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$GABA_A$ receptor subtypes in the mouse brain: Regional mapping and diazepam receptor occupancy by *in vivo* [^{18}F]flumazenil PET

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ABSTRACT

Classical benzodiazepines, which are widely used as sedatives, anxiolytics and anticonvulsants, exert their therapeutic effects through interactions with heteropentameric $GABA_A$ receptors composed of two α, two β and one γ2 subunit. Their high affinity binding site is located at the interface between the γ2 and the adjacent α subunit. The α-subunit gene family consists of six members and receptors can be homomeric or mixed with respect to the α-subunits. Previous work has suggested that benzodiazepine binding site ligands with selectivity for individual $GABA_A$ receptor subtypes, as defined by the benzodiazepine-binding α subunit, may have fewer side effects and may even be effective in diseases, such as schizophrenia, autism or chronic pain, that do not respond well to classical benzodiazepines. The distributions of the individual α subunits across the CNS have been extensively characterized. However, as $GABA_A$ receptors may contain two different α subunits, the distribution of the subunits does not necessarily reflect the distribution of receptor subtypes with respect to benzodiazepine pharmacology. In the present study, we have used *in vivo* [^{18}F]flumazenil PET and *in vitro* [^{3}H] flumazenil autoradiography in combination with $GABA_A$ receptor point-mutated mice to characterize the distribution of the two most prevalent $GABA_A$ receptor subtypes (α1 and α2) throughout the mouse brain. The results were in agreement with published *in vitro* data. High levels of α2-containing receptors were found in brain regions of the neuronal network of anxiety. The α1/α2 subunit combinations were predictable from the individual subunit levels. In additional experiments, we explored *in vivo* [^{18}F]flumazenil PET to determine the degree of receptor occupancy at $GABA_A$ receptor subtypes following oral administration of diazepam. The dose to occupy 50% of sensitive receptors, independent of the receptor subtype(s), was 1–2 mg/kg, in agreement with published data from *ex vivo* studies with wild type mice. In conclusion, we have resolved the quantitative distribution of α1- and α2-containing homomeric and mixed $GABA_A$ receptors *in vivo* at the millimeter scale and demonstrate that the regional drug receptor occupancy *in vivo* at these $GABA_A$ receptor subtypes can be determined by [^{18}F]flumazenil PET. Such information should be valuable for drug development programs aiming for subtype-selective benzodiazepine site ligands for new therapeutic indications.

Introduction

The γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors are heteropentamers consisting of members of several $GABA_A$ receptor subunit families. These are α1-6, β1-3, γ1-3, δ, ε, π, θ and ρ1-3 in humans, rats and mice. In the brain, a typical $GABA_A$ receptor consists of the counter-clock-wise (seen from the exoplasmic side) assembled subunits γ, β, α, β, α, where the individual subunit members depend on the regional and subcellular localization and on the development state (Fritschy and Möhler, 1995; Sigel and Steinmann, 2012). The inhibitory neurotransmitter GABA has two (agonist) binding sites located at the two extracellular interfaces between the adjacent α and β subunits. As ligand-gated chloride channels (GABA binding opens the channel), $GABA_A$ receptors regulate plasma membrane polarization and, therefore, neuronal activity (Knoflach et al., 2016). $GABA_A$ receptors are involved in the control of most if not all brain functions.

Besides the agonist binding sites, $GABA_A$ receptors offer several allosteric binding sites for pharmacological modulation, including the

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clinically important benzodiazepine binding site (Ernst et al., 2005; Sieghart, 2015). Benzodiazepines bind to the extracellular interface between the γ2 and the adjacent α subunit and act as positive or negative allosteric modulators by increasing or decreasing, respectively, the receptors affinity to GABA (Sigel and Steinmann, 2012). The γ2 subunit, which is primarily involved in synaptic $GABA_A$ receptors (Essrich et al., 1998; Nusser et al., 1998), is mandatory for the high-affinity binding of typical benzodiazepines. Of the six different α subunits, only α1, α2, α3 and α5 can form high affinity binding sites for the clinically relevant benzodiazepine site agonists such as diazepam. Diazepam binds to these sites in human, rat and mouse receptors with affinities (K_d, dissociation constant) in the single to low two-digit nanomolar range (Chiu and Rosenberg, 1979; Kammerman Sher and Machen, 1984; Pym et al., 2005; Ralvenius et al., 2016; Sieghart, 1995; Sigel and Steinmann, 2012). The benzodiazepine binding site antagonist flumazenil has a similar α-subunit selectivity profile as diazepam. It binds with a K_i (inhibition constant) of ~1 nM to human recombinant and mouse cortical membrane diazepam-sensitive $GABA_A$ receptors while its K_i to the human recombinant diazepam-insensitive α4α4 and α6α6 receptors is two orders of magnitude higher (Pym et al., 2005; Sieghart, 1995; Sigel and Steinmann, 2012; Zanotti et al., 1999). The structurally identical carbon-11 and fluorine-18 labeled flumazenil are established tracers for Positron Emission Tomography (PET) with 25 nM *in vivo* concentration in rat brain ([^{11}C]flumazenil) at 50% occupancy of the diazepam-sensitive $GABA_A$ receptors (Pike et al., 1993; Ryzhikov et al., 2005; Syvänen et al., 2011).

The last two decades on $GABA_A$ receptor research revealed that the individual pharmacological effects of benzodiazepines are mainly determined by the α subunits contained in the targeted receptor and their anatomical distribution in the nervous tissue. Sedation and anticonvulsive effects are mainly caused by benzodiazepine binding to the α1 subtype while anxiolytic effects were shown for the modulation of α2-containing and very recently for the α5-containing $GABA_A$ receptors (Atack, 2005; Behlke et al., 2016; Löw et al., 2000; Morris et al., 2006; Ralvenius et al., 2015; Rudolph and Knoflach, 2011; Rudolph and Möhler, 2014; Sigel and Steinmann, 2012). Muscle relaxation results from binding to the α2- and α3-$GABA_A$ receptor subtypes, and motor coordination is impaired by modulation of α1 or α3 receptor subtypes (Möhler et al., 2002; Newman et al., 2015; Ralvenius et al., 2015; Sigel and Steinmann, 2012). The α5 subunit is furthermore involved in learning and memory (Crestani et al., 2002; Ghafari et al., 2016). Finally, the recently discovered antihyperalgesic effect of benzodiazepines depends mainly on α2-containing $GABA_A$ receptors in the spinal cord (Knabl et al., 2008; Ralvenius et al., 2016, 2015).

Several of these findings are based on research with genetically modified mice carrying a point mutation in one or more $GABA_A$ receptor α subunits, rendering the respective receptors diazepam-insensitive. This is achieved by replacing a particular histidine within the extracellular domain, with an arginine (H- > R), resulting in the loss of high-affinity binding of diazepam and other typical benzodiazepine site agonists (Rudolph et al., 1999). The expression levels of the respective subunits, receptor assembly and function, as well as binding of alternative modulators were not appreciably affected in these single, double (point mutations in two α subunits) or triple point-mutated mice (Behlke et al., 2016; Crestani et al., 2002; Löw et al., 2000; Rudolph et al., 1999). This allowed identifying the α-subunits that are involved in the individual pharmacological and adverse effects of benzodiazepines as summarized above.

The growing awareness of the α-subtype selective benzodiazepine pharmacology revived the benzodiazepine drug research towards subtype-selective $GABA_A$ receptor modulators. Selectivity is achieved by subtype-dependent binding affinity or efficacy. The latter results from full or partial agonism at one subtype but full or partial antagonism, weaker agonism (or negative agonism) at other subtypes (Atack, 2005; Rudolph and Knoflach, 2011). Among the most advanced fields is the search for non-sedative anxiolytic drugs, targeting the subunit α2 while sparing the α1-subtype mediated effects including sedation, impairment of motor coordination, attention and memory deficits, and promotion of abuse (Atack, 2005; Atack et al., 2011; Rudolph and Knoflach, 2011). Respective ligands are currently evaluated in biological and preclinical research (Atack, 2010; Atack et al., 2011, 2006; Fischer et al., 2010; Ralvenius et al., 2016). Subtype-selectivity of $GABA_A$ receptor modulators is of further interest towards the pharmacological intervention in Down syndrome, epilepsy, depression, schizophrenia and autism (Rudolph and Möhler, 2014).

The H- > R point-mutated mice furthermore provided insight in the assembly of $GABA_A$ receptors regarding the positions of the two α subunits. Mixed receptors (*i.e.*, receptors with two different α subunits) of H- > R single point-mutated mice can contain both a mutated and a native α subunit. In such case, the latter is always favored to assemble adjacent to the γ2 subunit, forming a diazepam-sensitive $GABA_A$ receptor despite the presence of one point-mutated α subunit, and independent of the α subunit arrangement in the native receptor (Balic et al., 2009; Benke et al., 2004) but see (Baur and Sigel, 2005). With respect to the native α subunits, findings based on sequential immunoprecipitation experiments followed by analysis of the benzodiazepine site properties of isolated $GABA_A$ receptor subtypes suggested an α5 > α2 > α1 > α3 hierarchy of α subunits at the position adjacent to the γ2 subunit (Araujo et al., 1999, 1996; Balic et al., 2009; del Rio et al., 2001).

Considering the receptor-subtype dependent effects of $GABA_A$ receptor modulation, the regional and temporal patterns of the individual receptor subtypes are of high interest towards a better understanding of $GABA_A$ receptor functions, neuronal excitability in health and disease and the development of $GABA_A$ receptor subtype-selective modulators (Rudolph and Möhler, 2014). Information on the individual $GABA_A$ receptor subunit members on the mRNA and protein levels is available for various species (Fritschy and Möhler, 1995; Hörtnagl et al., 2013). However, the distribution of the individual α subunits does not necessarily reflect the distribution of receptor subtypes with respect to benzodiazepine pharmacology as this is determined by only one of the two α subunits in a receptor. The mapping of α subunit combinations in diazepam-sensitive $GABA_A$ receptors would provide more information in this respect. So far, the percentage of individual α subunit combinations was determined for whole brain and a few brain regions. The analyses were typically performed *ex vivo* and *in vitro*, involving immunoprecipitation, ligand binding assays, and autoradiography, involving wild type mice as well as the H- > R point-mutated mice (Balic et al., 2009; Benke et al., 2004; Ghafari et al., 2016; Ralvenius et al., 2015).

