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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

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A handwritten signature in black ink, appearing to read 'A. Kuckert', with a stylized flourish at the end.

Anika Kuckertz, Düsseldorf, 22. Oktober 2025

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Conference Contributions

Organization of Human Brain Mapping, July 22-26 2023, Montreal, Canada. Serotonin receptors in areas of the emotion regulation network in human and rat brains. (*Talk and Poster*).

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Summary

The rat is a key animal model in neuroscience research and is widely used in translational studies relating to human brain disorders. Analyzing the regional distribution patterns of neurotransmitter receptors provides information about the molecular organization of the brain and enables its functional segregation. Altered receptor distributions in the brain, such as muscarinic and serotonergic receptors, have been implicated in neurodegenerative and mood disorders, including Alzheimer's disease and depression. In translational research and *in vivo* neuroimaging, rat brain atlases are used for comparison with the human brain and to localize disorder-related alterations. However, translational research on mood disorders is challenging due to individual neuropathological mechanisms and symptoms. Therefore, the rat as model for studying human mood disorders must be carefully evaluated for each specific research question. The resolution of the developed rat brain atlases as references for *in vivo* neuroimaging, such as magnetic resonance imaging and positron emission tomography, remains too low for detailed identification of smaller brain regions. Thus, high-resolution, three-dimensional (3D) rat brain atlases are required. This doctoral thesis systematically compared parts of the serotonergic system between the human and rat brain to evaluate the translational potential of the rat for research of human mood disorders. Furthermore, novel maps and a 3D rat brain atlas, based on neurotransmitter receptor distributions, were developed as high-resolution reference resources.

The first autoradiographic study compared the regional and laminar distributions of 5-HT_{1A} and 5-HT₂ receptors in main components of the human emotion regulation network with those in homolog areas of the rat. Significant species differences were found mainly for the 5-HT_{1A} receptors (e.g., in the cingulate cortex and the hippocampus), whereas the 5-HT₂ receptors were more similarly distributed in most human and homolog rat brain areas. The second study used *in vitro* autoradiography and cell body staining to multimodally parcellate the entire rat brain iso- and proisocortex according to the molecular organization of cholinergic muscarinic M₂ receptors and cytoarchitectonic analysis, respectively. The resulting maps showed the detailed identification of 53 areas, which were published as a high-resolution, two-dimensional (2D) atlas. A third study developed a high-resolution 3D rat brain atlas based on reconstructing the previously published M₂ receptor maps of the iso- and proisocortex. The 3D atlas and corresponding data are openly accessible via the EBRAINS research infrastructure.

The autoradiographic visualization of 5-HT_{1A}, 5-HT₂, and M₂ receptor distributions in the rat brain provides high-resolution, multiscale (i.e., across the entire brain volume, specific regions and cortical laminas) and multidimensional (i.e., 2D maps and 3D reconstructions) references for interspecies comparisons with the human brain and *in vivo* neuroimaging. Furthermore, regional receptor distributions can be used to localize drug binding sites in future research. Our receptor data can be expanded to include additional data of e.g., glutamatergic and dopaminergic receptors, as well as brain regions such as the striatum and thalamus. This enables receptor- and brain region-specific analyses, which also allow comparisons with disorder-related alterations, e.g., in mood disorders and Alzheimer's disease.

Zusammenfassung

Die Ratte ist ein wichtiges Tiermodell in der neurowissenschaftlichen Forschung und wird in translationalen Studien zur Untersuchung von Erkrankungen des menschlichen Gehirns eingesetzt. Verteilungsmusteranalysen von Neurotransmitter-Rezeptoren liefern Informationen über die molekulare Organisation des Gehirns und ermöglichen funktionelle Kartierungen. Veränderte Neurotransmitter-Rezeptoren-Verteilungen, wie beispielsweise von muskarinischen und serotonergen Rezeptoren, werden mit neurodegenerativen Erkrankungen und affektiven Störungen, darunter Alzheimer und Depression, assoziiert. In der translationalen Forschung und der *in vivo* Neurobildgebung dienen Rattengehirnatlanten als Referenz für Vergleiche zum menschlichen Gehirn und zur Lokalisierung krankheitsbedingter Veränderungen. Aufgrund individueller neuropathologischer Mechanismen und Symptome ist die translationale Forschung affektiver Störungen beim Menschen herausfordernd. Deshalb ist es wichtig, die Ratte als Modell für jede spezifische Forschungsfrage zur Untersuchung affektiver Störungen sorgfältig zu evaluieren. Die Auflösung der entwickelten Rattengehirnatlanten für die *in vivo* Neurobildgebung, wie der Magnetresonanztomographie und der Positronen-Emissions-Tomographie, ist für eine detaillierte Identifizierung kleiner Gehirnregionen jedoch zu gering. Daher werden dreidimensionale (3D) Rattengehirnatlanten mit einer höheren Auflösung benötigt. In dieser Dissertation wurden Teile des serotonergen Systems zwischen dem Gehirn des Menschen und der Ratte systematisch verglichen, um somit das translationale Potenzial der Ratte für die Forschung affektiver Störungen im Menschen zu bewerten. Weiterhin wurden neuartige Karten und ein 3D-Atlas des Rattengehirns basierend auf der Verteilung von Neurotransmitter-Rezeptoren entwickelt, die als hochauflösende Referenzen dienen. In einer ersten autoradiographischen Studie wurden die regionalen und laminaren Verteilungsmuster der 5-HT_{1A}- und 5-HT₂-Rezeptoren in Hauptkomponenten des neuronalen Netzwerks der Emotionsregulation zwischen dem menschlichen Gehirn und homologen Regionen des Rattengehirns verglichen. Signifikante Spezies-spezifische Unterschiede zeigten sich vor allem bei den 5-HT_{1A}-Rezeptoren (z. B. im cingulären Kortex und im Hippocampus). Die 5-HT₂-Rezeptoren waren in den meisten Gehirnregionen zwischen Mensch und Ratte ähnlicher verteilt. In einer zweiten Studie wurde die *in vitro* Rezeptorautoradiographie und Zellkörperfärbungen genutzt, um den gesamten Iso- und Proisokortex des Rattengehirns multimodal, mittels Analyse der molekularen Organisation der cholinergen muskarinischen M₂-Rezeptoren und der Zytoarchitektur, zu parzellieren. Die resultierenden Karten zeigen die detaillierte Identifizierung von 53 Regionen die als hochauflösender, zweidimensionaler (2D) Rattengehirnatlas veröffentlicht wurden. Diese 2D-Karten wurden in einer dritten Studie zu einem 3D-Rattengehirnatlas des Iso- und Proisokortex rekonstruiert. Dieser 3D-Atlas sowie die dazugehörigen Daten wurden über die EBRAINS Forschungsinfrastruktur veröffentlicht.

Die autoradiographischen Visualisierungen der 5-HT_{1A}-, 5-HT₂- und M₂-Rezeptoren im Rattengehirn liefern hochauflösende, skalenübergreifende (d. h. entlang des gesamten Gehirnvolumens, spezifischer Regionen und kortikaler Schichten) und multidimensionale (d. h. 2D-Karten und 3D-Rekonstruktion) Referenzen für Interspezies-Vergleiche zum menschlichen Gehirn und die *in vivo* Neurobildgebung. Zudem können die regionalen Rezeptorverteilungen genutzt werden, um in zukünftigen Studien die Bindungsstellen von Medikamenten zu lokalisieren. Unsere Rezeptordaten können durch Integration zusätzlicher Daten, z. B. von glutamatergen und dopaminergen Rezeptoren, sowie Gehirnregionen wie Striatum und Thalamus, erweitert werden. Dies ermöglicht Rezeptor- und Region-spezifische Analysen, die auch für Vergleiche mit krankheitsbedingten Veränderungen, wie z.B. bei affektiven Störungen oder der Alzheimer-Krankheit, nutzbar sind.

List of Abbreviations

2D	two-dimensional
3D	three-dimensional
5-HT	serotonin
5-HT _{1A}	serotonin receptor type 1A
5-HT ₂	serotonin receptor type 2
CA1-CA3	regions 1, 2 and 3 of Cornu Ammonis of the hippocampus
Cb	cerebellum
Cg1'	cingulate cortex, area 1'
Cg2'd	cingulate cortex, area 2', dorsal part
Cg2'v	cingulate cortex, area 2', ventral part
CPu	caudate putamen
DBS	deep brain stimulation
DG	dentate gyrus
DTI	diffusion tensor imaging
Hipp	hippocampus
MRI	magnetic resonance imaging
Par1	primary parietal cortex
Par1BF	parietal cortex, area 1, barrel field representation
Par1DZ	parietal cortex, area 1, dysgranular zone
Par1DZO	parietal cortex, area 1, dysgranular zone, oral part
Par1Sh	parietal cortex, area 1, shoulder representation
Par1ULp	parietal cortex, area 1, upper lip representation
ParFL	parietal cortex, forelimb area
ParHL	parietal cortex, hindlimb area
Oc1B	binocular (nasal) primary occipital area
Oc1M	monocular (temporal) primary occipital area
Oc2Lc	caudolateral secondary occipital cortex
Oc2ML	mediolateral secondary occipital cortex
PET	positron emission tomography
WHS	Waxholm Space atlas of the Sprague Dawley rat brain

Content

1	Introduction	1
1.1	Rat Models for Translational Research of Human Mood Disorders and Comparative Analyses	1
1.2	Analyzing Neurotransmitter Receptor Distributions Across the Brain	3
1.3	Cyto- and Receptor Architecture for Detailed Multimodal Brain Mapping	6
1.4	Three-dimensional Receptor Atlas of the Rat Brain	8
1.5	Digital Infrastructure for Rat Brain Data	11
1.6	Ethical Approval and Animal Approval	13
1.7	Research Studies in this Dissertation	13
	Study 1	16
	Serotonin Receptors in Areas of the Emotion Regulation Network in Human and Rat Brains – A Comparative Autoradiographic Study (Kuckertz et al. 2025, Journal of Comparative Neuology)	16
	Study 2	35
	A New Map of the Rat Isocortex and Proisocortex: Cytoarchitecture and M₂ Receptor Distribution Patterns (Haghir, Kuckertz et al. 2023, Brain Structure and Function)	35
	Identification of the Somatotopic Representations in the Rat Parietal Cortex (Supplementary Results for Haghir, Kuckertz et al. 2023, Brain Structure and Function)	65
	Study 3	67
	3D Rat Brain Atlas Based on the Distribution Patterns of Cell Bodies and Cholinergic Muscarinic M₂ Receptors (Kuckertz et al., in Preparation)	67
2	Discussion	69
2.1	Rats in the Translational Research of Human Mood Disorders	70
2.2	Cholinergic Muscarinic M ₂ Receptor Maps Extent Histological Rat Brain Atlases	73
2.2.1	M ₂ Receptor Architectonic Analyses Provide Functionally Relevant Information	75
2.3	Development and Usage of the 3D Rat Brain Atlas	78

2.3.1	Reconstruction of M ₂ Receptor Maps into a 3D Rat Brain Atlas	81
2.3.2	A Digital and Openly Accessible Atlas for the Rat Brain	83
2.3.3	The 3D Rat Brain Atlas for <i>In Vivo</i> Neuroimaging	85
3	Conclusion and Outlook	92
	References	93

1 Introduction

1.1 Rat Models for Translational Research of Human Mood Disorders and Comparative Analyses

In neuroscience research, the rat is one of the most frequently used and probably the most extensively studied animal models (Keifer & Summers 2016). An update in *The Laboratory Rat* emphasized the high potential of rat models for studying human diseases (Hashway and Wilding, 2020). The authors highlighted the large amount of homologous genes and mutations shared by humans and rats, which are involved in the development of psychiatric disorders such as anxiety and depression (Olivier et al., 2008; Murphy and Moya, 2011; Hashway and Wilding, 2020; Szpirer, 2020). Interestingly, mice are often preferred for genetic modification modelling, rats are primarily used to assess disorder-related behavior and in pharmacological research (Cryan and Holmes, 2005). Furthermore, rats exhibit more sophisticated social behavior and share more functional similarities with humans than mice. Therefore, rat models appear to be more reliable for translational research of human mood disorders (Homberg 2013; Ryan et al. 2019).

The large number of patients suffering from mood disorders, such as major depression and anxiety, and the steady increase in the number of patients over the past decade, highlight the importance for ongoing research. The World Health Organization estimates that 5 % of the global adult population suffers from depression¹, and 4 % are affected by anxiety disorders². Another report by *Deutsches Ärzteblatt* stated that the number of patients in Germany with recurrent depression increased by 67 % between years 2012 and 2022 (Ärzteblatt 2023). This critical increase in patients with mood disorders requires (translational) research to understand the fundamental pathological mechanisms and to develop effective treatments.

Mood disorders, such as depression and anxiety, are strongly associated with dysfunctions in the serotonergic neurotransmission (Pourhamzeh et al. 2022; Albert & Blier 2023). Previous studies have found deficits in the binding of the serotonin (5-HT) transporter (Selvaraj et al. 2011; Bartlett et al. 2022) and alterations in the distribution

¹ <https://www.who.int/news-room/fact-sheets/detail/depression>

² <https://www.who.int/news-room/fact-sheets/detail/anxiety-disorders>

of 5-HT receptors, for example 5-HT_{1A} (Parsey et al. 2006; Savitz et al. 2009; Wang et al. 2016) and 5-HT₂ receptors (Biver et al. 1997; Fisher et al. 2009; Rosell et al. 2010) e.g., in the cingulate cortex and hippocampus in patients with depression and physical aggression disorder. To date, various rat models for human mood disorders, such as depression, have been developed and cover a broad range of investigations, including the involvement of environmental and genetic risk factors, as well as brain lesions and behavioral endpoints (Valvassori et al. 2017; Planchez et al. 2019). For example, the olfactory bulbectomized rat is a classical model of depression (Kelly et al. 1997), which has shown differences in the distribution of 5-HT_{1A} and 5-HT₂ receptors in the prelimbic areas and hippocampus (Watanabe et al. 2006; Sato et al. 2008; Sato et al. 2010).

However, the high variability of the neuropathological mechanisms and symptoms associated with human mood disorders requires ongoing evaluation of translational models (Belzung & Lemoine 2011; Planchez et al. 2019). Recent reviews highlighted the importance of minimizing the translational gap and failure rates between human patients and animal models (Ferreira et al. 2020; Uliana et al. 2022). Identifying similarities and difference between species in comparative analyses can facilitate the translational potential of animal models for human diseases (Hölter et al. 2024). Furthermore, analyzing disease conditions requires the identification of similarities and differences between healthy humans and rats, to enable unbiased interpretation of disorder-related alterations (Ryan et al. 2019; Hashway & Wilding 2020; Novati et al. 2022). Previous comparative studies have revealed similarities and conspicuous differences in the distribution of 5-HT_{1A} receptors in the healthy human and rat brains (Duncan et al. 1992; Duncan et al. 1998), as well as in human and mouse brains (Arnold & Hagg 2012). A study analyzing 5-HT_{2B} receptor binding also found species differences between healthy human and mouse brains (Manivet et al. 2002). However, the number of more recent comparative studies identifying similarities and differences in the serotonergic system between humans and rats remains limited.

In addition to comparative analyses, establishing standardized validation criteria (face, construct, and predictive validity) helps to evaluate the adequacy of animal models for neuropsychiatric disorders (Willner 1984). The continuous validation of both, newly developed and existing models using these criteria is essential (Belzung & Lemoine 2011; Planchez et al. 2019). Face validity aims to evoke similar symptoms in human and rat models, and includes the identification of biological markers that should be comparable between them (Zimmer & Descarries 2012; Planchez et al. 2019).

Construct validity identifies similarities, such as alterations in neuronal processing in the cingulate cortex and the hippocampus, between the patient and the animal model (Belzung & Lemoine 2011; Planchez et al. 2019). For example, alterations in the serotonergic system (e.g., the distribution of 5-HT_{1A} and 5-HT₂ receptors) have been found in the brains of patients with depression (Biver et al. 1997; Sargent et al. 2000; Meltzer et al. 2004; Neumeister et al. 2004; Wang et al. 2016; Gorwood et al. 2018), as well as in olfactory bulbectomized rats (Kelly et al. 1997; Watanabe et al. 2003; Sato et al. 2008). Predictive validity is defined as the extent to which the responsiveness and effects of treatments are comparable between human patients and the animal models (Planchez et al. 2019). Here, for instance, drugs targeting serotonergic receptors (e.g., the 5-HT_{1A} and 5-HT₂ subtypes) are used as classical targets for pharmacological treatments against mood disorders (Riad et al. 2004; Sato et al. 2008; Celada et al. 2013; Kaur Gill et al. 2019).

Comparative studies analyzing healthy human and rat brains under similar experimental and analytical conditions are crucial for providing reference data. Additionally, in translational research on neurodegenerative (e.g., Alzheimer's disease) and mood disorders (e.g., depression), comparative studies investigating the distribution patterns of neurotransmitter receptors, such as cholinergic muscarinic or serotonergic receptors, can provide fundamental data facilitating comparisons between healthy and diseased states (i.e., healthy control vs. patient or animal model) (Zilles et al. 1996; Rodríguez-Puertas et al. 1997; Duncan et al. 1998).

1.2 Analyzing Neurotransmitter Receptor Distributions Across the Brain

Neurotransmitters and their specific receptors are molecular structures that form key systems for the transmission of neuronal signals in the brain. These neurotransmitter receptor systems are essential for maintaining brain function by ensuring the balanced transmission of neuronal signals. Specifically, the activation or inhibition of distinct receptor types is being linked to different responses and behaviors. To date, numerous neurotransmitter receptor types and subtypes have been identified and classified based on their molecular structure, either ionotropic or metabotropic, and their neurochemical signaling properties, either excitatory or inhibitory.

The majority of neurotransmitter receptors are located in the neuropil, particularly on the synapses of neuronal dendrites (Zilles et al. 2004), with each synapse containing a variety of distinct receptor types. Over 20 years ago, *in vitro* receptor autoradiography was established as a technique for analyzing the receptor architecture. The *in vitro* receptor autoradiography visualizes and quantifies regional and laminar receptor distribution patterns across brain regions and cortical layers, respectively, by specifically labeling agonistic or antagonistic receptor binding sites using autoradiographically labeled ligands (Zilles et al. 1999; Zilles & Palomero-Gallagher 2001; Zilles, Schleicher, et al. 2002). To visualize the labeled receptor binding sites, the sections are exposed to radioactively sensitive film sheets, enabling them to be visualized through photographic development. Densitometric analysis of the gray values in the resulting receptor autoradiograms, and co-exposition of standards with known radioactive concentrations, enables the quantification of receptor binding sites as receptor densities in fmol/ mg protein. The resulting high-resolution autoradiograms (5 $\mu\text{m}/\text{pixel}$) allow detailed analysis of receptor architecture between distinct brain regions, as well as across cortical layers (Zilles, Schleicher, et al. 2002; Zilles et al. 2004; Zilles & Palomero-Gallagher 2017b).

In addition to brain mapping regarding the molecular organization, previous studies have shown that receptor architectonic analyses can be used for functional segregation of the brain. For example, it has been demonstrated that brain regions belonging to the same functional system exhibit a characteristic receptor architecture (Zilles, Palomero-Gallagher, et al. 2002; Zilles et al. 2004). Previous studies have described higher densities of cholinergic muscarinic M_2 , noradrenergic α_2 , and serotonergic 5-HT₂ receptors in primary sensory areas, including the primary somatosensory, primary auditory, and primary visual areas. Conversely, lower densities of these receptors have been found in the primary motor cortex (Geyer et al. 1996; Roland & Zilles 1998; Morosan et al. 2005; Zilles & Palomero-Gallagher 2017a). Normal brain function is maintained by a dynamic balance between excitatory and inhibitory signaling. Imbalances, such as those caused by alterations in receptor distributions, are strongly associated with dysfunction and the development of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, and neuropsychiatric disorders such as depression and anxiety. For example, motor impairments in Parkinson's disease mainly result from disturbances in dopaminergic neurotransmission (Brooks 2001; Rowe & Siebner 2012) and alterations in the

distribution of cholinergic muscarinic receptors, particularly the M₂ receptor type, are implicated in Alzheimer's disease (Lai et al. 2001; Lebois et al. 2018). Alterations in the serotonergic system, e.g., 5-HT_{1A} and 5-HT₂ receptor distributions, have been observed in the brains of patients with mood disorders such as depression, anxiety, panic disorder, and schizophrenia (Simpson et al. 1996; Sargent et al. 2000; Neumeister et al. 2004; Hurlmann et al. 2005; Drevets et al. 2007; Rosell et al. 2010; Wang et al. 2016; Bartlett et al. 2022; Pourhamzeh et al. 2022). Here, it is important to note that a wide range of neurotransmitter receptor types are involved in maintaining normal brain function, and that dysfunction is associated with alterations in multiple receptor types rather than a single receptor type. For example, in addition to alterations of 5-HT_{1A} receptor distribution, changes in muscarinic M₂ receptor levels were also found in patients with bipolar disorder and depression (Gibbons et al. 2016; Wang et al. 2016).

It is well known, that neurotransmitter receptors are effective targets in the pharmaceutical treatment of the disorders mentioned above. The serotonergic system, specifically the serotonin transporter and the 5-HT_{1A} and 5-HT_{2A} receptors are effective targets for the treatment of mood disorders (Celada et al. 2004; Roth et al. 2004; Ślifirski et al. 2021; Pourhamzeh et al. 2022). Also, muscarinic M₂ receptors have been used for the treatment of a variety of disorders, including Alzheimer's disease and schizophrenia (Haga et al. 2012; Kruse et al. 2013). Visualization of receptor binding sites using *in vitro* autoradiography localize the preferred (agonistic or antagonistic) binding sites of pharmaceutical drugs in specific brain regions. These fundamental information can serve as essential references for the development of agonistic or antagonistic drugs (Sato et al. 2008). Furthermore, receptor architectonic analyses, i.e., the distribution patterns of neurotransmitter receptors in the healthy brain, provide (quantifiable) reference data for comparison to disorder-related alterations in humans (Meltzer et al. 2004; Rosell et al. 2010) and in translational research using animal models (Watanabe et al. 2003; Benekareddy et al. 2010).

1.3 Cyto- and Receptor Architecture for Detailed Multimodal Brain Mapping

Over 100 years ago, Korbinian Brodmann pioneered the parcellation of the human cerebral cortex into distinct areas based on its heterogeneous neuroanatomical structure, known as cytoarchitecture (Brodmann 1909). Cytoarchitecture describes the organization, density, and shape of neuronal cell bodies. Differences of the cytoarchitecture can be used to identify distinct brain regions. During that time, the German neuroscientists Cécile and Oskar Vogt introduced the analysis of the organization of myelinated neuronal fibers across the brain, known as myeloarchitecture (Vogt & Vogt 1919). By combining these two methods, the Vogts expanded Brodmann's cytoarchitectonic maps by identifying more than 150 distinct brain regions using the myeloarchitecture. Thus, they showed that a multimodal approach (i.e., combining cyto- and myeloarchitecture) enables more detailed brain parcellation, identifying regions that could not always be distinguished by one technique alone.

Cécile and Oskar Vogt were also interested in how structural differences might relate to brain function. To investigate these structure-function relationships, they performed electrophysiological studies stimulating different neuroanatomical regions in the brain of the guenon monkey. They observed that stimulation of different neuroanatomical regions of the brain responded to movements of different body parts, such as the head, arms, and legs (Vogt & Vogt 1926). These experiments showed that neuroanatomically different brain regions differ also in the processed function, indicating functional specialization.

Since the early 2000s, novel approaches that combine analysis of brain structure with functional data have been developed. One well-established multimodal approach is to combine of cytoarchitectonic analysis with the distribution patterns of neurotransmitter receptors (receptor architecture) in postmortem brain sections (Zilles, Palomero-Gallagher, et al. 2002; Zilles & Amunts 2009). This multimodality is achieved through different laboratory experiments, computational analyses and the analysis of different neuronal components. Specifically, the majority of neurotransmitter receptors are located in the neuropil, particularly on the synapses of neuronal dendrites, and to a lesser extent on cell bodies (Zilles et al. 2004). Synapses and dendrites contain a broad range of various receptor types. Thus, combining cytoarchitecture and (multi)receptor

architecture provides information about brain structure and molecular organization (Zilles, Schleicher, et al. 2002).

To date, analyses of the architecture of several receptor types have been used to validate cytoarchitectonic borders, and this multimodal brain mapping strategy has contributed to the ongoing development of brain atlases (Rapan et al. 2022; Niu et al. 2024). For example, a study mapping the human hippocampus showed that borders identified by analyzing multiple receptor types were found at the same position as cytoarchitectonic borders (Palomero-Gallagher et al. 2020). A recent review describes that this correlation of cytoarchitecture and receptor architecture helps to understand the relevance of basic neuroanatomy to specific brain function and connectivity (Zachlod et al. 2023).

Cytoarchitecture added by receptor architectonic analyses provide functionally related information, i.e., the distribution of inhibitory and excitatory neurotransmitter receptors in single brain regions and in more complex regional networks. Specifically, it has been found that areas belonging to a functional network, such as the primary somatosensory, visual and auditory areas, have similar receptor distributions, which differ to those of areas belonging to distinct networks, e.g., the primary motor area (Zilles, Palomero-Gallagher, et al. 2002).

In addition to the regional receptor architecture, analyzing laminar receptor distribution patterns enables identification of cortical layers and comparable or different receptor architectures between them. Generally, the six cortical layers, named I to VI (from the pial surface to the white matter) have different responsibilities for receiving and transmitting distinct types of synaptic signals. Specifically, layers I-III (the supragranular or outer layers) are defined as the main input layers, and layers V-VI (the infragranular or deeper layers) are the main output layers. Layer IV is known to be the main thalamocortical input layer (Alloway 2007; Rockland 2019). A mapping study of Broca's region described that distinct receptor lamination patterns helped to detailly identify regional borders. For example, heterogenous laminar distributions of muscarinic M₂ receptors often indicate borders in the inferior frontal gyrus (Amunts et al. 2010). Furthermore, M₂ receptors have been described as a evolutionary conserved marker for primary somatosensory, visual and auditory areas in rodent, monkey and human brains, and a different distribution pattern was found in the motor cortex (Zilles & Palomero-Gallagher 2017a). Receptor architectonic analyses provide information about the molecular organization of the brain on a two-dimensional (2D) level.

However, three-dimensional (3D) neuroimaging techniques, such as positron emission tomography (PET) and Magnetic Resonance Imaging (MRI), require 3D, high-resolution brain atlases as references to represent neurotransmitter receptor distributions throughout the entire brain volume, and more specifically, along the extent of single regions. Such references can be provided by 3D reconstructing the receptor autoradiograms of postmortem brain sections obtained by *in vitro* autoradiography.

1.4 Three-dimensional Receptor Atlas of the Rat Brain

Rodents, especially rats, are the most commonly used animal models in neuroscience (Keifer & Summers 2016), and play a key role in translational research of human disorders and the development of therapies (Ryan et al. 2019; Novati et al. 2022). In addition to 2D brain mapping, 3D rat brain atlases are essential references for *in vivo* analyses. Neuroimaging techniques for *in vivo* studies, such as MRI (Leergaard et al. 2003; Papp et al. 2014; Kleven et al. 2023), diffusion tensor imaging (DTI) (Rumple et al. 2013; Kleven et al. 2023), and PET (Hayakawa et al. 2000; Stortz et al. 2018; Beliveau et al. 2020), require 3D, high-resolution rat brain atlases to accurately identify also smaller brain regions. The Waxholm Space (WHS) atlas of the Sprague Dawley rat brain is a 3D MRI/DTI-based atlas mapping 222 structures, which include mapping of 36 areas of the cerebral cortex (Papp et al. 2014; Kleven et al. 2023). Furthermore, atlas-based tools for segmenting and extracting rat PET data have been developed (Rubins et al. 2003; Schiffer et al. 2006; Lancelot et al. 2014). However, the relatively low spatial resolution of 1-2 mm/pixel in MRI, DTI and PET neuroimaging techniques limits precise identification of smaller subregions such as those within the cerebral cortex and subcortical structures, such as the amygdala and hypothalamus (Prieto et al. 2011; Kleven et al. 2023).

As an equivalent technique to *in vitro* receptor autoradiography, *in vivo* PET visualizes neurotransmitter receptor systems in the living organism, specifically, to capture their real-time responses and behavioral changes following stimulation and/ or drug administration (Meltzer et al. 2004; Riad et al. 2004; Schneck et al. 2021). *In vitro* receptor autoradiography analyses receptor binding sites in postmortem brain sections. Both techniques are used to visualize the localization of neurotransmitter receptor systems across the brain including the identification of regional differences

(Svarer et al. 2005; Bieszczad et al. 2012; Beliveau et al. 2017; Zilles & Amunts 2018; Palomero-Gallagher et al. 2020). Furthermore, PET and receptor autoradiography can be used to detect alterations in the receptor distribution related to disorders. For example, changes in receptor distribution were found in patients with neurological disorders (Mayberg et al. 1999; Toczek et al. 2003; Gibbons et al. 2016; Wang et al. 2016), as well as in animal models (Pellegrino et al. 2007; Döbrössy et al. 2012; Saigal et al. 2013; Vetel et al. 2020).

In vivo PET and *in vitro* receptor autoradiography are used to visualize neurotransmitter receptor binding sites through labeling with specific radioactive ligands (Bieszczad et al. 2012; Ravasi et al. 2012; Savli et al. 2012; Beliveau et al. 2020). However, some special considerations are required when comparing the results of these two techniques. It is important to note that different ligands can be used for PET and autoradiography to visualize the same receptor type. For example, FP-TZTP and Oxotremorine-M have been used to visualize the agonistic binding sites of muscarinic cholinergic M₂ receptors in PET and *in vitro* autoradiography, respectively (Zilles & Amunts 2009; Ravasi et al. 2012; Palomero-Gallagher & Zilles 2015). As classical markers for 5-HT_{1A} receptors, WAY 100635 is being used in PET and 8-OH-DPAT in *in vitro* autoradiography (Mathis et al. 1994; Simpson et al. 1996). However, WAY 100635 binds to the antagonistic binding site of the 5-HT_{1A} receptor and 8-OH-DPAT marks the agonistic binding site. This difference needs to be considered when comparing the results of *in vitro* receptor autoradiography with *in vivo* PET imaging, including in functional or pharmacological studies e.g., analyzing the neurochemical activation or suppression through the application of agonistic or antagonistic drugs.

Furthermore, there is a difference in the resolution of images obtained by *in vivo* PET and *in vitro* receptor autoradiography. Specifically, the spatial resolution of *in vivo* PET images is 1-2 mm/pixel, which is lower than the in-plane resolution of 5 µm/pixel within *in vitro* receptor autoradiograms. This limits the ability to accurately localize receptor binding, especially, the identification of smaller subregions, cortical layers and subcortical structures, such as those of the thalamus or amygdala (Sung et al. 2009; Moses 2011; Beliveau et al. 2017). In contrast, the higher resolution of *in vitro* receptor autoradiograms enables more detailed regional and laminar brain mapping (Prieto et al. 2011; Palomero-Gallagher & Zilles 2018), but they cannot capture the time domain.

To overcome the limitations of lower resolution of *in vivo* PET, and while maintaining the advantages of 3D neuroimaging, a computational pipeline was developed to reconstruct high-resolution receptor autoradiograms (obtained by *in vitro* autoradiography) and parcellation schemes (Funck et al. 2022; Funck et al. 2025). Registering the reconstructed 3D brain volumes in a standardized reference space, such as the WHS (Papp et al. 2014), enables precise identification of brain regions and their orientation in a common space. Nonetheless, a detailed, high-resolution, 3D rat brain atlas based on receptor architecture is still lacking. Such an atlas would serve as a reference for improving the identification of brain regions using *in vivo* neuroimaging methods, including MRI, PET and deep brain stimulation (DBS).

DBS is widely known as an effective *in vivo* treatment for various neurodegenerative and neuropsychiatric disorders (Wichmann & DeLong 2006). DBS of the subgenual cingulate area has been shown to have antidepressant and anti-anhedonic effects in patients with Alzheimer's disease (Mayberg et al. 2005) and depression (Johansen-Berg et al. 2008; Lozano et al. 2008). Despite these antidepressant effects, recent studies have reported that the efficiency of DBS is critically dependent on precise electrode placement (Treu et al. 2020; Andree et al. 2022). A less accurate electrode placement within the anatomical target region impairs the clinical outcome (Welter et al. 2014; Horn 2019).

Translational models are being used to investigate and improve the clinical outcomes of DBS. In unpredictably chronically stressed rats (a model of human depression), DBS of the infralimbic area, considered to be the rodent homolog of human subgenual cingulate area, showed antidepressant effects (Hamani et al. 2012; Lim et al. 2015; Jimenez-Sanchez et al. 2016). Studies in rats mostly verify electrode placement on 2D histological brain atlases (i.e., postmortem brain sections), such as those by Paxinos and Watson, rather than on 3D rat brain atlases (Hamani et al. 2012; Lim et al. 2015; Jimenez-Sanchez et al. 2016). However, DBS is an *in vivo* technique and therefore, the spatial orientation of brain regions cannot be taken into account when using 2D brain atlases for electrode placement within the brain volume (Andree et al. 2022).

1.5 Digital Infrastructure for Rat Brain Data

Digital infrastructures for brain mapping and atlases have been developed to collect, organize and visualize multimodal neuroimaging data within a common framework. Such infrastructures are essential for improving data sharing and analysis across the neuroscience community which includes diverse laboratories and methods (Mars & Palomero-Gallagher 2023). Over ten years ago, an atlas format for the storage of 2D brain delineations and a 3D brain atlas reconstructor has been developed. The reconstructor software was developed to create 3D volumes of brain structures based on 2D atlas data. However, the whole atlas format and reconstructor software are no longer available today (Majka et al. 2012).

EBRAINS is a leading, openly available European digital research infrastructure and has been developed as part of the Human Brain Project and is now undergoing further development³. The EBRAINS research infrastructure provides multivariate neuroimaging data from the human, monkey and rodent (i.e., mouse and rat) brains. The EBRAINS Knowledge Graph enables metadata to be stored and downloaded for interdisciplinary brain research⁴. EBRAINS also covers the Siibra software tool suite for visualizing and analyzing multiscale neuroimaging data obtained from various modalities, such as MRI, cytoarchitecture, fiber architecture and receptor architecture, as well as across multiple species (Amunts et al. 2022; Dickscheid et al. 2025).

