Retinoid Homeostasis in Depression and Schizophrenia

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List of abbreviations

BDI – Beck Depression Inventory
CRABP – cellular retinoic acid binding protein
CRBP – cellular retinol binding protein
CYP – cytochrome P450
CNS – central nervous system
DSM – Diagnostic and Statistical Manual of Mental Disorders
HPA – hypothalamus-pituitary-adrenal
HPLC – high performance liquid chromatography
MADRS – Montgomery-Åsberg Depression Rating Scale
MDD – major depressive disorder
mRNA – messenger ribonucleic acid
PANSS – Positive and Negative Syndrome Scale
PBMC – peripheral blood mononuclear cells
RA – retinoic acid
RAR – retinoic acid receptor
RBP – retinol binding protein
ROL – retinol
RXR – retinoid X receptor
SNP – short nucleotide polymorphism
STRA6 – stimulated by retinoic acid 6
SZ – schizophrenia
WHO – World Health Organization

Abstract

This thesis investigates Vitamin A homeostasis in patients with major depressive disorder (MDD) and in patients with schizophrenia (SZ). Contrary to many somatic disorders, parts of the pathophysiology and underlying molecular mechanisms in psychiatric disorders remain elusive. A wealth of research has identified retinoic acid (RA, the vitamin A metabolite mediating vitamin A's biological effects) as a highly potent molecule that regulates several essential neurobiological mechanisms and which may thus play a role in psychiatric disorders. There is a striking overlap between RA-regulated mechanisms and the typically occurring alterations of these mechanisms in psychiatric disorders – including synaptic plasticity, neurotransmission and neuroinflammation. Several preclinical studies and some epidemiological evidence link a dysregulation in retinoid homeostasis to depression and schizophrenia. A comprehensive assessment of retinoid homeostasis in patients with MDD, however, has never been performed. The role of retinoids in schizophrenia is more established but the potential influence of commonly used antipsychotic medication on retinoid homeostasis has not previously been investigated.

In two clinical observational trials, this dissertation project assessed several aspects of peripheral retinoid homeostasis in patients and healthy controls. In the RA-MDD study, comprising a cohort of 109 patients and controls, retinoid serum levels, the individual RA metabolism activity, and mRNA expression profiles of retinoid homeostasis-relevant genes were determined. In the RA-SZ study, the effects of the highly effective and widely used antipsychotic clozapine on RA catabolism was assessed. Retinoid serum levels and mRNA expression profiles were determined in patients with schizophrenia receiving clozapine medication, patients without clozapine medication, and healthy controls (N=24). Additionally, the effect of clozapine on RA catabolism was assessed in murine and human tissues, including brain tissue.

Patients with MDD had higher retinol (ROL) serum levels and greater RA synthesis activity than healthy controls. Interestingly, MDD-associated alterations in retinoid homeostasis were gender specific. ROL levels differed only between male patients and controls, while RA synthesis only showed a significant difference between female patients and controls. Results from the RA-SZ study showed that clozapine strongly inhibits RA catabolism and that this is mirrored in correspondingly higher RA serum levels in patients receiving clozapine medication compared to patients without clozapine. Compared to healthy controls, serum RA levels of both patient groups were significantly lower.

Taken together, this thesis delivers strong associative evidence of an altered retinoid homeostasis in depression and schizophrenia. The inhibitory effects of clozapine on RA catabolism and the respectively higher RA serum levels in patients with clozapine medication suggests that clozapine may, in part, act via regulation of the retinoid system. Both studies were the first to assess retinoid parameters in the respective patient cohorts and the comprehensive in vivo and in vitro assessments of retinoid homeostasis deliver valuable novel insights. A causal relationship between retinoid signaling and depression or schizophrenia cannot be inferred from these results. Nonetheless, the findings from this dissertation constitute a sound foundation on which (i) the putative pathophysiological contribution of a disrupted retinoid homeostasis in depression and (ii) the potential mediation of therapeutic effects of clozapine via the retinoid system can be explored further.