In this study, we employed positron emission tomography (PET) with [^{18}F]flumazenil to map the regional levels of α1- and α2-subunit containing diazepam-sensitive $GABA_A$ receptors as well as the occurrence of homomeric and mixed α1- and α2-containing receptors in the living mouse brain under healthy conditions. As flumazenil lacks subtype-selectivity, we used the single point-mutated mice, α1(H- > R, RHHH) and α2(H- > R, HRHH), and the triple point-mutated mice α1,α3,α5(H- > R, RHRR) and α2,α3,α5(H- > R, HRRR) (Ralvenius et al., 2015) besides the wild type mice (HHHH) to distinguish receptor subtype patterns. The regional relative densities of α1- and α2-containing and of α1α1 and α2α2 diazepam-sensitive $GABA_A$ receptors were estimated from the [^{18}F]flumazenil binding potentials (BP_{ND}) in the brains of the individual genotypes and from the differences between the genotypes (Table 1). The PET results were confirmed by *in vitro* autoradiography with [^{3}H]flumazenil. We furthermore evaluated the potency of [^{18}F]flumazenil PET to quantify region- and subtype-specific $GABA_A$ receptor occupancy in mice. We used diazepam as the model drug for this proof-of-concept receptor-occupancy study as its *in vitro* and *in vivo* affinities to individual receptor subtypes are well defined.

Table 1
Quantification of BP_{ND} (or kBq/cm^3) of individual α subunit combinations in diazepam-sensitive $GABA_A$ receptors.

Experimental genotype-specific BP_{ND} values or differences (region analysis or voxel-wise modelling)	Corresponding receptor-specific BP_{ND}[a]
BP_{ND}(HHHH)	BP_{ND}(αxαx)
BP_{ND}(HRRR)	BP_{ND}(α1αx)
BP_{ND}(RHRR)	BP_{ND}(α2αx)
BP_{ND}(HRHH)	BP_{ND}(αxαx but not α2α2)
BP_{ND}(RHHH)	BP_{ND}(αxαx but not α1α1)
BP_{ND}(HHHH) - BP_{ND}(RHRR)	BP_{ND}(α135α135)
BP_{ND}(HHHH) - BP_{ND}(RHHH)	BP_{ND}(α1α1)
BP_{ND}(HHHH) - BP_{ND}(HRRR)	BP_{ND}(α235α235)
BP_{ND}(HHHH) - BP_{ND}(HRHH)	BP_{ND}(α2α2)

[a] The numbers following an α symbol denote the possible α subunits. αx indicates that any α subunit capable of forming a high affinity benzodiazepine binding site is possible. The subunits α4 and α6 are not considered.

Material and methods

Animals

Animal housing and experiments were approved by the Veterinary Office of the Kanton Zurich (Switzerland) and were in accordance with the Swiss legislation on research with laboratory animals and with the EU Directive 2010/63/EU for animal experiments. Experiments complied with the ARRIVE guidelines. Mice were single H- > R point mutated (α1, designated RHHH and α2, HRHH, respectively) or triple H- > R point mutated (α1,3,5, RHRR, and α2,3,5, HRRR, respectively) on a 129X1/SvJ genetic background, as recently described (Ralvenius et al., 2015). The respective wild type mice are designated HHHH. Mice were transported to the PET facility at least 5 days before the experiments and were housed at a 12 h-12 h light-dark cycle and fed a normal chow and water at libitum.

In vitro *autoradiography*

Brains from wild type mice as well as mice containing the $GABA_A$ receptor α(H- > R) point mutations were rapidly dissected after euthanasia, immediately frozen in powdered dry ice, and stored at -80 °C until used. The distribution of diazepam-sensitive $GABA_A$ receptor subtypes was analyzed in 16 µm thick cryostat-cut sagittal sections. After removing from the freezer, the sections were immediately dried in a stream of cold air, washed two times for 10 min in assay buffer (50 mM Tris pH 7.4) to remove endogenous ligands and dried again. The sections were then incubated with 5 nM [^{3}H]flumazenil (1.85 TBq/mmol, ANAWA Trading SA, Wangen, Switzerland) diluted in assay buffer for 120 min on ice. After washing three times for 20 s in ice-cold assay buffer, the sections were dried and exposed along with tritium standards to a tritium-sensitive phosphorimaging screen (Cyclone Storage Phosphor Screen, Perkin Elmer, Waltham, MA). Quantification was done using the Optiquant software (Perkin Elmer). Non-specific binding was determined by co-incubating the sections with 10 µM clonazepam. Autoradiographs and data from the same series of experiments were recently published (Behlke et al., 2016).

PET experiments

A scheme of the PET study protocol is shown in the Inline Supplementary Fig. 1. For the PET experiments, mice weighed between 20.1 and 31.6 g. Vehicle (0.9% saline containing 1% Tween 80, baseline scans) or diazepam (Lipomed, Arlesheim, Switzerland) at the indicated dose, suspended in 200–320 µL vehicle was administered orally 30 min before tracer injection. The diazepam-containing formulations contained 20 kBq/kg [^{3}H]diazepam (3.119 TBq/mmol, PerkinElmer) in addition to confirm the administration and uptake of the complete diazepam dose by the analysis of blood plasma samples after the PET scan. About 10 min before tracer injection, anesthesia was induced by 5% isoflurane (Rothacher Medical, Heitenried, Switzerland) in oxygen/air (1:1) and was maintained at 2–5% isoflurane. The mouse was placed on the scanner bed and body temperature and respiratory rate were maintained at 36–37 °C by warm air and 50–70 per min by adjusting the dose of isoflurane, respectively. Sandiego et al. (2013) recently excluded a significant influence of isoflurane on [^{11}C]flumazenil PET in rhesus monkeys.

At time point zero, [^{18}F]flumazenil, synthesized from nitromazenil (ABX, Radeberg, Germany) according to Ryzhikov et al. (2005), was injected into a tail vein at a dose between 0.5 and 1.6 nmol (2.8–18 MBq). PET was acquired from 0 to 90 min after tracer injection in list mode with an Argus small animal PET/CT scanner (Sedecal, Madrid, Spain; axial field of view 4.8 cm; spatial resolution 1.6–1.7 mm (full width at half maximum) at the axial center of field of view and 5 mm radial offset (Goertzen et al., 2012)). After the PET scan, a computed tomography (CT) was registered for anatomical orientation. At the end of the scans, mice were euthanized by decapitation still under anaesthesia and for those scans with diazepam, plasma samples were analyzed in the beta (LS6500, Beckman Coulter, Brea, CA) and gamma counter (1480 Wizard 3", Perkin Elmer) to determine the concentrations of ^{3}H- (diazepam and its tritiated metabolites) and ^{18}F-labelled compounds (flumazenil and its radiometabolites), respectively.

PET data was reconstructed with Fourier rebinning followed by 2D ordered subset expectation maximization (FORE/2D-OSEM) with 2 iterations, 16 subsets, into time frames between 2 and 12 min, with corrections for single and random decays but not for attenuation. The nominal voxel size was 0.3875 mm (x and y directions) and 0.775 mm (z direction, along the symmetry axis of the bore). The total number of PET/CT scans was 35, of which 29 were used for further analysis (3 baseline scans each for HHHH, HRHH, HRRR, RHHH mice, 5 baseline scans for RHRR mice, 6 scans each after diazepam administration with HRRR and RHRR mice, respectively). The reasons for exclusion were failed tracer injection (1 scan), insufficient immobilization during the scan (4 scans) and exceptionally low plasma ^{3}H level after a 3 mg/kg diazepam administration (1 scan).

PET region-of-interest data analysis

Reconstructed PET data was processed with the PMOD software (PMOD, Zurich, Switzerland; v3.7). The PET data was first matched to the respective CT data set. The CT data was subsequently matched on an MRI template of mouse brain provided with the software package. The resulting transformation coordinates were used to match the PET data on the MRI template. In a final step, the matching between all PET data sets was confirmed and manually adjusted if required. The PMOD mouse brain regions template was used to define the regions of interest. If required, individual region outlines were adjusted manually to the averaged PET image of the HHHH mice (see Inline Supplementary Fig. 2). This adjusted template was then used for all PET data sets. Time-activity curves for the indicated regions were generated and used for further analysis. Standardized uptake values (SUV, dimensionless) were calculated from the image-derived radioactivity in Bq per cm^3 tissue divided by the radioactivity dose in Bq per g body weight, assuming a tissue density of 1 g/cm^3. For the calculation of region-specific distribution volume ratios (DVR) and BP_{ND} (DVR - 1), a reference region with negligible PET signal was defined in the brain stem (dorsal parts of pons and medulla) as shown in the Inline Supplementary Fig. 2. Its volume was 13.3 mm^3.

To validate the reference region as a surrogate for the blood plasma for quantitative PET, the reference-region ^{18}F concentration, as derived from the PET data (kBq/cm^3) and the specific radioactivity of [^{18}F]

flumazenil (GBq/μmol), was compared with the ^{18}F concentration in blood plasma, determined at the end of the scans with HRRR and RHRR mice receiving diazepam (Inline Supplementary Fig. 3). The ^{18}F concentration of the reference region, averaged from 78 to 90 min, correlated well with the blood plasma ^{18}F concentration determined at the end of the scans. The correlation remained when comparing the SUV values of the reference region averaged from 20 to 60 min ($SUV_{ref,20–60min}$) with the SUV of the blood plasma, calculated from the plasma ^{18}F radioactivity at the end of the scans. The correlations confirm the chosen reference region as a valid surrogate of the plasma input (Inline Supplementary Fig. 3). For an unknown reason, both reference region and plasma SUV were significantly higher in HRRR than RHRR mice. By trend, both the $SUV_{ref,20–60min}$ and plasma SUV at 100 min decreased with increasing diazepam concentration.