Rodent brain atlases serve as neuroanatomical references with a broad range of applications, including classical brain mapping and *in vivo* studies such as PET (Kim et al. 2022; He et al. 2024) and DBS (Hamani et al. 2012; Lim et al. 2015; Jimenez-Sanchez et al. 2016; López-Terrones et al. 2022). As previously mentioned, many recent *in vivo* studies have used 2D rat brain atlases to identify regions of interest. To overcome dimensional differences between 2D (i.e., in-plane resolution) and 3D (i.e., spatial resolution) atlases, high-resolution, 3D atlases are necessary to better represent the orientation and extent of regions across the brain volume. A group from Oslo (developer of the Waxholm Space Rat Brain Atlas) published digital rodent brain atlases and tools for integrating 2D and 3D imaging data (Hjornevik et al. 2007; Papp et al. 2018; Blixhavn et al. 2023; Kleven et al. 2023; Carey et al. 2025). For example,

³ <https://www.ebrains.eu/>

⁴ <https://search.kg.ebrains.eu/>

they developed a 3D atlas template based on the reconstruction of 2D histological atlas data from Paxinos and Watson (Paxinos & Watson 2006) and made this template available as an overlay for MRI and PET data (Hjornevik et al. 2007). Furthermore, the Schiffer rat brain atlas, is an MRI/PET-based 3D reference atlas that is used in PET studies (Schiffer et al. 2006; Kim et al. 2022; He et al. 2024). However, due to its spatial resolution of 1-2 mm/pixel, this atlas cannot be used for the identification of smaller brain regions, such as those of the frontal cortex or hippocampus (Sung et al. 2009; Kim et al. 2022; He et al. 2024). Additionally, the identification of cortical layers is limited by the lower resolution in PET and MRI neuroimaging.

Reconstructing high-resolution, 2D receptor autoradiograms obtained from rat brain sections into a 3D atlas template based the distribution of specific neurotransmitters throughout the brain, would provide a type of rat brain atlas that has not yet been fully developed. Furthermore, these 3D reconstructed neurotransmitter receptor (e.g., cholinergic muscarinic receptors) maps could serve as a valuable reference for PET analysis of similar receptor types (including the corresponding agonistic or antagonistic receptor binding sites) across the brain volume. Integrating such a receptor-based 3D atlas into the user-friendly Siibra tool would provide a novel resource for PET studies investigating the regional and, due to the high resolution of the atlas, also the laminar distribution patterns of neurotransmitter receptor systems.

Furthermore, a 3D receptor atlas of the rat brain could be used as a reference in translational research, for example to investigate altered neurotransmitter receptor distributions. Specifically, localizing dysfunctional neurotransmission within specific brain regions in disease models. The regional receptor densities can be extracted using *in vitro* receptor autoradiography to provide quantitative values as reference data in statistical analyses and comparisons with altered and pathological receptor distributions (Palomero-Gallagher et al. 2008; Rebholz et al. 2018). To date, quantitative receptor data for 30 human brain regions are available via the EBRAINS infrastructure and is integrated in the Siibra tool (Zilles et al. 2021; Ruland et al. 2022; Froudust-Walsh et al. 2023; Zhao et al. 2023).

A recent article emphasized that developments in simulation, artificial intelligence and computation are becoming increasingly important in brain research and medicine in the upcoming digital century (Amunts et al. 2024). Computational modelling of brain information processing includes analysis of complex datasets ranging from the molecular and cellular levels to whole brain structures, as well as integrated the

relationship between brain function and behavior (Dalla Porta et al. 2023; Amunts et al. 2024). Acetylcholine, for instance, is involved in attention and memory function and computational modelling of cholinergic neurotransmission (via cholinergic receptors) can provide information ranging from cellular firing and synaptic plasticity to large-scale behavioral outcomes (Yun et al. 2000; Newman et al. 2012; Hassan et al. 2018). Computational models have also been used to analyze and understand disorder-related alterations of neuronal signal transmission. Modelling dopaminergic processing in Parkinson's disease and schizophrenia, and the dysregulations of glutamatergic and serotonergic neurotransmission in depression and Alzheimer's disease (Rolls et al. 2008; Ramirez-Mahaluf et al. 2017; Joshi et al. 2020; Wiencke et al. 2020; Gandolfi et al. 2021). As neurotransmitter receptors are known to be effective targets for pharmacological treatments, understanding their distribution patterns and developing receptor-based computational models may help to predict and improve molecular interactions between drugs and receptor targets (Kumar et al. 2006; Li et al. 2010; Hassan et al. 2018; Joshi et al. 2020; Amunts et al. 2024).

1.6 Ethical Approval and Animal Approval

Human brains were obtained from the body donor program at the Department of Anatomy I, Medical Faculty, Heinrich Heine University Düsseldorf, Germany. All procedures were conducted in accordance with the ethical guidelines and were approved under study numbers 2023-2632 by the Ethics Committee of the Medical Faculty of Heinrich Heine University Düsseldorf. All donors had provided written informed consent in accordance with the Declaration of Helsinki.

All animal procedures were performed in accordance with the institutional animal welfare guidelines at the Research Centre Jülich (Germany), and followed the European Union guidelines (in line with NIH standards) for the use and care of laboratory animals. For our studies, brains were obtained from wild-type donor rats that had not been included in any experimental procedures.

1.7 Research Studies in this Dissertation

Multimodal cytoarchitectonic and receptor architectonic analyses enable precise brain mapping at structural and functional levels. Specifically, high-resolution autoradiographic images represent the distribution of neurotransmitter receptors and

provide fundamental information about the molecular organization associated with brain function, which further can be used for comparisons to disorder-related alterations. The rat is one of the most widely used animal models in neuroscience and translational research. Rat brain atlases are used as references to identify brain regions and alterations in disease models. However, the resolution of existing 3D rat brain atlases is too low to enable the precise identification of smaller regions in rodent *in vivo* neuroimaging. High-resolution, 3D atlases need to be developed to overcome this limitation. 3D reconstruction of receptor autoradiographic images offers a novel, high-resolution rat brain atlas and especially, for identifying specific brain regions and cortical layers throughout the entire brain volume.

Alterations in the distribution patterns of neurotransmitter receptors, e.g., muscarinic cholinergic M₂, serotonergic 5-HT_{1A} and 5-HT receptors, are often associated with neurogenerative and mood disorders, such as Alzheimer's disease and depression. The first study aimed to evaluate the use of the rat as a translational model for human mood disorders by systematically comparing the distribution patterns of 5-HT_{1A} and 5-HT receptors in key components of the human and rat emotion regulation network. Alternating sections of human and rat brains were used for cell body staining and *in vitro* autoradiographic visualization of 5-HT_{1A} and 5-HT₂ receptors. Specifically, similarities and differences in the distribution patterns of serotonergic 5-HT_{1A} and 5-HT₂ receptors in homolog components (i.e., the lateral prefrontal, the orbitofrontal, the anterior and midcingulate cortex, the hippocampal Cornu Ammonis regions 1-3 [CA1-3], the dentate gyrus [DG], and the central amygdaloid, accumbens, mediodorsal thalamic nuclei) of the emotion regulation network were determined and statistically evaluated. This comparative study aimed to assess the translational potential of the rat serotonergic system for comparisons with that of humans, and further to visualize the pharmacological (agonistic and antagonistic) drug binding sites of 5-HT_{1A} and 5-HT₂ receptors.

Cholinergic muscarinic M₂ receptors have been implicated in neurodegenerative disorders such as Alzheimer's disease. The second study aimed to analyze regional and laminar distribution patterns of M₂ receptors across the rat brain, and to develop novel maps of the isocortex and proisocortex. Alternating sections were used for *in vitro* autoradiography to visualize M₂ receptors and for cell body staining for

cytoarchitectonic analyses. The high-resolution images obtained of both M₂ receptors and cytoarchitecture were then used for the multimodal parcellation of the entire rat iso- and proisocortex. These maps were made available as a rat brain atlas, and furthermore, the quantitative M₂ receptor densities (fmol/ mg protein) were extracted for each parcellated area, which can be used for e.g., statistical analyses.

The third study aimed to describe the development of a novel, high-resolution, 3D rat brain atlas based on the distribution patterns of cholinergic muscarinic M₂ receptors (manuscript in preparation). A computational pipeline called BrainBuilder (Funck et al. 2025) was used to reconstruct the M₂ receptor autoradiograms and the isocortical and proisocortical maps from Study 2 into a 3D M₂ receptor volume and a 3D rat brain atlas, respectively. The resulting 3D volumes were then registered in the WHS coordinate system to enable analysis in a common reference space. To date, the 3D atlas and corresponding data, such as M₂ receptor densities (Kuckertz et al. 2024), the M₂ receptor volume (Kuckertz, Palomero-Gallagher, & Funck 2025), and the 3D atlas (Kuckertz, Haghiri, et al. 2025a) have been published via the EBRAINS research infrastructure. The 3D reconstruction of the cytoarchitectonic images to enable analysis of cell body distribution across the rat brain volume is in preparation.

Taken together, the three studies provide comprehensive information about the molecular organization of the rat brain and serve as important references for various analyses. Specifically, the species-specific distribution patterns of 5-HT_{1A} and 5-HT₂ receptors in components of the emotion regulation between healthy human and rat brains, determined in Study 1, help to evaluate the translational potential of the rat serotonergic system as model for human mood disorders. The provided reference data also enable comparisons to alterations in rat models and patients with mood disorders, and further the characterization of the pharmacological profiles of drugs targeting 5-HT_{1A} and 5-HT₂ receptors. The openly available digital 2D maps and 3D atlas of the rat brain developed in Studies 2 and 3, respectively, provide high-resolution reference resources for a broad range of applications, including 2D rat brain mapping, *in vivo* neuroimaging, such as PET, and comparisons with altered M₂ receptor distributions in e.g., Alzheimer's disease.

Study 1

Serotonin Receptors in Areas of the Emotion Regulation Network in Human and Rat Brains – A Comparative Autoradiographic Study

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The study was initiated and supervised by Prof. Nicola Palomero-Gallagher. My (Ms. Anika Kuckertz) contributions included performing the laboratory experiments, covering rat brain preparation, sectioning, histological cell body staining, and *in vitro* receptor autoradiography. I also performed the computational postprocessing of section digitalization and densitometric analyses of the receptor autoradiograms, as well as data preparation and analysis. The latter included the parcellation of the investigated brain regions and the extraction of receptor densities. The human receptor data were obtained from previous analyses performed by Prof. Palomero-Gallagher, while the receptor data of the human amygdala were provided by Dr. Olga Kedo. I prepared the figures and tables and wrote the manuscript, which was reviewed by all co-authors, with special contributions of Prof. Katrin Amunts and Prof. Palomero-Gallagher. The statistical analyses were performed in collaboration with Dr. Ling Zhao.

RESEARCH ARTICLE OPEN ACCESS

Serotonin Receptors in Areas of the Emotion Regulation Network in Human and Rat Brains—A Comparative Autoradiographic Study

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Keywords: emotion network | human | rat | receptor autoradiography | serotonin receptors | translational research

ABSTRACT

Serotonergic neurotransmission is crucial for emotion processing and is dysregulated in mood disorders. To analyze the pathophysiology of disease and develop effective pharmacological treatments, the suitability of the rat as a model for translational research must be continuously validated. In vitro receptor autoradiography was used to characterize (dis)similarities of regional and laminar serotonergic 5-HT_{1A} and 5-HT₂ receptor distributions between components of the human emotion regulation network and homologous rat areas, including areas of the lateral prefrontal, orbitofrontal anterior and midcingulate cortices, hippocampal cornu Ammonis (CA) and dentate gyrus (DG), and the accumbens, central amygdaloid, and mediodorsal thalamic nuclei. In both species, mean 5-HT_{1A} densities were highest in cingulate area 25/infralimbic cortex and the hippocampus, and lowest in the accumbens. Whereas human CA presented significantly higher 5-HT_{1A} density than DG, the opposite was found in rats. Across the cortical depth, in humans, layers I–III and V contained the highest and lowest 5-HT_{1A} densities, respectively. In rats, layers I–II contained the lowest and layers V–VI the highest 5-HT_{1A} values. Mean 5-HT₂ densities were lower than 5-HT_{1A} densities in all areas of both species, whereby layers III and VI contained the highest and lowest 5-HT₂ densities, respectively. Rats presented a more widespread range of significant differences concerning the ratio between 5-HT_{1A} and 5-HT₂ receptors across examined areas than did humans. Concluding, this comparative study reveals species differences in 5-HT_{1A} and 5-HT₂ receptor densities in components of the emotion regulation network, which should be considered when using the rat as a model in the translational research of mood disorders.

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1 | Introduction

Serotonin is involved in the modulation of physiological and behavioral processes such as blood pressure, food intake, and the regulation of emotion and mood (Kohn et al. 2014; Lesch and Waider 2012). The emotion regulation network has been defined on the basis of functional imaging studies and encompasses areas involved in managing and controlling emotional responses. That is, it regulates which emotions arise and when this happens, how long they last, and how these emotions are experienced and expressed (for reviews, see Gross 2014; Morawetz et al. 2020; Palomero-Gallagher and Amunts 2022; Underwood et al. 2021). The emotion regulation system covers a complex brain network composed of cortical and subcortical structures. Key areas in this network are prefrontal areas 9 and 10, orbitofrontal areas 11 and 47, and cingulate areas 25, 24, 24', and 32, as well as the hippocampus (Gilbert et al. 2010; Kohn et al. 2014; Palomero-Gallagher and Amunts 2022; Rolls 2004, 2019; Stevens et al. 2011; Vogt 2005; Wolf et al. 2018) (Figure 1). Subcortical structures most frequently associated with the control of emotions include the accumbens (Acb), central amygdaloid (Ce), and mediodorsal thalamic (MDT) nuclei.

Serotonin, one of the phylogenetically oldest neurotransmitters, exerts its effects via activation of both ionotropic and metabotropic receptors (Pourhamzeh et al. 2022; Żmudzka et al. 2018). There are six families of metabotropic receptors (5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆, 5-HT₇), which encompass additional subtypes and, depending on the second messenger systems to which they are coupled, have an excitatory or inhibitory effect. The 5-HT₃ receptor is a ligand-gated Na⁺ and K⁺ ion channel and is thus excitatory in nature. Dysfunctions in serotonergic neurotransmission have been associated with the pathophysiology of mood disorders (Albert and Blier 2023; Asan et al. 2013; Drevets et al. 2007; Hedlund 2009; Pourhamzeh et al. 2022; Rebholz et al. 2018; Tauscher et al. 2001; Walstab et al. 2010; Wang et al. 2016). Neuroimaging studies described altered distribution patterns of 5-HT_{1A} and 5-HT₂ receptors in areas of the emotion regulation network in patients (Arora and Meltzer 1991; Biver et al. 1997; Drevets et al. 1999; Schneek et al. 2021; Wang et al. 2016) and in rat models of depression (Topic et al. 2007; Watanabe et al. 2003; Zaniewska et al. 2010). Hippocampal 5-HT_{1A} receptor distribution patterns are also altered in patients with anxiety (Drevets et al. 1999; Mayberg et al. 1999). Although there is strong evidence from preclinical rodent models of mood disorders that 5-HT₃ and 5-HT₄ receptors are involved in depression and anxiety, studies in humans remain inconclusive (Bétry et al. 2011; Gupta et al. 2016; Rebholz et al. 2018; Walstab et al. 2010). Preclinical studies on the role of 5-HT₃ receptors in depression have been hampered by the lack of selective ligands, and results concerning the 5-HT₆ and 5-HT₇ receptors remain inconclusive, since both their activation and blockade have been associated with antidepressant effects (Hedlund 2009; Jastrzębska-Więsek et al. 2015; Wesolowska and Nikiforuk 2007; Żmudzka et al. 2018).

Thus, pharmacological treatment of mood disorders mainly targets serotonergic receptors with a predominance of the 5-HT_{1A} and 5-HT₂ subtypes, because antidepressant effects, and thus clinical relevance, of other subtypes are not yet fully understood (Artigas et al. 1996; Hedlund 2009; Kaur Gill et al. 2019; Meyer

et al. 1999; Pourhamzeh et al. 2022; Roth et al. 2004; Sargent et al. 2000; Walstab et al. 2010).

The success rate of translational neuroscience lies below 1% and thus requires continuous validation of translational models (Austin 2021; Keifer and Summers 2016). Although mice are preferred as genetic models, the rat remains one of the most widely used species for pharmaceutical research (Cryan and Holmes 2005). In this framework, comparative studies are important to establish whether humans and rats present comparable serotonergic innervation and receptor distribution patterns in regions known to be involved in the neuropathology of mood disorders.

Serotonergic innervation patterns have been extensively studied in rodents (Awasthi et al. 2021; Bonn et al. 2013; Cropper et al. 1984; Lidov et al. 1980; Linley et al. 2013; Maddaloni et al. 2017; Moore and Halaris 1975; Oleskevich and Descarries 1990), less in macaques (Berger et al. 1988; O'Rourke and Fudge 2006; Raghanti et al. 2008), and only relatively sparsely in humans (Lew et al. 2019; Raghanti et al. 2008). Although only a few of these studies were comparative in nature, the combined information they provide highlights the existence of regional and laminar differences in the density of serotonergic terminals between rodent, nonhuman primate, and human brains. Higher 5-HT_{1A} receptor densities were found in the supragranular than in the infragranular layers of humans, and the opposite was described for rats. Furthermore, in humans, hippocampal 5-HT_{1A} densities (Duncan et al. 1998; Hoyer et al. 1986a; Pazos and Palacios 1985; Pazos et al. 1987a) and serotonergic innervation (Moore and Halaris 1975; Oleskevich and Descarries 1990; Wilson and Molliver 1991) were higher in CA than DG, and the contrary holds true in rats. In contrast, laminar 5-HT₂ receptor distribution patterns are comparable in rats (Pazos et al. 1985; Pompeiano et al. 1994) and humans (Burnet et al. 1995; Hoyer et al. 1986b; Palomero-Gallagher et al. 2013; Pazos et al. 1987b). Furthermore, they did not necessarily cover all areas of the emotion regulation network, and more importantly, they used diverse methods and compounds for the analyses of receptor distribution patterns. Thus, the adequacy of the rat as a model for diseases associated with serotonergic neurotransmission remains a question of interest for translational neuroscience (Valvassori et al. 2013).

To fill this gap, we applied quantitative *in vitro* receptor autoradiography to characterize the regional and laminar distribution patterns of 5-HT_{1A} and 5-HT₂ receptors in key components of the human emotion regulation network (areas 11, 47, 25, 24a, 24b, 24a', 24b', and 32, the cornu Ammonis [CA] and dentate gyrus [DG] regions of the hippocampus, as well as the Acb, Ce, and MDT) and compared them with homologous areas of the rat brain (i.e., MO, LO, IL, Cg2d, Cg1, Cg2'd, Cg1', Cg3, CA, DG, Acb, Ce, and MDT, respectively). We aimed to determine whether the pattern of significant differences between homologous areas in the human and rat brain was the same for 5-HT_{1A} and 5-HT₂ receptors. Furthermore, given that each brain area is characterized by a specific receptor balance subserving its function (Zilles et al. 2002), we also aimed to determine whether the ratio between 5-HT_{1A} and 5-HT₂ receptors across these areas was the same in human and rat brains. We provide a quantitative characterization of (dis)similarities in the serotonergic system of

humans and rats, enabling assessment of the adequacy of the rat as a model for translational research on disorders pertaining to this neurotransmitter in humans.

2 | Materials and Methods

2.1 | Subjects and Tissue Processing

Human receptor data were taken from previously published studies (Kedo et al. 2018; Palomero-Gallagher et al. 2015, 2020, 2008; Palomero-Gallagher and Zilles 2009, 2019; Zilles and Palomero-Gallagher 2017) and included six human brains (three males, three females; 61–79 years of age) obtained from the body donor program of the Department of Anatomy of the University of Düsseldorf, Germany, in compliance with their Ethics Committee. Donors had no history of neurological or psychiatric disorders or long-term drug treatment.

Three adult male Wistar rats were anesthetized by isoflurane inhalation and decapitated, and then, their brains were extracted. Animal procedures were performed in accordance with the institutional animal welfare committee at the Research Centre Jülich, Germany (RRID:SCR_023416) and the European Union (National Institutes of Health) guidelines for the use and care of laboratory animals.

Brains were shock-frozen in isopentane (-50°C) and stored at -80°C until further processing. Two of the brains were serially sectioned coronally and the third in the sagittal plane (thickness 20 μm) using a cryostat microtome (Leica Microsystems, Germany, RRID:SCR_008960), and the sections were thaw-mounted on glass slides. Neighboring sections were used for the visualization of cell bodies (Merker 1983; Palomero-Gallagher et al. 2008) and receptors.

2.2 | Receptor Autoradiography

Binding experiments for 5-HT_{1A} and 5-HT₂ receptor visualization were the same as those performed for human tissue and followed previously published protocols (Palomero-Gallagher and Zilles 2018).

2.2.1 | 5-HT_{1A} receptors

Sections were preincubated for 30 min at 22°C in 170 mM Tris-HCl buffer (pH 7.7) for rehydration and removal of endogenous substances. The main incubation was performed in 170 mM Tris-HCl buffer (pH 7.7) plus 0.01 % ascorbic acid and 4 mM CaCl₂ (monohydrate) for 60 min at 22°C . It comprised parallel incubations with adjacent sections for the visualization of total and nonspecific binding sites. Total 5-HT_{1A} receptor binding was determined by adding [³H]8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) (BIOTREND Chemikalien GmbH, Germany, RRID:SCR_012423) (humans: 1 nM, K_D : 0.5 nM; rats: 0.3 nM, K_D : 0.3 nM) to the buffer. Nonspecific binding was determined by adding [³H]8-OH-DPAT (0.3 nM) plus serotonin (1 μM) as a displacer to the main incubation buffer. Finally, sections were rinsed in 170 mM Tris-HCl buffer (pH 7.7) for 5 min at 4°C and dipped three times into distilled

water for 2 s each at 4°C to stop the binding process and eliminate unbound ligand. [³H]8-OH-DPAT is a highly selective agonist that labels both, the presynaptically located autoreceptors and the postsynaptic binding sites of the 5-HT_{1A} receptor (De Vry et al. 2004; Mineur et al. 2015; Pazos et al. 1987a; Sotelo et al. 1990). Additionally, [³H]8-OH-DPAT has an agonistic effect on the 5-HT₇ receptor, though with a much lower potency than at the 5-HT_{1A} receptor (Hedlund et al. 2004; Lovenberg et al. 1993; Ruat et al. 1993).

2.2.2 | 5-HT₂ receptors

The 170 mM Tris-HCl buffer (pH 7.7) was used for all experimental steps. Sections were preincubated for 30 min at 22°C . The main incubation was performed for 120 min at 22° . Total binding was determined using [³H]ketanserin (BIOTREND Chemikalien GmbH, Germany, RRID:SCR_012423) (1.14 nM, K_D : 1.14 nM), and for the nonspecific binding experiment, the displacer mianserin (10 μM) was also included in the main incubation buffer. Rinsing was performed at 4°C for two 10-min periods, followed by three dips in distilled water at 4°C for 2 s each. [³H]Ketanserin is a prototypical 5-HT₂ receptor antagonist, mainly to the 5-HT_{2A} subtype, and it also recognizes the 5-HT_{2C} subtype (Sharpley et al. 1994) and the 5-HT_{1C} receptor (Herndon et al. 1992), though with lower affinities. Additionally, ketanserin displays weak adrenergic α_1 receptor blocking properties, whereby it reduces the central sympathetic tone and thus exerts a vasodilatory effect (Biegon et al. 1986; Israilova et al. 2002; Pazos et al. 1985, 1987b).

Specific binding was calculated by the difference between total and nonspecific binding and amounted to 95% for both receptor types. Radioactively labeled sections were co-exposed with [³H]-standards (Amersham, Germany) of known radioactivity concentrations against tritium-sensitive films for 15 weeks. The ensuing autoradiographs represent regional and laminar binding site distribution patterns.

2.3 | Digitization of Autoradiographs

Autoradiographs were digitized as 8-bit images with an in-plane resolution of 5 $\mu\text{m}/\text{pixel}$ size using an image analysis system AxioVision (Zeiss, Germany, RRID:SCR_002677) connected with a digital CCD camera (Axiocam MRm, Zeiss, Germany) and an S-Orthoplanar 60-mm macro lens (Zeiss, Germany) to enable densitometric measurement of receptor binding sites (Palomero-Gallagher and Zilles 2018; Zilles et al. 2002). The co-exposed [³H]-standards were used to compute nonlinear calibration curves, which were used to linearly transform the autoradiographs of the radiolabeled sections into a concentration of receptor binding sites (in fmol/mg protein) using in-house developed MATLAB (MathWorks, Inc., Natick, MA, RRID:SCR_001622) scripts. Linearized autoradiographs were pseudo-color-coded for visualization purposes (Palomero-Gallagher and Zilles 2018).

Histological sections from human brains were digitized using a Huron scanner (TissueScope, CA, RRID:SCR_024996) and rat sections using a light microscope (Axio Imager Vario.A2, Zeiss, Germany) combined with a stage device and a CCD camera (Axiocam 506 mono, Zeiss, Germany). All resulting 8-bit images have an in-plane resolution of 1 $\mu\text{m}/\text{pixel}$.

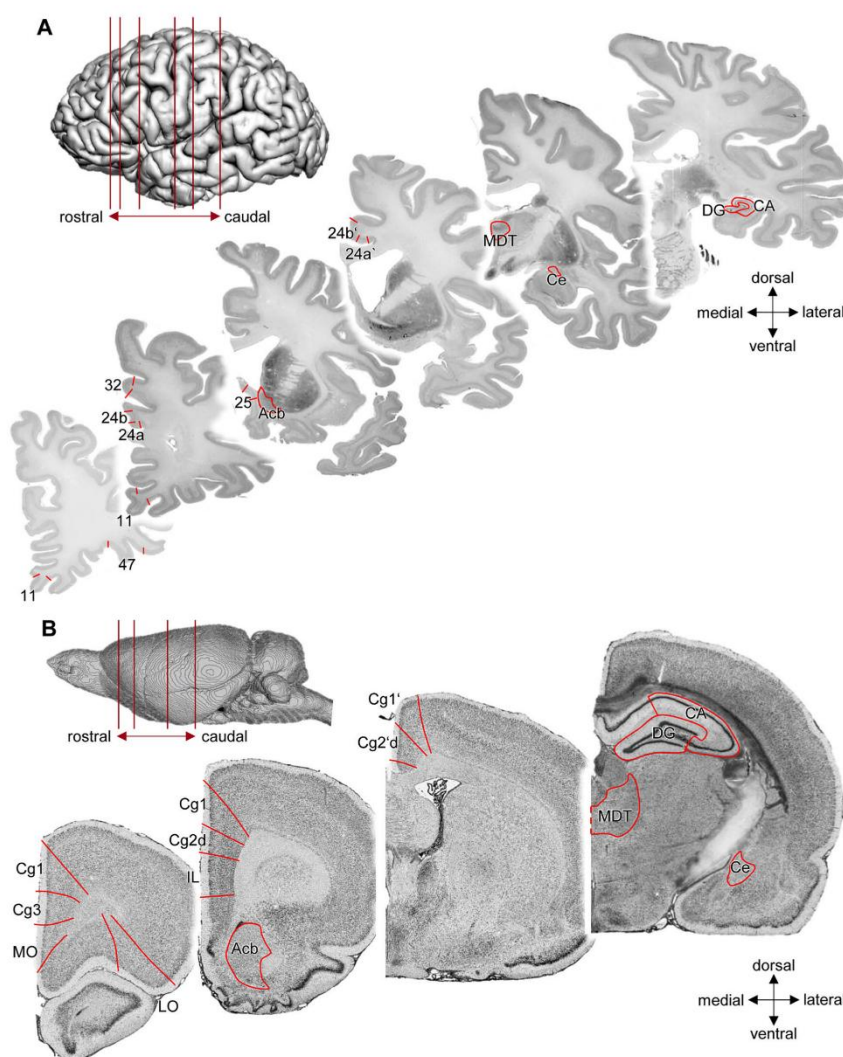


FIGURE 1 | Localization of the investigated human and rat brain areas. (A) Lateral views and serial coronal sections (right hemisphere) of the Big Brain (RRID:SCR_001593) provided by the Human Brain Project (RRID:SCR_002241) (Amunts et al. 2013) and Jülich brain atlas (RRID:SCR_023277) (Amunts et al. 2020) and (B) the Waxholm Space atlas of the Sprague Dawley rat brain (Papp et al. 2014). Red lines indicate the rostrocaudal level from which the exemplary cell body-stained sections were taken to demonstrate the location and cytoarchitecture of the analyzed areas in the human (Amunts et al. 2013) and rat brain (Haghir et al. 2023). Nomenclature of brain regions is provided in Table 1.

2.4 | Regions of Interest

We investigated regions identified in human functional imaging studies as being part of the emotion regulation network (Kohn et al. 2014; Quirk and Beer 2006; Stevens et al. 2011; Timbie and Barbas 2015; Wager et al. 2003): medial and lateral orbitofrontal cortex (areas 11 and 47, respectively); anterior (25, 24a, 24b, and 32) and midcingulate (24a' and 24b') areas; hippocampus (DG

and CA); and the subcortical Acb, Ce, and MDT. In the rat brain, we analyzed the homologous areas MO (medial orbitofrontal) and LO (lateral orbitofrontal); areas IL, Cg2d (cingulate area 2, dorsal part), Cg1 (cingulate area 1), and Cg3 (cingulate area 3); midcingulate areas Cg2'd and Cg1'; DG and CA regions; and Acb, Ce, and MDT (Gabbott et al. 1997; Groenewegen 1988; Heilbronner et al. 2016; Lucas-Neto et al. 2013; Olucha-Bordonau et al. 2015; Öngür and Price 2000; Price 2007; Vogt et al.

2013; Vogt and Paxinos 2014). We restricted our analysis to the posterior portion of the body of the human hippocampus and its correlate in the rat brain (i.e., the dorsal hippocampus), since depression-related alterations in hippocampal volume are most pronounced in the posterior than in the anterior hippocampus (Schermy et al. 2011), and fMRI studies have shown that the functional connectivity between the posterior hippocampus and brain regions involved in emotion regulation is altered in depression (Ge et al. 2019; Tura and Goya-Maldonado 2023). To ensure a comparable level of granularity to in vivo neuroimaging studies (Meltzer et al. 2004; Wacker et al. 2009; Wang et al. 2016), we here only report the mean density over the entire CA region, and we do not distinguish between the core and shell portions of the Acb.

Regions of interest were identified according to previously established cytoarchitectonic criteria (Haghir et al. 2023; Palomero-Gallagher and Zilles 2015; Paxinos and Watson 2013; Vogt and Paxinos 2014) in the sections processed for visualization of cell bodies (Figure 1) (Amunts et al. 2013, 2020; Haghir et al. 2023). The delineations were then transferred to the adjacent receptor autoradiographs, and mean densities were extracted from the autoradiographs using the in-house developed software AnaRec (Impieri et al. 2019). Depending on the size of the area, we analyzed three to five sections per brain and receptor type.

Mean receptor densities of all areas were visualized for both species as polar plots, and to facilitate comparisons of the human and rat fingerprints, we used the same axis scaling for each receptor type and identical positions for homologous areas. Relative 5-HT_{1A} and 5-HT₂ receptor densities were calculated for each area as a ratio (%) between the density of the area (R_{area}) and a receptor type-related averaged density (R_{mean}) calculated across all investigated human or rat areas using Equation (1):

$$\text{Ratio (\%)} = \frac{(R_{area} - R_{mean})}{R_{mean}} \times 100. \quad (1)$$

2.5 | Statistical Analyses

The experimental design consists of three variables: species (humans, rats), receptor type (5-HT_{1A}, 5-HT₂), and region (13 areas per species). This mixed design analysis of variance was analyzed with linear mixed-effects models (Baayen et al. 2008). Analyses were performed with the lmerTest package (Kuznetsova et al. 2017) based on the statistical programming environment R (version: 4.0.4) (<https://www.r-project.org/>, RRID:SCR_015656).

The linear mixed model was constructed by defining species, receptor type, and region as fixed factors, while subject (brain) was set as a random factor (Equation 2) (Barr et al. 2013; Bates et al. 2015). The resulting omnibus test provided statistical information concerning the main effects of species, receptor type, and region; the interaction effects between them; their fixed effects; and the random effects of subject, which included the random intercept. As the interaction effect between species, receptor type, and region was found to be significant, the simple effects and post hoc test were further analyzed, and the false discovery rate (FDR) approach (Benjamini and Hochberg 1995)

was used for multiple comparisons in both cases.

$$RD_{arb} = \alpha_0 + \alpha_1 S_{sb} + \alpha_2 R_{rb} + \alpha_3 A_{ab} + \alpha_4 S_{sb} R_{rb} A_{ab} + \alpha_5 B, \quad (2)$$

where RD is the receptor density, S is species, A is area, R is receptor type, and B is brain.

Hierarchical clustering and principal component analyses (PCAs) were performed for human and rat areas separately using in-house developed MATLAB (MathWorks, Inc., Natick, MA, Rel. R2019a, RRID:SCR_001622) scripts. For equal weighting of the two receptors, densities were normalized by z-scores separately for the 5-HT_{1A} and 5-HT₂ receptors by computing the difference between the density in a given area and the average of that receptor over all areas, then dividing that difference by the standard deviation. Euclidean distances and the Ward linkage algorithm were used to define the degree of (dis)similarity in the hierarchical cluster analyses. The number of stable clusters was determined by *k*-means analysis combined with the elbow method (Rousseeuw 1987).

3 | Results

3.1 | Comparison of Mean Receptor Densities

The 5-HT_{1A} and 5-HT₂ receptors present regional differences in their expression levels in the human and the rat brain (Figure 2A; Tables S1 and S2). The highest and lowest 5-HT_{1A} receptor densities were found in the human CA and MDT, respectively, but in the rat DG and Acb, respectively. Concerning the 5-HT₂ receptors, the highest and lowest densities were measured in human area 25 and Ce, respectively, and in the rat Acb and CA, respectively. For both species, the magnitude of the difference between the highest and lowest densities is considerably larger for the 5-HT_{1A} than the 5-HT₂ receptors. Consequently, human and rat 5-HT₂ receptor fingerprints are relatively “rounder” than those of the 5-HT_{1A} receptors. This is particularly true for the human brain because in rats, there are two peaks resulting from the relatively higher 5-HT₂ density in Cg3 and Acb than in Cg1, DG, CA or Ce (Figure 2A). In the human brain, 5-HT_{1A} receptor densities were generally higher than those of 5-HT₂ receptors, but the opposite holds true for the rat brain. Additionally, the difference in size between the 5-HT_{1A} and 5-HT₂ receptor fingerprints was larger in the human than in the rat brain (Figure 2A).

In line with these qualitative observations, significant differences were found for both 5-HT_{1A} and 5-HT₂ receptor densities between the human and rat brain (Tables 1, S3, and S4). Specifically, six of the 13 analyzed areas contained significantly higher 5-HT_{1A} densities in humans compared to rats (Table 1, SET of species; Table S1). Notably, 5-HT_{1A} density was significantly lower in the human than in the rat DG. Concerning 5-HT₂ receptors, we only found a significant difference for the Acb, which presented lower values in humans than in rats.

