NeuroImage 150 (2017) 279–291

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/neuroimage

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

Adrienne Müller Herde[a], Dietmar Benke[b], William T. Ralvenius[b], Linjing Mu[c], Roger Schibli[a], Hanns Ulrich Zeilhofer[a,b], Stefanie D. Krämer[a,*]

[a] *Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences (D-CHAB), ETH Zurich, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland*
[b] *Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*
[c] *Department of Nuclear Medicine, University Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland*

ARTICLE INFO

Keywords:
$GABA_A$ receptor
Alpha subunit
Selective
Benzodiazepine
Receptor occupancy

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

* Corresponding author.
E-mail address: Stefanie.Kraemer@pharma.ethz.ch (S.D. Krämer).

http://dx.doi.org/10.1016/j.neuroimage.2017.02.022
Received 28 October 2016; Accepted 9 February 2017
Available online 10 February 2017
1053-8119/

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.