Using the above described reference region, regional DVR were calculated with a Logan reference plot, not correcting for k_2 (Krämer, 2015; Logan et al., 1996) with a t^* (start of analysis of the linear phase in the Logan reference plot) of 20 min. PET data was included for 60 min scan duration as two mice died between 60 min and the end of the scans. The effect on DVR of setting t^* to 5 or 30 min was < 4%. Including data up to 90 min changed the DVR by maximally 4%. Representative Logan plots are shown in the Inline Supplementary Fig. 4. For comparison, we averaged the SUV between 0 and 60 min ($SUV_{0–60min}$) or 20 and 60 min ($SUV_{20–60min}$) after scan start (integrated SUV divided by the duration of the respective time window).

To investigate the distribution of $GABA_A$ receptor α subunits which are involved in diazepam-sensitive $GABA_A$ receptors in mouse brain, we assumed equal affinity of radiolabelled flumazenil to α1, α2, α3 and α5 $GABA_A$ receptor subtypes, as was shown for the human and rat homologues (Pym et al., 2005; Sieghart, 1995). The average injected mass of [^{18}F]flumazenil for the baseline PET scans was 28.1 ± 10.3, 31.6 ± 1.1, 32.3 ± 12.5, 30.1 ± 1.5 and 33.7 ± 11.4 nmol/kg for HHHH, HRHH, HRRR, RHHH and RHRR mice, respectively, without a significant difference between the groups (uncorrected t-test). The average concentration of non-displaceable [^{18}F]flumazenil ($C_{ND,FMZ}$) between 20 and 60 min p.i. as calculated from the image-derived kBq/cm^3 of the reference region, averaged between 20 and 60 min, and the respective specific radioactivity in GBq/μmol, was on average 11.6 ± 5.4 nM. Compared with the [^{11}C]flumazenil concentration at half maximal $GABA_A$ receptor occupancy determined in rat brain, *i.e.*, $K_{d,FMZ}$=25.1 nM (Syvänen et al., 2011), this would result in 32 % receptor occupancy on average by the tracer (this $K_{d,FMZ}$ was confirmed for [^{18}F]flumazenil in mouse brain in Section 3.3). Considering the similar tracer doses of the five groups, we did not correct the data in this part of the study for the receptor occupancy by the tracer. The constant ratios between time-activity curves of regions of interest and reference region over time indicate that saturation phenomena can be neglected for these scans (Inline Supplementary Fig. 5).

PET voxel-wise modelling and difference plots

BP_{ND} were calculated voxel-wise with the PXMod module of PMOD by means of the implemented reference Logan model and the reference region defined in the Inline Supplementary Fig. 2. The parameter t^* was between 0 and 20 min, k_2 was fixed at 5 min^{-1} to reduce its impact as it was unknown (Krämer, 2015; Müller Herde et al., 2015). Individual BP_{ND} maps were averaged for each genotype (5 for RHRR, 3 each for HHHH, HRRR, HRHH and RHHH) and difference plots were generated from these data sets with the PMOD PFUS module.

Abundancy of subunit combinations

The abundancy of individual α subunit combinations was calculated from the BP_{ND} of baseline [^{18}F]flumazenil PET scans as shown in Table 1 or in analogy from the kBq/mg specific binding of the autoradiography analyses. The possible α subunits of a particular receptor are indicated by the numbers following the two α symbols. For example, α1α135 indicates that the respective BP_{ND}, kBq/mg or % includes diazepam-sensitive $GABA_A$ receptors with an α1 in one of the positions and an α1, α3 or α5 in the second position. An index x indicates that all α subunits which can form a high affinity benzodiazepine binding site are possible. The percentage of a particular subunit combination with respect to all diazepam-sensitive receptors was calculated as the respective BP_{ND} (or kBq/mg in autoradiography) divided by the BP_{ND} (or kBq/mg) of the HHHH genotype, multiplied with 100 %.

We did not consider any involvement of the subunits α4 or α6 in the diazepam-sensitive receptors. These two subunits are not capable of forming high-affinity benzodiazepine binding sites but can be present in diazepam-sensitive $GABA_A$ receptors in the position distant to the γ2 subunit. They are involved in both γ2 and the diazepam-insensitive extrasynaptic δ receptors (Farrant and Nusser, 2005; Glykys et al., 2007; Kasugai et al., 2010; Nusser et al., 1998). The expression of α6 is restricted to cerebellum in mouse brain (Hörtnagl et al., 2013). In our analyses, α4 and α6 could replace one α1 or α2 subunit in the receptors designated α1α1 and α2α2, respectively, the correct designations would be α1α146 and α2α246. In addition, heteromeric subunit combinations in Table 1 would be modified accordingly to α135α13546, α235α23546.

Predicted abundancy of α1α1, α2α2 and α1α2 receptors

Eqs. (1)–(3) were applied to predict the relative abundancy of α1α1, α2α2 and α1α2 receptors with respect to the total of diazepam-sensitive receptors. The equations follow a model of random distribution of the α1 and α2 subunits between the diazepam-sensitive $GABA_A$ receptors. They are furthermore based on the concept that in a receptor with a point-mutated and a native α1 or α2 subunit, the native subunit is involved in benzodiazepine and thus [^{18}F]flumazenil binding (Balic et al., 2009). According to this concept, the fraction of α1 subunits with respect to all α subunits in diazepam-sensitive receptors equals the sum of the signals (BP_{ND} or kBq/mg) from α1αx (HRRR) plus α1α1 (HHHH-RHHH, to account for the second α1 subunit in α1α1 receptors) divided by twice the signal of the HHHH mice (corresponding to all α subunits involved in diazepam-sensitive receptors, Eq. (1)). Note that the term (BP_{ND}(HHHH)/2×BP_{ND}(HHHH)) was replaced by 0.5 in the Equations.

The probability of the occurrence of α1α1 receptors should consequently equal the square of the above described fraction if the α subunits are distributed randomly between the receptors (Eq. (1)). The probability of the occurrence of α2α2 receptors would be analogous (Eq. (2)) and the respective probability of heteromeric α1α2 diazepam-sensitive $GABA_A$ receptors would equal twice (to take into account both α1α2 and α2α1) the product of the fractions of α1 and α2 (Eq. (3)). The index rnd refers to the assumption of random distribution of α1 and α2 between the receptors. Note that α1α1 includes α1α46 and α2α2 includes α2α46. As the wild type diazepam-sensitive $GABA_A$ receptors would also include α4 and α6, the respective error is cancelled out in Eqs. (1)–(3).

$$\%\ \alpha1\alpha1_{rnd} = 100\% \times \left(\frac{BP_{ND}(HRRR) - BP_{ND}(RHHH)}{2 \times BP_{ND}(HHHH)} + 0.5 \right)^2 \quad (1)$$

$$\%\ \alpha2\alpha2_{rnd} = 100\% \times \left(\frac{BP_{ND}(RHRR) - BP_{ND}(HRHH)}{2 \times BP_{ND}(HHHH)} + 0.5 \right)^2 \quad (2)$$

$$\%\ \alpha1\alpha2_{rnd} = 200\% \times \left(\frac{BP_{ND}(HRRR) - BP_{ND}(RHHH)}{2 \times BP_{ND}(HHHH)} + 0.5 \right) \times \left(\frac{BP_{ND}(RHRR) - BP_{ND}(HRHH)}{2 \times BP_{ND}(HHHH)} + 0.5 \right) \quad (3)$$

In case the α1 and α2 subunits are indeed distributed randomly between the diazepam-sensitive $GABA_A$ receptors as assumed in Eqs. (1–3), % $\alpha1\alpha1_{rnd}$ equals 100%×(BP_{ND}(HHHH)-BP_{ND}(RHHH))/BP_{ND}(HHHH), *i.e.*, the percentage of experimentally observed α1α1 diazepam-sensitive $GABA_A$ receptors, according to Table 1. The comparison is analogous for the percentage of α2α2 receptors. The percentage of experimentally observed α1α2 diazepam-sensitive $GABA_A$ receptors is equal to or higher than 100%×((BP_{ND}(RHRR)+BP_{ND}(HRRR)-BP_{ND}(HHHH))/BP_{ND}(HHHH). This is the percentage of BP_{ND} (or kBq/cm^3) exceeding the BP_{ND} of HHHH mice, when BP_{ND} values of α1- and α2-containing receptors are summed up, *i.e.*, the minimal percentage of receptors containing both subunits at the same time. In case of random distribution of α1 and α2 between the diazepam-sensitive $GABA_A$ receptors, this percentage is equal or lower than % $\alpha1\alpha2_{rnd}$.

Receptor occupancy

$GABA_A$ receptor occupancy by diazepam was analyzed for HRRR and RHRR mice from the DVR values plotted *versus* the diazepam dose of the respective scan. The diazepam doses were 0.03, 0.3, 1, 3, 10, 30 mg/kg for the 6 HRRR mice and 0.3, 1, 3, 3, 10, 30 mg/kg for the 6 RHRR mice. The 3 HRRR and 5 RHRR baseline scans of the receptor distribution study were included in the analysis. In the receptor occupancy study, $C_{ND,FMZ}$ (20–60 min) reached values in the range of $K_{d,FMZ}$ of flumazenil for $GABA_A$ receptors determined in rat brain *in vivo* (25.1 nM) (Syvänen et al., 2011). To take the partial receptor occupancy by the tracer into account, the diazepam doses (D_{DZP}) in the DVR/D_{DZP} plots were corrected by the addition of D_{50}/$K_{d,FMZ}$×$C_{ND,FMZ}$, where D_{50} is the finally fitted diazepam dose (mg/kg) at half-maximal receptor occupancy. The objective function to fit the data was accordingly adapted as shown in Eq. (4), where n_H is the Hill coefficient.

$$DVR=(DVR_{max}-DVR_{min})\times\frac{{D_{50}}^{n_H}}{{D_{50}}^{n_H}+\left(D+\frac{D_{50}}{K_{d,FMZ}}\times C_{ND,FMZ}\right)^{n_H}}+DVR_{min} \quad (4)$$