Concerning the ratio between 5-HT_{1A} and 5-HT₂ receptors across all examined areas, we found a more widespread range of significant differences in the rat than in the human brain (Table 1, SET of receptors). In humans, significant differences were restricted to CA, with a higher density of 5-HT_{1A} than of 5-HT₂ receptors,

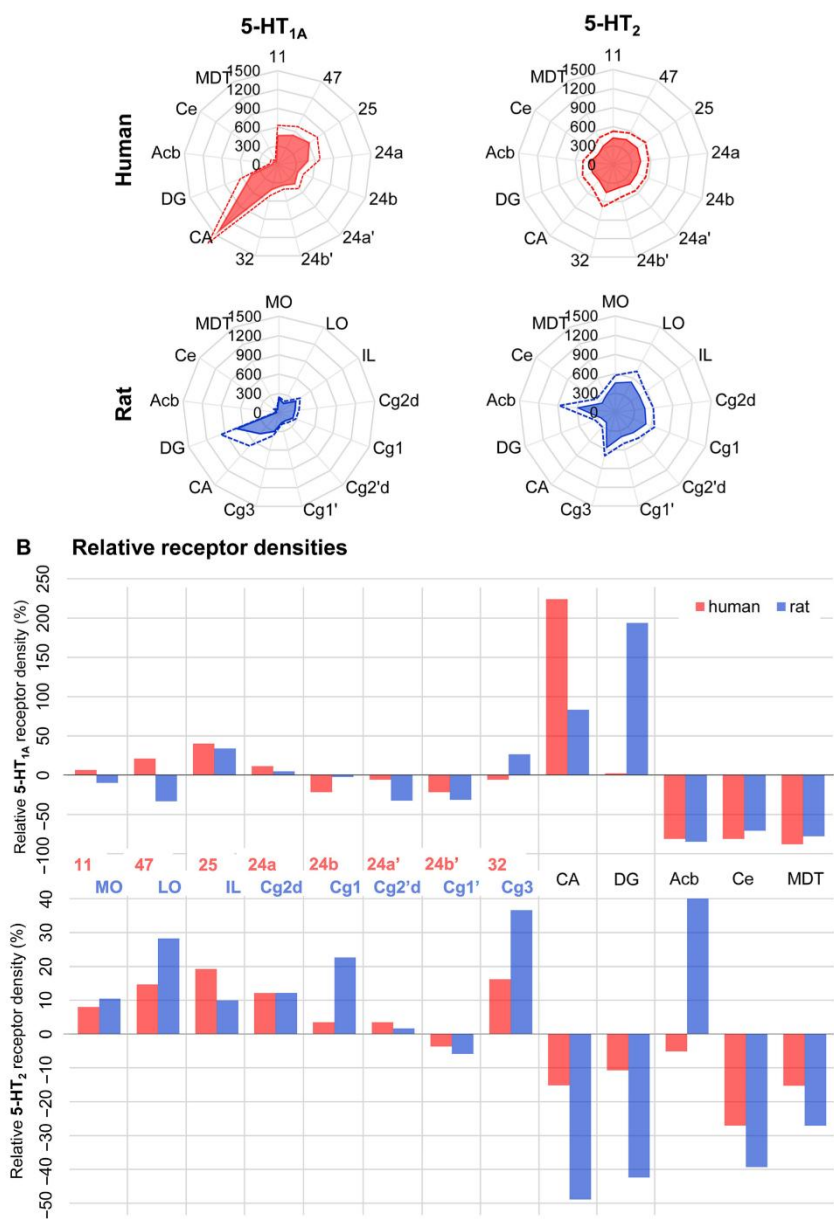


FIGURE 2 | Quantitative values of 5-HT_{1A} and 5-HT₂ receptor distributions. (A) Polar coordinate plots depicting the mean densities (colored surfaces $\pm$ standard deviation, dashed lines) in fmol/mg protein of 5-HT_{1A} (left) and 5-HT₂ (right) receptors in the analyzed human (red) and rat (blue) areas. Homologous areas in the human and rat brains are located at the same position in the plot (e.g., human area 11 and its rat homolog MO are both found at “12 o’clock”), and axis scaling is identical for all four plots. (B) Bar charts showing relative 5-HT_{1A} (top) and 5-HT₂ (bottom) receptor densities (in %) across human (red) and rat (blue) areas. Nomenclature of brain regions is provided in Table 1, and numerical values are provided in Tables S1 (for A) and S2 (for B).

TABLE 1 | Results of the statistical analyses determining species- or receptor-specific differences.

Region		SET of species		SET of receptors	
		5-HT _{1A}	5-HT ₂	Human	Rat
		Human vs. rat	Human vs. rat	5-HT _{1A} vs. 5-HT ₂	5-HT _{1A} vs. 5-HT ₂
11	MO	0.01	0.77	0.59	0.00
47	LO	0.00	0.39	0.21	0.00
25	IL	0.00	0.79	0.02	0.09
32	Cg3	0.20	0.23	0.31	0.00
24a	Cg2d	0.01	0.82	0.67	0.00
24b	Cg1	0.25	0.27	0.19	0.00
24a'	Cg2'd	0.00	0.95	0.92	0.00
24b'	Cg1'	0.04	0.98	0.39	0.00
CA	CA	0.00	0.15	0.00	0.01
DG	DG	0.01	0.09	0.32	0.00
Ce	Ce	0.73	0.53	0.00	0.01
Acb	Acb	0.51	0.00	0.00	0.00
MDT	MDT	0.96	0.57	0.00	0.00

Note: The table shows the results of the simple-effect tests (Hawrylycz et al. 2011) performed (following the significant results of the Omnibus test, Table S4) with the mixed-effects model to detect significant ($p < 0.05$) species-specific (SET of species) or receptor-specific (SET of receptors) density differences. For the species-specific comparison, cells highlighted in orange indicate significantly ($p < 0.05$) higher densities in the human than in the rat brain, and cells highlighted in blue signify the opposite situation. For the receptor-specific comparison, cells highlighted in orange indicate significantly higher ($p < 0.05$) 5-HT_{1A} than 5-HT₂ densities, and cells highlighted in blue signify the opposite situation. The p -values are FDR-corrected.

and the subcortical nuclei, all of which presented lower 5-HT_{1A} than 5-HT₂ receptor densities. In contrast, the rat brain presented significant differences in all examined areas except for IL. Rat CA and DG both contained a higher density of 5-HT_{1A} than of 5-HT₂ receptors, and the opposite held true for the remaining areas. Finally, we determined whether human and rat brains presented comparable relative distribution patterns of 5-HT_{1A} and 5-HT₂ receptors and found a striking species-specific difference concerning the relative 5-HT_{1A} receptor densities in CA and DG (Figure 2B). Specifically, significantly higher densities were found in the human CA than the DG, and the opposite held true for the rat hippocampus (Table S4). This was mainly due to the higher 5-HT_{1A} receptor density in the molecular layer of DG but lower concentration in the CA3 region of rats compared to humans (Figure 3). In contrast, human and rat areas presented a comparable relative distribution pattern of the 5-HT₂ receptors, with the notable exception of the Acb, which showed a lower value than the mean in humans but a higher density than the mean in rats (Figure 2A).

3.2 | Comparison of Laminar Receptor Distributions

Human orbitofrontal and cingulate areas were characterized by the highest 5-HT_{1A} receptor densities in layers I–III, the lowest values in layers IV (when present) and V, and slightly higher densities in layer VI (Figures 3A and S1). Homologous areas in the rat brain not only showed a principally different 5-HT_{1A} receptor distribution but also region-specific variations in this

pattern. Areas MO and LO presented a single maximum in layers V and III, respectively, and lower densities in the superficial than the deeper layers. Areas Cg1, Cg2d, and Cg2'd presented a similar 5-HT_{1A} pattern to that of the orbitofrontal areas, but with a broader maximum, which was centered over layers III and Va in Cg1 and Cg2'd, but encompassed layers III–VI in Cg2d. Area Cg1' presented a 5-HT_{1A} receptor maximum in layer V, and its superficial and deeper layers contain comparable values (Figures 3A and S1).

Laminar distribution of 5-HT₂ receptors was more similar between humans and rats, with most areas presenting a single maximum centered over layer III and lowest densities in layer VI. Human area 25 constitutes the notable exception, due to a particularly broad maximum that spans layers I–III (Figures 3A and S1). Both species displayed considerable laminar differences within the hippocampus, since human CA presented a distinct laminar distribution, with the highest 5-HT₂ receptor densities in the pyramidal layer, and a homogeneous distribution pattern across the rat hippocampus (Figure 3B).

3.3 | Multivariate Analyses

In humans, the hierarchical clustering analysis seems to be driven by the striking differences in the relative 5-HT_{1A} receptor densities, particularly between the CA and DG. CA was characterized by the highest 5-HT_{1A} and one of the lowest 5-HT₂ receptor densities and segregated from all other areas (cluster 1; Figure 4). This was mainly driven by the second principal component of the

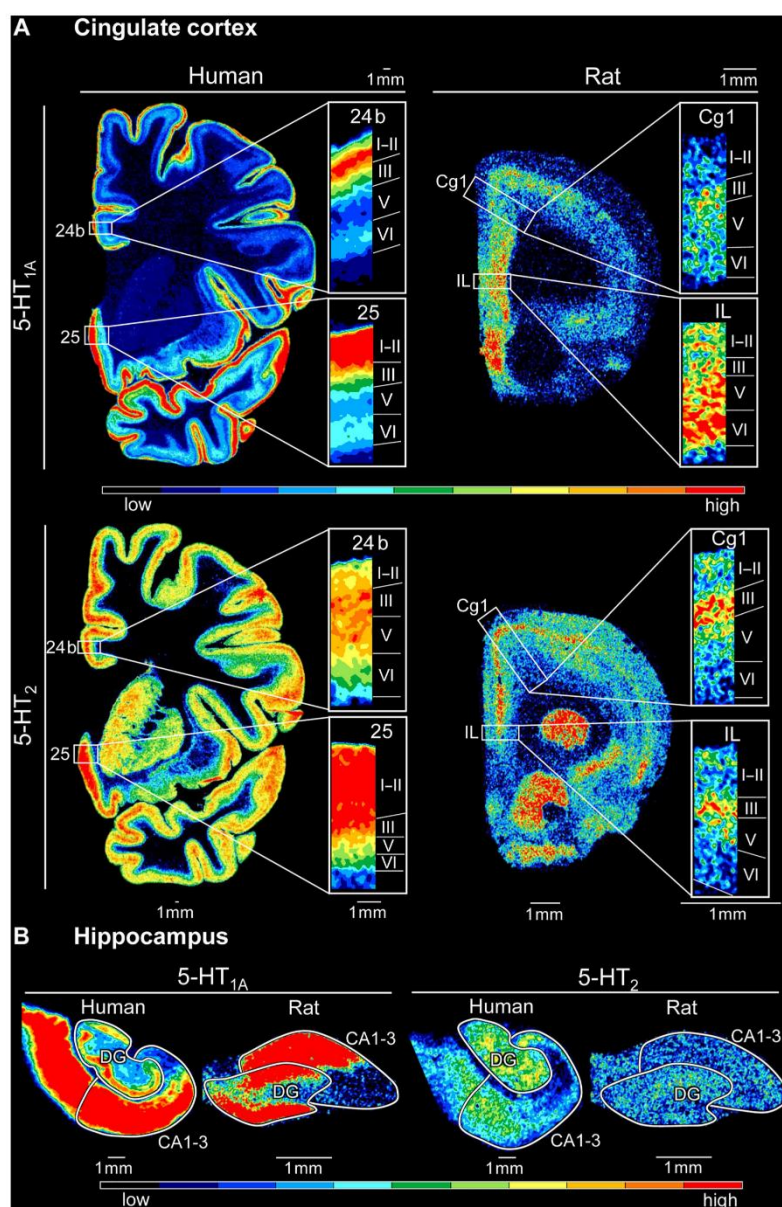


FIGURE 3 | Autoradiograms depicted regional and laminar 5-HT_{1A} and 5-HT₂ receptor distributions. (A) Representative coronal sections showing laminar 5-HT_{1A} (top) and 5-HT₂ (bottom) receptor distribution patterns in human (left) and rat (right) cingulate areas. Laminar distribution patterns of all investigated areas are provided in Figure S1. (B) Representative snippets showing differences in the regional distribution of 5-HT_{1A} (left) and 5-HT₂ (right) receptors within the human and rat hippocampus. Roman numerals indicate cortical layers, and numerical values of receptor densities in fmol/mg protein are provided in Table S1. Nomenclature of brain regions is provided in Table 1.

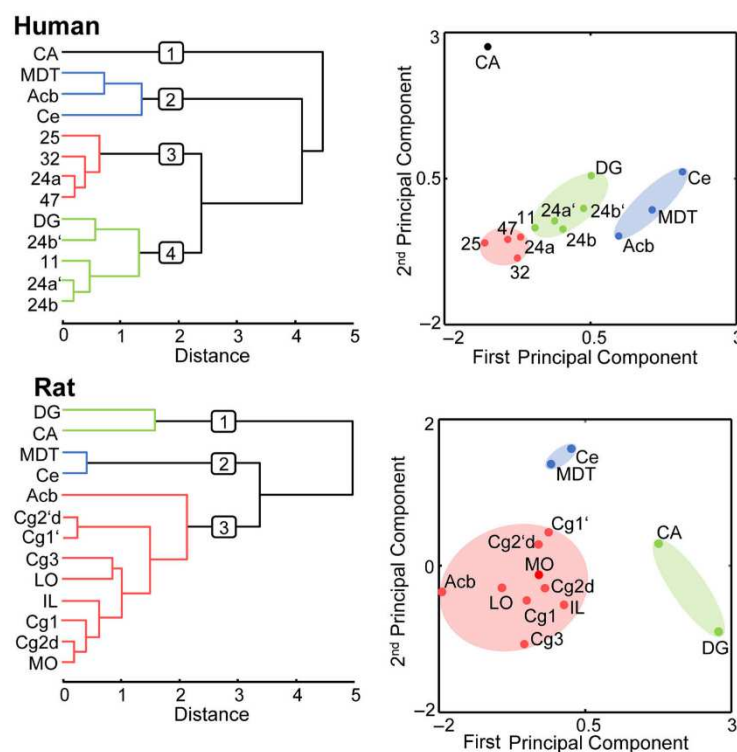


FIGURE 4 | Multivariate analyses of 5-HT_{1A} and 5-HT₂ receptor distributions. Hierarchical clustering (left) and principal component analysis (right) (Ellison-Wright and Bullmore 2010) to determine clustering of human and rat areas based on (dis)similarities in their serotonin receptor architecture. The *k*-means clustering revealed four and three as the optimal number of clusters for human and rat areas, respectively. Nomenclature of brain regions is provided in Table 1.

PCA, whereas the separation of clusters 2, 3, and 4 was driven by the first principal component. Cluster 2 encompassed the three subcortical nuclei, all of which display very low 5-HT_{1A} and 5-HT₂ receptor densities. Cluster 3 contains lateral orbitofrontal area 47 and the anterior cingulate areas, except for 24b, which was found in cluster 4 together with midcingulate areas 24a' and 24b', medial orbitofrontal area 11, and the DG.

In rats, the differences in 5-HT_{1A} receptor densities between CA and DG were significantly smaller than in humans. Both regions displayed the highest and the lowest 5-HT_{1A} and 5-HT₂ receptor densities, respectively, measured in all examined areas of the rat brain. This similarity in receptor architecture was reflected in their grouping together in cluster 1 separately from the remaining areas, and its segregation was mainly driven by the first principal component of the PCA. The subcortical nuclei MDT and Ce were in cluster 2, whereas the Acb, characterized by higher 5-HT₂ densities, was in cluster 3 together with the remaining rat areas. Segregation of clusters 2 and 3 was driven by the second principal component of the PCA and can be explained by the low 5-HT_{1A} and 5-HT₂ densities measured in Ce and MDT.

4 | Discussion

Our quantitative comparative study systematically analyzed (dis)similarities of 5-HT_{1A} and 5-HT₂ receptor distributions in components of the human emotion regulation network and homologous areas in rats. We provide reliable reference data in healthy brains and contribute to assessing the adequacy of the rat as an appropriate model for translational research of human mood disorders.

4.1 | Laminar Serotonin Innervation and Receptor Distribution Patterns

Serotonergic neurons are located exclusively in the raphe nuclei of the mammalian hindbrain, but their highly collateralized axons innervate every region of the central nervous system, allowing them to simultaneously modulate numerous brain areas (Soiza-Reilly and Gaspar 2020). Development of the brain's serotonergic innervation pattern commences during the early embryonal phase and is completed within the first postnatal month (D'Amato et al. 1987; Lidov and Molliver 1982; Wallace

and Lauder 1983), and this neurotransmitter system plays an essential role in normal developmental processes (Gaspar et al. 2003; Lauder 1993). However, the density and complexity of serotonergic innervation patterns do not remain static throughout adulthood but are susceptible to changes in brain serotonin levels, thus highlighting the importance of a balanced serotonin homeostasis to preserve serotonergic circuitry (Pratelli et al. 2017).

The few studies addressing serotonergic innervation in the human brain revealed immunoreactive terminals in all analyzed cortical areas, with higher densities in the superficial than the deep layers, and a conspicuous horizontally running plexus in layer I (Raghanti et al. 2008; Trottier et al. 1996). This pattern reflects the distribution we described for the 5-HT_{1A} receptors, which show uniquely high densities in layers I–III and low values in layers V–VI, as well as that of the 5-HT₂ receptors, which present the highest densities in layer III.

The rat cortex receives a dense regional and layer-specific heterogeneous serotonergic innervation, with a decreasing rostrocaudal gradient in fiber density and highest values in the medial prefrontal cortex (Audet et al. 1989; Beaudet and Descarries 1978; Lidov et al. 1980; Reader 1981; Vertes 1991; Wilson and Molliver 1991), which we also found to contain relatively high 5-HT_{1A} and 5-HT₂ densities. Although in most areas, layers I and VI present the strongest and weakest serotonergic innervation, respectively, we found layer I to present the lowest 5-HT_{1A} and 5-HT₂ densities. Our laminar distribution patterns for both species and receptors are in accordance with previously published autoradiographic and immunohistochemical reports, as well as with the visualization of 5-HT_{1A} receptor mRNA (Burnet et al. 1995; Chalmers and Watson 1991; Hoyer et al. 1986a, 1986b; Jansson et al. 2001; López-Giménez et al. 1998; Palomero-Gallagher et al. 2019, 2008; Palomero-Gallagher and Zilles 2015; Palomero-Gallagher et al. 2013; Pazos et al. 1985; Pazos and Palacios 1985, 1987a, 1987b; Pompeiano et al. 1992, 1994; Willins et al. 1997). Interestingly, laminar differences in serotonergic innervation were also found between human and nonhuman primate brains and are thought to indicate a human-specific shift in the role of serotonin modulation of cortical input and output functions (Raghanti et al. 2008).

Humans and rats present a higher serotonergic axonal density in CA than in DG (Moore and Halaris 1975; Oleskevich and Descarries 1990; Wilson and Molliver 1991). Interestingly, confirming previous observations, we found significantly higher 5-HT_{1A} densities in CA than in DG of humans and the opposite situation in rats. Our results pertaining to the 5-HT₂ receptor are in line with the low detection of 5-HT_{2(A)} receptor mRNA in both species (Burnet et al. 1995; Pompeiano et al. 1994).

Rat Ce and MDT are almost as strongly innervated by serotonergic neurons as are cortical areas (Asan et al. 2013; Cropper et al. 1984; Vertes et al. 1999) but display some of the lowest 5-HT_{1A} and 5-HT₂ receptor densities measured in the present study, a finding in accordance with previously published reports (Chalmers and Watson 1991; Pazos et al. 1985; Pazos and Palacios 1985; Pompeiano et al. 1992, 1994). In contrast, the Acb presents, together with Cg3, the highest 5-HT₂ density in the rat brain. In line with previous studies (Pazos et al. 1985, 1987b), we found a significantly higher 5-HT₂ density in the rat than in the human Acb. Also in line

with previous publications, we determined comparable laminar 5-HT₂ receptor distributions between homologous cortical areas of the human (Hoyer et al. 1986b; Palomero-Gallagher et al. 2008; Palomero-Gallagher and Zilles 2009; Pazos et al. 1987b) and rat brain (Pazos et al. 1985; Pompeiano et al. 1994), but considerable differences for the 5-HT_{1A} receptor (Duncan et al. 1998).

4.2 | Differential 5-HT_{1A} and 5-HT₂ Receptor Balance in Human and Rat Brains

Brain areas are each characterized by a unique combination of receptor types, their receptor fingerprints. This specific balance between different receptors modulates synaptic connectivity, signal integration, and contribution of the area to network dynamics subserving normal brain function (Palomero-Gallagher and Zilles 2018; Zachlod et al. 2023; Zilles et al. 2002). Changes in receptor fingerprints are associated with brain disorders such as epilepsy, hepatic encephalopathy, and progressive supranuclear palsy (Chiu et al. 2017; Graebnitz et al. 2011; Palomero-Gallagher et al. 2012; Palomero-Gallagher and Zilles 2013), and alterations in serotonergic neurotransmission have been associated with depression (Bartlett et al. 2022; Biver et al. 1997; Drevets et al. 1999; Hrdina et al. 1993; Mann 1999; Schneek et al. 2021; Wang et al. 2016), schizophrenia (Dean et al. 1999; Hurlmann et al. 2005; Joyce et al. 1993; Laruelle 1993; Simpson et al. 1996), and eating disorders (Bailer et al. 2004; Gorwood et al. 2018), highlighting the importance of understanding the relationship between receptor expression levels for the development of effective therapeutic strategies.

Rats are extensively used as animal models, including the field of mood disorders (Planchez et al. 2019), due to their ease of manipulation and well-characterized behaviors (Hashway and Wilding 2020; Keifer and Summers 2016; Wang et al. 2017). However, we here found humans and rats to differ significantly in the laminar distribution patterns of 5-HT_{1A} and 5-HT₂ receptors. Furthermore, we also found significant differences in the balance between 5-HT_{1A} and 5-HT₂ densities in the majority of analyzed cortical areas, but not in the subcortical nuclei, where both species consistently presented significantly lower 5-HT_{1A} than 5-HT₂ receptor concentrations. The differential balance resulted from the fact that (i) most human areas presented higher 5-HT_{1A} densities than their rat homologs, but both species contained comparable 5-HT₂ densities, and (ii) most rat cortical areas contained significantly lower 5-HT_{1A} than 5-HT₂ densities, which we did not observe in humans.

A further divergent aspect between humans and rats was found when analyzing the relative distribution of 5-HT_{1A} and 5-HT₂ receptors across all examined areas. Here again, the hippocampus was particularly conspicuous because, despite its evolutionary conserved neuroanatomy (Zilles 2005), in humans we found a significantly higher 5-HT_{1A} density in CA than DG, but the opposite held true for rats.

In contrast, we found a good correspondence in the relative distribution patterns of both receptors in human area 25 and its rat homolog IL. Areas 25 and IL were characterized by higher densities of both receptors than the surrounding cortex, thus replicating previously published observations (Palomero-

Gallagher et al. 2008; Pazos et al. 1985; Pazos and Palacios 1985, 1987a, 1987b; Santana and Artigas 2017; Várnäs et al. 2004) and supporting species-overarching inhibitory functions via 5-HT_{1A} receptors in areas 25 and IL. We also found a good correspondence concerning relative 5-HT_{1A} and 5-HT₂ distributions in the Ce and MDT, though not in the Acb of both species. Notably, deep brain stimulation of the Acb had antidepressant and antianhedonic effects in patients with treatment-resistant depression (Bewernick et al. 2010) and in a rat model of depression (Hamani et al. 2012; Lim et al. 2015).

These species-specific differences in the relative distribution of 5-HT_{1A} and 5-HT₂ receptors resulted in a different clustering pattern of the analyzed areas, with four and three stable clusters identified in the human and rat brains, respectively. The DG clustered with CA in the rat brain but with anterior midcingulate areas in the human brain, and this seems to be driven by the striking species-specific difference in the relative distribution pattern of 5-HT_{1A} receptors in CA and DG. Given that DG and CA constitute the main input and output regions of the hippocampus, respectively (Amaral et al. 2007), this clustering pattern could be indicative of a differential involvement of the serotonergic system in the modulation of hippocampal input and output mechanisms in humans and rats, since activation of 5-HT_{1A} receptors is crucial for the coordination of DG and CA3 neuronal firing during novelty detection processes (Luo et al. 2011; Meeter et al. 2006; Nolan et al. 2011). Interestingly, the human and rat DG differ in the neuroanatomical structure of their principal cell type, that is, the granule cells. Specifically, adult human granule cells have apical and basal dendrites, but rat granule cells only present apical dendrites (Seress and Pokorny 1981; Seress and Mrzljak 1987).

We found the most prominent species differences in the CA and DG regions of the hippocampus, ventromedial area 24, dorsomedial area 32, orbitofrontal area 47, and the Acb. Activation of these areas is associated with behavioral control, the subprocess of emotion regulation enabling the subject to monitor, evaluate, and modulate their emotional response to an external stimulus (Phillips et al. 2008). Cognitive neuroscientists postulated a model of emotion regulation whereby these processes may occur in an automatic (i.e., subconscious) or a controlled (i.e., conscious) manner (Gross 2014; Gross and Thompson 2007; Phillips et al. 2008). The hippocampus and area 32 mediate the so-called voluntary (conscious) behavioral control, whereas ventromedial area 24, orbitofrontal area 47, and the Acb are involved in automatic behavioral control (Etkin et al. 2011; Merz et al. 2011; Phillips et al. 2008). Interestingly, the cognitive processes associated with voluntary behavioral control are significantly less developed in rodents than the automatic behavioral control strategies, and, with regard to emotion regulation, most differences between healthy controls and patients with major depression are found in areas subserving the automatic regulation of emotions (Rive et al. 2013).

Most important for future studies assessing potential novel drugs in rat models, our findings can provide an explanation as to why compounds such as Vilazodone, Robalzotan, and Gepirone (Arborelius et al. 1999; Drossman et al. 2008; Kaur Gill et al. 2019; Mucke 2000; Zareifopoulos and Dylja 2017) failed in clinical trials and have been discontinued (Hughes et al. 2005; Zareifopoulos and Dylja 2017). They all specifically target the

5-HT_{1A} receptor, for which we found considerable differences between humans and rats. Thus, it is not surprising that, although they had been classified as highly promising for the treatment of depression after studies in rat models (Arborelius et al. 1999; Kaur Gill et al. 2019; Page et al. 2002), they showed no significant advantage in remission rates and were associated with severe side effects and high placebo response rates (Drossman et al. 2008; Kirsch 2015; Zareifopoulos and Dylja 2017). Thus, our findings question the value of rats as a translational model to test drugs targeting serotonergic receptors. They also highlight the necessity of assessing the adequacy of rats as animal models of disorders involving receptors of other neurotransmitters such as dopamine.

5 | Conclusions

Concluding, our systematic comparative analysis demonstrates that humans and rats differ significantly in the balance between 5-HT_{1A} and 5-HT₂ receptors both within and across areas involved in the emotion network. The provided quantitative reference data of healthy brains are useful for comparisons with alterations in patients and rat models, including the development of pharmacological treatments. Furthermore, it highlights the necessity of comparative analyses of humans and species commonly used in neuroscientific research as one approach to facilitate and accelerate translational neuroscience.

Author Contributions

Anika Kuckertz: data acquisition, visualization, and interpretation, writing—original draft, writing—review and editing. **Ling Zhao:** data analysis and visualization. **Olga Kedo:** data acquisition and mapping. **Katrin Amunts:** writing—review and editing. **Nicola PalomeroGallagher:** conception and design, data curation and interpretation, writing—review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data supporting this study are provided in the Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Table 1: Mean (in fmol/mg protein) 5-HT_{1A} and 5-HT₂ receptor densities $\pm$ standard deviations in areas of the emotion regulation network in the human brain and their homologs in rats. **Supplementary Table 2:** Numerical values of relative 5-HT_{1A} and 5-HT₂ receptor densities (in %) in areas of the emotion regulation network in the human brain and their homologs in rats. **Supplementary Table 3:** Results of the ANOVA test performed with a mixed-effects model. **Supplementary Table 4:** The results of the simple effect test with mixed-effects model. **Supplementary Fig. 1:** Laminar 5-HT_{1A} and 5-HT₂ receptor distributions between the human and rat orbitofrontal and cingulate areas.

Study 2

A New Map of the Rat Isocortex and Proisocortex: Cytoarchitecture and M₂ Receptor Distribution Patterns

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Prof. Hossein Haghir and I (Ms. Anika Kuckertz) contributed equally as first authors to this publication. The Study was initiated by Prof. Karl Zilles[†] and Prof. Nicola Palomero-Gallagher and Prof. Haghir, from the Department of Anatomy and Cell Biology at the School of Medicine in Iran, later joined the team.

The experimental procedures - including brain preparation and sectioning, cell body staining, and *in vitro* receptor autoradiographic binding of M₂ receptors - were performed in the laboratories of INM-1. Profs. Zilles, Palomero-Gallagher and Haghir began the cytoarchitectonic and M₂ receptor architectonic parcellation of coronal rat brain sections. I continued and finalized the parcellation of the entire rat brain. Prof. Haghir and I further contributed by adding parcellations of horizontal and sagittal rat brain sections, while Prof. Palomero-Gallagher and I prepared the 2D atlas (see supplementary material, the link is provided above).

The statistical analyses I have carried out in collaboration with Dr. Ling Zhao. Prof. Palomero-Gallagher and I prepared all figures and tables and the text was written by Prof. Palomero-Gallagher, Prof. Haghir and me.

Furthermore, Prof. Palomero-Gallagher and I prepared a corresponding dataset containing the regional M₂ receptor densities which we made openly available through the EBRAINS research infrastructure (Kuckertz et al. 2024).



A new map of the rat isocortex and proisocortex: cytoarchitecture and M₂ receptor distribution patterns

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The authors would like to dedicate this article to the memory of Professor Karl Zilles, deceased on the 26th of April 2020, and to whom we are all deeply indebted. Professor Zilles was fully involved in the planning of the present study and spent many hours analyzing the correlation between cytoarchitecturally identified cortical and subcortical structures and the heterogeneous distribution of M₂ receptors throughout the rat brain. We would also like to express our gratitude to him for the time he dedicated to numerous instructive conversations and discussions on both scientific and non-scientific topics. His neuroscientific legacy will remain for generations to come.

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Abstract

Neurotransmitters and their receptors are key molecules in information transfer between neurons, thus enabling inter-areal communication. Therefore, multimodal atlases integrating the brain's cyto- and receptor architecture constitute crucial tools to understand the relationship between its structural and functional segregation. Cholinergic muscarinic M₂ receptors have been shown to be an evolutionarily conserved molecular marker of primary sensory areas in the mammalian brain. To complement existing rodent atlases, we applied a silver cell body staining and quantitative in vitro receptor autoradiographic visualization of M₂ receptors to alternating sections throughout the entire brain of five adult male Wistar rats (three sectioned coronally, one horizontally, one sagittally). Histological sections and autoradiographs were scanned at a spatial resolution of 1 µm and 20 µm per pixel, respectively, and files were stored as 8 bit images. We used these high-resolution datasets to create an atlas of the entire rat brain, including the olfactory bulb, cerebellum and brainstem. We describe the cyto- and M₂ receptor architectonic features of 48 distinct iso- and proisocortical areas across the rat forebrain and provide their mean M₂ receptor density. The ensuing parcellation scheme, which is discussed in the framework of existing comprehensive atlases, includes the novel subdivision of mediomedial secondary visual area Oc2MM into anterior (Oc2MMa) and posterior (Oc2MMp) parts, and of lateral visual area Oc2L into rostralateral (Oc2Lr), intermediate dorsolateral (Oc2Lid), intermediate ventrolateral (Oc2Liv) and caudolateral (Oc2Lc) secondary visual areas. The M₂ receptor densities and the comprehensive map of iso- and proisocortical areas constitute useful tools for future computational and neuroscientific studies.

Keywords Brain mapping · Acetylcholine · Receptor architecture · Amygdala · Hippocampus · Basal ganglia · Thalamus · Brainstem

Introduction

Rodents, in particular rats, are the most widely used animal models in neuroscience research (Keifer and Summers 2016). Thus, the rat cerebral cortex is probably the most extensively studied cortical structure. Due to the structural and functional heterogeneity of the cerebral cortex, it is

crucial to have a detailed and reliable map to enable precise localization.

Brodmann (1909) identified two principal subdivisions within the mammalian cerebral cortex based on differences in laminar developmental timelines: the homogenetic cortex, in which areas display the basic six-layered structure both in embryonic and adult stages, and the heterogenetic cortex, with a different layering in early and adult stages. Homogenetic and heterogenetic cortex correspond to the isocortex and allocortex, respectively, as defined by Vogt (1910) based on histological criteria, and are separated by the transitional mesocortex. The proisocortex constitutes the portion of the mesocortex which directly abuts the

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isocortex, whereas the periallocortex is adjacent to the allocortex (Brodman 1909; Stephan 1975).

The rat iso- and proisocortex have been parcellated into numerous areas based mostly on cell body stained sections (Palomero-Gallagher and Zilles 2015; Paxinos and Watson 2013; Swanson 2004; Zilles 1985), and the most widely used atlas presents both Nissl- and acetylcholinesterase-stained sections (Paxinos and Watson 2013). The resulting maps, however, are mainly based on the analysis of a single modality, frequently of Nissl-stained sections. Differences in cytoarchitecture are often very difficult to be detected by researchers with less experience in this special anatomical field. This restriction becomes particularly obvious when the available cortical maps are compared. The comparison shows similarities in the parcellation of many brain regions, although often different names were used for the same cortical area (Table 1). However, there are also differences, and the main reason for the discrepancies is not the nomenclature used by different authors, but the different delineation criteria leading to different numbers of cortical areas.

To overcome the difficulties caused by the nearly exclusive cytoarchitectonic maps, the present observation shows the quantitative distribution of a key molecule of signal transmission, i.e. the cholinergic muscarinic binding site of the M_2 receptor, in serial sections throughout the complete isocortex and proisocortex of the rat. To bridge the gap between previous cytoarchitectonic maps and the current parcellation scheme, the M_2 receptor distribution is compared with alternating Nissl-stained sections of the same brains. In contrast to all presently available atlases of this part of the rat cortex, the receptor-based parcellation has several advantages:

- It is based on visualization of a functionally relevant molecule of signal transmission, i.e. on the heterogeneous regional and laminar distribution of the M_2 receptor, which has been proven in previous studies of rodent brains (Palomero-Gallagher and Zilles 2015; Schubert et al. 2016).
- The M_2 receptor clearly highlights the thalamo-cortical input layers of the primary visual, somatosensory and auditory areas at least in all primate brains (Zilles and Palomero-Gallagher 2017a), and thus may help to identify those areas also in the rat brain, by its exceptionally high density in those areas.
- This receptor is also heterogeneously distributed throughout other isocortical areas, and thus provides delineation criteria depending on its differential quantitative binding of the specific ligand. This enables detailed parcellations and a mapping of all isocortical areas, which exceeds and improves previous attempts while simultaneously providing functionally relevant information.

- Quantitative differences in receptor density enable an objective description of the similarities and differences between the mapped cortical areas.

Aim of the present study was to provide a detailed parcellation of the rat iso- and proisocortex using the heterogeneous distribution of the cholinergic muscarinic M_2 receptor densities, a comparison with cytoarchitectonic parcellations, and a uniform and easy to understand topographical nomenclatural system, which does not presume functional identifications. The latter aspect does not, however, exclude comparisons with functional data. It simply avoids a premature confusion of anatomically based names of cortical areas with those cortical units which had been identified by other methods.

