0.001, t-test). **(B).** Extracellular noradrenaline levels after acute alcohol (2 g/kg, i.p.) treatment in the NAcc and **(C).** In the CPu (*p≤0.05, **p≤0.01, ***p≤0.001 vs. baseline).

Figure 3: The role of Rasgrf2 in alcohol effects on serotonin activity. **(A).** Serotonin basal levels in the nucleus accumbens (NAcc) and caudate putamen (CPu) prior to alcohol treatment (***p<0.001, t-test). **(B).** Extracellular serotonin levels after acute alcohol (2 g/kg, i.p.) treatment in the NAcc and **(C).** In the CPu (*p<0.05 vs. baseline).

Figure 4: The role of Rasgrf2 in alcohol-induced changes in mRNA expression of **(A.** and **B.)** β1-adrenoceptor, **(C.** and **D.)** β2-adrenoceptor, **(E.** and **F.)** α2-adrenoceptor, and **(G.** and **H.)** the noradrenaline transporter (NAT) in the brain of Rasgrf2$^{-/-}$ and wild type mice. Higher deltaCT values represent lower mRNA expression rate (mean ± SEM; FC - frontal cortex, VStr - ventral striatum, DStr - dorsal striatum, Hipp – hippocampus, Cerr – cerebellum; * p<0.05,**p<0.01, ***p<0.001).

Figure 5: The role of Rasgrf2 in alcohol-induced changes in mRNA expression of **(A.** and **B.)** β1-adrenoceptor, **(C.** and **D.)** β2-adrenoceptor, **(E.** and **F.)** α2-adrenoceptor, and **(G.** and **H.)** the noradrenaline transporter (NAT) in the blood of Rasgrf2-/- and wild type mice (mean ± SEM). Higher deltaCT values represent lower mRNA expression rate.

Table 1: The correlation between blood and brain area mRNA expression in saline and alcohol treated Rasgrf2-/- (MT) and wild type (WT) mice (*$p<0.05$).