The function in Eq. (4) was fit to the experimental DVR values, with the variables D_{50}, DVR_{max} and DVR_{min}. $K_{d,FMZ}$ and n_H were fit from the whole brain DVR of the RHRR mice. Alternatively, n_H was fixed at 1.0, based on the findings of previous *ex vivo* studies with n_H=1.11 ± 0.16 (Facklam et al., 1992). DVR values were transformed to % receptor occupancy (RO(%)) according to Eq. (5). Eq. (6) was used to calculate RO(%) by diazepam from its dose and the fit D_{50}. Analyses were performed for whole brain and the individual brain regions drawn in the Inline Supplementary Fig. 2.

$$RO(\%)=\frac{DVR_{max}-DVR}{DVR_{max}-DVR_{min}}\times 100\% \quad (5)$$

$$RO(\%)=\frac{D^{n_H}}{D^{n_H}+{D_{50}}^{n_H}}\times 100\% \quad (6)$$

In an alternative approach, the receptor occupancy after diazepam administration was estimated from the Lassen plot (Cunningham et al., 2010) of the $SUV_{20–60min}$. In brief, $SUV_{20–60min}$ of the baseline scans of HRRR or RHRR mice were averaged and the averaged $SUV_{20–60min}$ of the individual brain regions were plotted on the x-axis. For each diazepam dose, the differences between the baseline $SUV_{20–60min}$ and the dose-dependent $SUV_{20–60min}$ per region were plotted on the y-axis. The slope of the linear regression corresponds to the receptor occupancy (as a fraction of 1) at the respective dose. The (in theory) common x-value at y=0 of all lines (all doses) equals the $SUV_{20–60min}$ of the non-displaceable tracer. D_{50} was fit from the slopes and respective diazepam doses with the objective function slope=D_{DZP}/(D_{DZP}+D_{50}). For this analysis, receptor occupancy by the tracer was neglected and n_H was assumed 1.

Statistics

Mean values are shown with their standard deviations (SD) and were compared by a two-tailed homoscedastic student's t-test or by one-way ANOVA with Bonferroni post-hoc correction, as indicated. Differences with *p*-values below 0.05 were considered significant as indicated by an asterisk. Data fitting in the receptor occupancy study was performed with the MATLAB analysis functions fitnlm and lsqcurvefit (versions 2014b and 2016b, Matworks, Natick, MA). Fit parameters are given with their estimated standard errors (SEM) as indicated. Fit D_{50} values ($D_{50,i}$ and $D_{50,j}$ in Eq. (7)) were compared according to Eq. (7) and *p* values were revealed from the resulting *t* values and the degree of freedom with the tdist function of Microsoft Excel (Office 2013).

$$t=\frac{D_{50,i}-D_{50,j}}{\sqrt{SEM_i^2-SEM_j^2}} \quad (7)$$

Results

Distribution of diazepam-sensitive $GABA_A$ receptor subtypes in the mouse brain

The regional distribution patterns of $GABA_A$ receptor α-subtype combinations as revealed by the PET experiments is shown in Fig. 1. The averaged voxel-wise BP_{ND} and their differences are proportional to the density of the indicated diazepam-sensitive $GABA_A$ receptors. To confirm these *in vivo* data, we performed *in vitro* autoradiography with [^{3}H]flumazenil. Representative autoradiographs are shown in Fig. 2. Fig. 3 shows the results of the region-of-interest analysis from the PET and autoradiography experiments, in BP_{ND} and kBq/mg, respectively. Representative ^{18}F time-activity curves of the respective baseline PET scans of each genotype are shown in the Inline Supplementary Fig. 5.

In addition to BP_{ND}, we calculated the relative diazepam-sensitive $GABA_A$ receptor densities, in analogy to Fig. 3A, from $SUV_{0-60min}$ and $SUV_{20–60min}$, respectively. The $SUV_{20-60min}$ are shown in Inline Supplementary Fig. 6. In contrast to the BP_{ND} values, SUV values do not depend on a reference region but depend on tracer plasma concentrations and distribution to the brain. The density pattern calculated from the BP_{ND} (Fig. 3A) were in better agreement with the results from the *in vitro* autoradiography study (Fig. 3B) than were the patterns calculated from $SUV_{0–60min}$ (data not shown) or $SUV_{20–60min}$ (Inline Supplementary Fig. 6), confirming the validity of BP_{ND} for this analysis. Based on this comparison and the evaluation of the reference region (Inline Supplementary Fig. 3), we used the BP_{ND} for the further analyses.

For both *in vivo* and *in vitro* analysis, the density of flumazenil-binding receptors in HHHH mice was highest in hippocampus, superior and inferior colliculus and cerebral cortex, while brain stem had the lowest density (Figs. 1–3). Tracer accumulation in HRHH and HRRR mice, which have a wild type α1 but mutated α2 (HRHH) or mutated α2, α3 and α5 (HRRR) was similar as in the HHHH mice. There was no significant difference between these genotypes in the region-of-interest analyses both in PET and autoradiography (Fig. 3). This indicates that the majority of diazepam-sensitive $GABA_A$ receptors contain at least one α1 subunit throughout the mouse brain.

Despite the high occurrence of α1, mice with the mutated α1 subunit (RHHH and RHRR) still accumulated [^{18}F]- and [^{3}H]flumazenil *in vivo* and *in vitro*, respectively, albeit to reduced degrees compared to HHHH, HRHH and HRRR mice (Figs. 1 and 2). The reduction was significant for all investigated brain regions in both assays (Fig. 3). The signals were substantially higher than the small or

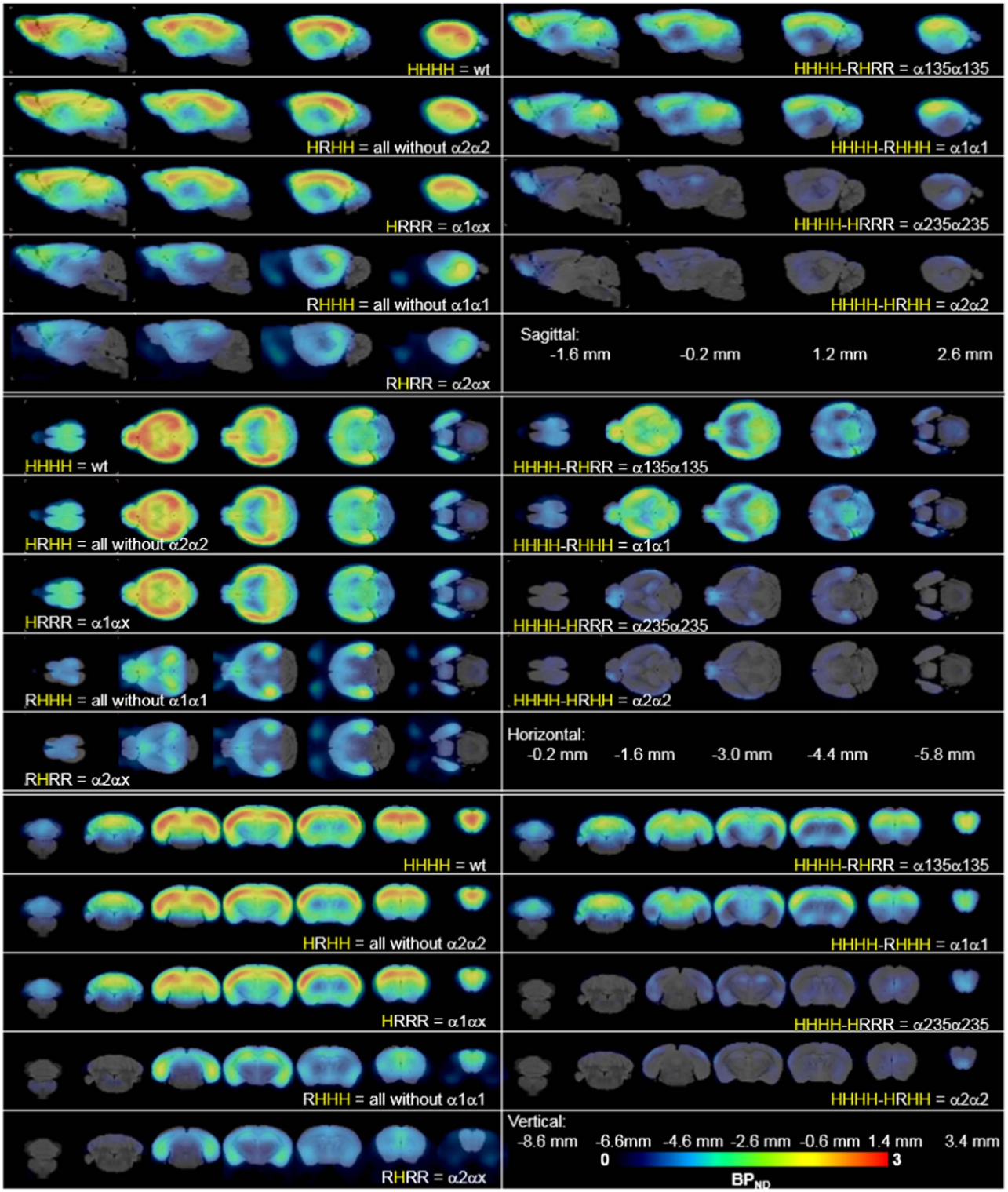

Fig. 1. PET images (BP_{ND}) of the investigated genotypes and corresponding difference plots, as indicated. BP_{ND} images were averaged from 3 or 5 (RHRR) scans each. The numbers following the two α indicate the (possible) α subunits of the involved receptors (an index x indicates that all α subunits are possible; α4 and α6 are not explicitly indicated); wt, wild type. Plane positions (in mm) relative to the Bregma are indicated. The quantitative regions-of-interest analysis is shown in Figs. 3 and 4.

absent difference between HHHH and HRHH or HRRR mice (Fig. 3). The fact that the sum of BP_{ND} or kBq/mg of HRRR and RHRR (or RHHH) mice exceeded the respective signal in HHHH mice indicates the presence of mixed α1α2 (or α1α235 in the case of RHHH) diazepam-sensitive $GABA_A$ receptors. As concluded from the signals of RHRR mice, all regions except of cerebellum contained significant levels of α2.