Materials and methods

Five adult male Wistar rats (250 g) were decapitated under isoflurane narcosis, the brains were carefully removed from the skull, immediately deep frozen in isopentane at $-50\text{ }^{\circ}\text{C}$, and placed in pre-cooled plastic bags and stored in a deep freezer at $-80\text{ }^{\circ}\text{C}$ until further processing. All animal procedures and husbandry were approved by the institutional animal welfare committee at the Research Centre Jülich and were carried out in accordance with the guidelines of the European Communities Council Directive for the care and use of animals for scientific purposes.

Each rat brain was serially sectioned at $20\text{ }\mu\text{m}$ thickness using a cryostat microtome (Leica Microsystems, Germany) at -18 to $-20\text{ }^{\circ}\text{C}$. Blockface images were obtained for one of the rats (R8) during sectioning (Schubert et al. 2016). Three of the brains were sectioned in the coronal plane (R8, R865, R5), one in the sagittal plane (R874) and another horizontally (R875). Brains to be sectioned in the coronal plane were attached with Tissue Freezing Medium (Leica) to the cryotome chuck by the brainstem and most posterior portion of the cerebellum. The brain to be sectioned in the sagittal plane was attached by the cortex of the right hemisphere containing the posterior portion of Par2 and anterior part of the temporal region, and the brain to be sectioned in the horizontal plane was attached by the brainstem and cortex containing the basal forebrain. Rats R8, R874 and R875 were sectioned in their entirety, yielding a total of 1370, 553 and 312 sections, respectively. The forebrain of R865 was also entirely processed, but sections were only obtained from the rostral portion of the cerebellum, yielding a total of 897 sections. All sections were thaw-mounted onto pre-cooled silanized glass slides. The sections of R8 were organized in series of adjoining triplets of which the first section was used for visualization of cell bodies and cytoarchitecture approach, the second one was processed for

Table 1 Correspondences between the areas identified in the present study and those shown in the maps of Paxinos and Watson (2013) and of Swanson (2004)

	Present map	Paxinos & Watson (2013)		Swanson (2004)	
dorsolateral frontal	Fr2	M2		MOs	
	Fr1	M1			
	Fr3	Fr3		MOP	
parietal	Par1	S1	TeA (caudal part)	SSp	
	ParVr				
	ParFL				S1FL
	ParHL				S1HL
	Par2	S2		SSs	
	ParVc			VISC	
	ParPd	PtPD		PTLp	
	ParPv	PtPR			
temporal	Te1	Au1		AUDp	
	Te2d	AuD (caudal third)		AUDpo	
	Te2v	AuV (caudal third)		TEa (caudal part)	
	Te3r	AuD (rostral two-thirds)		AUDd	
	Te3v	AuV (caudal two-thirds)		AUDv	
occipital	Oc1B	V1B		VISp	
	Oc1M	V1M			
	Oc2MMp	V2MM		VISpm	
	Oc2MMa			VISpa	
	Oc2ML	V2ML			
	Oc2Lr	V2L		VISrl	
	Oc2Lid			VISal, VISlm	
	Oc2Liv			VISall, VISll	
	Oc2Lc			VISli, VISpl, VISx	
cingulate	IL	A25		ILA	
	Cg3	A32D (rostral two-thirds)		PL	
	Cg1	A32D (caudal third), A24b		ACAd	
	Cg1'	A24b'			
	Cg2d	A24a		ACAv	
	Cg2'd	A24a'			
	Cg2v	A33			
	Cg2'v				
retrosplenial	RSA	A30		RSPagl	
	RSGc	A29c		RSPd	
	RSGb	A29b		RSPv	
	RSGa	A29a			
orbital	DLO	DLO		Ald (rostral part)	
	LO	LO		ORBl	
	VLO				
	VO	VO		ORBv	
	MO	MO, part of A32V		ORBm	
insular	DI	DI		DI	
	Gla	GI		GU	
	Glp				
ectorhinal	EctD	TeA (rostral part)		TEa (rostral part)	
	EctV	Ect		ECT	
	EctP				
perirhinal	PRhD	PRh (rostral part)		PERI (rostral part)	
	PRhV				
postrhinal	PoRhD	PRh (caudal part)		PERI (caudal part)	
	PoRhV				

quantitative in vitro receptor autoradiography for M_2 receptors, and the last one was preserved unprocessed. Each of the adjoining series into which sections of R865 were organized encompassed 24 sections, of which the 7th and 23rd were processed for the visualization of M_2 receptors and of cell bodies, respectively. Sections from R874 and R875 were also organized into groups of 24, but here every 9th and 21st section were processed for the visualization of M_2 receptors and of cell bodies, respectively. Thus, there were 40 μm between each section processed for M_2 receptor visualization in R8, but 480 μm in R865, R874 and R875. For brain R5 we processed groups of sections, each containing 18 sections, obtained from 19 rostro-caudal levels spread throughout the brain. Of these levels, three were taken from the olfactory bulb, 14 from the forebrain and the remaining two from the cerebellum.

The histological sections were stained with a silver staining technique for cytoarchitectonic analysis (Merker 1983), which results in a higher contrast and more intense visualization of cell bodies than the widely used cresyl-violet stain of Nissl substance. The procedure of quantitative in vitro receptor autoradiography used in this study to demonstrate the densities (in fmol/mg protein) of the agonistic receptor binding sites of the cholinergic muscarinic M_2 receptor, has been published in detailed previously (Palomero-Gallagher and Zilles 2018). In summary, during a 20 min pre-incubation step at room temperature in 20 mM HEPES-Tris (pH 7.5) containing 10 mM MgCl_2 and 300 nM pirenzepine, sections were rehydrated and endogenous substances removed. Sections were then incubated 60 min at room temperature in the main incubation buffer with 1.7 nM [^3H]-oxotremorine-M (PerkinElmer, USA) to visualize the total agonistic binding sites of the M_2 receptor. Finally, the binding procedure was stopped and surplus tritiated ligand as well as buffer salts were eliminated by a rinsing step (2 $\times$ 2 min) in ice-cold buffer followed by a dip in distilled water. Sections were air dried and exposed together with plastic scales of increasing and known radioactivity concentrations against beta-radiation-sensitive films, which were developed after 15 weeks.

Cell body stained sections were scanned using a light microscope (Axioplan 2 imaging, ZEISS, Germany) equipped with a motor-operated stage controlled by the Axiovision (Zeiss, Germany) image analyzing system applying a 6.3 $\times$ 1.25 objective (Planapo®, Zeiss, Germany), and a CCD camera (Axiocam MRm, ZEISS, Germany), resulting in images with an in-plane resolution of 1 μm per pixel, and 8-bit grey resolution (Palomero-Gallagher and Zilles 2018). M_2 receptor autoradiographs were digitized using an image acquisition and processing system Axiovision (Zeiss, Germany) consisting of a source of homogenous light and a high resolution CCD-camera (Axiocam MRm, Zeiss, Germany) with an S-Orthoplanar 60-mm macro lens (Zeiss, Germany)

corrected for geometric distortions, and resulting in images with an in-plane resolution of 5 μm per pixel and 8-bit grey resolution. Quantification of receptor densities was performed using the in house software AnaRec (Impieri et al. 2019), which enables computation of the mean grey value of all pixels encompassed by a specific cortical area, and transformation of this value into a receptor concentration per unit protein (fmol/mg protein). For further details see Palomero-Gallagher and Zilles (Palomero-Gallagher and Zilles 2018). For visualization purposes, autoradiographs were subjected to linear contrast enhancement, colour coding and median (Palomero-Gallagher and Zilles 2018).

Receptor densities were extracted separately for the left and right hemisphere. We used a non-parametric permutation test (1000 permutations; (Nichols and Holmes 2002) and an FDR correction (Benjamini and Hochberg 1995) to identify possible significant interhemispheric differences in receptor densities.

Results

All topographical relations of the rat brain parcellation are depicted in the map presented in Fig. 1A–D. Figure 2 and levels c1–c39 of the Supplementary Material display overviews of coronal sections through different rostro-caudal levels of the rat brain, and which were processed for the visualization of cell bodies (Fig. 2A; top panel in each level of the Supplementary Material) or of regional and laminar differences in the densities of the M_2 receptor (Fig. 2B; bottom panel in each level of the Supplementary Material). With a comparable organization, levels s1–s5 and h1–h9 of the Supplementary Material display overviews of sections through different medio-lateral and dorso-ventral levels, respectively, processed for the visualization of M_2 receptor and cell body distribution patterns. Labelling of the muscarinic cholinergic M_2 receptors with [^3H]-oxotremorine-M revealed their heterogeneous distribution throughout the rat brain, although without significant interhemispheric differences (Fig. 3 and Table 2), with highest densities found in the olfactory bulb (Supplementary Material, levels c1–c3, s5, h6–h9) and lowest ones in the cerebellum, including the dentate (DN) and interposed (IP) nuclei (Supplementary Material, level c36, s1–s5, h1–h8). Differences in M_2 receptor densities not only reveal borders between isocortical areas (the focus of this manuscript), but also enable identification of layers in the superior colliculus (SC; Supplementary Material, levels c25–c30, s4–s5, h2–h4) and nuclei in the amygdala (e.g., higher densities in the anterior basolateral nucleus [BLA] than in the central [Ce] nuclei; Supplementary Material, levels c18–c22), thalamus (e.g., high densities in the anteroventral [AV], rhomboid [Rh], reuniens [Re]

Fig. 1 Lateral (A), medial (B), dorsal (C) and ventral (D) schematic views of the brain of rat #8 with the colour-coded map of isocortical areas and neighboring mesocortical region (proisocortical and periallocortical areas). The allocortex is not shown. Dashed black line indicates the course of the rhinal fissure. Dotted black line highlights the border between the nasal and temporal portions of the primary visual area (Oc1B and Oc1M, respectively). Dashed grey lines and numbers indicate the positions of sections shown in Fig. 2 and in the Supplementary Material. To avoid cluttering, the numbers have been placed above the lines with short dashes, and below the lines with long dashes. For abbreviations see the Supplementary Material

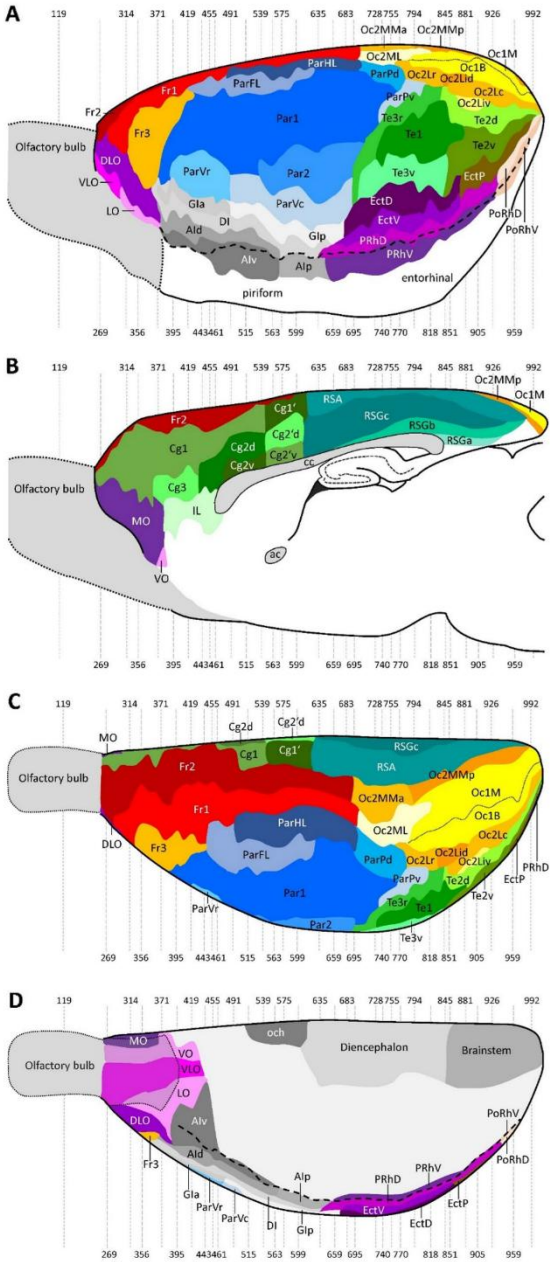
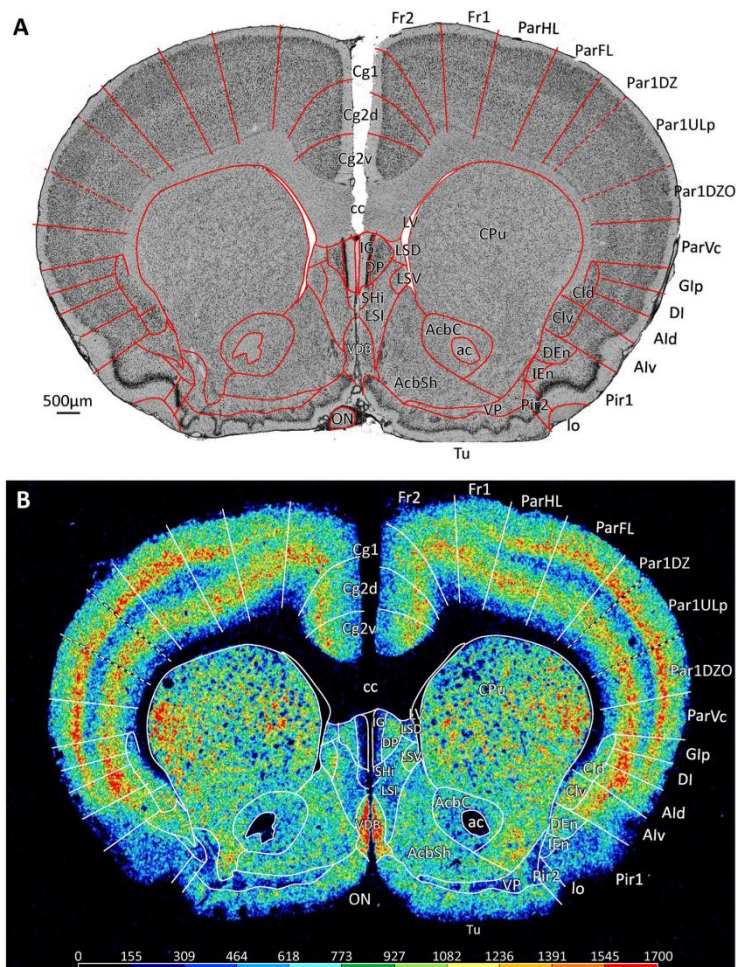


Fig. 2 Coronal sections through the brain of rat #8 showing the distribution of (A) cell bodies in section #355, and of (B) the cholinergic muscarinic M_2 receptor in section #356. Colour scale indicates receptor binding site concentrations in fmol/mg protein. Dotted lines mark artificial tissue loss. For abbreviations and for all 39 sections through the rat brain, including the olfactory bulb and the cerebellum, see the Supplementary Material



and mediodorsal [MD] thalamic nuclei, and low densities in the medial geniculate nucleus [MGN] as well as in the ventral anterolateral- [VAL], posterolateral [VPL] and posteromedial [VPM] nuclei; Supplementary Material, levels c17-c28, s4-s5, h5-h8), basal ganglia (e.g., higher densities in the caudate putamen [CPu] and low densities in the globus pallidus; Supplementary Material, levels c8-c25, s2-s3, h5-h6) or brainstem (e.g., high densities in the pontine gray [PG] hypoglossal nucleus [XII], and low densities in the pontine [PnC] and tegmental reticular nuclei [TRN], as well as in the spinal nucleus of the trigeminal nerve [SpV]; Supplementary Material, levels c30-c39, s5), and of regions within the hippocampal

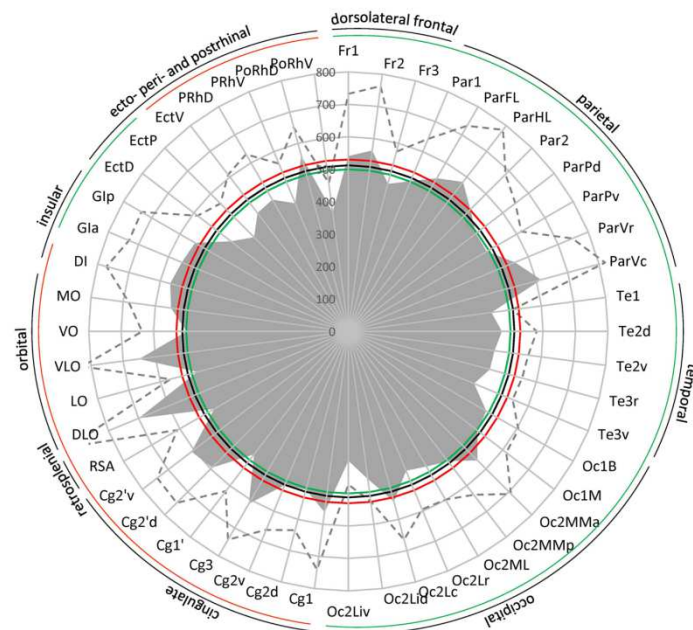
formation (e.g., increasing density gradient when moving from the dentate gyrus through the CA3-CA1 regions to the subicular complex; Supplementary Material, levels c18-c31, s1-s5, h3-h8).

Frontal cortex

The frontal cortex comprises the

- Dorsolateral frontal cortex with isocortical areas Fr1, Fr2 and Fr3

Fig. 3 Mean M_2 receptor density plus standard deviation (dashed line) in fmol/mg protein. Values averaged over all cortical layers and all sections containing a given area are displayed as a polar coordinate plot. The thick black line indicates the average receptor density across all areas, the green and red lines designate the mean receptor density across the isocortical and proisocortical areas, respectively



- Cingulate cortex with proisocortical areas Cg1, Cg1', Cg2d, Cg2'd, Cg2v, Cg2'v and Cg3
- Orbital cortex with proisocortical areas DLO, LO, MO, VO and VLO.

Topography

Areas Fr1–Fr3 belong to the dorsolateral frontal cortex (Fig. 1A–C) and are characterized by the lack of a prominent layer IV (dysgranular cortex). They are thus clearly delineable from the adjacent parietal areas, which have a distinct and thick layer IV (Fig. 2A; Supplementary Material, levels c2–c20, s3–s4, h1–h6). The Fr-region is found on the dorsolateral and dorsomedial surfaces of the hemisphere and occupies its frontal third. This region is surrounded by the agranular cingulate cortex medially, and the agranular orbital cortex basolaterally (Fig. 1A–C). The occipital cortex follows caudally. Areas Fr1–Fr2 are visible as two longitudinal stripes. Fr2 is the medial and longer strip, which is placed on the dorsomedial surface of the hemisphere and extends from the frontal pole to the occipital cortex. Fr2 is bordered medially by cingulate areas Cg1 and Cg1'. Laterally, Fr2 is bordered mainly by Fr1, except for the most rostral part, where it abuts the dorsolateral orbital cortex (DLO). Fr1 is a long cortical strip located on the dorsolateral surface of the hemisphere,

where it adjoins Fr2 rostromedially and extends parallel to Fr2 towards the occipital cortex. Laterally, Fr1 is delimited from anterior to posterior by DLO, Fr3, and parietal areas ParFL and ParHL, respectively. Therefore, Fr3 is the most postero-lateral area of the frontal cortex, and is bordered laterally by DLO and caudally by parietal (Par1 and ParFL) and insular dysgranular (DI) cortices.

The cingulate cortex roughly spans the rostral half of the medial surface of the hemisphere, where it occupies the most dorsal part of the cortex and is delimited caudally by the retrosplenial cortex (Fig. 1B, C). Ventrally, the rostral portion of the cingulate cortex is delimited by orbital area MO and the infralimbic cortex (IL), and the posterior portion by the corpus callosum. The most dorsal portion of the cingulate cortex is occupied rostrally by Cg1 and caudally by Cg1', and both areas encroach over a short distance on the most medial part of the dorsal hemispheric surface. Cg1 is located ventro-medial to Fr2, and extends rostro-caudally from the rostral pole to the end of the rostral fourth of the corpus callosum, where it is followed caudally by area Cg1'. Cg1 is bordered ventrally (from anterior to posterior) by MO, Cg3 and Cg2d. Cg1' is delimited dorso-laterally by frontal area Fr2 and ventrally by Cg2'd. The ventral portion of the cingulate cortex is occupied (from rostral to caudal) by areas Cg3, Cg2 and Cg2', whereby Cg3 does not extend to the rostral

Table 2 Mean absolute M_2 receptor density (in fmol/mg protein; $\pm$ standard deviation) separately for the left and right hemispheres, and averaged over both

	Left	Right	p-value	Mean
Fr1	536 $\pm$ 196	545 $\pm$ 193	0.4625	540 $\pm$ 193
Fr2	551 $\pm$ 207	574 $\pm$ 197	0.3656	562 $\pm$ 200
Fr3	466 $\pm$ 111	476 $\pm$ 99	0.4336	470 $\pm$ 103
Par1	492 $\pm$ 142	510 $\pm$ 145	0.3626	501 $\pm$ 142
ParFL	533 $\pm$ 207	556 $\pm$ 171	0.3866	545 $\pm$ 187
ParHL	579 $\pm$ 221	582 $\pm$ 194	0.5195	580 $\pm$ 203
Par2	520 $\pm$ 136	544 $\pm$ 172	0.3477	532 $\pm$ 153
ParPd	502 $\pm$ 180	490 $\pm$ 145	0.5145	495 $\pm$ 154
ParPv	466 $\pm$ 111	523 $\pm$ 137	0.1728	492 $\pm$ 123
ParVr	535 $\pm$ 202	576 $\pm$ 203	0.3047	556 $\pm$ 192
ParVc	608 $\pm$ 192	620 $\pm$ 231	0.4685	614 $\pm$ 205
Te1	445 $\pm$ 76	443 $\pm$ 57	0.4895	444 $\pm$ 66
Te2d	473 $\pm$ 115	472 $\pm$ 107	0.5035	473 $\pm$ 108
Te2v	455 $\pm$ 112	452 $\pm$ 105	0.5155	454 $\pm$ 106
Te3r	437 $\pm$ 108	463 $\pm$ 101	0.2777	451 $\pm$ 103
Te3v	409 $\pm$ 150	429 $\pm$ 113	0.3936	419 $\pm$ 129
Oc1B	479 $\pm$ 126	502 $\pm$ 107	0.3257	490 $\pm$ 115
Oc1M	498 $\pm$ 125	506 $\pm$ 100	0.4605	502 $\pm$ 112
Oc2MMa	559 $\pm$ 150	565 $\pm$ 167	0.4865	562 $\pm$ 147
Oc2MMp	503 $\pm$ 136	505 $\pm$ 135	0.5864	504 $\pm$ 131
Oc2ML	468 $\pm$ 97	503 $\pm$ 160	0.3327	481 $\pm$ 118
Oc2Lr	456 $\pm$ 139	474 $\pm$ 131	0.4256	464 $\pm$ 130
Oc2Lc	558 $\pm$ 134	529 $\pm$ 119	0.2967	542 $\pm$ 122
Oc2Lid	460 $\pm$ 82	456 $\pm$ 67	0.4785	458 $\pm$ 71
Oc2Liv	383 $\pm$ 94	420 $\pm$ 25	0.3367	398 $\pm$ 74
Cg1	565 $\pm$ 178	558 $\pm$ 192	0.5015	561 $\pm$ 180
Cg2d	512 $\pm$ 137	497 $\pm$ 130	0.4106	504 $\pm$ 131
Cg2v	514 $\pm$ 153	512 $\pm$ 157	0.5145	512 $\pm$ 150
Cg3	603 $\pm$ 126	621 $\pm$ 139	0.3896	612 $\pm$ 129
Cg1'	461 $\pm$ 150	496 $\pm$ 156	0.2977	477 $\pm$ 144
Cg2'd	594 $\pm$ 166	593 $\pm$ 153	0.4545	593 $\pm$ 155
Cg2'v	606 $\pm$ 154	611 $\pm$ 119	0.5195	608 $\pm$ 134
RSA	468 $\pm$ 133	486 $\pm$ 133	0.3347	476 $\pm$ 132
DLO	653 $\pm$ 251	748 $\pm$ 215	0.2218	698 $\pm$ 232
LO	472 $\pm$ 81	492 $\pm$ 99	0.3616	481 $\pm$ 87
VLO	652 $\pm$ 195	645 $\pm$ 170	0.4945	649 $\pm$ 179
VO	498 $\pm$ 144	498 $\pm$ 144	0.5525	498 $\pm$ 138
MO	549 $\pm$ 143	552 $\pm$ 134	0.4955	551 $\pm$ 131
DI	558 $\pm$ 207	578 $\pm$ 207	0.4006	568 $\pm$ 204
Gla	541 $\pm$ 193	569 $\pm$ 171	0.4076	555 $\pm$ 177
Glp	553 $\pm$ 190	538 $\pm$ 203	0.4486	545 $\pm$ 192
EctD	450 $\pm$ 132	462 $\pm$ 132	0.4116	456 $\pm$ 130
EctP	405 $\pm$ 162	413 $\pm$ 148	0.4635	410 $\pm$ 148
EctV	451 $\pm$ 161	468 $\pm$ 141	0.4076	459 $\pm$ 150
PRhD	468 $\pm$ 167	469 $\pm$ 163	0.5045	468 $\pm$ 162
PRhV	425 $\pm$ 132	429 $\pm$ 140	0.4985	427 $\pm$ 133
PoRhD	540 $\pm$ 71	579 $\pm$ 111	0.2348	560 $\pm$ 92
PoRhV	380 $\pm$ 121	361 $\pm$ 85	0.4306	369 $\pm$ 93

Nominal p-values resulting from the analysis of left–right differences in receptor densities are provided since there were no significant interhemispheric differences even before correction for multiple comparisons

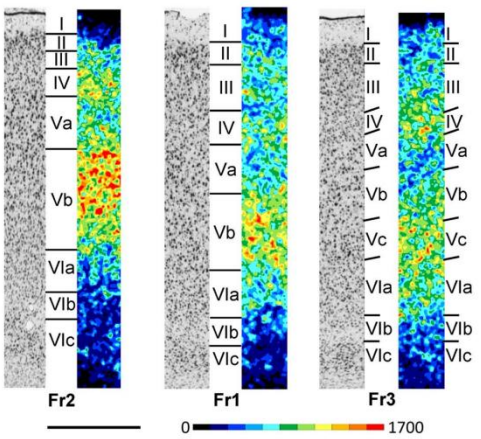


Fig. 4 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and of M_2 receptor binding sites (right side) in areas Fr1, Fr2 and Fr3 of the rat dorsolateral frontal cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m

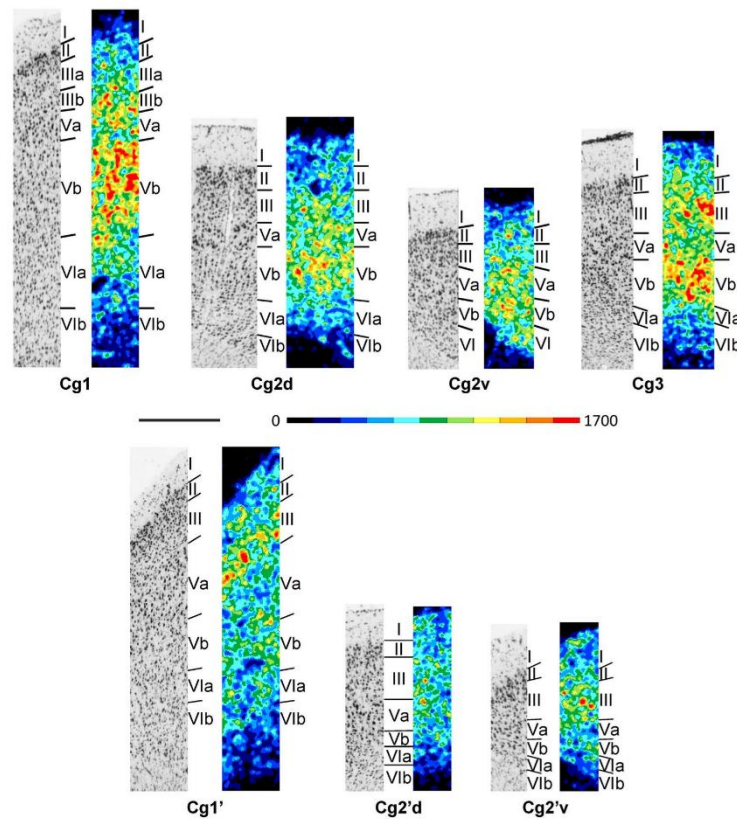
pole. Areas Cg2 and Cg2' can each be subdivided into dorsal and ventral parts (Cg2d/Cg2v and Cg2'd/Cg2'v, respectively). Cg3 is a small area located between Cg1 and IL, and delimited caudally by Cg2d. IL is a periallocortical area and thus not topic of the present study. A very thin indusium griseum, which is not shown on our map, is located below Cg2v and separates it from the corpus callosum.

The orbital cortex occupies the ventral surface of the frontal pole and encroaches onto the ventral part of the medial and lateral surfaces (Fig. 1A, D). From lateral to medial, it consists of 5 areas DLO, LO, VLO, VO and MO. Rostro-caudally, LO extends from the middle of the orbital region toward its caudal end; therefore, in the anterior part of the orbital cortex LO is absent. DLO is found on the ventrolateral surface of the hemisphere. It is bordered dorsally by frontal areas Fr1–Fr3 and ventrally by VLO in the anterior, and LO in the posterior part of the orbital cortex. MO is located on the medial aspect of the hemisphere below the cingulate cortex and medial to VO. VLO occupies the central position between VO medially and LO laterally.

Cytoarchitecture

The most conspicuous feature of the dorsolateral frontal cortex is its highly dysgranular cytoarchitecture, i.e. an incipient layer IV is clearly identifiable, and the presence of large and densely packed pyramidal cells in layer V (Figs. 2A and 4; Supplementary Material, levels c2–c20, s3–s4, h1–h6). Layer IV is particularly thin and inconspicuous in areas Fr1 and

Fig. 5 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and of M_2 receptor binding sites (right side) in areas Cg1, Cg2d, Cg2v, Cg1', Cg2'd, Cg2'v and Cg3 of the rat cingulate cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m



Fr2 (Fig. 4). The infragranular stratum (layers V–VI) of these areas is consistently broader than the supragranular layers (layers I–III). Layer II has a higher cell packing density in Fr1 and Fr2 compared to Fr3. Layer III can be distinguished from layer II by the lower cell packing density of layer III. Layer V can be subdivided in Fr1 and Fr2 into sublayers Va–Vb by the occurrence of the largest pyramidal cells in layer Va. Fr3 is characterized by a tripartite layer V, with larger pyramids in Va and Vb than in Vc, and a slightly lower cell packing density in Vb than in Va. Layer VI can be subdivided in all Fr areas into sublayers VIa–VIc by the lower packing density of neurons in sublayer VIb.

The thickness of the cortical ribbon of the cingulate cortex notably varies between its areas (Figs. 2A and 5; Supplementary Material, levels c4–c16, s4–s5, h1–h5). The broadest cortex is found in Cg1 and Cg1', the narrowest in Cg2v and Cg2'v. The thickness of Cg3 is comparable to that of Cg2d, and both areas can be distinguished by the overall higher packing density in the former than in the latter area, and by

the slightly smaller layer Va pyramids in Cg3 than Cg2d. In all areas of the cingulate cortex layer V comprises larger neurons, but with a lower packing density, than does layer VI. Layer II of Cg1 is narrow and cell dense, and thus can be clearly delineated from layer III. In contrast, in Cg1' the border between layers II and III is blurred. Layers V and VI of Cg1 and Cg1' can be subdivided into two sublayers. In both areas layers Va and VIa have larger neurons than Vb and VIb, respectively, though this is more obvious in Cg1 than in Cg1'. Areas Cg2d, Cg2v, Cg2'd, and Cg2'v have a relatively broader layer II than that of Cg1 or Cg1', and its border to layer III is blurry. Layer VI can be subdivided into a cell sparser VIa and cell denser VIb in Cg2d and Cg2'd, but not in Cg2v or Cg2'v. The most conspicuous differences between anterior (i.e., Cg1, Cg2d, Cg2v) and posterior (i.e., Cg1', Cg2'd, Cg2'v) subdivisions of the cingulate cortex are found in the densities of their M_2 receptors, which will be described below.

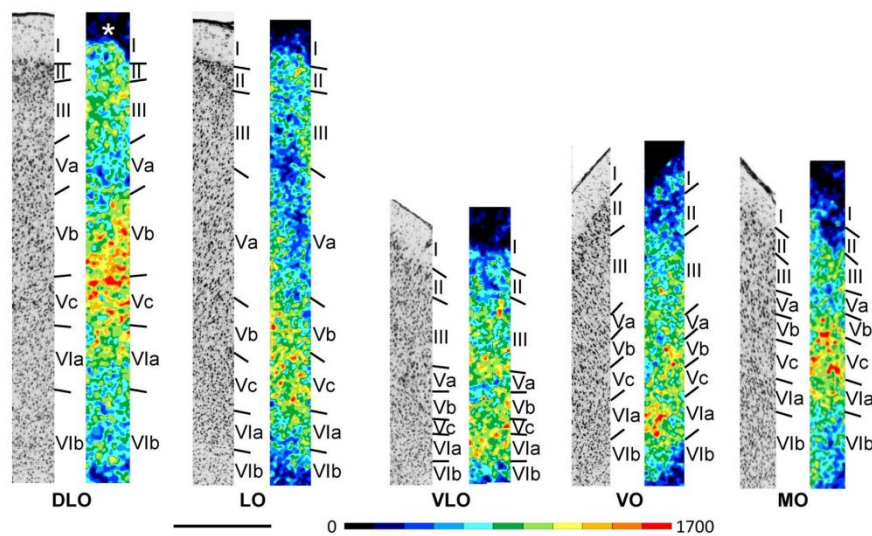


Fig. 6 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and of M_2 receptor binding sites (right side) in areas DLO, LO, VLO, VO, and MO of the rat orbital cortex. Scale

bar 500 μ m. Roman numerals indicate cortical layers. Asterisk indicates missing tissue in layer I of DLO

In the rat orbital cortex (Figs. 2A and 6; Supplementary Material, levels c2–c7, s4–s5, h5–h7), it is difficult to provide data on absolute cortical thickness and the relative width of the different layers. The real width of the cortex and its layers cannot be measured in the histological sections because the plane of sectioning runs obliquely to the cortical surface due to the bending of this cortical region. Layer II of all orbital areas has a higher cell packing density compared to layer III, this difference in cell density is least prominent in MO and VLO. Sublayer Va contains the largest pyramidal cells of all orbital cortical areas, and sublayer Vb a lower cell packing density than that of Vc. Layer VI has two parts: sublayer VIa with a high cell packing density and sublayer VIb with a low packing density. A clear delineation of the different orbital areas is difficult in cell-body stained sections. Therefore, the presented parcellation of these areas is mainly based on differences in the expression of M_2 receptors.