1 Conceptual and empirical background

Although the phenotypical manifestations of different psychiatric disorders are distinct, there is a large overlap of psychological, social and neurobiological aspects between them. Tremendous progress has been made in elucidating the neurobiological underpinnings of psychiatric disorders. Yet, the final molecular mechanisms and their intricate interconnections remain incompletely understood. Increasing evidence points to a central role for the signaling molecule retinoic acid (RA)[1] in the neurobiology of psychiatric disorders.

Well-established neurobiological disease models of psychopathology identify five major processes or systems that are most commonly affected – though to varying degrees – in disorders such as depression and schizophrenia. These include (i) monoaminergic neurotransmission (ii) neuroendocrine processes, (iii) neuroinflammation, (iv) neurotrophic signaling and neurogenesis, and (v) cellular and synaptic plasticity. Although these systems are infinitely complex and they appear diverse at first sight, RA may represent a unifying factor as it is an upstream regulator of a range of mechanisms within these systems and is crucial for maintaining normal brain functioning. This makes RA a highly interesting target of investigation, possibly contributing to an improved understanding of the pathophysiology and potential therapeutic targets for psychiatric disorders.

The following paragraphs, will focus on how precisely RA is involved in the neurobiology of schizophrenia and depression. But first, the metabolism and signaling pathways of RA will be briefly described to deliver an understanding of the different factors involved in maintaining the retinoid system in homeostatic balance.

1.1 Retinoid homeostasis

Vitamin A (retinol, ROL) and its metabolites – the retinoids – regulate multiple mechanisms peripherally and within the central nervous system (CNS), that support functions including vision, fertility, immunity, lipid metabolism, as well as cell growth, differentiation, and survival. Retinoids have most prominently been investigated for their role as morphogens in embryonic and early post-natal development but their role in the adult CNS is now well recognized. Of all the retinoid metabolites, RA is the biologically most active, which means it mediates most of the biological effects of vitamin A. Given the highly potent effects of RA, maintaining its levels under tight temporal and spatial control is essential. Studies on RA toxicity and teratogenicity,

[1] Unless noted otherwise, “RA” refers to *all-trans* retinoic acid, the most potent of the RA isomers.

as well as investigations of the effects of vitamin A deprivation, show the devastating physiological and downstream behavioral effects of a retinoid system that is out of balance (McCaffery et al., 2003). The text and figure below give an overview of the parameters that are involved in maintaining the retinoid system in balance and mediating RA signaling.

1.1.1 Retinoic acid metabolism and signaling pathways

Synthesis. Retinoids are derived from the diet in the form of animal-derived retinyl esters or plant-derived β-carotene. From the liver, which is the main retinoid storage site in the body, retinol bound to the transport proteins retinol-binding protein 4 (RBP4) and transthyretin (TTR), is transported to target tissue (Step 1). Upon uptake of circulating ROL into the cell via free diffusion (Step 2) or via the membrane protein STRA6 (Step 3), ROL is bound to an intracellular transport protein (Step 4) and can be synthesized to *all-trans* retinoic acid (*at*-RA) via a two-step enzymatic process. In a first, reversible step, *all-trans* retinol is converted to *all-trans* retinaldehyde (Steps 5-7) by enzymes called retinol dehydrogenases (RDH). The irreversible conversion from retinal to *at*-RA occurs via retinaldehyde dehydrogenases (RALDH; Step 8; Napoli, 2012). Newly synthesized *at*-RA (Step 9) can either be translocated to the nucleus (Step 10), exhibit non-genomic functions (Step 12), diffuse to neighboring cells (Step 18), or be catabolized by cytochrome P450 (CYP450) enzymes into more inert metabolites (Step 19).