The hippocampus was the only region that showed a significant difference between RHHH and RHRR mice in the PET study (Figs. 1 and 3; not significant in the autoradiography study). This indicates that hippocampus contains a significant fraction of diazepam-sensitive receptors with one or both subunits different from α1 and both subunits different from α2, i.e., α135α35. Because α5 is stronger expressed in the hippocampus than α3 (Hörtnagl et al., 2013), this

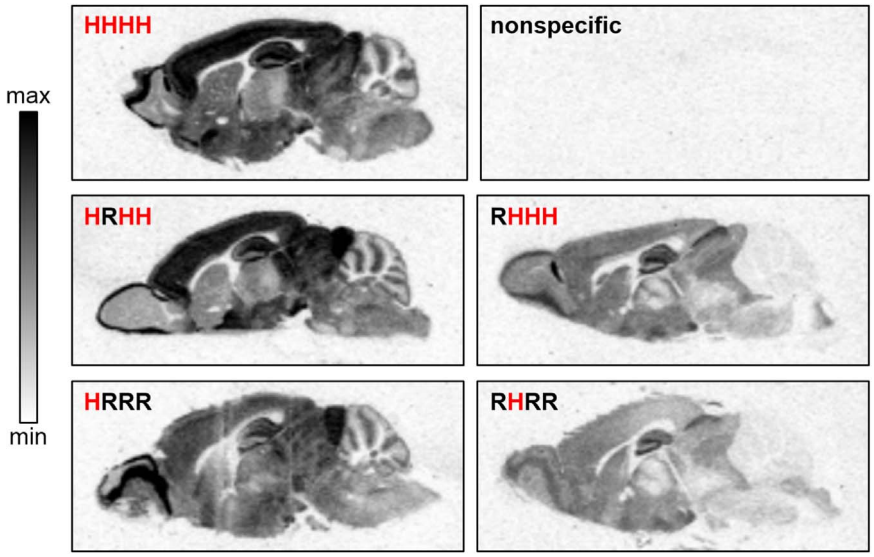

Fig. 2. *In vitro* autoradiography with [^{3}H]flumazenil with sagittal brain slices of mice with the indicated genotype. The [^{3}H]flumazenil concentration was 5 nM. Nonspecific binding was negligible as tested with 10 μM clonazepam. See Figs. 3 and 4 for quantitative regions-of-interest analysis. Scale bar, minimal (min) to maximal (max) radioactivity accumulation.

suggests the presence of diazepam-sensitive $GABA_A$ receptors with either α1α5 or α5α5 subunit combinations.

Irrespective of whether differences were significant or not between the genotypes, we estimated the percentage of subtype combinations from the differences between the BP_{ND} (or kBq/mg) of the investigated genotypes. The voxel-wise differences from the PET BP_{ND} are shown in

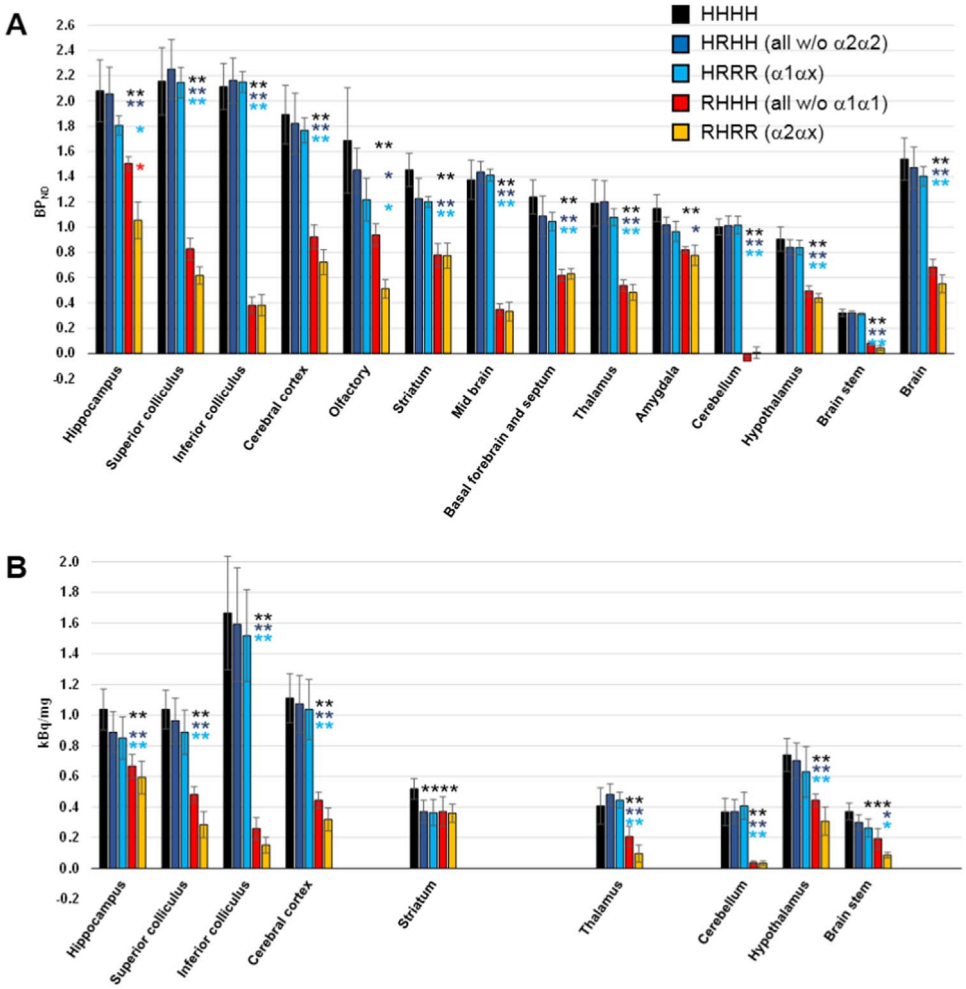

Fig. 3. Quantitative region-of-interest analysis of [^{18}F]- and [^{3}H]flumazenil binding in brains of mice with different genotypes as indicated. A) PET BP_{ND}, n=5 (RHRR) or 3; B) *in vitro* autoradiography kBq/mg, n=7 brains, 5–7 analyzed sections each. Mean values with standard deviations. *, $p < 0.05$ (one-way ANOVA, Bonferroni-corrected), the location of the asterisk above a particular bar and its color identify the compared genotypes. Note that part of the autoradiography data was published before (Behlke et al., 2016).

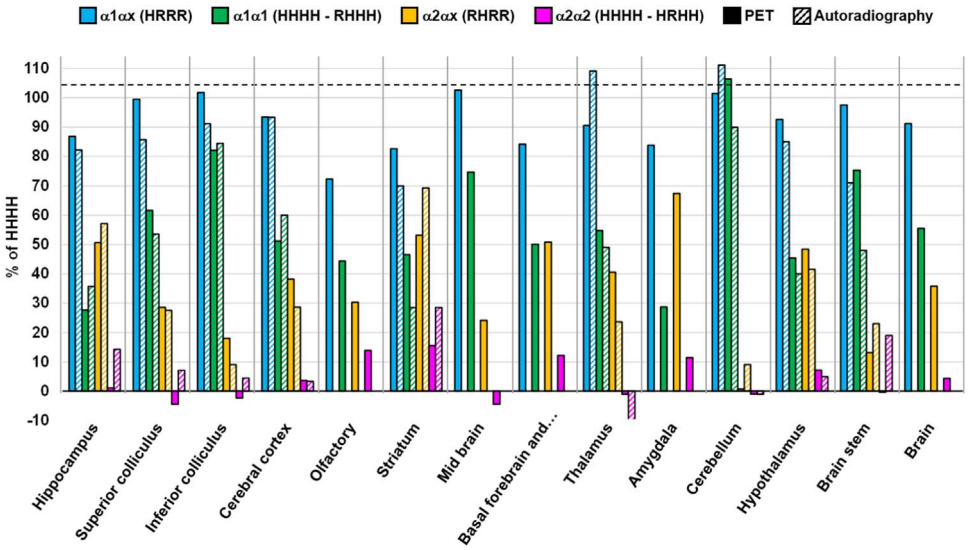

Fig. 4. Percent contribution of α1- and α2-containing $GABA_A$ receptors to the total pool of diazepam-sensitive $GABA_A$ receptors in mouse brain regions. Estimated from PET with [^{18}F] flumazenil (solid fills) and from *in vitro* autoradiography with [^{3}H]flumazenil (pattern fills). Color legend in the Figure. This data is included in Table 2.

Table 2
Estimated relative regional occurrence of individual $GABA_A$ α subunit combinations in mouse brain (in % of total diazepam-sensitive receptors per region). The three highest values comparing the regions of interest are in bold font, the three lowest are underlined.