Receptor architecture

The dorsolateral frontal, cingulate and orbital regions can be easily delineated from each other based on differences in mean and laminar M_2 receptor densities (Figs. 3, 4, 5 and 6). Area Fr3 presents a lower mean M_2 receptor density than that of laterally adjacent orbital area DLO, and this difference is particularly obvious in deeper layer V. The

M_2 receptor density in Fr2 is lower than that of medially adjacent cingulate area Cg1, particularly due to the higher supragranular density in Cg1 than in Fr2. Orbital area MO contains a clearly lower M_2 receptor density than either Cg1 or Cg3.

In the dorsolateral frontal cortex (Figs. 2B and 4; Supplementary Material, levels c2–c20, s3–s4, h1–h6), the mean M_2 receptor density is higher in Fr2 than in Fr1 or Fr3 (Fig. 3), and this is particularly true for layer Vc (Fig. 4). In all Fr-areas, layer Vb shows the highest, and layers VIb and VIc the lowest M_2 receptor density. Layer IV is distinguishable in all three areas by its higher density than that of adjacent layers, particularly of Va, though this difference is least prominent in Fr1 and most clearly visible in Fr3. Fr3 differs from Fr1 and Fr2 by the lowest receptor density in the superficial part of layer V. Within frontal areas Fr1 and Fr2, an increasing gradient in the density of M_2 receptors was observed when moving caudally, that could be explained by motor somatotopic organization.

In the cingulate cortex (Figs. 2B and 5; Supplementary Material, levels c4–c16, s4–s5, h1–h5), the highest and lowest mean density of M_2 receptors is found in Cg3 and Cg1', respectively (Fig. 3), and this is particularly obvious in layer Vb (Fig. 5). Whereas in areas Cg1, Cg2d, and Cg2v layer Vb contains a higher M_2 receptor density than layer III, the opposite holds true for their caudal counterparts

Cg1', Cg2'd, and Cg2'v, respectively. Interestingly, Cg3 has equally high receptor densities in layers III and Vb. The densities of layers I and II of Cg3 are similar. In the infra-granular stratum of Cg3, layer V shows a higher density than layer VI in all cingulate areas.

All areas of the orbital cortex (Figs. 2B and 6; Supplementary Material, levels c2-c7, s4-s5, h5-h7) present a lower M_2 receptor density in layer Va than in layers Vb or Vc, and this difference is particularly obvious in LO. The most lateral of the orbital areas, DLO, contains the highest mean M_2 receptor density, and medially adjacent area LO the lowest (Fig. 3). VLO differs from all other orbital areas by its layer III receptor density, which is comparable to that found in its layers Vb and Vc. Layer Vc of MO contains a higher M_2 receptor density than that of laterally adjacent VO.

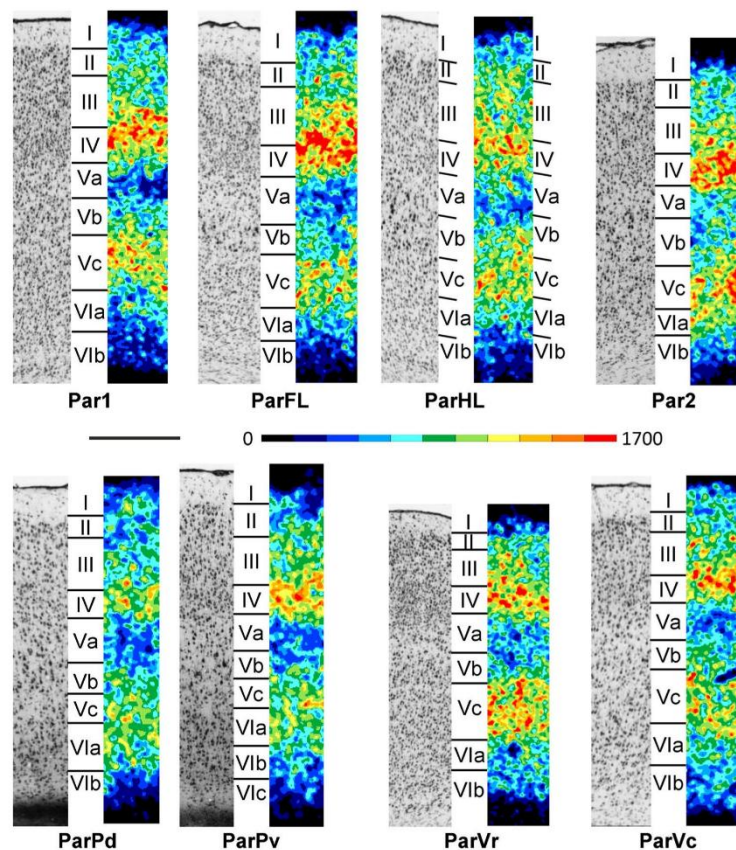
Parietal cortex

Topography

The parietal isocortex of the rat occupies more than half of the dorsolateral surface of the hemisphere (Fig. 1A–C). It is delimited rostro-medially by the frontal cortex and ventrally by the insular cortex. It has common borders with the temporal cortex postero-laterally, the occipital cortex postero-dorsally, and cingulate area Cg1' and the retrosplenial agranular cortex medially (Fig. 1A–C). The parietal cortex can be divided into three regions:

- A large central parietal region comprising the highly granular primary somatosensory cortex with areas Par1, ParFL (forelimb area) and ParHL (hindlimb area), and the second somatosensory cortex with area Par2. Par1 is delimited rostrally by Fr3, ventrally (from rostral to cau-

Fig. 7 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and M_2 receptor binding sites (right side) in parietal areas of the rat brain. The magnification for Par1 was taken from the region of the upper lip representation (Par1ULp). Roman numerals indicate cortical layers. Scale bar 500 μ m



dal) by dysgranular insular area DI, rostrally and caudally by ventral parietal areas (ParVr and ParVc, respectively) and Par2. Dorsally, Par1 shares borders with ParFL and ParHL, whereas caudally it is delimited by posterior parietal areas ParPd and ParPv as well as temporal area Te3r. ParHL occupies a thin strip of cortex on the dorsolateral portion of the hemisphere medial to ParFL and lateral and caudal to Fr1. ParHL is delimited caudally by ParPd. Par2 is surrounded by Par1, ventrocaudal parietal area ParVc and temporal area Te3r.

- A posterior parietal region, ParP, with dorsal and ventral subdivisions (ParPd and ParPv, respectively), which occupies a narrow strip of cortex running in a dorsoventral direction caudal to the primary parietal areas Par1 and ParHL. ParPd is located lateral to medial occipital area Oc2ML and is delimited medio-caudally by lateral occipital area Oc2Lr, and ventrally by Par1 and ParPv. Oc2Lr is found dorsal to ParPv, which is located rostral and dorsal to temporal area Te3r.
- A ventral parietal region between the central parietal region and the insular cortex comprises rostral area ParVr and caudal area ParVc. ParVr is bordered rostrally and ventrally by Par1 and granular insular area GIa, respectively, and is followed caudally by ParVc, which abuts the ventrally located granular insular area GIp and caudally located areas Te3r and EctD.

Cytoarchitecture

In general, a well-developed layer IV is the most conspicuous cytoarchitectonic feature of all areas of the isocortical parietal region (Fig. 7). Additionally, layer V is divided into three sublayers: Va shows the lowest and Vc the highest packing density of all three sublayers; Va presents small to medium sized pyramidal cells, Vb contains the largest and Vc the smallest pyramids. The entire layer V is broader and contains more sparsely packed and larger neurons than layer VI. Layer VI is subdivided into a sublayer VIa with more and larger cells than VIb. The thickness of the cortical ribbon of the parietal cortex notably varies only slightly between its areas, whereby ParVr is clearly narrower than the remaining areas (Figs. 2A and 7; Supplementary Material, levels c6-c25, s1-s3, h1-h7).

The areas of the central region show the prototypical cytoarchitecture of the primary sensory cortex in the rat brain, i.e. a wide granular layer IV (Figs. 2 and 7; Supplementary Material, levels c6-c25, s1-s3, h1-h7). This layer can be delineated from layers II-III by its distinctly higher cell packing density. Layer IV also differs from sublayer Va, which appears as a pale stripe with a low packing density of small to medium sized pyramidal cells. The thickness of layer IV changes in the different parts of Par1, and thus may lead to further somatotopically-related subdivisions such as

that of the barrel field (Par1BF; Supplementary Material, levels c16-c23). Within Par1BF, layer IV is characterized by a patch-like appearance due to modular differences in the packing density of granule cells (Supplementary Material, levels c16-c23). Area Par2 also has a conspicuous layer IV, though less prominent than in Par1, and with slightly larger cells. Additionally, layers III and V of Par2 are cell sparser than those of Par1 (Fig. 7; Supplementary Material, levels c13-c21). Furthermore, layer V pyramids of Par2 are larger than those of Par1. Layer IV of ParFL and ParHL is less prominent compared to that of Par1, and this is particularly true for ParHL. Layer Vb is thicker in ParHL than ParFL, reaches a slightly higher cell density, as does layer Va, and contains larger pyramids (Fig. 7; Supplementary Material, levels c8-c20).

The posterior parietal region contains areas ParPd and ParPv, which present a narrower supragranular and a broader infragranular layers than the rostrally adjoining parietal areas (Supplementary Material, levels c21-c25, s3, h1-h4). Furthermore, posterior parietal areas are characterized by a low cell packing density in layer II, which is considerably broader in ParPv than in ParPd (Fig. 7). Layer IV is not conspicuous, and this is particularly true for ParPd. Layers Va and Vc are more cell sparse in ParPd than in ParPv. Layer VI presents evenly spaced middle-sized neurons in both ParPd and ParPv.

The ventral region consists of areas ParVr and ParVc (Supplementary Material, levels c21-c25, h7). This region is characterized by a relatively thin supragranular layers, which distinguishes it from the central parietal region, particularly from Par2 (Fig. 7). Layer II is more easily separable from layer III in ParVc than ParVr because of the lower cell

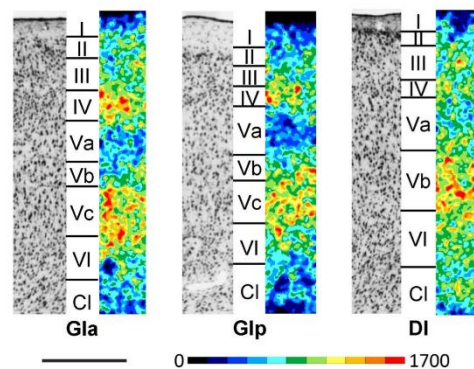


Fig. 8 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and M_2 receptor binding sites (right side) in areas GIa, GIp, DI, AIa, AIv and AIp of the rat insular cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m

packing density in layer III of ParVc. ParVr has a higher cell density in layers IV and VI than does ParVc. Layer V of ParVc contains more sparsely distributed and larger neurons than that of ParVr. Layers Va, Vb and Vc are more conspicuous in ParVr than in ParVc. ParVr differs from Par2 by its slightly higher cell density in the supragranular layers. ParVr can be distinguished from the ventrally adjoining insular cortex (area GIa; Fig. 8) by its higher cell density in sublayer Va and smaller cells in sublayers Vb/c. ParVc is difficult to segregate cytoarchitecturally from GIp, but the receptor distribution between both areas is clearly different (see below).

Receptor architecture

The parietal region mainly differs from the rostrally adjoining frontal region by its higher density of M_2 receptors in layer IV (Fig. 2; Supplementary Material, levels c6–c20, s3, h1–h4). Additionally, receptor density is lower in layer Va of the parietal compared with the frontal areas. Layer Va of the parietal cortex is also wide and clearly visible as an obvious stripe of low density (Fig. 7). The temporal cortex has a generally lower receptor density in layer IV than the parietal cortex (Supplementary Material, levels c20–c32, s1–s2, h3–h7). The low-density stripe in layer Va is even more obvious when progressing from the parieto-frontal border to the insular cortex (Fig. 2B; Supplementary Material, levels c6–c18). The occipital cortex shows a lower density in layer IV than the parietal cortex, with the notable exception of the anterior occipital areas (Supplementary Material, levels c20–c25, s3, h1–h3).

All areas of the *central parietal region* show a trilaminar receptor density pattern in layer V, with a very low density in layer Va, a moderate one in layer Vb and a relatively high density in layer Vc (Figs. 2B and 7; Supplementary Material, levels c6–c23, s1–s3, h1–h7). Although somatotopic representations within Par1 are associated with variations of M_2 receptor densities in layer IV (Supplementary Material, levels c6–c23, s1–s3, h1–h7), in general terms these values are higher than those found in layer IV of the adjacent areas. Sublayer Vb of ParHL has a higher density than that of ParFL. Layer IV of Par2 also presents slight variations in M_2 receptor densities, though not as conspicuous as those seen in layer IV of Par1.

Areas of the posterior parietal region are characterized, as those of the central and ventral regions, by a broad band with a low M_2 receptor density which corresponds with cytoarchitectonic layer Va (Fig. 7; Supplementary Material, levels c21–c24, s2–s3, h1–h4). ParPd and ParPv are also characterized by the lowest overall concentrations within the parietal region, and this is particularly true for ParPv (Figs. 3, 7).

Areas ParVr and ParVc of the ventral parietal region are receptor architecturally similar to Par2, but their layer IV

receptor density is slightly lower (Fig. 7). Furthermore, the difference in receptor densities between supragranular and infragranular layers is not as large in ParVr and ParVc as in the remaining parietal areas. Mean M_2 receptor density is higher in ParVc than in ParVr (Fig. 3).

Insular cortex

The insular cortex, also known as claustrum, contains granular to dys- and agranular areas (GI, DI and AI, respectively), thus encompassing isocortical (GI), proisocortical (DI), and periallocortical (AI) areas (Fig. 1A). All insular areas are underlied by the claustrum.

Topography

The insular cortex of the lissencephalic rat is located on the ventrolateral surface of the hemisphere, neighbours the anterior part of the rhinal sulcus, and extends from the orbitofrontal cortex rostrally to the perirhinal and ectorhinal cortices caudally (Fig. 1A). It is bounded dorsally mainly by ventral parietal areas ParVr and ParVc. However, at its most rostral portion, it shares a small border with areas Fr3 and Par1. Ventrally, the insular cortex is delimited by the piriform cortex. The insular cortex can be divided dorsoventrally into three longitudinal stripes according to its cytoarchitectonic criteria:

- A dorsal granular strip which can be further subdivided into an anterior area (GIa) located below ParVr and a posterior, slightly larger, area (GIp) found below ParVc. GIp is delimited caudally by the ectorhinal cortex.
- A middle dysgranular strip composed of a single area (DI), which in its most rostral portion wraps around GIa and thus also shares a (very short) common border with Par1.
- A ventral agranular part adjacent to the piriform cortex. The rostral two-thirds of the agranular insular cortex are further subdivided into a dorsal (AId) and a ventral (AIV) agranular cortex, but the posterior third constitutes a single region (AIP). Thus, AId is placed between DI dorsally and AIV ventrally. AIV wraps around the rhinal fissure, including both its dorsal and ventral banks. Areas AId, AIV and AIP belong to the periallocortex and will therefore not be further discussed in this study.

Cytoarchitecture

The granular insular cortex (GI) has a well-developed layer IV, which is visible as a dark band of closely packed granular cells which is in sharp contrast to the cell sparse superficial

part of layer V directly beneath it (Figs. 2A and 8; Supplementary Material, levels c6-c18, s1-s3, h5-h6). Layers II, III and IV of GI tend to become narrower when they approach the border of the ventrally placed DI. These layers have a higher cell packing density in GIa than GIp, and layer I is broader in GIp than GIa. Furthermore, layer Vb pyramids are smaller in GIp than in GIa. Thus, interface between layers Vb and Vc is easier to identify in the rostral than in the caudal portion of GI.

The cytoarchitecture of DI is comparable to that of AI, but an incipient layer IV can be defined by the presence of some scattered granular cells at the interface between layers III and V, which contain large pyramidal neurons (Fig. 8). The border between layers II and III of DI is blurry, and the very thin and discontinuous layer IV of DI gradually disappears when it reaches the border with the agranular insular cortex. Layer V has two distinct parts, a superficial layer Va with a lower, and a deep layer Vb with a higher cell density.

Receptor architecture

Insular areas can be distinguished from the dorsally and ventrally adjacent parietal and piriform cortex, respectively, by their higher M_2 receptor density in the deeper layers (Figs. 2B; Supplementary Material, levels c6-c18, s1-s3, h5-h6). Interestingly, granular, dysgranular and agranular insular areas differ in their M_2 laminar distribution patterns (Fig. 8). GIa and GIp present comparably high M_2 receptor densities in layers IV and Vc, and a conspicuously lower density in layer Va. The mean M_2 receptor density of GIa is higher than that of GIp. The M_2 receptor density of layer IV in DI is clearly lower than that of Vb, and only slightly higher than that of Va.

Temporal cortex

Five isocortical areas have been defined within the temporal cortex: primary auditory area Te1 and secondary auditory areas Te2d, Te2v, Te3r and Te3v.

Topography

The temporal cortex occupies the posterior third of the dorso-lateral surface of the rat cerebral hemisphere (Fig. 1A and C). It extends between the occipital and ecto-rhinal cortex and lies caudal to ParVc, and caudo-ventral to Par1, Par2 and ParPv. Area Te1 occupies a central position in the anterior half of the temporal cortex and is completely surrounded by the secondary temporal cortex, which encompasses the subdivisions of areas Te2 and Te3. Area Te3r is found rostral and dorsal to Te1, and is delimited rostrally (from ventral to dorsal) by ParVc, Par2 and Par1, rostro-dorsally by ParPv, and dorsally by occipital area Oc2Lr. Area Te3V is ventral to Te1, dorsal to ecto-rhinal area EctD, and delimited rostrally and caudally by Te3r and Te2v, respectively. Area Te2d is found dorso-caudal to Te1; it is delimited by Te3r rostrally, and by lateral occipital areas Oc2Lid, Oc2Liv and Oc2Lc dorsally. The most posterior portion of Te2d shares a common border with post-rhinal area PoRhD. Area Te2v is delimited rostrally by Te3v and ventrally mainly by ecto-rhinal area EctP, although most rostrally it shares a border with EctD.

Cytoarchitecture

Cortex of the temporal region is characterized in general by narrower supragranular than infragranular layers and the presence of a prominent layer IV (Fig. 9; Supplementary Material, levels c20-c32, s2-s2, h3-h7). Layer V contains large pyramidal neurons and can be subdivided into sublayers Va with a lower, and Vb with a higher cell

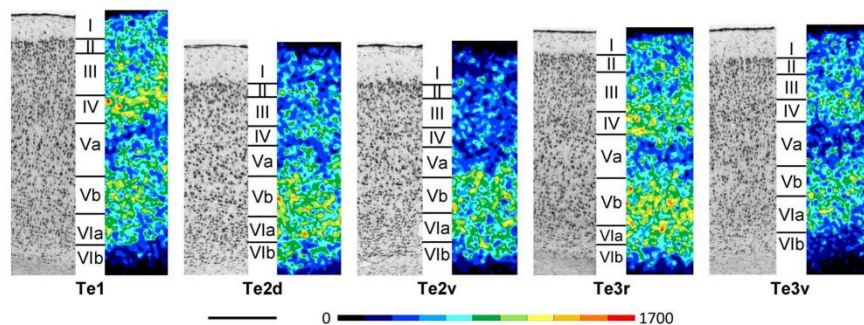


Fig. 9 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and M_2 receptor binding sites (right side) in areas Te1, Te2d, Te2v, Te3r and Te3v of the rat temporal cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m

packing density. Layer VI contains smaller neurons than layer V and can also be subdivided into sublayers VIa with a higher, and VIb with a lower cell packing density. The border between layers II and III is faint and definable based on a difference in neuronal size rather than cell packing density. Layer IV of Te1 is broader and more densely packed than that of Te2 or Te3. Layers III and V of Te1 present a higher and lower cell packing density, respectively, than those of the remaining temporal areas. Layer III pyramids are larger in Te1 than in Te2. Layer V pyramids are larger in Te2d than in Te2v, but smaller than those of Te3r or Te3v. The latter areas differ by their layer Vb, which is more densely packed in Te3r, as well as their layers IV and Va, which are more densely packed in Te3v.

Receptor architecture

In general, the density of M_2 receptors decreases in both a dorsoventral and a rostro-caudal direction in the temporal cortex (Fig. 9; Supplementary Material, levels c22–c32,

s2–s2, h3–h7), so that Te2d contains the highest and Te3v the lowest mean density values (Fig. 3). Layer IV of Te1 and Te3r presents a conspicuously higher M_2 receptor density than that of areas Te2d, Te2v or Te3v. The deeper layers of Te3r present a higher M_2 receptor density than those of Te1, but the superficial layers of Te1 contain more M_2 receptors than those of Te3r or Te3v. Te3r differs from caudally adjacent Te2d by the lower M_2 receptor density in the latter region, particularly in its supragranular layers. Whereas the superficial and deep layers of Te3v contain comparable M_2 receptor densities, in caudally adjacent Te2v the deeper layers present a higher concentration of M_2 receptors.

Occipital cortex

Topography

The occipital isocortex is located on the caudal third of the dorsal and dorsolateral surfaces of the hemisphere, and close

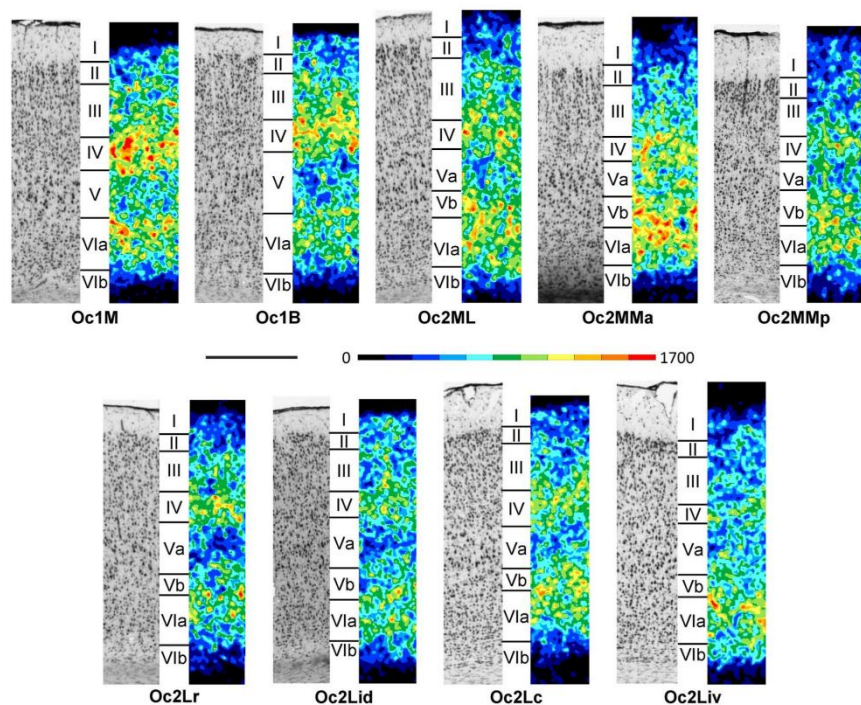


Fig. 10 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and M_2 receptor binding sites (right side) in areas Oc1M, Oc1B, Oc2ML, Oc2MMa, Oc2MMp, Oc2Lr, Oc2Lid, Oc2Lc, Oc2Liv,

Oc2Lc and Oc2Liv of the rat occipital cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m

to the occipital pole also encroaches onto its medial surface (Fig. 1A–C). It can be divided into a primary occipital cortex (Oc1), which is surrounded by the secondary occipital cortex (Oc2). Oc1 encompasses the monocular primary visual area (Oc1M) and laterally adjacent binocular primary occipital area (Oc1B). Oc2 can be divided into *three medial* (Oc2ML mediolateral secondary occipital cortex, and Oc2MM mediomedial secondary occipital cortex with anterior and posterior portions [Oc2MMA and Oc2MMp, respectively]) and four lateral areas (Oc2Lr rostrolateral secondary occipital cortex, Oc2Lc caudolateral secondary occipital cortex, Oc2Lid intermediate dorsolateral secondary occipital cortex, and Oc2Liv intermediate ventrolateral secondary occipital cortex).

Medial to the rostral tip of Oc1 lies area Oc2ML, which is delimited rostrally by frontal area Fr1, medially by areas Oc2MMA and Oc2MMp, and laterally (from rostral to caudal) by areas ParPd, Oc2Lr and Oc1B. Oc2MMA is delimited rostrally by Fr1 and Fr2, medially by the agranular retrosplenial cortex (RSA) and caudally by Oc2MMp, which occupies a long strip of occipital cortex extending between RSA and Oc1M.

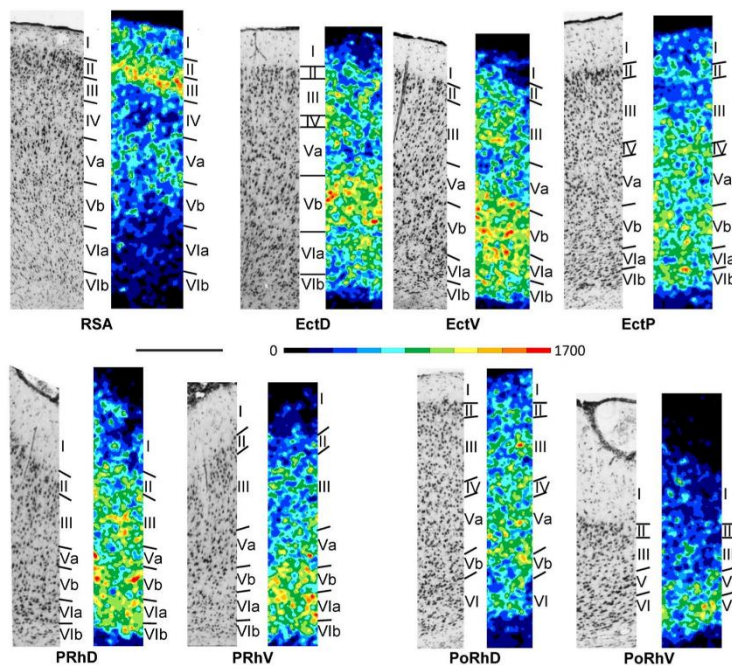
Occipital cortex lateral to Oc1B is occupied mainly by areas Oc2Lr and Oc2Lc, which are separated from each other by the small wedge-shaped area Oc2Lid. Thus, Oc2Lid

is delimited medially by Oc1B, rostrally by Oc2Lr, laterally by Te2d, and latero-caudally by Oc2Lc. A further small occipital area, Oc2Liv, is intercalated between Oc2Lc and temporal area Te2d. Area Oc2Lr abuts ParPd rostrally, and shares common borders medially with Oc2ML and Oc1B, and laterally with areas ParPd, ParPv and Te3r. The most rostral portion of Oc2Lc is delimited medially by Oc2Lid, and laterally (from rostral to caudal) by Te2d and Oc2Liv. The caudal half of Oc2Lc is located between Oc1B and Te2d.

Cytoarchitecture

Areas of the occipital cortex are characterized in general by one of the broadest layer IV of the rat brain. Within the occipital cortex, Oc1 has the most conspicuous inner granular layer (Fig. 10; Supplementary Material, levels c20–c33, s3, h1–h3). The packing density of granule cells in layer IV of Oc1M is a little higher than that in Oc1B, and cell bodies tend to be generally larger in Oc1M than in Oc1B. Layers II and III are not easily distinguishable from each other in either Oc1 subdivision, but layers II/III are thinner in Oc1B than in Oc1M. Layer V of Oc1M has a lower cell packing density than that of Oc1B.

Fig. 11 Magnifications depicting the laminar distribution pattern of cell bodies (left side) and M₂ receptor binding sites (right side) in the agranular retrosplenial area (RSA), in the dorsal (EctD), ventral (EctV) and posterior (EctP) subdivisions of the ectosplenial cortex, as well as in the dorsal and ventral subdivisions of the perirhinal (PRhD, PRhV, respectively) and postrhinal (PoRhD, PoRhV, respectively) cortex. Roman numerals indicate cortical layers. Scale bar 500 μ m



Areas Oc2ML and Oc2MM of the medial secondary occipital cortex (Fig. 1) have a narrower and less cell dense layer IV than that of Oc1 areas, as well as a sublaminate layer V, with larger pyramids in Va than in Vb (Fig. 10). Granule cells are larger and more densely packed in layer IV of Oc2ML than in those of Oc2Mma or Oc2MMp, and the opposite holds true for layer V pyramids. Layer II is slightly denser than layer III in the three areas, and this difference is more obvious in Oc2ML. The main differences between Oc2Mma and Oc2MMp are the higher cell packing density in layer II of Oc2MMp and the larger layer V pyramids in Oc2Mma.

Areas of the lateral secondary occipital cortex are characterized by relatively small layer III pyramids, resulting in a blurred border with layer IV, and the presence of a layer Va with larger pyramids than those in Vb (Fig. 10). Layer VI can be also subdivided into a sublayer VIa with higher and a sublayer VIb with lower cell packing density and smaller neurons. Layer IV is broadest in Oc2Lc and narrowest in Oc2Liv. Oc2Lc also presents the largest layer Va pyramids of all lateral secondary occipital areas. The overall cell packing density in Oc2Lr is lower than that of Oc2Lid, though this feature is most obvious in Va. In contrast to Oc2Lc, Oc2Liv has a fairly well-defined layer II, which can be distinguished from layer III due to the lower cell packing density in the latter layer.

Receptor architecture

All occipital areas show a bilaminar distribution of the M_2 receptor with one maximum in layer IV and a second one in layer VIa or in layers Vb and VIa (Fig. 10; Supplementary Material, levels c20–c33, s3, h1–h3). Throughout the secondary occipital cortex layer Va presents a lower M_2 receptor density than does Vb. Interestingly, M_2 densities are higher in layer Vb of medial than of lateral Oc2 areas. Medial occipital areas are generally characterized by a higher mean M_2 receptor density than that of lateral occipital areas (Fig. 3). Layers IV, V and VIa of Oc1M present a higher M_2 receptor density than those of Oc1B. In Oc2ML the two M_2 receptor maxima reach comparable values, whereas in Oc2Mma and Oc2MMp layers Vb and VIa present a higher density than does layer IV. Furthermore, M_2 receptor densities are lower in layer Va of Oc2ML than in those of Oc2MM subdivisions. Oc2Mma and Oc2MMp can be identified by the higher density of M_2 receptors in the former than in the latter area. When moving from Oc2Lr over Oc2Lid to Oc2Lc, M_2 receptor densities show an increasing gradient in layer VIa (Fig. 10). Layers III and IV of Oc2Liv present a lower M_2 receptor density than those of Oc2Lc.

Retrosplenial cortex

The retrosplenial cortex constitutes a mesocortical brain region which in the rat encompasses the so-called agranular (RSA) and granular (RSG) retrosplenial areas. RSA is classified as proisocortex, whereas RSG is a periallocortical area, and is thus not subject of the present analysis.

Topography

RSA lies on the dorsomedial surface of the rat hemisphere and is delimited medially by RSG, rostrally by cingulate area Cg1' and laterally (from rostral to caudal) by Fr2 and Oc2MM (Fig. 1B, C). Close to the occipital pole RSA is replaced caudally by area Oc2MM.

Cytoarchitecture

Layer II of RSA is broad and its border with layer III is blurred. Layer IV is dysgranular, with loosely packed medium-sized granular shaped pyramids (Fig. 11; Supplementary Material, levels c17–c32, s3–s5-, h1–h4). Layer V is subdivided into sublayers Va and Vb clearly identifiable by the much larger pyramids in the former than in the latter sublayer. Layer VI of RSA is also sublaminate, whereby VIb contains larger but less densely packed neurons than does VIa.

Receptor architecture

Layer III is characterized by a conspicuously higher M_2 receptor density than any other layer of RSA, thus enabling its clear delineation from layer II (Fig. 11; Supplementary Material, levels c17–c32, s3–s5-, h1–h4). Layers IV–VI present only moderate-to-low M_2 receptor densities, which do not emphasize the sublaminate of layers V or VI.

Ecto-, peri-, and postrhinal cortex

Topography

This region of the rat brain comprises ventrolateral cortex along the posterior half of the rhinal sulcus. It extends in the rostro-caudal direction from the insular cortex to the occipital pole. The rhinal region is bordered dorsally mainly by areas Te3v and Te2v (rostrally and caudally, respectively), except for its most rostral part, which is adjoined by Te3r and ParVc. Ventrally, the rhinal cortex is neighbored from anterior to posterior by the piriform and entorhinal cortices, respectively (Fig. 1A, D). The rhinal cortex can be divided into two longitudinal stripes:

- The dorsal longitudinal stripe extends from the insular cortex to the occipital pole and contains the ectorhinal cortex (Ect), which encompasses isocortical and proisocortical areas.
- The ventral longitudinal stripe wraps around the rhinal fissure, including both its dorsal and ventral banks and is occupied by proisocortical areas. The anterior two-thirds of this stripe constitute the perirhinal cortex (PRh). Its posterior third is occupied by the postrhinal cortex (PoRh).

The anterior half of the length of the ectorhinal cortex (Ect) is further subdivided into a dorsal (EctD) and a ventral ectorhinal area (EctV). Its posterior half is formed by a single posterior ectorhinal area (EctP). Dorsally EctD is mainly delimited by Te3v, though its most rostral portion borders with Te3r and ParVc, and its most caudal portion abuts Te2v. The ventral ectorhinal cortex (EctV) lies between EctD and PRh. PRh can be further subdivided into a dorsal part (PRhD), which lies ventral to EctV and EctP, and a ventral part (PRhV), which is located dorsal to the piriform (part of the allocortex) and entorhinal (part of the periallocortex) cortex. *PoRh* can also be further subdivided into a dorsal part (PoRhD), which lies mainly ventral to EctP except for its most caudal part, which neighbours Te2v and then Te2d, and a ventral part (PoRhV), which is located dorsal to the entorhinal cortex. The postrhinal cortex rises steeply and wraps obliquely around the caudal pole of the hemisphere. Thus, its detailed analysis is difficult to perform in coronal sections because most of it is obliquely arranged and thus, tangentially sectioned.

Cytoarchitecture

Layer IV in the ectorhinal cortex is generally inconspicuous and fades away when moving ventrally (Fig. 11; Supplementary Material, levels c19–c32, s1–s2, h4–h7). Thus, EctD and EctP can be classified as a dysgranular isocortical areas, whereas EctV is agranular and part of the proisocortex. EctD has a broader but less densely packed layer II and a slightly more prominent layer IV than does EctP. Layer III of EctP is broader than that of EctD and characterized by a slight increasing gradient in the packing density of its pyramids. Pyramids in layer Vb of EctD present a slight columnar organization, which is not visible in the corresponding layers of EctV or EctP.

The perirhinal cortex is agranular and differs from the ectorhinal cortex mainly by its thinner layer V and broader layer I (Fig. 11). Additionally, layers II and III are distinctly separated in the ectorhinal cortex, whereas in the perirhinal cortex the border between them is blurred. PRhD differs from PRhV by its patchy layer II (its cells form clumps), lower packing density of neurons in the deep part of layer III (which gives

the appearance of a cell-sparse gap between layers III and Va) and smaller pyramidal neurons in layers Va, Vb and VIa.