Genotypes (alpha subunit combinations)	Method	Brain region[a]													
		Hip	SC	IC	Ctx	Olf	Str	Mb	BFS	Tha	Amy	Cb	Hyp	BS	Brain
HHHH	PET, AR[b]	100	100	100	100	100	100	100	100	100	100	100	100	100	100
HRRR (α1αx)	PET	87	100	**102**	93	72	83	**103**	84	91	84	**101**	93	98	91
	AR	82	86	91	**93**		70			**109**		**111**	85	71	
HHHH-RHHH[c] (α1α1)	PET	28	62	**82**	51	44	47	**75**	50	55	29	**106**	45	**75**	56
	AR	36	54	**84**	**60**		29			49		**90**	40	48	
$\alpha1\alpha1_{rnd}$[d]	PET	33	65	84	52	34	42	79	45	53	32	108	48	75	54
	AR	35	49	77	59		24			63		101	39	35	
(HHHH-RHHH)/HRRR (α1α1 of α1αx)	PET	32	62	**81**	55	61	56	73	60	61	34	**105**	49	**77**	61
	AR	43	63	**93**	64		41			45		**81**	47	**68**	
RHRR (α2αx)	PET	**51**	29	18	38	30	**53**	24	**51**	41	**67**	1	48	13	36
	AR	**57**	28	9	29		**69**			24		9	**42**	23	
HHHH-HRHH (α2α2)	PET	1	-4	-2	4	**14**	**16**	-4	**12**	-1	**12**	-1	7	0	4
	AR	**14**	7	4	3		**29**			-18		-1	5	**19**	
$\alpha2\alpha2_{rnd}$[d]	PET	7			4	5	12		10		16		8		4
	AR	13	3		3		24						5	4	
(HHHH-HRHH)/RHRR (α2α2 of α2αx)	PET	2	< 1	< 1	10	**46**	**29**	< 1	**24**	< 1	17	< 1	15	< 1	12
	AR	25	26	**49**	12		**41**			< 1		< 1	12	**83**	
RHRR+HRRR-HHHH (min α1α2)	PET	**37**	28	20	32	3	36	27	35	31	**51**	2	**41**	11	27
	AR	**39**	13	0.2	22		**39**			23		9	**27**		
$a1a2_{rnd}$[d]	PET	30			30	26	44		42		44		38		29
	AR	42	24	12	25		48						29	25	
RHHH-RHRR (α135α35)	PET	**22**	10	0	11	**25**	0	1	-1	5	4	-7	6	**12**	9
	AR	7	**19**	6	11		2			**27**		1	19	**29**	
HRHH-HRRR (α235α35)	PET	**12**	5	1	3	**14**	2	2	4	**10**	5	-1	0	3	4
	AR	4	7	4	3		1			**9**		-10	**10**	**10**	
HRRR-(HHHH-RHHH)-RHRR (min α1α35)	PET	**8**	**9**	2	4	-2	-17	4	-17	-5	-12	-6	-1	**9**	0
	AR	-11	**5**	-2	5		-30			**36**		**12**	4	0	

[a] Hip, hippocampus; SC, superior colliculus; IC, inferior colliculus; Ctx, cerebral cortex; Olf, olfactory bulb; Str, striatum; Mb, mid brain; BFS, basal forebrain and septum; Tha, thalamus; Amy, amygdala; Cb, cerebellum; Hyp, hypothalamus; BS, brain stem.
[b] AR, autoradiography.
[c] Difference between the two indicated genotypes.
[d] Predicted, assuming a random (rnd) distribution of α1 and α2 subunits between the diazepam-sensitive $GABA_A$ receptors. Part of the data is shown in Fig. 4.

Fig. 1 and the region-of-interest analyses of the PET and autoradiography data in Fig. 4 and Table 2.

The regions with > 90% α1-containing $GABA_A$ receptors in both analyses were inferior colliculus, cerebellum, cerebral cortex and thalamus. Of these regions, inferior colliculus and cerebellum contained > 80% receptors with exclusively α1 as binding-site forming subunit (designated α1α1, HHHH-RHHH). Note that a significant fraction of the diazepam-sensitive receptors in cerebellum designated α1α1 may be α1α6, as α6 levels are high in this brain region (Hörtnagl et al., 2013). In the PET analysis, hippocampus and amygdala were the regions with lowest fractions of α1α1 (< 30%). In the autoradiography analysis, striatum showed the lowest α1α1 fraction (< 30%; 47% in the PET analysis). The α1α1 fraction in whole brain was 56%.

The percentage of α2-containing $GABA_A$ receptors was > 50% in hippocampus and basal forebrain and septum and was highest with 67% in amygdala (PET) and 69% in striatum (autoradiography). The

percentage of α2-containing diazepam-sensitive receptors was lowest in inferior colliculus and cerebellum, the regions with the highest α1α1 fractions. Receptors with exclusively α2 as the binding-site forming α subunit (α2α2, HHHH-HRHH) were present in several regions, reaching >10% in hippocampus (autoradiography but only 1% by PET), olfactory bulb, striatum, basal forebrain and septum, amygdala and brain stem (autoradiography but no evidence by PET). In olfactory bulb, close to 50% (by PET) and in striatum 29/41% (PET/autoradiography) of the α2-containing diazepam-sensitive receptors were α2α2 receptors. Averaged for whole brain (PET), 12% of the α2-containing diazepam-sensitive receptors had exclusively α2 as the binding-site forming α subunit.

As discussed above, a substantial fraction of the α1-containing diazepam-sensitive $GABA_A$ receptors were α1α2 receptors. The estimated minimal percentage of α1α2 receptors is shown in Table 2. It was highest in hippocampus, striatum, basal forebrain and septum, amygdala and hypothalamus (all >35% by PET).

Regions with higher accumulation in HRHH than HRRR, or in RHHH than RHRR mice, must contain $GABA_A$ receptors with at least one α3 or α5 subunit. This fraction was <30% in all regions and was <3% in e.g., striatum and cerebellum. It was significant (RHHH-RHRR) for hippocampus by PET as reported earlier in this Section. The estimation of the minimal fraction of α1α35 receptors (HRRR-(HHHH-RHHH)-RHRR) was not conclusive comparing PET and autoradiography data. PET suggested their existence (≥8%) in hippocampus, superior colliculus and brain stem. Autoradiography suggested ≥12% in thalamus and cerebellum (Table 2).

Predictability of the occurrence of α1α1, α2α2 and α1α2 receptors

Based on the experimental data, we calculated the percentage of α1α1, α2α2 and α1α2 receptors which would be expected if the respective α subunits were distributed randomly between the diazepam-sensitive $GABA_A$ receptors. The correlations between these predicted and the experimentally determined % of the individual combinations are shown in Fig. 5. In the case of α1α2, the predicted fractions are compared to the minimally occurring fractions estimated from the experimental data. The correlations of all three receptor types are in agreement with the model of random α1- and α2-subunit distribution. In particular for α1α1, the near identity of predicted and observed values is in strong favor of a random distribution. The predicted fractions for the individual brain regions are shown in Table 2 (indicated with the index rnd).

Receptor occupancy by diazepam

Next, we investigated whether the receptor occupancy by diazepam and in particular the dose at half-maximal occupancy D_{50} depended on the binding-site forming α subunit in the receptors. We, therefore, performed receptor occupancy experiments by [^{18}F]flumazenil PET with HRRR and RHRR mice and compared the D_{50} between the genotypes and between the various brain regions. The Inline Supplementary Fig. 7 shows the time-activity curves of the respective scans.

To confirm the successful administration of diazepam, the diazepam formulation was spiked with [^{3}H]diazepam and tritium levels were determined in plasma at the end of the scans. The correlation between plasma concentrations and diazepam dose of the included scans is shown in Fig. 6A. According to Eq. (4), the % RO depends on the affinity and concentration of both diazepam and [^{18}F]flumazenil. The range of [^{18}F]flumazenil concentrations in the reference region of the scans with RHRR mice (3.4 to 15.5 nM as calculated from the PET data and the specific radioactivity) allowed to fit the respective [^{18}F]flumazenil concentration at half-maximal occupancy ($K_{d,FMZ}$ in Eq. (4)) together with the D_{50}. The fitted $K_{d,FMZ}$ was 22.5 nM (SEM 6.7 nM) for whole brain, close to the 25.1 nM determined for rat brain with [^{11}C]flumazenil (Syvänen et al., 2011). Fitting $K_{d,FMZ}$ in the HRRR mice did not converge to a meaningful solution. We, therefore, used the fit $K_{d,FMZ}$ value of the RHRR mice for the analysis of the scans with HRRR mice, in particular as the value determined with RHRR mice was close to the value determined in wild-type rats (Syvänen et al., 2011). The Hill coefficient n_H, fit from the same data set (whole brain RHRR mice), was 0.56 (SEM 0.45). As fitting n_H did not substantially improve the fits as compared to n_H=1.0 (as used above to fit $K_{d,FMZ}$), n_H was fixed at 1.0 for the further analysis, in agreement with previous findings (Facklam et al., 1992). The respective sums of the squared residuals were 7.4×10^{-3} (n_H fitted) and 8.8×10^{-3} (n_H=1.0).

Fig. 6B shows the dose-dependent % RO for whole brain of HRRR and RHRR mice. The fit D_{50} values were 1.66 (SEM 1.16) and 1.85 (SEM 0.39) mg/kg, respectively, without significant difference according to Eq. (7). D_{50} of the individual brain regions (Inline Supplementary Fig. 8) ranged from 0.71 for olfactory bulb of HRRR mice (note high spill-over from Harderian glands) to 2.2 mg/kg for striatum in the same genotype (the cerebellum of RHRR mice was not considered as tracer accumulation was negligible). Statistical analysis according to Eq. (7), without correction for multiple comparisons, revealed only one region pair with p faintly lower than 0.05 (0.048; inferior colliculus and striatum in RHRR mice) but did otherwise not reveal any significant difference between any two D_{50} of the analyzed regions, independent of the genotype. For comparison, the D_{50} estimated from the Lassen plots drawn from the $SUV_{20\text{-}60min}$ were 3.1 mg/kg and 1.7 mg/kg for the HRRR and RHRR mice, respectively (Inline Supplementary Fig. 9).

Receptor occupancy in brain at sedative and antihyperalgesic doses of diazepam

In a recent study, half-maximal sedation was achieved in HRRR mice at 0.33 ± 0.05 mg/kg diazepam which resulted in 46.9 ± 5.5% RO in the brain. In RHRR mice, half-maximal antihyperalgesia was reached at 7.6 ± 0.6 mg/kg and 70.9 ± 1.7% RO in spinal cord (Ralvenius et al., 2015). The respective % RO were determined *ex vivo* in tissue homogenates of mice euthanized 90 min after diazepam administration. The results of the current PET study allowed to estimate the respective % RO in brain *in vivo*. The two doses for half-maximal effects as determined in the previous study (Ralvenius et al., 2015) are indicated as vertical lines in Fig. 6B (whole brain) and the Inline Supplementary Fig. 8 (individual brain regions). Receptor occupancy at 0.33 mg/kg was 16.6 and 15.2 % in HRRR and RHRR mice, respectively. At 7.6 mg/kg it was 82.1 and 80.5%, respectively. When the data was fit with n_H fit from the RHRR mice (0.56, SEM 0.44), the RO% were 36.9 and 32.5% at 0.33 mg/kg and 77.2 and 73.5% at 7.6 mg/kg, respectively.

Discussion

Distribution of α1, α2 and α5-containing diazepam-sensitive receptors

The distribution patterns of α1- and α2-containing diazepam-sensitive $GABA_A$ receptors revealed by [^{18}F]flumazenil PET and *in vitro* autoradiography with [^{3}H]flumazenil are in good agreement with the previously published immunohistochemistry results for the individual α subunits in mouse (Hörtnagl et al., 2013) and rat brain (Fritschy and Möhler, 1995). This was expected as the majority of $GABA_A$ receptors contain the benzodiazepine-binding site forming γ2 subunit and a substantial fraction of the α1 and α2 subunits is involved in diazepam-sensitive receptors, and, therefore, binds [^{18}F]- and [^{3}H]flumazenil (Olsen and Sieghart, 2008).

In analogy to previous findings (Benke et al., 2004; Ralvenius et al., 2015), the sum of specifically accumulated [^{18}F]- or [^{3}H]flumazenil in a particular brain region of HRRR and RHRR mice

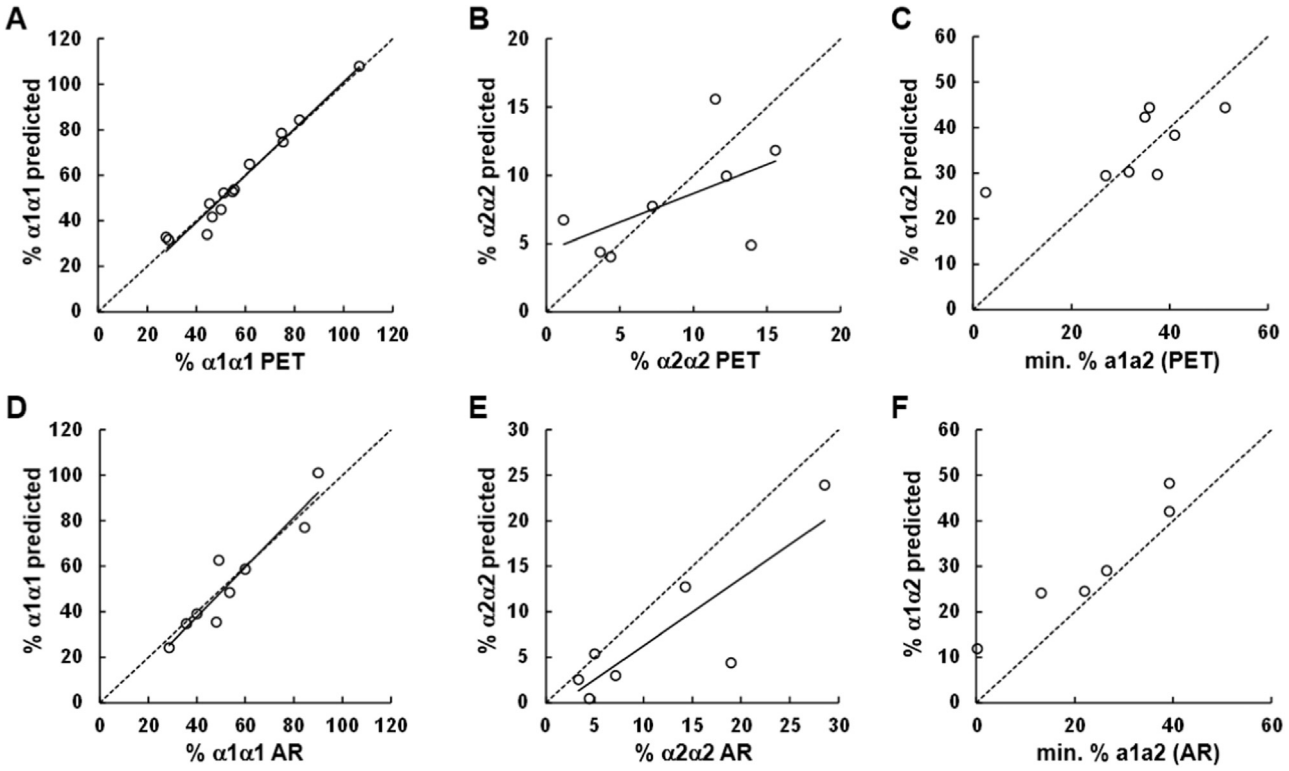

Fig. 5. The occurrence of α1α1, α2α2 and α1α2 receptors is in agreement with a model of random distribution of the α1 and α2 subunits between the diazepam-sensitive $GABA_A$ receptors. A, D) Percentage of α1α1 of total diazepam-sensitive $GABA_A$ receptors predicted from the PET (A) and autoradiography data (D) of various brain regions (y-axis), in comparison with the observed occurrence (x-axis). The prediction assumed a random distribution of the detected α1 and α2 subunits between the diazepam-sensitive $GABA_A$ receptors. B, E) Predicted percentage of α2α2 based on PET (B) and autoradiography (E) data compared to the observed occurrence (negative values for experimental α2α2 in Table 1 were excluded from the analysis). C, F) Predicted % α1α2 compared to the minimal occurrence of α1α2 determined by PET (C) and autoradiography (F). Broken lines, lines of identity. Solid lines, linear regressions between predicted and observed occurrences (%predicted=slope×% observed+intercept). Slope, intercept and Pearson's *r* were A) 1.03, -1.8%, 0.98; B) 0.42, 4.5%, 0.55; D) 1.09, -5.9 %, 0.94; E) 0.74, -1.2 %, 0.85. No regression analysis for C and F as the x-axes show minimally occurring percentages, *i.e.*, individual values may be higher than indicated (shifted to the right).

(BP_{ND}(HRRR)+BP_{ND}(RHRR)) exceeded the accumulation in the respective HHHH brain region (BP_{ND}(HHHH)). This confirms the previous observations that in a receptor with mixed α subunits (α1, α2, α3 or α5) of which one has a H- > R point mutation, the native subunit will assemble next to the γ2 subunit and render the $GABA_A$ receptor diazepam-sensitive, independent of the α subunit arrangement in the wild-type receptor. As a consequence, mixed α1α2 diazepam-sensitive $GABA_A$ receptors were detected with all investigated genotypes in this study, and α1α5 were detected in HRRR as well as HRHH mice, independent of whether α1 or α5 is adjacent to the γ2 subunit in the native receptor.

Owing to this characteristic and as performed *in vitro* with whole mouse brain before (Balic et al., 2009; Benke et al., 2004; Rudolph et al., 1999), our data allowed estimating the occurrence of α1- and α2-

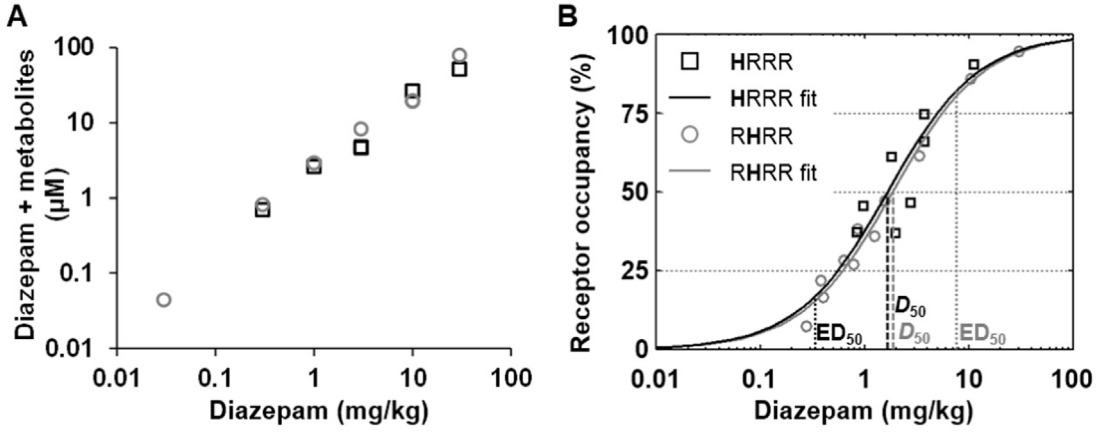

Fig. 6. Receptor occupancy of $GABA_A$ receptors by diazepam in HRRR and RHRR mice. A) Plasma concentrations of diazepam and metabolites included in the ^3H balance, 100 min after oral administration of the indicated dose. Pearson's *r* for the correlation between logarithmic plasma concentration and logarithmic diazepam dose (HRRR and RHRR mice) was 0.991. B) Receptor occupancy of flumazenil-sensitive $GABA_A$ receptors by diazepam in whole brain of HRRR (black squares) and RHRR (grey circles) mice. The *x* axis corresponds to the diazepam dose+$D_{50}/K_{d,FMZ}\times C_{ND,FMZ}$ (or diazepam alone in the absence of flumazenil). Solid lines, fit functions for HRRR (black) and RHRR (grey) mice. The fitted maximal and minimal DVR were 3.05 (SEM 0.21) and 1.45 (SEM 0.20) for HRRR mice and 1.77 (SEM 0.02) and 0.99 (SEM 0.03) for RHRR mice. The Hill coefficient was fixed at 1.0 for the fits. Broken vertical lines indicate fit D_{50} for HRRR (black) and RHRR (grey) mice. The dotted vertical lines indicate the recently determined diazepam doses at half-maximal sedation in RHHH mice (ED_{50} 0.33 mg/kg, black), and the dose at half-maximal antihyperalgesia in HRHH mice (ED_{50} 7.6 mg/kg, grey) (Ralvenius et al., 2015).

containing diazepam-sensitive receptors averaged for whole brain (PET), averaged for the regions of interest (PET and autoradiography) and voxel-wise (PET). They furthermore allowed estimating the receptor fractions containing exclusively α1 or α2 subunits as the binding-site forming subunits, throughout the living mouse brain. In general, the results agreed with recent *in vitro* studies with whole mouse brain. We found that 91% of the diazepam-sensitive $GABA_A$ receptors in whole brain contained one or two α1 subunits, close to the contribution of combined α1α1, α1α2 and α1α3 receptors to total diazepam-sensitive $GABA_A$ receptors of 89% determined with mouse brain membranes in binding assays (Benke et al., 2004). In the PET study, we identified 56 % of the diazepam-sensitive $GABA_A$ receptors as α1α1 (or α1 in combination with α4 or α6), similar to the 59% and 61% detected with brain membranes (Benke et al., 2004; Rudolph et al., 1999). In agreement with the former *in vitro* studies (Benke et al., 2004), the majority of the α1-containing diazepam-sensitive $GABA_A$ receptors were identified as homomeric α1α1 receptors and their regional distribution pattern matched former *in vitro* autoradiography results revealed from HHHH and RHHH mice (McKernan et al., 2000).