The postrhinal cortex contains a dorsal dysgranular area PoRhD and a ventral agranular area PoRhV. Both areas differ from the perirhinal cortex by their thicker layer II, higher packing density in layers V and VI, and smaller pyramidal cells in layer V (Fig. 11).

Receptor architecture

The ecto-, peri and postrhinal cortex can also be divided into distinct areas when analysing the density and laminar distribution pattern of M_2 receptors (Fig. 11; Supplementary Material, levels c19–c32, s1–s2, h4–h8). Within this brain region, area PoRhD presents the highest, and PoRhV the lowest mean M_2 receptor density (Fig. 3). In EctD and EctV, sublayer Va appears as a distinct low-density band in M_2 receptor autoradiographs; layers II–III (and IV when present) above this low-density band show a moderate and homogenous receptor density, which cannot be subdivided into distinct layers. In both areas sublayer Vb shows a high receptor density. The most prominent difference between EctD and EctV is found in layer VIa, which presents a higher receptor density in the latter than in the former area. Furthermore, in EctV sublayer VIa shows a higher receptor density than sublayer VIb, whereas EctV presents a homogenous receptor density throughout layer VI. EctP contains a lower receptor density, particularly in layer Vb, compared to EctD and EctV.

EctD and EctP differ from Te2v by its lower M_2 receptor density in the infragranular layers. ParVc and Glp can be distinguished from EctD by their very prominent and high receptor density in layer IV. DI in comparison with EctV shows a higher receptor density in layer Va.

The perirhinal cortex differs from EctV by its laminar distribution of M_2 receptors, particularly in its lower density in layers Va and VIb. The most obvious difference between PRhD and PRhV is the lower receptor density in layer III of PRhV than PRhD.

The postrhinal cortex differs from the perirhinal cortex by an overall lower receptor density, which is particularly obvious in layers V–VI. PoRhD has a higher receptor density in layers I–V, but a lower density in layer VI compared to PoRhV.

Discussion

The expression of transmitter receptors is a powerful tool for mapping the cerebral cortex in the human brain (for a comprehensive review see (Palomero-Gallagher et al. 2015)). The pattern of the regionally heterogeneous receptor distribution

frequently matches cytoarchitectonic subdivisions, or can lead to even finer parcellations. Particularly, the cholinergic muscarinic M_2 receptor is an excellent indicator of cortical segregation, since it not only shows borders of cortical areas at exactly the same places as cytoarchitectonic analysis, but can additionally distinguish between cortical types (e.g. isocortex vs. allocortex and sensory vs. motor vs. association cortex), indicate the hierarchical position of an area within a specific functional system, and provide valuable information on the evolutionary constancy of cholinergic neurotransmission in primary sensory areas (Palomero-Gallagher and Zilles 2019; Zilles and Palomero-Gallagher 2017a, b). Since the muscarinic M_2 receptor is an important molecule for improving the signal-to-noise ratio in cortical areas (Lucas-Meunier et al. 2003; Sillito and Kemp 1983), this receptor is also an important modulator of brain functions. The relationship between the distribution of multiple neurotransmitter receptors, including that of the M_2 receptor, and the cyto- and myeloarchitectonic segregation throughout the rat isocortex has been subject of previous studies (Palomero-Gallagher and Zilles 2004, 2015). However, these studies were based on a sparser covering of the isocortex than that chosen for the present study and did not comprehensively cover the proisocortex. Thus, our knowledge of the cortical distribution of the M_2 receptor in the rat brain remains incomplete. The present study maps for the first time the regional and laminar distributions of this receptor in serial sections through the rat entire isocortex and its neighbouring proisocortical areas. It also serves as a validation of cytoarchitectonic mapping.

For the areas described in this study, we have coined a relatively simple nomenclature which builds on topographical relationships as initially proposed by Zilles (1985) and expanded in Palomero-Gallagher and Zilles (Palomero-Gallagher and Zilles 2004, 2015), which strictly avoids any functional implications. In the past, various nomenclatures have been used for the cortical areas of the rat (Palomero-Gallagher and Zilles 2015; Paxinos and Watson 1986, 2013; Swanson 2004; Zilles 1985). Different names for the same cortical area were applied, which emphasized either its histological, topographical or functional aspect. This can lead to confusions and misinterpretations because the location of the functional data is frequently not mapped to architectonical findings in the same brain. Since the present observation is solely based on structural (cyto- and chemoarchitectonic) data, we apply a nomenclatural system which is based on the simple topographical division of the rat cerebral cortex in frontal, orbital, insular, cingulate, parietal, temporal, rhinal and occipital regions without inferring functional implications by the name of the areas. This does not mean, however, that the areas identified in the present study cannot be tentatively interpreted by comparing the structural with functional data (see below).

Frontal cortex

Dorsolateral frontal areas Fr1, Fr2 and Fr3 of the present study were found to be dysgranular, i.e. an incipient inner granular layer (layer IV) is clearly identifiable, and is most prominent in Fr3. Therefore, areas Fr1–Fr3 do not match the typical agranular appearance of motor cortical areas in primates. Interestingly, granularity of the rodent motor cortex has been subject of debate, as has that of the primate motor cortex (Garcia-Cabezas and Barbas 2014). Although some authors consider the rodent motor cortex to lack a layer IV (Beaulieu 1993; Donoghue and Parham 1983; Donoghue and Wise 1982; Swanson 2004), our identification of an incipient layer IV in areas Fr1–Fr3 provides further support for the classification of this brain region as being dysgranular in nature (Cho et al. 2004; Krieg 1946a; Palomero-Gallagher and Zilles 2015; Skoglund et al. 1997; Yamawaki et al. 2014; Zilles and Wree 1995).

Lateral frontal areas Fr1 and Fr3 and medial frontal area Fr2 are comparable to the lateral precentral (PrCl) and medial precentral (PrCm) areas of Krettek and Price (1977), respectively, by location and extent, and were identified as motor areas. Interestingly, Swanson (2004) classifies this cortex as somatomotoric in nature, as PrCl and PrCm correspond to his primary somatomotor (MOp) and secondary somatomotor (MOs) areas, respectively. Donoghue and Wise (1982) named these two parts of frontal cortex lateral agranular (AGl) area and medial agranular (AGm) area according to their architectonic appearance as agranular cortex, whereas Paxinos and Watson (2013) identified three different architectonical fields in the frontal cortex of the rat and used a mixture of functional (primary motor, M1 and secondary motor, M2) and topographical (Fr3) nomenclatural terms (Table 1).

AGl and AGm have been considered as parts of the primary motor cortex because both project to the spinal cord (Wise et al. 1979). However, stimulation evoked movements revealed representation fields for all body parts in AGl but not in AGm (Donoghue and Wise 1982; Neafsey et al. 1986). Neafsey et al. (1986) showed that the part of AGm which extends rostral to AGl is probably the premotor and supplementary motor cortex, while the part of AGm which lies medial to AGl is the frontal eye field and the vibrissae motor area. Thus, the frontal eye field, which is located in the medial frontal cortex, would occupy the posterior part of our Fr2, whereas its anterior part would contain the premotor and supplementary motor cortex. Although Donoghue and Wise (1982) and Neafsey et al. (1986) noticed that there are some features within AGl that would allow its subdivision into two parts, they proposed to consider the cytoarchitectonic differences (agranular vs. dysgranular) as signs of somatotopic subdivisions of the primary motor cortex. This observation is in accordance with our findings

of variations in M_2 receptor densities within Fr1 and Fr2, which we also interpret as reflecting somatotopic motor subdivisions. As in the present analysis, Zilles (1985) and Palomero-Gallagher and Zilles (Palomero-Gallagher and Zilles 2004, 2015) subdivided AGI into two cortical fields, namely Fr1 and Fr3, based first on differences in cyto- and myeloarchitecture, and in the latter studies also including a receptor architectonic analysis. Fr1 and Fr3 areas would constitute two subdivisions of AGI, and represent two different somatotopic subfields of the primary motor cortex in electrophysiological studies (Donoghue and Wise 1982). The motor head field, including tongue, lips and jaw, are represented in a region of the lateral frontal cortex coextensive with Fr3 in the present map (Fabri and Burton 1991a; Hall and Lindholm 1974; Neafsey et al. 1986; Tandon et al. 2008). The representation field for wrist and digits of the forelimb, called rostral forelimb area (RFA), is located medial to the head field of Fr3 in Fr1 (Neafsey and Sievert 1982). Medial to RFA is the motor representation field of the vibrissae. A second forelimb area for the proximal parts (shoulder and elbow) was found caudal to RFA (Neafsey et al. 1986; Tandon et al. 2008). Trunk, neck and hind-limb are represented in Fr1 caudal to CFA. The medial frontal cortex is represented by Fr2 in the present study. This area is most probably the equivalent of AGm (Donoghue and Wise 1982) and PrCm (Zilles et al. 1980).

In contrast to the situation found in the sensory functional systems, in which the primary areas show the highest density of M_2 receptors, the primary motor cortex (coextensive with our areas Fr1 and Fr3) shows a lower M_2 receptor density than the premotor and supplementary motor cortices (located within our area Fr2). This feature is not unique to the rat brain, but has also been described in the human and macaque monkey brains (Geyer et al. 1998; Rapan et al. 2021; Zilles and Palomero-Gallagher 2017a, b), and thus seems to constitute an evolutionary constant in the molecular organization of networks subserving sensory and motor functions.

Within the cingulate cortex we identified areas Cg1, Cg1', Cg2d, Cg2v, Cg2'd, Cg2'v and Cg3. Area Cg3 is comparable in location to the rostral two thirds of area A32D of Paxinos and Watson (Paxinos and Watson 2013), whereas the caudal third of A32D, together with their A24b, corresponds to our area Cg1. Areas Cg2d and Cg2v constitute dorsal and ventral subdivisions of our previously identified area Cg2 (Palomero-Gallagher and Zilles 2015), and are the equivalent of A24a and A33, respectively, of Paxinos and Watson (2013). Likewise, Cg2'd and Cg2'v are found within our previously described Cg2' (Palomero-Gallagher and Zilles 2015), and correspond to A24a' and A33, respectively, of Paxinos and Watson (2013). We found the distribution of M_2 receptors

to clearly segregate Cg2d and Cg2v from Cg2'd and Cg2'v, respectively, with considerably higher densities in the former than in the latter areas. In the mouse brain areas Cg2d and Cg2v differ from their caudal counterparts in the density and topography of their connectivity patterns (Fillinger et al. 2017, 2018). Vogt and Paxinos (2014) proposed that the portion of rodent cingulate cortex occupied by areas A24a' and A24b' should be considered homolog of the primate midcingulate cortical region (Vogt et al. 2003, 2005). Interestingly, a recent meta-analysis indicates a specific role of rodent midcingulate cortex in the regulation of task monitoring and visuospatial orientation, functions which have also been associated with the human midcingulate cortex (van Heukelum et al. 2020).

In accordance with numerous previously published maps of the rat *orbital cortex* (e.g., (Kondo and Witter 2014; Krettek and Price 1977; Murphy and Deutch 2018; Price 2007; Ray and Price 1992; Van De Werd and Uylings 2008), we identified five proisocortical areas based on differences in cytoarchitecture and M_2 receptor distribution patterns. Interestingly, Paxinos and Watson (2013) only identify four areas, whereby our area DLO is comparable in location and extent to their equally named area, but their MO is smaller than ours, because it is replaced by their area A32V, which reaches much further rostrally than does our area Cg3. Importantly, we identify area VLO between VO and LO, whereas Paxinos and Watson (2013) do not, so that their areas VO and LO share a common border. Based on the location, extent and relative size of these two areas in the map of Paxinos and Watson (2013), it seems that their LO encompasses our areas VLO and LO. It is more plausible that this region of the rat orbital cortex covers three areas (rather than two) given that VO, VLO and LO not only differ in their cytoarchitecture as revealed by classical histological stainings and in their molecular structure as determined by immunohistochemical and receptor autoradiographic analyses (present study; (Kondo and Witter 2014; Linley et al. 2013; Van De Werd and Uylings 2008), but also in their connectivity patterns (Kondo and Witter 2014; Krettek and Price 1977; Murphy and Deutch 2018; Price 2007; Ray and Price 1992).

Parietal cortex

The parietal cortex is named granular (or in parts dysgranular) cortex because it is, histologically, characterized by the presence of a more or less prominent inner granular layer (layer IV). The parietal cortex receives, processes, integrates and interprets sensory information pertaining touch, vibration, pain and temperature from skin and joints, and thus constitutes the somatosensory cortex of the rat (for a comprehensive review see (Ebner and Kaas 2015).

The central parietal region

Among the three currently existing anatomical rat brain atlases (Paxinos and Watson 2013; Swanson 2004; Zilles 1985), that of Swanson (2004) has the simplest parcellation for parietal cortex; which he divided into two areas: SSp (primary somatosensory) and SSs (secondary somatosensory). SSp in Swanson's map encompasses our areas Par1, ParHL and ParFL whereas SSs is equivalent of Par2 in our map. Zilles (1985) defined four areas in the central parietal region: Par1, FL, HL and Par2, whereby areas FL and HL are equivalent of ParFL and ParHL in our map. Paxinos and Watson (2013) provided the most detailed of the three parcellation maps, with identification of all somatotopic representations within the central parietal regions, and although it is an anatomical rat brain atlas, the parcellation and nomenclature systems used are mostly derived from electrophysiological studies (e.g., Chapin and Lin 1984; Dawson and Killackey 1987; Welker 1971). Paxinos and Watson (2013) divided the central parietal region into two main areas: primary somatosensory area S1 and secondary somatosensory area S2, which are equivalent of Par1 and Par2 in our map, respectively. Within S1, they identified and named somatotopic representations for different body parts based on electrophysiological studies, as well as two narrow dysgranular areas which were delineated and named according to histological and topographical features.

Par1 is the topographical equivalent of the electrophysiologically identified S1. S1 in rats, like other mammals, contains a complete and inverted somatotopic representation of the contralateral half of body, located along the mediolateral extension of the parietal cortex (Krubitzer et al. 2011; Santiago et al. 2007). Because of the higher sensory receptor density in the head region, including vibrissae, lips and tongue, there is a huge magnification of the representation for these areas on the parietal cortex which has been denominated "barrel field" (Chapin and Lin 1984; Santiago et al. 2007). This area has a characteristic feature in histological Nissl staining, the so-called "barrels" (Woolsey and Van der Loos 1970; Woolsey et al. 1975), which are separated from each other by cell-sparse dysgranular regions called septa (Santiago et al. 2007; Woolsey and Van der Loos 1970; Woolsey et al. 1975). It is of note that rat's whiskers not only compensate for the poverty of visual inputs to the rat brain (Petersen 2007), but can be used to manipulate objects in order to recognize them (stereognosia), a function for which in primates use their hands (Krubitzer et al. 2011). Par1 is characterized by conspicuously higher M_2 receptor densities in layer IV but lower densities in layer Va than adjacent areas. Furthermore, the somatotopic representations are associated with variations in the density of M_2 receptors in layer IV, which are particularly high in the vibrissae representation area Par1BF. The dysgranular zones described by

Chapin and Lin (1984) can also be clearly identified due to their thinner layer IV, which presents a and slightly higher M_2 receptor density than that of adjacent portions of Par1.

ParFL and ParHL are somatotopic representations of the forelimb and hindlimb within Par1, respectively. However, they are classified as distinct areas, unlike the other sensory somatotopic representations in Par1, because electrophysiological studies have demonstrated an overlap within these regions between sensory and motor representations of the forelimb and hindlimb (Hall and Lindholm 1974; Krubitzer et al. 2011; Santiago et al. 2007).

Par2 is equivalent of S2 in electrophysiological maps (Benison et al. 2007; Brett-Green et al. 2004; Fabri and Burton 1991a; Remple et al. 2003; Welker and Sinha 1972). Electrophysiological studies revealed that S2 lies just below the vibrissae, nose and upper lips representations in S1, and contains a somatotopic representation of the contralateral body which is a mirror reversal of the somatotopic map in S1. Thus, in contrast to S1, the somatotopic map in S2 is upright (Fabri and Burton 1991a; Krubitzer et al. 2011; Remple et al. 2003). Interestingly, although layer IV of Par2 presents variations in M_2 receptor density, they are not as pronounced as those described for layer IV of Par1, and we were not able to establish a correlation with somatotopic representations. This could be due to the fact that receptive fields in S2 are generally larger and less responsive in the anesthetized rat than those in S1 (Krubitzer et al. 2011; Remple et al. 2003). Par2 has a generally lower M_2 receptor density than that of Par1. Indeed, the fact that the primary sensory areas show significantly higher M_2 receptor densities than surrounding areas is an evolutionarily conserved characteristic of mammalian sensory cortices (Zilles & Palomero-Gallagher 2017a).

The ventral parietal region

The ventral parietal region (ParV) and its subdivisions (ParVr and ParVc) were identified and named by Palomero-Gallagher and Zilles (Palomero-Gallagher and Zilles 2004, 2015) based on cyto- and receptor architectonic features. Our ParVr is located within the ventro-rostral portion of area S1 of Paxinos and Watson (2013) and SSp of Swanson (2004). Our ParVc occupies the same topographic location as the visceral area (VISC) of Swanson (2004), and as the most ventral part of area S2 of Paxinos and Watson (2013). We believe that our classification of this band of cortex as belonging to the parietal region rather than to the insular cortex is correct, because ParVr and ParVc do not cover the claustrum. Furthermore, their M_2 receptor distribution pattern, in particular the conspicuously high density in layer IV, is more similar to that of the remaining parietal than of the insular areas. Additionally, our identification further supports the existence of a third representation of the

contralateral body just lateral to S2, which was characterized based on electrophysiological and microinjection tracing studies and named the parietal ventral area (PV; Fabri and Burton 1991a; Remple et al. 2003). The somatotopic map in PV is similar in size and shape to that of S2, but it is inverted, as is the case in S1 (Fabri and Burton 1991a; Krubitzer et al. 2011; Remple et al. 2003). The PV regions of forelimb and hindlimb are continuous with those described for S2, and are found at a location occupied by our area ParVc. The representation area for upper and lower incisors located rostral to S2 and to the upper lip representation in PV (Remple et al. 2003) would be equivalent of our ParVr, which lies ventral to ParI.

The posterior parietal region

Caudal to ParI we identified a posterior parietal region which corresponds to area PTLp of Swanson (2004) and areas PtPD and PtPR of Paxinos and Watson (2013). The posterior parietal region in our map is also in topographical agreement with the posterior parietal cortex of physiological studies, which was found to act as a multimodal association cortex (Lee et al. 2011; Reep and Corwin 2009). There are some homologies between the posterior parietal region of the rat and the posterior parietal cortex of primates in location, functions and connections (Bushara et al. 1999; Reep et al. 1994; Reep and Corwin 2009; Torrealba and Valdes 2008). It receives extensive afferents from dysgranular zones within S1 cortex (Fabri and Burton 1991b; Lee et al. 2011), visual cortex and non-primary sensory nuclei of thalamus such as lateral dorsal, lateral posterior and posterior nuclei (Reep et al. 1994). The posterior parietal cortex has reciprocal connections with retrosplenial, visual and motor cortices (Corwin and Reep 1998; Reep et al. 1994), and is interconnected with Par2 in a topographically organized manner (Olsen et al. 2019). Single neuron recordings in freely behaving rats have demonstrated the involvement of the posterior parietal cortex in navigating the environment (Nitz 2009) and in spatial attention, as has been established for the posterior parietal cortex in primates (Bucci 2009; Corwin and Reep 1998; Reep and Corwin 2009; Torrealba and Valdes 2008). Furthermore, lesions to posterior parietal cortex were used to successfully develop a rat model of human hemi-neglect syndrome (Reep et al. 2004).

Insular cortex

We divided the insular cortex rostro-caudally into two subregions and dorsoventrally into three longitudinal stripes according to cytoarchitectonic criteria and differences in M₂ receptor distribution patterns: a dorsal granular part with areas GIa and GIp, a middle dysgranular part composed of area DI, and a ventral agranular part with areas AId, AIv and

AIp, whereby the granular and agranular cortex can each be divided into rostral and caudal portions. In the primate brain, only the granular part of the insular cortex has been classified as being isocortical, whereas the dysgranular and agranular portions are considered to belong to the proisocortex and periallocortex, respectively (Mesulam and Mufson 1985). Areas GIa and GIp occupy the part of insular cortex identified as gustatory area (GU) by Swanson (2004) and granular insular area (GI) by Paxinos and Watson (2013) and Van De Werd and Uylings (2008), and area DI is the equivalent of the equally named areas of these authors. Our parcellation of AId, AIv and AIp is in complete agreement with that of Swanson (2004) and Paxinos and Watson (2013). The parcellation of the agranular insular cortex by Van De Werd and Uylings (2008) differs slightly in nomenclature, since their areas AId1 and AId2 correspond to our areas AId and AIv, respectively.

The insular cortex plays a crucial role in the processing of interoceptive, gustatory, olfactory, auditory, somatosensory, and nociceptive stimuli, as well as in the coding of emotional and affective states (for a comprehensive review see (Livneh and Andermann 2021)). The cytoarchitectonic segregation of the insular cortex is reflected in its connectivity and functional topography, since two rostro-caudally arranged distinct subregions have been identified, each of which contains granular, dysgranular and agranular areas, which differ in their connectivity patterns (Mathiasen et al. 2015; Shi and Cassell 1998b). The anterior portion of the insula is involved in higher associative sensory functions associated e.g., with food reward value, pain modulation or short-term memory for odour location (DeCoteau et al. 1997; Di Pietro et al. 2004; Jasmin et al. 2003; Ragozzino and Kesner 1999; Saper 2002), whereas the posterior part receives direct input from the gustatory and visceral thalamic nuclei (in particular area DI) and is involved in viscerosensory perception (Cechetto and Saper 1987; Saper 2002; Shi and Cassell 1998b). Shi and Cassell (1998a, b) further subdivided the posterior insular cortex into a more rostrally located “posterior” and a more caudally located “parietal” insular domain based on a differential connectivity with the anterior insular areas and the secondary somatosensory cortex. However, these differences do not seem to be associated with changes in cortical architecture. Although visceral and sensory input from the tongue are topographically organized within area DI, there is a considerable degree of overlap between the representations of these two sensory modalities (Cechetto and Saper 1987; Kosar et al. 1986; McDonald et al. 1999).

Temporal cortex

We here identified a primary temporal area (TeI) in the central position within the anterior half of the temporal cortex and surrounded by secondary and association temporal

cortex (areas Te2 and Te3), which can be further divided into dorsal (areas Te2d and Te3r) and ventral (Te2v and Te3v) components. This differs from our previous map of the temporal cortex (Palomero-Gallagher and Zilles 2015) in that we then identified a further temporal area, TeV, which (as discussed below) in the present map we have redefined as ectorhinal cortex.

Although the three currently existing anatomical rat brain atlases (Paxinos and Watson 2013; Swanson 2004; Zilles 1985) agree in that the temporal cortex includes a “core and belt complex”, there are discrepancies concerning the number, extent and nomenclature of belt areas. Our area Te1 corresponds to Au1 of Paxinos and Watson (2013) and AUDp of Swanson (2004) and is the equivalent of the functionally defined primary auditory cortex. Our Te3r and Te3v cover approximately the rostral two-thirds of areas AuD and AuV of Paxinos and Watson (2013), (2004) respectively, the caudal third being occupied by the most rostral portion of our areas Te2d and Te2v, respectively. Areas Te2d and Te2v continue further caudally along cortex which in the map of Paxinos and Watson (2013) is occupied completely by the posterior portion of their area TeA (Table 1). In the map of Swanson (2004) areas Te3r and Te3v are found at a location which topologically corresponds to his AUDd and AUDv, respectively, whereas Te2d and Te2v would correspond to his AUDpo and the posterior portion of his TEa, respectively. The rostral part of area TeA/TEa of Paxinos and Watson (2013) and Swanson (2004) is occupied by our ectorhinal area EctD (Table 1).

In contrast to the primate brain, where all primary sensory areas are characterized by the highest density of M_2 receptors (Zilles and Palomero-Gallagher 2017a), in the rat temporal cortex M_2 receptor densities decrease continuously in the dorsoventral direction. Thus, Te2d has the highest, Te1 has only moderate, and Te2v has the lowest density of M_2 receptors, and this situation could be due to the considerably higher degree of myelination of Te1 compared to Te2 or Te3 (Palomero-Gallagher and Zilles 2015; Zilles 1985), whereas in the human cortex areas surrounding the primary auditory cortex also present a high degree of myelination (Palomero-Gallagher and Zilles 2019).

Occipital cortex

The occipital cortex contains the visual cortex of the rat and can be divided into a primary occipital area (Oc1) or primary visual area (V1) in the centre of the occipital cortex, which is surrounded by a secondary occipital cortex (Oc2) or secondary visual cortex (V2). Similar to the situation described for the auditory system, while all currently existing comprehensive maps of the rat cortex are in agreement with the relative location of the primary visual

cortex, however, there are considerable differences regarding how the secondary occipital cortex lateral and medial to Oc1 is organized. All maps depict a subdivision of the primary visual cortex into two areas which form two rostro-caudally oriented parallel bands along the posterior third of the dorsal surface of hemisphere: our areas Oc1M and Oc1B correspond to the equally named areas of Zilles (1985) and Palomero and Zilles (2015), areas V1M and V1B of Paxinos and Watson (2013), respectively, and area VISp of Swanson (2004). Area Oc1M, which lies medial to Oc1B, receives visual input from the temporal part of the contralateral visual hemifield just through the contralateral eye and thus constitutes the monocular portion of the primary visual cortex, while Oc1B receives visual input from the nasal part of the contralateral visual hemifield through both eyes, and constitutes the binocular portion of the primary visual cortex (Zilles et al. 1984). As described for the primate brain (Zilles and Palomero-Gallagher 2017a), the rat primary visual cortex is characterized by a considerably higher density of muscarinic cholinergic M_2 receptors. However, in the rat brain these higher densities are restricted to layer IV, the main recipient of thalamo-cortical projections, and which is also particularly targeted by cholinergic innervation (Eckstein et al. 1988).

Analysis of the present dataset, which provides a much tighter sampling of the M_2 receptors than previously available (Palomero-Gallagher and Zilles 2004, 2015), led to the subdivision of Oc2MM into areas Oc2MMa and Oc2MMp, and confirmed the existence of Oc2ML. Oc2Mma and Oc2MMp correspond to area V2MM of Paxinos and Watson (2013), and Oc2ML to their V2ML. Interestingly, our Oc2MMp is comparable to VISpm of Swanson (2004), and our areas Oc2Mma and Oc2ML to his VISpa. Electrophysiological and tracer studies revealed the existence of three retinotopically organized fields medial to the primary visual cortex and with distinct connectivity patterns (Espinoza and Thomas 1983; Montero 1993): posteromedial (PM), anteromedial (AM), and anterior (A) areas, which would be functional correlates of our Oc2MMp, Oc2Mma and Oc2ML, respectively.

The present study also resulted in considerable changes concerning the parcellation and classification of cortex located lateral to Oc1. Whereby at this location we previously only identified a single occipital area, namely Oc2L (Palomero-Gallagher and Zilles 2004, 2015; Zilles 1985), which corresponds to V2L of Paxinos and Watson (2013), we have now defined areas Oc2Lr, Oc2Lid, Oc2Liv and Oc2Lc (Table 1). Area Oc2L of Zilles (1985) encompasses Oc2Lr, Oc2Lc and Oc2Lid, whereas Oc2Lr occupies cortex which we previously identified as ParPC (Palomero-Gallagher and Zilles 2004, 2015). Oc2Lr was previously classified as a parietal area because its layer V pyramids

are clearly larger than those of occipital areas, and only slightly smaller than those of the adjacent parietal cortex. However, the tighter sampling of M_2 receptors revealed that it shares more similarities with occipital than with parietal areas based on relative differences in laminar receptor densities. Most importantly, layer Va of Oc2Lr does not stand out by its conspicuously lower density of M_2 receptors than adjacent layers, as is the case in the parietal cortex. Thus, as is the case in occipital areas, the border between layers Va and Vb of Oc2Lr is not clearly revealed by the M_2 receptor. Furthermore, the considerably lower M_2 density in layer IV of Oc2Lr than of parietal areas further warrants its classification as occipital cortex. Our area Oc2Lr most probably corresponds to rostrolateral visual area (VISrl) of Swanson (2004), whereas our Oc2Lc covers cortex occupied by his intermediolateral (VISli), posterolateral (VISpl) and VISx visual areas. Our area Oc2Lid encompasses the anterolateral (VISal) and mediolateral (VISlm) visual areas of Swanson (2004), whereas our area Oc2Liv covers his anterior laterolateral (VISall) and laterolateral (VISll) visual areas (Table 1). Our parcellation of the lateral occipital cortex is in accordance with the results of electrophysiological and connectivity studies (Espinoza and Thomas 1983; Montero 1993; Olavarria and Montero 1984), which identified antero-lateral (AL), lateromedial (LM) and laterolateral (LL) areas comparable in location to our Oc2Lr, Oc2Lid and Oc2Liv, respectively. We were not, however, able to identify architectonic correlates of their laterointermediate (LI) and posterolateral (PL) areas (Espinoza and Thomas 1983; Montero 1993; Olavarria and Montero 1984), since both areas are located within our Oc2Lc.

Retrosplenial cortex

On the dorsomedial surface of the hemisphere, medial to the secondary occipital area Oc2MM and the most posterior portion of Fr2, we identified RSA, the proisocortical portion of the retrosplenial cortex, and equivalent of A30 of Paxinos and Watson (2013), and RSPagl of Swanson (2004). RSA is involved in spatial learning processes (Pothuizen et al. 2009) and has also been designated as the dysgranular subdivision of the retrosplenial cortex (Pothuizen et al. 2009; Vogt and Peters 1981), since a layer IV is present, although variable in thickness and invaded by layer III and layer V pyramids. RSA presents a conspicuous stripe of high M_2 receptor density in layer III, but very low values in layers IV–VI. The superficial layers of RSA are characterized, together with layers V and IV, by a high density of cholinergic terminals (Eckenstein et al. 1988), and also receive projections from RSG (Tsai et al. 2022), the postsubiculum and the laterodorsal nucleus (van Groen and Wyss 1992).

Ecto-, peri-, and postrhinal cortices

Cortex around (and dorsal to) the posterior half of the rhinal sulcus encompasses areas interconnecting the hippocampal formation and entorhinal cortex with the lateral occipito-temporal and medial prefrontal association cortex, and are involved in memory and sensory perception processing (Burwell and Amaral 1998; Burwell et al. 1995; de Curtis and Pare 2004; Doan et al. 2019; Fernandez and Tendolkar 2006; Hwang et al. 2018). In the primate brain, Brodmann (1909) defined a perirhinal area 35 and an entorhinal area 36 in the vicinity of the rhinal sulcus and mentioned the existence of homologous areas in the rodent brain. Krieg (Krieg 1946b, c) adopted this nomenclature for the rat brain, where he identified two longitudinally oriented strips—36 dorsally and 35 ventrally—with distinct cytoarchitectonic features. Differences in connectivity later resulted in the subdivision of Krieg's area 35 into a rostral portion which was named perirhinal area and a posterior portion designated postrhinal area (Deacon et al. 1983).

Interestingly, the maps of Paxinos and Watson (2013) and of Swanson (2004) depict an entorhinal area (Ect and ECT, respectively) dorsally and a single perirhinal area (PRh and PERI, respectively) ventrally, whereas Burwell et al. (1995) propose a principal rostro-caudal subdivision of this brain region rather than a dorsoventral one. They identified a perirhinal region rostrally which includes a dorsal area 36 and a ventral area 35, and a posterior region composed of the postrhinal area, which they consider to be the rat homolog of macaque parahippocampal areas TF and TH (Burwell et al. 1995). More recently, Burwell (2001) described cytoarchitectonic and histochemical criteria enabling the definition of dorsal and ventral subdivisions within area 35 (35d and 35v, respectively) and the postrhinal cortex (PORd and PORv, respectively), as well as a tripartition of area 36 into one posterior (36p) and two rostral (36d dorsally and 36v ventrally) subdivisions. Our identification of dorsal and ventral subdivisions within the perirhinal and the postrhinal regions is in accordance with areas 35d/35v and PORd/PORv, respectively, identified by Burwell (2001). We delineated three areas within the entorhinal cortex: EctP covering its caudal half, and delimited rostrally by EctD and EctV. Both EctD and EctP are dysgranular, and classified as part of the isocortex, whereas EctV is an agranular proisocortical area (Burwell et al. 1995). Our areas EctD, EctV and EctP correspond to areas 36d, 36v and 36p of Burwell (2001), respectively, whereby EctP extends slightly further caudally than does 36p.

In our previous map (Palomero-Gallagher and Zilles 2015) we had identified cortex now occupied by the entorhinal areas as area TeV, since its M_2 receptor distribution pattern more closely resembles that of the dorsally adjacent temporal areas than that of the perirhinal cortex, and

Brodman (1909) and Krieg (Krieg 1946b, c) had assigned their area 36 to the temporal region, and not to the rhinal region. However, we here decided to apply the widely used term *ectorhinal* because this cortical region seems to share more connectivity and functional similarities with the perirhinal and postrhinal areas than with temporal areas Te2v or Te3v. Indeed, the *ectorhinal* and *perirhinal* areas, but not the temporal association cortex (which roughly corresponds to our areas Te2v, Te2d, and Te3v), are found in the top 20th percentile of the degree, strength, betweenness and closeness centrality measures as revealed by the strength of intra-hemispheric connectivity of rat brain areas (Swanson et al. 2017). Thus, *ectorhinal* and *perirhinal* areas are thought to constitute hubs in the rat macro-connectome together with AIP, the entorhinal cortex, and the lateral and basolateral amygdalar nuclei (Swanson et al. 2017). Functionally, the *ectorhinal* and *perirhinal* areas have been associated in rats with the processing of visual and tactile stimuli (Nishio et al. 2018; Ramos 2014), whereas the temporal association cortex is involved in higher order auditory but not visual processes (Davis and McDaniel 1993; Ellard 1998; Feigin et al. 2021), and is also activated by auditory-driven maternal behaviour (Tasaka et al. 2020).

Concluding, the present study provides a comprehensive parcellation of the entire rat iso- and *proisocortex* based on the analysis of cell bodies and of the muscarinic cholinergic M₂ receptor as a functionally relevant molecule and evolutionarily conserved marker of primary sensory areas (Zilles and Palomero-Gallagher 2017a). We describe the cyto- and M₂ receptor architectonic features enabling the identification of 48 distinct areas across the frontal, parietal, temporal, occipital, cingulate, retrosplenial, orbital, insular, *ectorhinal*, *perirhinal*, and *postrhinal* regions and provide the mean M₂ receptor densities extracted from each of the identified areas. This data, together with the atlas provided in the Supplementary Material, in which we have also labelled components of the hippocampal formation as well as multiple subcortical and brain stem nuclei, constitutes a useful tool for future computational and experimental neuroscientific studies.

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Author contributions HH and AK wrote the manuscript text. HH, AK, LZ and NPG performed the formal analysis. HH, AK, LZ and JH prepared figures. NPG and Karl Zilles (deceased 2020) designed the study. All authors reviewed the manuscript.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors have no conflicts to declare.

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Identification of the Somatotopic Representations in the Rat Parietal Cortex (Supplementary Results for Haghiri, Kuckertz et al. 2023, Brain Structure and Function)

Study 2 was published in 2023 (Haghiri et al. 2023) and the provided rat brain atlas includes cytoarchitectonic and M₂ receptor architectonic mapping of the somatotopically-related subdivisions within the rat primary parietal cortex (Par1). In the maps, the regional borders are indicated by dashed lines (see Supplementary Material levels c7-c23 of (Haghiri et al. 2023)).

Here, I have complemented the information on the parcellation schemes of the five somatotopic subdivisions: Par1DZ (parietal cortex area 1, dysgranular zone); Par1ULp (parietal cortex area 1, upper lip representation); Par1DZO (parietal cortex area 1, dysgranular oral zone); Par1BF (parietal cortex area 1, barrel field) and Par1Sh (parietal cortex area 1, shoulder representation), regarding multimodal analyses of the cytoarchitecture and M₂ receptor distribution patterns. Additionally, the M₂ receptor densities of each area have been calculated and are shown in Table 1. Further details on the Materials and Methods can be found in our publication (Haghiri et al. 2023).

Variations in the thickness and cell packing density of inner granular layer IV, as well as differences in M₂ receptor densities across the cortical layers of Par1, enabled the identification of the somatotopically-related subdivisions (Figure 1). Cytoarchitectonic analyses showed that layer IV was clearly identifiable in all subdivisions, and was slightly thinner in Par1DZ, Par1DZO and Par1Sh. The cell packing density in layer V was highest in Par1DZ, slightly lower in Par1ULp, Par1DZO, and Par1BF, and lowest in Par1Sh. Par1Sh presented the most cell-sparse layer Vb compared to the other subdivisions.

The highest and lowest mean M₂ receptor densities were found in Par1DZO and Par1Sh, respectively (Table 1). Further differences in the laminar M₂ receptor distribution patterns were found between somatotopic subdivisions. Generally, higher M₂ receptor densities were found in layers IV and Vc and lowest values in layer Va in all five subdivisions. Variations in M₂ receptor densities in layers IV and Vc were used to distinguish between the five subdivisions. Specifically, very high M₂ receptor densities were found in layer IV of Par1BF, followed by slightly lower values in Par1DZ and Par1DZO, and the lowest densities in Par1ULp and Par1Sh. Par1BF also showed a high M₂ receptor density in layer III, which was lower in the other subdivisions.

Analysis of layer Vc revealed the highest M₂ receptor densities in Par1BF and Par1DZO, and the lowest in Par1ULp.

Table 1. Mean absolute M₂ receptor densities (in fmol/mg protein $\pm$ standard deviation, SD) across the five somatotopic representations. Mean values represent the average receptor density across all cortical layers and both rat brain hemispheres.

	Mean $\pm$ SD
Par1DZ	612 $\pm$ 194
Par1ULp	645 $\pm$ 200
Par1DZO	677 $\pm$ 204
Par1BF	625 $\pm$ 190
Par1Sh	590 $\pm$ 186

Par1BF parietal cortex, area 1, barrel field; *Par1DZ* parietal cortex, area 1, dysgranular zone; *Par1DZO* parietal cortex, area 1, dysgranular oral zone; *Par1Sh* parietal cortex, area 1, shoulder representation; *Par1ULp* parietal cortex, area 1, upper lip representation.

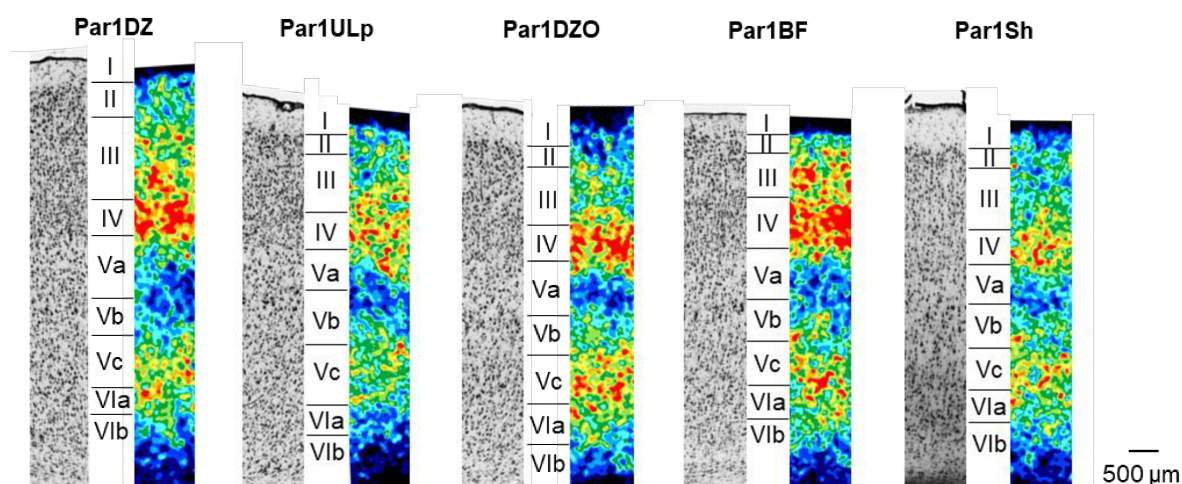


Figure 1. Somatotopic subdivisions of the rat primary parietal cortex. Representative snippets represent cytoarchitecture (left) and laminar M₂ receptor distributions (right) within five somatotopic subdivisions of the rat primary parietal cortex. The color scale bar indicates M₂ receptor binding site density in fmol/mg protein. Roman numerals indicate cortical layers. Region abbreviations correspond to those shown in Table 1.

Study 3

3D Rat Brain Atlas Based on the Distribution Patterns of Cell Bodies and Cholinergic Muscarinic M₂ Receptors

In Preparation

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This publication includes datasets that we have published via the EBRAINS research infrastructure. One dataset is currently being prepared.:

Kuckertz, A., Haghiri, H., Palomero-Gallagher, N., & Funck, T. (2025). *Parcellation scheme of rat iso- and proisocortical areas in Waxholm space (v2)* EBRAINS. 10.25493/WR29-H4S

Kuckertz, A., Haghiri, H., Palomero-Gallagher, N., & Funck, T. (2025). *Parcellation scheme of rat iso- and proisocortical areas in Waxholm space (v1)* EBRAINS. 10.25493/B4DF-8WF

Kuckertz, A., Palomero-Gallagher, N., & Funck, T. (2025). *Atlas of muscarinic M₂ receptor distributions in the rat brain (v2)* EBRAINS. 10.25493/GF7X-2AU

Kuckertz, A., Haghiri, H., Zilles, K., & Palomero-Gallagher, N. (2024). *Muscarinic acetylcholine M2 receptor densities in areas of the rat iso- and proisocortex (v1)* EBRAINS. 10.25493/XSGR-HXQ

Kuckertz, A., Palomero-Gallagher N., Schubert N., Funck T. *Atlas of regional and laminar distribution of silver stained cell bodies in the rat brain (v1)*. 2025. EBRAINS (*in preparation*).

The study was supervised by Prof. Palomero-Gallagher and Prof. Amunts. The provided datasets were published in the knowledge graph of the EBRAINS research infrastructure, which led by Prof. Amunts. Dr. Thomas Funck developed the BrainBuilder reconstruction pipeline and performed the 3D reconstructions.

My (Ms. Anika Kuckertz) contributions included the preparation of the images for the 3D reconstructions, preparation of the corresponding datasets (i.e., data descriptors and metadata) for EBRAINS. In addition, I am currently continuing to write the manuscript and prepare the tables and figures for the submission of the manuscript of Study 3.

2 Discussion

This dissertation includes research studies, that analyzed the distribution patterns of neurotransmitters receptors in the rat brain and provide multimodal (i.e., cyto- and receptor architecture) and multidimensional (i.e., 2D maps and 3D reconstructions) reference resources for neuroimaging studies. Specifically, the first comparative study identified quantitative similarities and differences in the distribution patterns of the serotonergic 5-HT_{1A} and 5-HT₂ receptors in key components of the human emotion regulation network and in homologous areas of the rat brain (Kuckertz, Zhao, et al. 2025). These results provide a systematic evaluation of the translational potential of the rat serotonergic system for the research of human mood disorders, including the visualization of the pharmacological binding sites of 5-HT_{1A} and 5-HT₂ receptors.

In the second study, novel, high-resolution rat brain maps of the isocortex and proisocortex, based on cytoarchitecture and the distribution patterns of muscarinic cholinergic M₂ receptors were developed and published as a multimodal, 2D rat brain atlas (Haghir et al. 2023). The third study contained the reconstruction of the previously published (Study 2) M₂ receptor maps into a novel, high-resolution 3D rat brain atlas. To date, the following data have been published through the EBRAINS research infrastructure: the M₂ receptor volume; the 3D rat brain atlas; and a dataset containing iso- and proisocortical M₂ receptor densities (Table 2). The 3D reconstruction of cell body-stained sections and the manuscript describing the 3D reconstruction pipeline, including the data used, are currently being prepared. Our high-resolution imaging data serve as references for precise parcellations of smaller regions, identification of specific cortical layers and also functional networks in the rat brain, and for *in vivo* studies.

Table 2. Overview of the datasets obtained in studies 2 and 3. Four datasets are openly available on the EBRAINS infrastructure (i.e., the EBRAINS Knowledge Graph), each with a separate DOI. One dataset is currently being prepared.

Dataset title	Dataset DOI
Atlas of muscarinic M ₂ receptor distributions in the rat brain (v2)	https://doi.org/10.25493/GF7X-2AU
Parcellation scheme of rat iso- and proisocortical areas in Waxholm space (v2)*	https://doi.org/10.25493/WR29-H4S
Parcellation scheme of rat iso- and proisocortical areas in Waxholm space (v1)	https://doi.org/10.25493/B4DF-8WF
Muscarinic acetylcholine M ₂ receptor densities in areas of the rat iso- and proisocortex (v1)	https://doi.org/10.25493/XSGR-HXQ
Atlas of regional and laminar distribution of silver stained cell bodies in the rat brain (v1)	In preparation

*Version 2 (v2) of 'Parcellation scheme of rat iso and proisocortical areas in Waxholm space' (Kuckertz, Haghir, et al. 2025b) is a correction of v1 (Kuckertz, Haghir, et al. 2025a) and consists of a simple translation of 6 pixels along the z-axis to better align the parcellation to the stereotaxic reference space of the Waxholm Rat Brain.

2.1 Rats in the Translational Research of Human Mood Disorders

Various rat models have been developed for the translational research of human mood disorders, such as depression (Valvassori et al. 2017; Planchez et al. 2019). Our comparative study systematically analyzed the distribution patterns of serotonergic 5-HT_{1A} and 5-HT₂ receptors in key structures of the human emotion regulation network and their homologous areas in rats (Kuckertz, Zhao, et al. 2025). The results revealed fundamental similarities and differences between healthy human and rat brains, which contribute to evaluate the rat, particularly two commonly implicated 5-HT receptor types, as a translational model for human mood disorders. Specifically, 5-HT₂ receptors were found to be more similar distributed (e.g., highest densities in layer III), whereas unique differences were found for 5-HT_{1A} receptors (e.g., CA1-3 and DG of the hippocampus) between humans and rats. These findings provide interesting insights into a potential conservation of the serotonergic system, as well as species-specific characteristics in humans and rats. The similarities between the species found for 5-HT₂ receptors may indicate that their distribution seemed to be more conserved during evolution than that of the 5-HT_{1A} receptors. A previous study described that 5-HT_{2A} receptor signaling on neuronal progenitor cells appears to be

crucial for the expansion of the human transmodal cortex during evolution (Luppi et al. 2023).

Interestingly, 5-HT_{1A} receptors showed different distribution patterns between humans and rats, with higher densities in the hippocampal CA1-3 regions in humans and in the DG in rats (Kuckertz, Zhao, et al. 2025). The hippocampal neuroanatomy is generally homolog between humans and rats (i.e., the subregions CA1, CA1, CA3 and the DG, which contains CA4 and fascia dentata), and 5-HT_{1A} receptors play a crucial role in adult hippocampal neurogenesis (Santarelli et al. 2003; Banasr et al. 2004; Lavenex 2012). However, the distinct distribution of 5-HT_{1A} receptors between the human and rat hippocampal regions (Kuckertz, Zhao, et al. 2025) may indicate a species- and regional-specific involvement of 5-HT_{1A} receptors in hippocampal neurogenesis (Banasr et al. 2004; Klempin et al. 2010).

Mood disorders are well-known to be associated with alterations in the serotonergic system, including 5-HT synthesis and 5-HT_{1A} and 5-HT₂ receptor distribution patterns in the brain. Such alterations have been observed in the cingulate and orbitofrontal cortices, the hippocampus, the amygdala, the thalamus, and the basal ganglia in patients with depression (Yates et al. 1990; Arora & Meltzer 1991; Drevets et al. 1999; Sargent et al. 2000; Wang et al. 2016; Schneck et al. 2021), as well as in corresponding regions of translational rodent models (Earley et al. 1994; Watanabe et al. 2003; van der Stelt et al. 2005; Topic et al. 2007; Zaniewska et al. 2010). Despite altered 5-HT_{1A} and 5-HT₂ receptor distributions being found in homolog areas (e.g., the human subgenual cingulate area and the rat infralimbic area) in patients with mood disorders and rat models, recent publications have described the need to minimize the translational gap of rat models investigating human depression (Slaney et al. 2018; Ferreira et al. 2020; Uliana et al. 2022). Comparative studies analyzing fundamental similarities and differences in the distribution of serotonergic receptors between the brains of healthy humans and rats can provide data for evaluating the application of the rat's serotonergic system as a model for researching human mood disorders. To date, the number of comparative studies analyzing species similarities and differences of 5-HT_{1A} and 5-HT₂ receptors, as well as of other 5-HT receptor types, between the healthy human and rat brain are still limited (Duncan et al. 1992; Cloëz-Tayarani et al. 1997; Duncan et al. 1998; Setola & Roth 2003). Such studies providing fundamental information of the healthy brain are further used for comparisons to disorder-related alterations in the distribution of e.g., 5-HT_{1A} and 5-HT₂ receptors in mood disorders.

To enable a standardized validation of translational animal models of human mood disorders, three validation criteria (face, construct, and predictive validity) have been established (Willner 1984; Belzung & Lemoine 2011). The results of our comparative study contribute to these validation criteria. For example, no significantly different 5-HT₂ receptor distribution patterns were found in most of the investigated human and homologous rat brain areas (e.g., human cingulate area 32 vs. rat cingulate Cg3; human orbital area 11 vs. rat orbital area MO) (Kuckertz, Zhao, et al. 2025). These interspecies similarities may serve as comparable markers between the healthy human and rat brain, which are covered by the face validity. Furthermore, species similarities in the distribution patterns of 5-HT₂ receptor binding sites in healthy brains can be used as reference data for comparisons to disorder-related alterations in brains of patients and rat models, which belong to the construct validity. Previous studies have shown that reference data from healthy human and rat brains, such as those provided in our study (Kuckertz, Zhao, et al. 2025), are used to identify altered 5-HT₂ receptor binding sites in patients (Arora & Meltzer 1991; Biver et al. 1997; Hurlemann et al. 2005) and in animal models (Watanabe et al. 1993; Sato et al. 2008; Benekareddy et al. 2010). Identifying species differences, especially different regional 5-HT_{1A} receptor distributions across the hippocampus and layers of the cingulate and orbital areas, can help to minimize false-positive assumptions when comparing altered 5-HT_{1A} receptor distributions in patients and translational rat models (Kuckertz, Zhao, et al. 2025). A meta-analysis showed a decrease in 5-HT_{1A} receptors in the anterior cingulate cortex and hippocampus of patients with depression (Wang et al. 2016), which was also observed in olfactory bulbectomized rats (Sato et al. 2008). It is important to compare such disorder-related alterations with reference data on 5-HT_{1A} receptor distributions in healthy brains, and to distinguish the comparison specifically between humans and rats (Kuckertz, Zhao, et al. 2025).

Additionally, the data from our comparative study are relevant to pharmacological research. 5-HT_{1A} and 5-HT₂ receptors are classical pharmacological targets for the treatment of mood disorders (Celada et al. 2004; Zaniewska et al. 2010; Artigas 2013; Ślifirski et al. 2021; Pourhamzeh et al. 2022). For example, hippocampal neurogenesis is reduced in depression and anxiety and activation of 5-HT_{1A} receptors via agonistic compounds has been demonstrated to enhance neuronal cell proliferation (Santarelli et al. 2003; Banasr et al. 2004). 5-HT₂ receptor antagonists have antipsychotic effects in patients with schizophrenia (Correll et al. 2020). Thus, visualizing and quantifying

the agonistic 5-HT_{1A} (via [³H]-8-OH-DPAT) and antagonistic 5-HT₂ (via [³H]-ketanserin) receptor binding sites in the healthy brain (Kuckertz, Zhao, et al. 2025) provides fundamental information about the regional and laminar localization of drug binding sites. This information could help to optimize future therapies involving agonistic 5-HT_{1A} and antagonistic 5-HT₂ receptor drugs.

2.2 Cholinergic Muscarinic M₂ Receptor Maps Extent Histological Rat Brain Atlases

The second study included the parcellation of the entire rat isocortex and proisocortex using multimodal analyses of the cytoarchitecture and the distribution patterns of cholinergic muscarinic M₂ receptors. The resulting maps, including detailed information about the topography and cyto- and receptor architectonic parcellation schemes of 48 distinct areas, are openly available (Haghir et al. 2023).

Here, I have expanded the previously published maps by adding parcellation schemes for five somatotopic subdivisions of the rat primary parietal cortex (Figure 1, Identification of the Somatotopic Representations in the Rat Parietal Cortex). The differentiation of the somatotopic subdivisions was revealed by variations in the M₂ receptor densities, mainly in layers IV and Vc. Regional differences were less clear in cytoarchitectonic analyses alone. Par1DZ was found to be the most rostral subdivision, showing a thinner layer IV and a lower M₂ receptor density. A thinner layer IV and a lower M₂ receptor density were also found in Par1DZO. A study analyzing projections arising in the dysgranular zones of the rat primary somatosensory cortex found that deep somatic inputs are processed mainly in the dysgranular zones, which differ from the forelimb area (Lee & Kim 2012). The vibrissae representation area, Par1BF, is marked by the highest M₂ receptor density in layer III and a thick layer IV. Layer IV of Par1BF contains the barrel cells, each of which receives strong input from one whisker (Welker 1976). The upper lip and shoulder representation areas (Par1ULp and Par1Sh, respectively) have lower M₂ receptor densities, with the lowest values in layer Vc of Par1ULp and layer IV of Par1Sh (Figure 1, Identification of the Somatotopic Representations in the Rat Parietal Cortex). Overall, identifying the somatotopic representations provides information on the functional segregation of the rat brain regarding the distribution patterns of M₂ receptors, which are one of the main

contributors to sensory processing (Alenda & Nuñez 2007; Hoff et al. 2010; James et al. 2019).

There are some differences between our cyto- and M₂ receptor atlas and the rat brain atlas of Paxinos and Watson. Paxinos and Watson mapped the rat brain using unimodal analyses of the cytoarchitecture with cresyl violet staining (Paxinos & Watson 2006; Paxinos & Watson 2013). Additionally, Paxinos and Watson identified 40 regions belonging to the entire rat iso- and proisocortex, whereas our maps include detailed parcellations of 53 distinct regions (Haghir et al. 2023). This demonstrates, that receptor architectonic analyses (in our case of cholinergic muscarinic M₂ receptors) can expand cytoarchitectonic brain atlases by identifying additional subregions and making it a powerful technique for more detailed brain mapping.

A further difference between our maps and those of Paxinos and Watson relates to the number of serial sections that were parcellated and the spacing between them. Specifically, the rat brain atlas by Paxinos and Watson shows parcellation of adjacent sections, with 120 µm of spacing between each section (Paxinos & Watson 2013). We mapped neighboring sections (obtained by cell body staining and M₂ receptor autoradiography) with a thickness of 20 µm along the entire rostrocaudal axis of the rat brain. For the multimodal analyses, the rat brain sections were sorted into triplets, with each third section used for a distinct modality (Haghir et al. 2023). This resulted in the mapping of 246 high-resolution images obtained from autoradiographic visualization of M₂ receptor binding sites, with gaps of only 40 µm between each section. The identified borders were cytoarchitectonically validated by neighboring cell body-stained sections. To date, this is the most dense characterization of the entire rat iso- and proisocortex, as other mapping approaches use a smaller number serial sections. This comprehensive mapping on the 2D level is crucial for the development of a high-resolution 3D rat brain atlas. Such a high-resolution 3D rat brain atlas was developed in Study 3 and a manuscript describing the used images and reconstruction pipeline is currently being prepared. A dataset of the 3D rat brain atlas was published through the EBRAINS infrastructure (Kuckertz, Haghir, et al. 2025b).

The *in vitro* autoradiographic approach involves computational postprocessing to generate high-resolution images (5 µm/pixel), which are used for brain mapping. Furthermore, densitometric analysis and a the in-house-developed AnaRec software (Impieri et al. 2019) enable the extraction of quantitative receptor binding sites (i.e. receptor densities in fmol/ mg protein) for each parcellated region (Zilles, Palomero-

Gallagher, et al. 2002; Zilles, Schleicher, et al. 2002; Impieri et al. 2019). A dataset of M₂ receptor densities for each of the 48 iso- and proisocortical areas has been published via the EBRAINS infrastructure (Kuckertz et al. 2024). In this thesis, I have added M₂ receptor densities for the five somatotopic subdivisions (Par1BF, Par1DZ, Par1DZO, Par1Sh, Par1ULp) of the rat primary parietal cortex (see Table 1, Identification of the Somatotopic Representations in the Rat Parietal Cortex). Such reference data, specifically M₂ receptor densities from the healthy rat brain, can be used for comparisons with disorder-related alterations. For example, a previous study found differences of M₂ receptor densities and distribution patterns in the motor cortex (representative frontal cortex areas Fr1, Fr2 and Fr3) between a rat model of hyperkinetic movement disorder compared to wild-type rats. In addition to M₂ receptor densities of Fr1, Fr2 and Fr3, our provided dataset also includes data on further key regions, such as subdivisions of the cingulate and parietal cortices, which showed implications in other diseases, including post-traumatic stress disorder and bipolar disorder (Aykaç et al. 2012; Gibbons et al. 2016).

2.2.1 M₂ Receptor Architectonic Analyses Provide Functionally Relevant Information

In addition to showing regional receptor distributions, high-resolution autoradiograms also reveal laminar receptor distribution patterns, providing information about the molecular organization between brain regions and cortical layers. Over 20 years ago, researchers discovered that brain regions belonging to the same functional system or regional network tend to have similar receptor architectures, as evidenced by the distribution patterns of various neurotransmitter receptor systems (Zilles, Palomero-Gallagher, et al. 2002; Zilles et al. 2015). In accordance with previous studies in monkeys and humans (Geyer et al. 1997; Morosan et al. 2005; Zilles & Palomero-Gallagher 2017b; Palomero-Gallagher & Zilles 2019), the primary sensory areas (i.e., somatosensory, auditory and visual cortices) of the rat showed similar M₂ receptor distribution patterns. Our second study revealed comparable M₂ receptor distributions in the primary sensory areas: Par1 (parietal cortex, area 1), Te1 (temporal cortex, area 1), Oc1B (binocular primary visual area), and Oc1M (monocular primary visual area) in the rat. These regions were characterized by the highest M₂ receptor densities in layer IV, and the lowest in layer Va (for Par1 and Te1) or layer V (for Oc1B and Oc1M)

(Haghir et al. 2023). Interestingly, these distribution patterns differed from those found in the primary motor area (i.e., area Fr1 in our Study 2), which contained a lower mean M₂ receptor density and the highest values in layer Vb, and lower densities in layer IV (Haghir et al. 2023). This distinct regional and laminar M₂ receptor architecture may indicate a different involvement of cholinergic signaling through M₂ receptors in sensory and motor processing.

Analyzing the laminar distribution patterns of M₂ receptors further enables the identification of possible layer-specific preferences in signal transmission. The high M₂ receptor density in layer IV of rat primary sensory areas (e.g., Par1, ParFL, ParHL, Te1, Oc1M, Oc1B, Oc2ML and Oc2Lc), which is known as the main layer for receiving thalamocortical inputs (Ito 1985; Ray & Price 1992; Miller et al. 2001; Viaene et al. 2010; Bopp et al. 2017), may indicate a strong involvement of M₂ receptor-mediated inhibition of thalamocortical inputs (Beierlein et al. 2000; Eggermann & Feldmeyer 2009; Picciotto et al. 2012; Cox 2014). As an example of functional segregation, the rodent barrel field cortex (referred to as Par1BF in our Study 2) receives dense thalamocortical inputs from the vibrissae, and M₂ receptors appear to play an important role in regulating tactile whisker function (Woolsey & Van der Loos 1970; Sachdev et al. 1998).

Furthermore, lower M₂ receptor densities were found in layer Va of the sensory areas, e.g., Par1, Te1 and Oc1B (with the lowest values in the primary parietal areas, including the somatotopic subdivisions; Figure 1 Identification of the Somatotopic Representations in the Rat Parietal Cortex) (Haghir et al. 2023). Layer Va pyramids are involved in intracortical sensory processing, for example, the modulation of tactile stimuli for object localization (Aroniadou & Keller 1993; Ahissar et al. 2000; Schubert et al. 2005). Lower expression of M₂ receptors in layer Va seemed to indicate minor involvement in the regulation of intracortical processing of sensory information, and preferential contribution of M₂ receptor signaling in thalamocortical inputs in layer IV (Hooks et al. 2013). This suggests layer-specific cholinergic processing of sensory information, however, further research on the specific role of M₂ receptors is needed (Obermayer et al. 2017; James et al. 2019).

As described above, M₂ receptors showed a distinct laminar pattern in motor areas compared to the primary somatosensory, auditory and visual areas (Haghir et al. 2023). The apical dendrites of layer Va and Vb pyramidal cells project up to layer I, providing intracortical connections (Radnikow & Feldmeyer 2018). Layer Vb pyramids

are involved in motor function, e.g., motor skill learning and differentially respond to muscarinic acetylcholine receptors (Anderson et al. 2010). Further studies investigating (sub)layer-specific involvement of muscarinic acetylcholine receptors in motor function would be interesting (for review see (Obermayer et al. 2017)). However, it should be noted that analyses of the cholinergic innervation patterns are not fully represented by M₂ receptors alone, as other muscarinic subtypes such as M₁ and M₄ receptors are also responsible for cholinergic signaling (Obermayer et al. 2017).

Investigations of receptor architecture go beyond neuroanatomical parcellation based solely on cytoarchitecture. That is, receptor architectonic analyses provide knowledge about the molecular organization of brain regions, including indications of layer-specific signaling patterns. It is important to note that functional segregation of the brain by receptor architecture (e.g., identification of areas belonging to a functional network) requires analyses of multiple neurotransmitter receptors, since brain function is maintained by the balance and dynamic adaptation of inhibitory and excitatory neurotransmitter systems. Imbalances in these systems, for example caused by changes in the distribution patterns of muscarinic cholinergic or serotonergic receptors, are strongly associated with the development of neurodegenerative and neuropsychiatric disorders, such as Parkinson's disease, Alzheimer's disease and depression (Reisine et al. 1977; Dewar et al. 1990; Yates et al. 1990; Drevets et al. 2007; Cremer et al. 2015; Wang et al. 2016; Khan et al. 2022).

For example, rats with the hyperkinetic movement disorder showed altered distribution patterns of the M₂ receptors, but also for GABA_A and adenosine A_{2A} receptors (Palomero-Gallagher et al. 2008; Palomero-Gallagher et al. 2010). Furthermore, altered densities of cholinergic, serotonergic and adrenergic receptors were found in the lateral amygdaloid nucleus of epileptic brains (Graebenitz et al. 2011). The here provided rat brain maps and atlas data, specifically, the regional and laminar distribution patterns of M₂, 5-HT_{1A} and 5-HT₂ receptors, as well as receptor densities (Haghir et al. 2023; Kuckertz et al. 2024; Kuckertz, Haghir, et al. 2025b; Kuckertz, Palomero-Gallagher, & Funck 2025; Kuckertz, Zhao, et al. 2025), enabled multi-receptor analyses of two key transmitter systems implicated in neurodegenerative and neuropsychiatric disorders (Lai et al. 2001; Hurlemann et al. 2005; Rosell et al. 2010; Wang et al. 2016; Khan et al. 2022). The rat brain atlas and corresponding data can be expanded to include data and analyses of additional neurotransmitter systems, such as dopaminergic and glutamatergic receptors, and

further structures e.g., the striatum, hippocampus and thalamus. As described above, multimodal approaches enable more detailed brain mapping by analyzing different neurobiological characteristics. Data obtained from further methods e.g., histology and functional neuroimaging can also be included in the here provided rat brain atlas.

2.3 Development and Usage of the 3D Rat Brain Atlas

In this doctoral thesis a rat brain atlas for both *in vitro* and *in vivo* neuroimaging studies was developed. Five datasets have been prepared and four are openly available via the EBRAINS Knowledge Graph (Table 2 and Figure 2). The first dataset contains the 3D reconstruction of high-resolution (5 $\mu\text{m}/\text{pixel}$) M₂ receptor autoradiograms, depicting the heterogeneous distribution patterns of M₂ receptors across the 3D rat brain volume (Kuckertz, Palomero-Gallagher, & Funck 2025). The second and third datasets contain the reconstructions of our previously published iso- and proisocortical maps (Haghir et al. 2023) into a 3D rat brain atlas (Kuckertz, Haghir, et al. 2025b, 2025a). Version 2 (v2) of the dataset contains a corrected version of version 1 (v1) achieved by a simple translation of six pixels along the z-axis to better align the parcellation to the stereotaxic reference space of the WHS (Kuckertz, Haghir, et al. 2025b). The fourth dataset contains M₂ receptor densities (in fmol/ mg protein) for each of the 48 iso- and proisocortical areas providing directly usable data for statistical analyses (Kuckertz et al. 2024). A fifth dataset containing the 3D reconstruction of high-resolution images (1 $\mu\text{m}/\text{pixel}$) of cell body-stained sections for cytoarchitectonic analyses is currently being prepared.

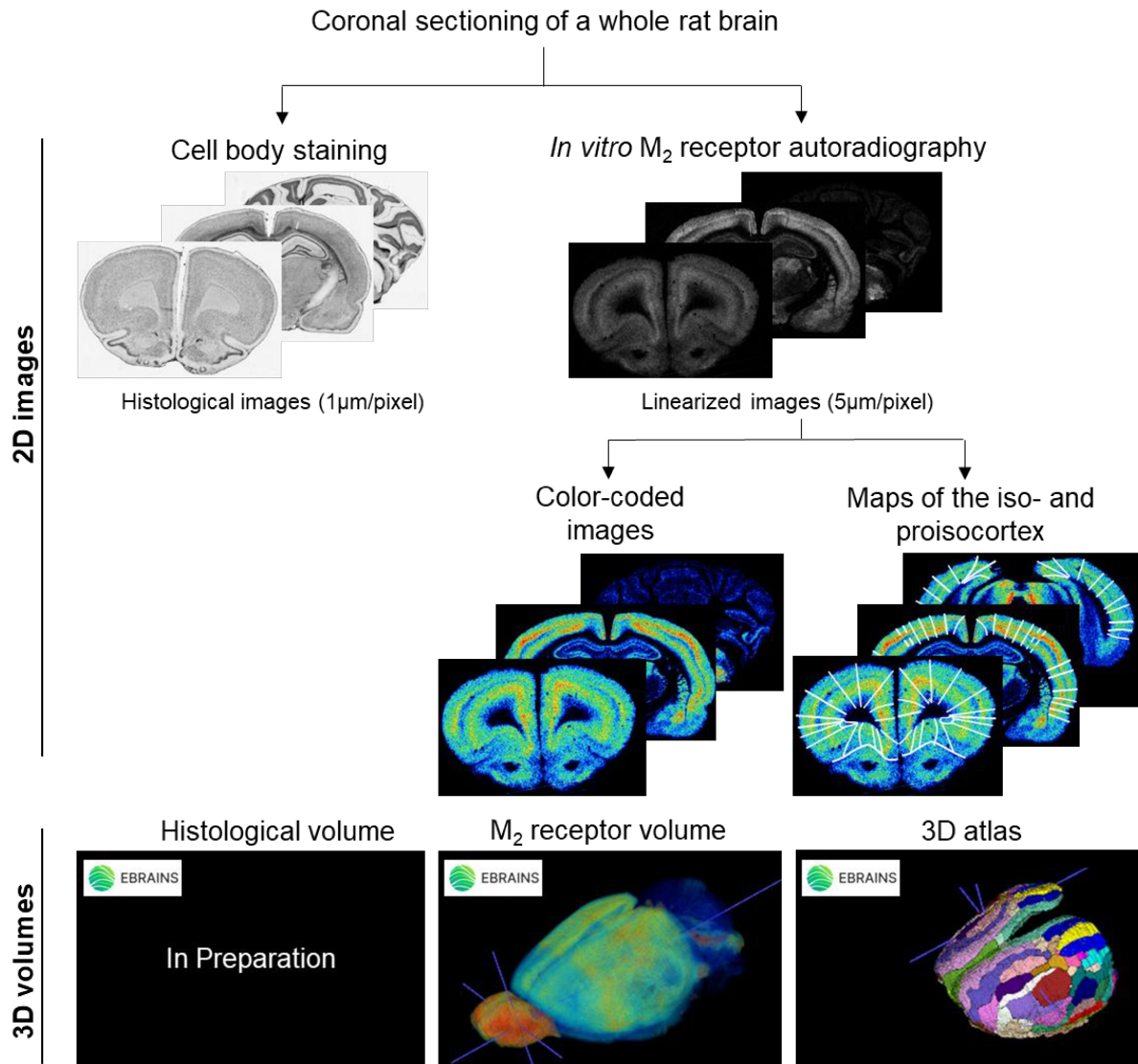


Figure 2. Overview of multimodal 2D and 3D neuroimaging data of the rat brain. After coronal sectioning (20 µm thickness) of a whole rat brain, alternating sections were used for staining of cell bodies and visualizing cholinergic muscarinic M₂ receptor binding sites using *in vitro* receptor autoradiography. Scanning the sections provided 2D histological images with an in-plane resolution of 1 µm pixel. Computational postprocessing of the M₂ receptor autoradiograms produced linearized images with an in-plane resolution of 5 µm/pixel. For visualization purposes, the linearized images were color-coded, with blue tones indicating lower and red tones indicating higher M₂ receptor densities. These images were used to map the entire rat iso- and proisocortex (Haghir et al. 2023). The 3D reconstruction of the histological cell body-stained images is in preparation. The linearized images and the maps of the iso- and proisocortex were reconstructed as 3D volumes of the rat brain and registered to the WHS (Papp et al. 2014). The corresponding data were published via the EBRAINS research infrastructure (Kuckertz et al. 2024; Kuckertz, Haghir, et al. 2025a, 2025b; Kuckertz, Palomero-Gallagher, & Funck 2025).