The α2-subunit containing diazepam-sensitive $GABA_A$ receptors are of high interest because of their anxiolytic action and involvement in schizophrenia and other diseases (Heldt and Ressler, 2007; Lewis et al., 2005; Rudolph and Möhler, 2014). The PET experiments identified 36% of diazepam-sensitive $GABA_A$ receptors in whole brain as α2-containing receptors. In comparison, the sum of α1α2, α2α2 and α2α3 receptors was 25% in the recent experiments with brain membranes (Benke et al., 2004). In our PET study, the percentage of α2-containing diazepam-sensitive $GABA_A$ receptors was highest in the amygdala, in agreement with previous findings on the high relative occurrence of the α2 subunit in this region (Löw et al., 2000; Marowsky et al., 2004). The amygdala significantly contributes to the regulation of fear and anxiety. Hyperexcitability of its neuronal activity is closely related to anxiety and evidence is strong that the GABAergic system is crucial for its regulation (Marowsky et al., 2004; Nuss, 2015; Prager et al., 2016; Skorzewska et al., 2015). Besides the amygdala, striatum, hippocampus, hypothalamus and basal forebrain and septum had high fractions of α2-subunit containing diazepam-sensitive $GABA_A$ receptors in our PET study. All these regions are suggested to be involved in the neuronal network of anxiety (Lago et al., 2016). Besides the data from the region-of-interest analysis, the images of the voxel-wise mapping of α2-containing receptors are in good agreement with the suggested circuits of anxiety (Lago et al., 2016).

The contribution of α2α2 to all diazepam-sensitive $GABA_A$ receptors was 4% in the PET study but 12% in former studies (Benke et al., 2004; Löw et al., 2000). In the current work, the quantification of the α2α2 diazepam-sensitive $GABA_A$ receptor fraction was based on a parameter difference which was not significant, in both PET and autoradiography and was accordingly inaccurate, also explaining the discrepancy between the PET and autoradiography data in this work. This shows the limitation of the suggested PET method for the analysis of the distribution patterns of receptor subtypes with low occurrence.

In agreement with recent reports (Araujo et al., 1999; Ghafari et al., 2016; Olsen and Sieghart, 2008), we found in the hippocampus substantial fractions of diazepam-sensitive $GABA_A$ receptors containing α3 or α5 subunits (significant by PET). As α5 levels prevail over α3 levels in mouse hippocampus (Hörtnagl et al., 2013), we assume that a high fraction of these receptors are α5-containing diazepam-sensitive $GABA_A$ receptors. This would be in accordance with the recently estimated fraction of 16% α5-containing diazepam-sensitive $GABA_A$ receptors in the hippocampus (Rudolph and Möhler, 2004). The levels of α5 and α1, probably assembled as α1α5 diazepam-sensitive $GABA_A$ receptors, increased further in mouse hippocampal areas after a spatial learning task (Ghafari et al., 2016). As α5 is the predominating benzodiazepine-binding subunit in α5-subunit containing diazepam-sensitive $GABA_A$ receptors (Balic et al., 2009; del Rio et al., 2001), it is certainly relevant for benzodiazepine pharmacology in this brain region, despite its relatively low levels compared to α1. The selective modulation of α5-containing diazepam-sensitive $GABA_A$ receptors is currently explored in preclinical research and clinical trials to improve cognitive functions (Bailey et al., 2002; ClinicalTrials.gov, 2016; Ling et al., 2015; Rudolph and Möhler, 2014).

Homomeric and mixed diazepam-sensitive α1 and α2 $GABA_A$ receptors

Our data confirmed the strong evidence from *in vitro* experiments that diazepam-sensitive $GABA_A$ receptors with mixed α subunits reach relevant levels in the mouse brain (Benke et al., 2004). Both the PET and autoradiography data support a model of random distribution of α1 and α2 between the respective diazepam-sensitive receptors. This allowed us estimating the % of mixed α1α2 receptors in all brain regions, although these numbers were not directly available from the experimental data. We estimated that 29% of all diazepam-sensitive receptors in whole brain are α1α2 receptors, similar to the minimal expected fraction estimated by direct differences between the genotypes (27%) but higher than the 13% revealed *in vitro* before (Benke et al., 2004).

Based on our conclusion that the α1 and α2 subunits are randomly distributed between the respective diazepam-sensitive $GABA_A$ receptors and considering the privileged position of α2 over α1 at the benzodiazepine-binding site, α2-selective drugs may still be effective in brain regions with an excess of α1 subunits. Contrary, high α2 subunit levels may prevent α1-selective pharmacology. As an example, an α1:α2 subunit ratio of 9:1 would correspond to ratios of α1α1, α1α2 and α2α2 diazepam-sensitive receptors of 81:18:1 (9^2:(9×1+1×9):1^2), resulting in ~20% receptors with α2 pharmacology (α1α2+α2α2). The inverse ratio, 1:9 for α1:α2 would result in a ratio of 1:100 α1 to α2 pharmacology (α1α1 : (α1α2+α2α2). A ratio of α1:α2=1:1 should provide 25% receptors with α1 pharmacology and 75% receptors with α2 pharmacology. In our analysis, α1-subunit containing diazepam-sensitive $GABA_A$ receptors exceeded the fraction of α2-subunit containing receptors in all analyzed brain regions. However, on the microscopic level, brain nuclei exist with higher α2 than α1 subunits, such as the central nucleus of the amygdala (Marowsky et al., 2004). Based on our study, the ratio of α2 to α1 pharmacology was highest in this brain region (amygdala), with a 2:1 ratio, ($\alpha1\alpha2_{rnd}$+$\alpha2\alpha2_{rnd}$):($\alpha1\alpha1_{rnd}$). Besides amygdala, the suggested regions involved in anxiety discussed in Section 4.1 (*Distribution of α1, α2 and α5-containing diazepam sensitive receptors*) all had predicted α2/α1 pharmacology ratios above or equal to 1. The ratio was 0.6 for whole brain, for comparison, and 0.7 for the cerebral cortex.

This agreement between the observed anxiolytic effect of α2-selective $GABA_A$ receptor modulation and the by PET predicted prevailing pharmacology in the brain regions involved in anxiety is encouraging towards the prediction of further specific effects of α-subtype selective $GABA_A$ receptor modulators. According to the model of randomly distributed α1 and α2 subunits, the regional pharmacology may be predicted from the individual subunit levels, determined by any quantitative method. Comparing the two methods used in this work, PET has the advantages that living animals can be investigated in longitudinal studies and that 3-dimensional data sets are generated including the complete brain. Autoradiography has the advantage of a higher spatial resolution. This offers the possibility to focus on individual nuclei or cell layers in the brain, as seen, e.g., for the hippocampus in this study. Whether the α3 and α5 subunits are distributed randomly between the diazepam-sensitive $GABA_A$ receptors or not remains, however, to be investigated.

Receptor occupancy by diazepam

In the receptor occupancy study with diazepam in HRRR and RHRR mice, we explored the possibility to determine region- and

receptor subtype-specific receptor occupancies in the living mouse brain by PET. D_{50} values of diazepam were not significantly different between the HRRR and RHRR mice. This was expected based on the similar K_D values for recombinant α1α1 and α2α2 $GABA_A$ receptors for both diazepam (Ralvenius et al., 2016; Sieghart, 1995) and flumazenil (Pym et al., 2005). Atack et al. (1999) found similar displacement curves with [^{3}H]flumazenil as a function of intraperitoneally administered diazepam dose in cerebellum and spinal cord of mice, concluding that the affinities of both compounds is similar to $GABA_A$ receptors containing α1, which is high in cerebellum, and receptors containing α2 or α3, which are high in spinal cord. The ID_{50} values were around 2 mg/kg, similar to the ID_{50} determined 30 min after oral administration of diazepam to wild type mice (Facklam et al., 1992) and similar to the D_{50} values determined in this PET study. In our study, regional D_{50} values were independent of the predominant α1/α2 subunit combinations in the individual brain regions, suggesting that the affinity of diazepam to α1α2 mixed receptors is similar as to the homomeric receptors of α1 and α2.

We compared our PET results with a previous study involving the same mouse model and protocol for diazepam administration. In accordance with the previous study, we found that ED_{50} of sedation was reached at less than half-maximal α1-receptor occupancy while at ED_{50} for antihyperalgesia more than half-maximal occupancy of the α2-containing receptors in brain was reached, similar to the findings for spinal cord in the previous study (Ralvenius et al., 2015).

Conclusions

Our results demonstrate that preclinical PET with [^{18}F]flumazenil allows mapping and quantifying diazepam-sensitive $GABA_A$ receptor subunits and their combinations in the living mouse brain. We quantified the regional distribution of α1α1, α2α2 and suggest the distribution of α1α2 diazepam-sensitive $GABA_A$ receptors. Our data support a model of random distribution of the detected α1 and α2 subunits between the diazepam-sensitive receptors, allowing the prediction of the regional predominating subtype-specific pharmacology of α1/α2-targeting benzodiazepines. A receptor-occupancy study in mouse brain revealed similar results as a recent study for spinal cord, namely that the occupancy of α2-containing receptors is above 50% under diazepam-induced antihyperalgesia, while less than 50% occupancy is required at α1 receptors for sedation. The *in vivo* affinity of α1- and α2-containing receptors for diazepam is similar, independent of the brain region and whether homomeric or mixed receptors predominate. This study suggests [^{18}F]flumazenil PET as a potent tool to predict $GABA_A$ receptor subtype-specific pharmacology and to determine the occupancy of diazepam-sensitive $GABA_A$ receptors in individual brain regions of interest. This could support the development of subtype-selective $GABA_A$ receptor modulators.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuroimage.2017.02.022.

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