Rat *in vivo* neuroimaging techniques require high-resolution brain atlases on the 3D level. In the third study, a high-resolution, i.e., 50 µm/pixel, 3D rat brain atlas was developed based on the reconstruction of our previously published M₂ receptor maps (Haghir et al. 2023; Kuckertz, Haghir, et al. 2025b). The higher spatial resolution of our

3D atlas enables more precise identification of smaller brain regions than the 20-times lower resolution of 1-2 mm/pixel within *in vivo* neuroimaging techniques such as MRI (Schwarz et al. 2006; Kjonigsen et al. 2015; Zhao et al. 2020), PET (Sung et al. 2009; Prieto et al. 2011; Bieszczad et al. 2012; Ravasi et al. 2012; He et al. 2024) and DBS (Hamani et al. 2014; Lim et al. 2015; Jimenez-Sanchez et al. 2016; Andree et al. 2022). Compared to the MRI-based WHS atlas of the Sprague Dawley rat brain, which encompasses 36 areas of the cerebral cortex (Kleven et al. 2023), our 3D atlas offers a more detailed parcellation of 53 iso- and proisocortical regions (Kuckertz, Haghiri, et al. 2025b).

As described by Kleven et al. (2023), the cerebral cortex appears homogeneous in the MR images used for the WHS rat brain atlas (Kleven et al. 2023). Figure 3 shows the difference in resolution between MRI of the WHS on the left and the M₂ receptor volume on the right, with the precise identification of cortical layers provided by the M₂ receptor volume. Compared to the lower resolution of the WHS rat brain atlas, our M₂ receptor-based atlas enabled the identification of further subdivisions, for example areas 1 and 2 of the cingulate and the lateral visual cortex. Specifically, the anterior and posterior, dorsal and ventral cingulate regions, as well as rostral and caudal subdivisions of the lateral secondary occipital area were identified (Haghiri et al. 2023). The rostral and caudal subdivisions of the lateral secondary occipital area have not previously been parcellated in histological atlases. (Swanson 2004; Paxinos & Watson 2013). Images on the right in Figure 3 showed our 3D atlas overlaid on the M₂ receptor volume, representing the delineation of posterior cingulate area 1 (Cg1'), and the dorsal and ventral subdivisions of posterior cingulate area 2 (Cg2'd and Cg2'v, respectively). These subdivisions were not identified in the WHS rat brain atlas.

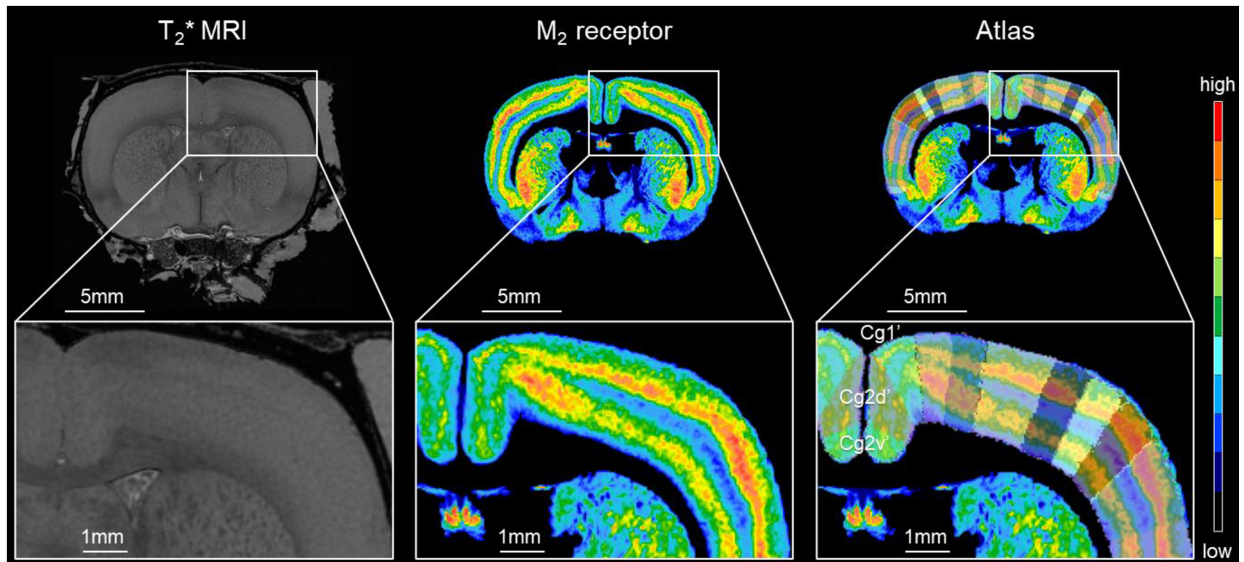


Figure 3. Spatial resolution difference between MR imaging and M_2 receptor autoradiography. Representative images of a *in vivo* T_2^* -weighted MRI scan obtained from the WHS Sprague Dawley rat brain atlas (left), the 3D volume of color-coded M_2 receptor autoradiograms (middle) and the 3D rat brain atlas (right) in the coronal plane (Kuckertz, Haghir, et al. 2025b; Kuckertz, Palomero-Gallagher, & Funck 2025) are shown. The M_2 receptor volume and the 3D atlas were registered to the WHS (Papp et al. 2014). Exemplary snippets with 4-times higher magnification were shown in the bottom row. Scale bars indicate 5 mm and 1 mm for lower and higher magnification, respectively. The color scale bar indicates lower and higher M_2 receptor densities in blue and red tones, respectively. *Cg1'* cingulate cortex, area 1', *Cg2'd* cingulate cortex, area 2', dorsal part, *Cg2'v* cingulate cortex, area 2', ventral part

2.3.1 Reconstruction of M_2 Receptor Maps into a 3D Rat Brain Atlas

The 3D reconstructions of the M_2 receptor autoradiograms (Kuckertz, Palomero-Gallagher, & Funck 2025) and the iso- and proisocortical maps (Kuckertz, Haghir, et al. 2025b) (shown in Figure 2) were performed using the BrainBuilder pipeline (Funck et al. 2025). BrainBuilder has been successfully applied for the reconstruction of 2D receptor autoradiograms from the human and monkey brains (Rapan et al. 2021; Funck et al. 2022; Niu et al. 2024). The pipeline has the great advantage of being individually adaptable to brain sections from various species, including those of mice and rats, and is compatible with images obtained from multimodal experiments (e.g., cell body and myelin staining, and various neurotransmitter receptor types). A manuscript detailing the 3D reconstruction pipeline is being preparation.

The M_2 receptor volume and the 3D atlas have a spatial isotropic resolution of 50 $\mu\text{m}/\text{pixel}$, which was achieved through various experimental and computational factors, as described below. In addition to the steps of the BrainBuilder pipeline (Funck

et al. 2025), experimental factors, such as the usage of nearly every brain section and the processing of high-resolution images, are crucial to achieve a high spatial resolution of the 3D reconstruction. Specifically, as described in the 2D mapping study, 1,370 serial coronal microtome sections (20 μm thickness) spanning the entire rostrocaudal axis of the rat brain were processed (Haghir et al. 2023). Processing of serial, high-quality brain sections without loss of tissue and free of sectioning artifacts, is essential for high-resolution reconstruction of the entire rat brain. The sections were grouped into triplets for multimodal analyses. The first section of each triplet was used for cell body staining; the second for *in vitro* autoradiographic visualization of M₂ receptors; and the third was stored as an unprocessed backup (Haghir et al. 2023). This yielded into 451 cell body-stained sections and 454 M₂ receptor autoradiograms, which were used for the reconstruction of the histological volume for cytoarchitectonic analysis (a dataset is in preparation) and the M₂ receptor volume (Kuckertz, Palomero-Gallagher, & Funck 2025), respectively. For the development of the 3D atlas, we parcellated 246 sections covering the entire iso- and proisocortex along the rostrocaudal axis approximately from Bregma +5.64 mm to -9.12 mm (Paxinos & Watson 2013). Parcellating such a large number of serial neighboring sections is essential for the development of a detailed rat brain atlas. Using adjacent sections enables more precise identification of the extent of specific brain regions and especially, the transition zones between different regions along the rostrocaudal, as well as the dorsoventral axes. Regions and regional borders can occur within few millimeters in the rat brain. For example, the rostral and caudal borders of the rostromedial secondary occipital area (Oc2Lr) were identified within a range of only 1 mm (Kuckertz, Haghir, et al. 2025b).

In addition to the high number of 20 μm -thick serial sections, small gaps of only 40 μm between each third section (Haghir et al. 2023) resulted in a higher spatial resolution of our M₂ receptor volume and the 3D rat brain atlas than that of other 3D reconstructions (Hess et al. 1998; Prieto et al. 2011). For example, Prieto and colleagues performed a 3D reconstruction using receptor autoradiograms from every fifth 40 μm -thick section, which resulted in 200 μm gaps (Prieto et al. 2011). In order to obtain a fully reconstructed 3D brain volume, the gaps between the sections must be interpolated using pixel intensity values from the autoradiographic images. The smaller the gaps (e.g., 40 μm vs. 200 μm), the less interpolation is needed, thereby increasing the spatial resolution of the reconstructed volume.

In addition to the experimental factors of section quality and the large number of parcellated sections, the improved quality of the 3D reconstruction is due to the higher alignment accuracy obtained by computational processing of BrainBuilder. The BrainBuilder pipeline includes methods for quantifying the accuracy of the 2D section to reference volume alignments (Funck et al. 2025). Specifically, alignment accuracy can be quantified using the Dice score, where a score of 1 indicates an ideal alignment in the reconstruction (Dice 1945). Dice scores above 0.90 are considered to represent highly accurate and ideal (i.e. high-quality) results (Hsu et al. 2021). The Dice scores were calculated for the alignments of the 3D reconstructions of the M₂ receptor volume and the iso- and proisocortical atlas. The Dice score of the histological volume will be calculated in the dataset of the 3D reconstruction, which is currently being prepared. The Dice scores were averaged across all aligned 2D sections (Funck et al. 2025). The Dice scores for the M₂ receptor volume included the alignment of all sections along the entire rostrocaudal rat brain axis, i.e., covering the olfactory bulb, the cerebrum and the cerebellum. The calculated Dice score ($\pm$ standard deviation) of the reconstructed M₂ receptor volume (Kuckertz, Palomero-Gallagher, & Funck 2025) was 0.92 ± 0.18 . Our 3D rat brain atlas included sections covering the entire iso- and proisocortex approximately from Bregma +5.64 mm to -9.12 mm (Paxinos & Watson 2013; Kuckertz, Haghir, et al. 2025b) and the Dice score was 0.97 ± 0.01 . These Dice scores demonstrate very high alignment accuracies of our 3D reconstructions. The 3D reconstructions were provided in the NIfTI (Neuroimaging Informatics Technology Initiative) format as nii.gz-files and can be opened using ITK-SNAP⁵ (Kuckertz, Haghir, et al. 2025b; Kuckertz, Palomero-Gallagher, & Funck 2025). A manuscript detailing the 3D reconstruction procedures and the development of the 3D atlas of the rat iso- and proisocortex, including all datasets, is in preparation.

2.3.2 A Digital and Openly Accessible Atlas for the Rat Brain

To date, there has been a lack of a common, standardized atlas system for multimodal, whole-brain imaging data of rats. A recent review emphasized the importance of registering multimodal neuroimaging data within a unified framework to facilitate a more comparable and better sharable system across studies (Mars & Palomero-

⁵ <http://www.itksnap.org/>

Gallagher 2023, 2024). For example, the Siibra software tool contains the MRI-based WHS of the Sprague Dawley rat brain (Papp et al. 2014; Kleven et al. 2023). As described above, we have registered our 3D rat brain atlas to the standardized stereotaxic space of the WHS (Kuckertz, Haghir, et al. 2025b). This will further enable the integration of our 3D rat brain atlas into the Siibra software tool, allowing for the standardized visualization and analyses of multiple neuroimaging data (e.g., regional and laminar M₂ receptor distribution patterns) of the rat brain within a common reference space. Our 3D rat brain atlas and related data (i.e., M₂ receptor volume and regional M₂ receptor densities) extend the currently available MRI-based WHS atlas by including a greater amount of brain regions belonging to the iso- and proisocortex. Additionally, the published dataset of iso- and proisocortical M₂ receptor densities (Kuckertz et al. 2024) can be integrated into the Siibra software tool, as ‘data feature’ linked to each brain region. To date, neurotransmitter receptor densities for 30 cytoarchitectonic areas of the human brain are available in EBRAINS and have been integrated into Siibra (exemplary EBRAINS datasets (Zilles et al. 2021; Ruland et al. 2022)).

Furthermore, a digital rat brain atlas system incorporating openly accessible neurotransmitter receptor data can facilitate the development of computational rat brain models. For example, computational modeling of cholinergic neurotransmission may help to understand the influence and dynamics of acetylcholine on neuronal firing and cognitive functions (Hasselmo & Schnell 1994; Newman et al. 2012). Additionally, models of cholinergic neurotransmission can provide insights into neuropathological mechanisms such as the depletion of cholinergic input and its impacts on working memory (Newman et al. 2012) and the effects in the hippocampus of Alzheimer’s disease (Menschik & Finkel 1998). However, computational models require high-quality reference data on molecular processes from healthy brains, and such neuroimaging data are currently lacking in existing models (Khan et al. 2022). The here developed atlas based on cholinergic muscarinic M₂ receptors and the related data, will serve as references from healthy brains in the development and application of computational models of cholinergic signal transmission. Furthermore, computational modelling approaches can be used to validate and select animal models with the greatest translational potential (Gratz et al. 2018). Data on the regional and laminar distribution patterns M₂ receptors throughout the rat brain (Haghir et al. 2023; Kuckertz, Palomero-Gallagher, & Funck 2025) provide fundamental and functionally relevant

information for developing computational simulations and models of M₂ receptor neurotransmission. These information may expand our understanding of M₂ receptor signaling patterns in the healthy rat brain, which further enable comparisons to alterations of M₂ receptor distribution or signaling patterns in rat models of e.g., Alzheimer's disease.

In the context of pharmacological treatments targeting neurotransmitter receptors, computational models can be used to predict receptor-drug interactions, thereby improving therapeutic efficacy (Kumar et al. 2006; De Benedetti & Fanelli 2018; Joshi et al. 2020). Localizing receptor binding sites, which are used as pharmacological drug targets, can contribute to identify potential hotspots of drug action in the brain, such as specific brain regions and cortical layers. For example, muscarinic cholinergic agonists, such as acetylcholinesterase inhibitors, seemed to attenuate cognitive impairments (Bubser et al. 2012; Gholami et al. 2022; Ismail et al. 2025). In our study, [³H]-oxotremorine-M was used to visualize agonistic M₂ receptor binding sites across the rat brain. High densities of M₂ receptor binding sites were found in specific regions and cortical layers, such as layer IV of the primary parietal cortex (Haghir et al. 2023). These findings can contribute to provide information of regional preferences for agonistic M₂ receptor drug binding, thereby contributing to localize drug action in the brain and the stimulated effects on the evoked M₂ receptor mediated neurotransmission. Such information are fundamental for computational modeling of agonists binding to M₂ receptors, especially the difference between brain regions.

2.3.3 The 3D Rat Brain Atlas for *In Vivo* Neuroimaging

Our 3D rat brain atlas is intended to serve as a high-resolution molecular reference for the precise identification of regions shown in *in vivo* neuroimaging, such as MRI and PET. For example, Atlas3D was developed as a tool to provide 3D visualization of 62 rat brain structures in tomographic PET and MRI images (Hjornevik et al. 2007). The 3DAtlas is based on the reconstruction of histological parcellations obtained from the Paxinos and Watson Rat Brain Atlas (Paxinos & Watson 2006; Hjornevik et al. 2007). As described above, our M₂ receptor-based atlas of iso- and proisocortical regions expanded the purely histological atlas to include additional regions, such as the lateral secondary occipital cortex (Haghir et al. 2023; Kuckertz, Haghir, et al. 2025b), which was not identified in the current version of the Rat Brain Atlas from Paxinos and Watson

(Paxinos & Watson 2013). Furthermore, compared to the cytoarchitectonic atlas, the 3D M₂ receptor atlas enables more accurate comparisons with PET, specifically analyzing neurotransmitter receptor distribution patterns. This is explained in more detail below.

PET is an *in vivo* technique offering the advantage of visualizing neurotransmitter receptor distribution patterns in the living brain (Schiffer et al. 2006; Sung et al. 2009; Bieszczad et al. 2012; Vidal et al. 2018), as opposed to the analysis of receptor distributions in postmortem sections using *in vitro* autoradiography. However, despite their ability to represent neurotransmitter receptor distributions, there is a considerable difference in the spatial resolution between these techniques (Schmidt & Smith 2005). Specifically, the spatial resolution is 1-2 mm/ pixel *in vivo* PET images (Jacobs & Cherry 2001; Moses 2011; Beliveau et al. 2017; Stortz et al. 2018), and 5 µm/pixel for *in vitro* autoradiograms (Zilles, Schleicher, et al. 2002; Palomero-Gallagher & Zilles 2018; Haghiri et al. 2023). It is important to note that *in vitro* autoradiograms are images on the 2D level, whereas the 3D rat brain atlas here developed, based on the reconstruction of 2D M₂ receptor autoradiograms, has a spatial resolution of 50 µm/pixel. This is 20- to 40- times higher than that of *in vivo* PET (Kuckertz, Palomero-Gallagher, & Funck 2025). Figure 4 shows the difference in resolution between *in vivo* PET images and *in vitro* receptor autoradiograms in exemplary images. Both, the PET and autoradiographic images, showed the visualization of the agonistic M₂ receptor binding sites using [¹⁸F]FP-TZTP and [³H]Oxotremorine-M, respectively. The PET images were obtained from an exemplary open-access publication (Ravasi et al. 2012), and the corresponding autoradiographic images are provided by the developed 3D M₂ receptor atlas (Kuckertz, Palomero-Gallagher, & Funck 2025). Due to the limited spatial resolution of PET images, *in vivo* analyses of changes in receptor distribution or activation patterns can only be localized to relatively large brain areas (Sung et al. 2009; Prieto et al. 2011). In particular, accurate differentiation of subregions, e.g., in the frontal and somatosensory cortex and the hippocampus, as well as of subcortical structures such as the caudate putamen (CPu), is limited in *in vivo* PET (Casteels et al. 2006; Bieszczad et al. 2012; Ravasi et al. 2012). The higher resolution of the M₂ receptor volume enables the distinction of such subregions belonging to e.g., the somatosensory cortex and the hippocampus, as well as enabling further detailed identification of the cortical layers (Figure 4).

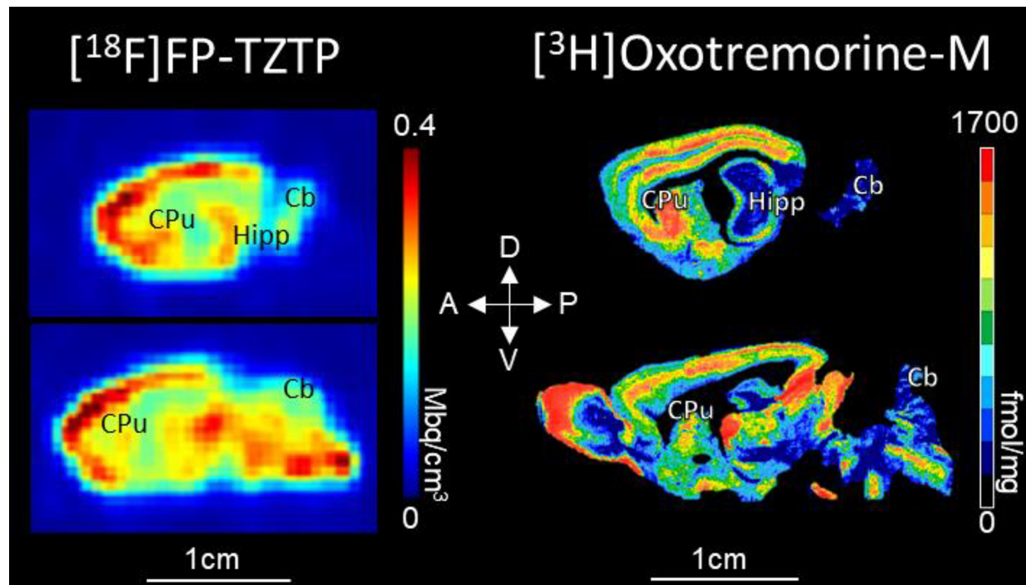


Figure 4. Resolution difference between *in vivo* PET images and *in vitro* receptor autoradiograms. Exemplary images of rat brains in two sagittal levels depict agonistic M₂ receptor binding sites by *in vivo* PET with [¹⁸F]FP-TZTP (left) (Ravasi et al. 2012) and *in vitro* autoradiography with [³H]Oxotremorine-M (right) (Kuckertz, Palomero-Gallagher, & Funck 2025). The sagittal levels are comparable between the two methods. PET images were taken with permission from an exemplary publication (Ravasi et al. 2012). The color scales bars indicate lower and higher M₂ receptor densities in blue and red tones, respectively. Note, PET and autoradiographic images have been scaled differentially (i.e., Mbq/cm³ and fmol/mg). Scale bars indicate 1 cm. Cb Cerebellum, CPu caudate-putamen (striatum), Hipp Hippocampus, A anterior, D dorsal, P posterior, V ventral.

Figure 4 showed the unique difference in the spatial resolution between *in vivo* PET and the 3D reconstructed *in vitro* autoradiograms. In addition to enabling precise differentiation of cortical areas, our *in vitro* autoradiographic M₂ receptor volume allowed identification of the cortical layers. For example, the lower M₂ receptor densities in the middle layer and higher values in the adjacent outer and deeper layers are visible in the *in vitro* autoradiograms (right-hand images in Figure 4). However, it is important to note that some more criteria should be considered when comparing the results between these neuroimaging techniques (Prieto et al. 2011). For example, PET results show the distribution of receptors in the living brain, which is influenced by factors, such as blood flow and the kinetic metabolism of the ligand used. Whereas *in vitro* autoradiography analyzes receptor distributions in postmortem brain sections in a steady state, independent of kinetic processes. Both [¹⁸F]FP-TZTP and [³H]Oxotremorine-M are used to visualize agonistic binding sites of M₂ receptors in *in vivo* PET and *in vitro* autoradiography, respectively. But their specific binding affinities (represented by the dissociation constant of the ligand) and the *in vivo* vs. *in vitro* conditions result in different values of absolute receptor densities, which are indicated

by distinct units (see the color scale bars in Figure 4). To date, *in vitro* receptor autoradiography has been used to evaluate receptor binding and visualization characteristics of PET ligands (Ettrup et al. 2010; Lemoine et al. 2010), including their use in translational animal models (Saigal et al. 2013; Shimochi et al. 2022; Fu et al. 2025) and preclinical studies (Vidal et al. 2018; Ikenuma et al. 2023; He et al. 2024; Yang et al. 2025). The results of our *in vitro* autoradiographic studies (Haghir et al. 2023; Kuckertz et al. 2024; Kuckertz, Zhao, et al. 2025) provide reference data on regional and laminar receptor distributions, including quantitative receptor density values in the rat brain. These data can be used to evaluate and validate PET ligands, especially those targeting M₂, 5-HT_{1A} and 5-HT₂ receptors.

To date, the Schiffer Sprague Dawley Rat Brain Atlas (Schiffer et al. 2006) has been used as a standard reference atlas for PET analyses (Prieto et al. 2011; Balsara et al. 2014; Kim et al. 2022; Tang et al. 2025). However, with a spatial resolution of 1.5-2 mm/pixel, it is approximately 40-times lower than that of our 3D rat brain atlas, which offers a spatial resolution of 50 μ m/pixel (Kuckertz, Haghir, et al. 2025b). Figure 5 shows an exemplary rat brain MRI scan onto which our 3D atlas can be overlaid and both brain volumes have been registered to the coordinate system of the WHS (Papp et al. 2014). Our 3D rat brain atlas can be used as a high-resolution reference for *in vivo* images, enabling the precise identification of smaller subregions and surpassing the spatial specificity of the Schiffer rat brain atlas. Specifically, our atlas enables the identification of brain regions along the entire rostro-caudal axis and the three dimensions (i.e., coronal, sagittal and horizontal planes). In the future, our rat brain atlas overlay will also be compatible and usable with further *in vivo* neuroimaging techniques, such as PET.

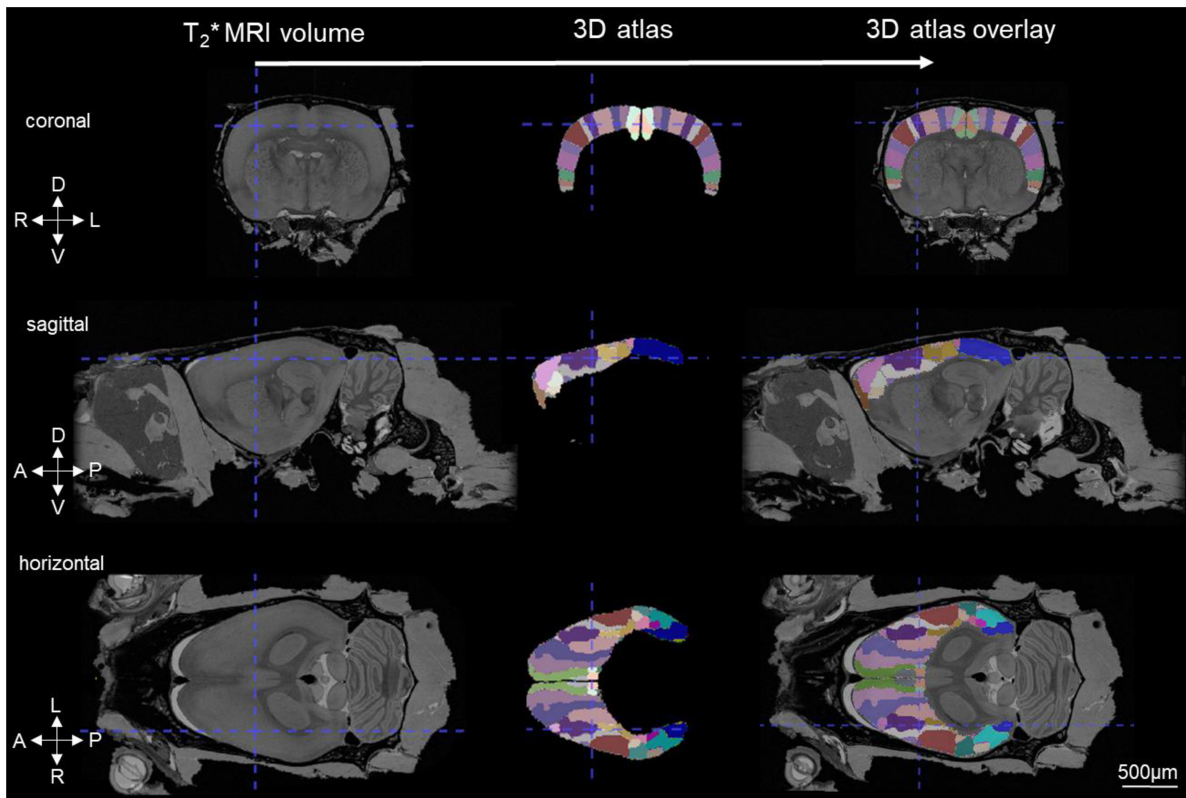


Figure 5. A novel 3D rat brain atlas for *in vivo* studies. Exemplary MRI scans of a rat brain in the coronal, sagittal, and horizontal planes were integrated into the WHS and overlaid with our 3D rat brain atlas for *in vivo* identification of iso- and proisocortical brain regions (Kuckertz, Haghir, et al. 2025b). Here, exemplary levels of coronal, sagittal and coronal planes (i.e., the displayed section/ total number of brain sections) were shown. Coronal level: 620/ 1024, sagittal level: 354/ 512, horizontal level: 381/ 512 (visualized with ITK-SNAP). A anterior, D dorsal, L left, P posterior, R right, V ventral.

In addition to parcellations of the entire iso- and proisocortex, our 2D maps also provide detailed delineations of other key regions of the rat brain, such as the hippocampus, thalamus, and amygdala. These can be found in the supplementary material of our previous publication (Haghir et al. 2023). The here used BrainBuilder reconstruction pipeline, can be applied to include maps of additional brain regions (Funck et al. 2025). This can expand the 3D rat brain atlas of the iso- and proisocortex and the application for techniques such as DBS.

Over the past 20 years, research has shown the significant role of DBS in treating various neurological and neuropsychiatric disorders, such as Parkinson's disease, depression and anxiety (Mayberg et al. 2005; Bewernick et al. 2010; Velasques et al. 2014; Hariz & Blomstedt 2022). For example, DBS of the subgenual cingulate area showed antidepressant effects in patients with treatment-resistant depression (Mayberg et al. 2005; Johansen-Berg et al. 2008). Additionally, DBS of the infralimbic

area, which is considered to be the rodent homolog of the human subgenual cingulate area, has demonstrated antidepressant effects in the chronic unpredictable stress rat model of depression (Hamani et al. 2012; Lim et al. 2015). Translational rat models help to investigate and improve the therapeutic outcome of DBS in humans, including to deeper understand precise neurobiological mechanisms such as neurotransmitter depletion and synaptic inhibition, which are not well understood today (Jimenez-Sanchez et al. 2016). To translate information about the DBS outcome from rats to humans, it is important to stimulate and analyze homolog regions of human and rat brain. Here in the first study, defined homolog regions of the human and rat emotion regulation network and found unique species differences and similarities in the distributions of 5-HT_{1A} and 5-HT₂ receptors (Kuckertz, Zhao, et al. 2025). This study provides fundamental reference data (from parts of the serotonergic system) of 13 homologous regions in the healthy human and rat brain that may serve as comparable target areas for DBS in both species.

Furthermore, precise placement of the stimulation electrode in the target brain region is one of the most critical factors for effective *in vivo* DBS (Treu et al. 2020; Andree et al. 2022). To date, most DBS studies in rats have used 2D histological brain atlases for orientation in the brain (Paxinos & Watson 2013; Swanson 2018), to identify the target region, and to place the electrodes (Hamani et al. 2014; Lim et al. 2015; Jimenez-Sanchez et al. 2016; Zhao et al. 2020). However, 2D atlases do not accurately depict the spatial orientation of the region of interest in the brain, compared to 3D atlases (Papp et al. 2014; Kuckertz, Haghir, et al. 2025b). Precise electrode placement is important to stimulate the region of interest rather than adjacent areas. For example, electrical stimulation of the ventromedial prefrontal cortex, specifically the prelimbic and infralimbic areas, has been shown to have antidepressant and anxiolytic-like effects (Hamani et al. 2012; Lim et al. 2015; Papp et al. 2018; Shimizu et al. 2018). However, previous studies have shown that stimulating adjacent areas such as the prelimbic and infralimbic areas, which are direct neighbors, can result in different neuronal mechanisms (Hamani et al. 2014; Shimizu et al. 2018; López-Terrones et al. 2022). Additionally, according to the very small size of some rat brain regions, more detailed reference atlases are required for precise regional localization. For example, the prelimbic area, which corresponds to Cg3 in our atlas, covered only about 1.8 mm along the rostrocaudal axis (Haghir et al. 2023). Stimulating both directly adjacent and very small rat brain areas highlights the need for detailed 3D rat brain atlases in order

to accurately identify the stimulated region and the borders between adjacent regions along the brain volume (i.e., the x, y and z dimensions). The here provided rat brain mapping and 3D atlas covered 53 areas of the entire iso- and proisocortex and the supplementary material provided in the second study includes maps of further regions, such as the infralimbic area and the hippocampal regions, as well as the localization of thalamic nuclei (Haghir et al. 2023; Kuckertz, Haghir, et al. 2025b). As described above, our 3D rat brain atlas can be expanded by reconstructing of further regions.

In addition to precise electrode placement, the 3D atlas can help to localize the activation patterns induced by DBS in specific rat brain regions, as well as in cortical laminas (Yazdan-Shahmorad et al. 2011; Andree et al. 2022). For example, stimulating the subthalamic nucleus in rats has led to activation of layer V neurons in the primary motor cortex (Li et al. 2012) and a distinct polarization has been found at the transition zone between layer IV and V after intracortical stimulation (Yazdan-Shahmorad et al. 2011). Our 3D rat brain atlas, which is based on the reconstruction of high-resolution M₂ receptor autoradiograms, enables the precise identification of cortical layers (Figure 3) (Kuckertz, Palomero-Gallagher, & Funck 2025). In the future, our atlas can be expanded by parcellations of cortical layers, providing a reference also for identifying specific layers.

Overall, our high-resolution 3D rat brain atlas provides a reference to precisely localize the target region (e.g., Cg3) for accurate placement of the stimulation electrode, specifically, by considering the 3D shape of the brain and the spatial extent of single brain regions (Figure 3) (Kuckertz, Haghir, et al. 2025b). Beyond the coronal plane, our reconstructed 3D volumes and the 3D rat brain atlas can also be displayed in the sagittal and horizontal planes (Figure 5). This enables multiplanar visualization to facilitate the precise identification of brain structures located in the center of the brain, such as the nucleus accumbens and the hippocampus, which are further regions commonly targeted in DBS (Bewernick et al. 2010; Hamani et al. 2012; Perez et al. 2013; Lim et al. 2015).

3 Conclusion and Outlook

Analyses of the distribution patterns of serotonergic and cholinergic muscarinic receptors in the human and rat brains can be used to define the molecular organization of specific regions and cortical layers. The high-resolution maps and the 3D atlas of the rat brain provide reference resources for various investigations, including the identification of species differences and disorder-related alterations in patients and translational rat models of neuropsychiatric and neurodegenerative disorders, such as depression and Alzheimer's disease.

Specifically, the first comparative study revealed similarities and differences in the distribution patterns of 5-HT_{1A} and 5-HT₂ receptors between areas of the human emotion regulation network and their homolog areas in the rat brain, and provide:

- (i) data for interspecies comparisons, highlighting potential evolutionary conservations;
- (ii) systematic reference data for identifying altered receptor distributions in patients with mood disorders and in translational rat models;
- (iii) data for evaluating the rat's serotonergic system as translational model for studying human mood disorders; and
- (iv) data on the regional localization of agonistic 5-HT_{1A} and antagonistic 5-HT₂ receptor binding sites, relevant to pharmacological research.

Our M₂ receptor-based maps and the developed 3D atlas of the iso- and proisocortex will serve as high-resolution references for neuroimaging studies of the rat brain. Particularly, they will facilitate the precise identification of brain regions and cortical layers in *in vivo* studies using MRI, PET or DBS. The 3D rat brain atlas and corresponding data are openly available via the EBRAINS research infrastructure.

The 3D rat brain atlas is designed to be extended with additional neuroimaging data from further brain regions and neurotransmitter receptor binding experiments. We plan to integrate our 3D rat brain atlas of the iso- and proisocortex, including the corresponding receptor density data for the 53 defined regions, into the Siibra software tool in the future. As shown, our 3D atlas can be overlaid onto volumetric images, and we intend to make our atlas available for the registration of PET images.

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