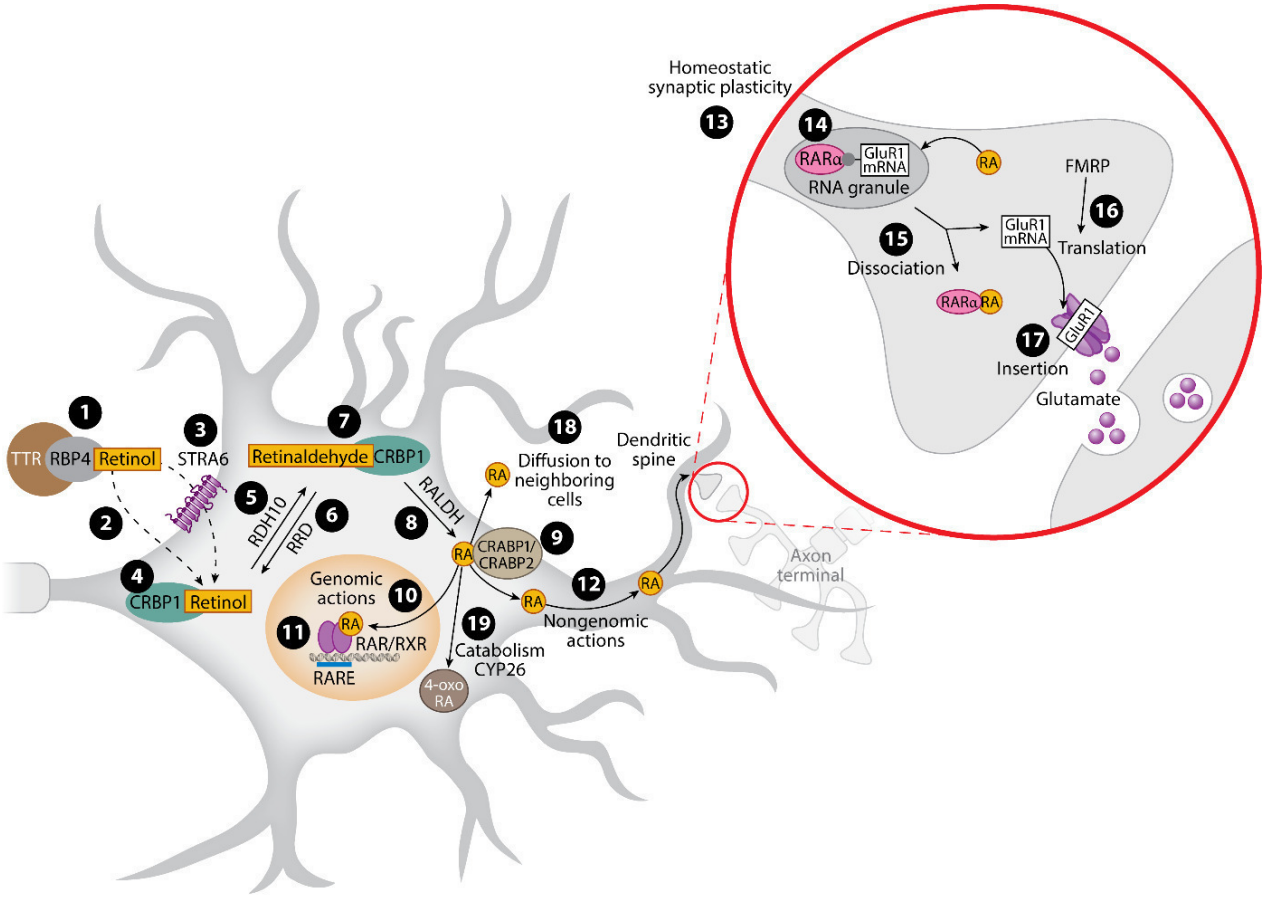
Transcription Activator. Bound to cellular retinoic acid binding proteins (CRABP), *at*-RA can enter the nucleus of the cell. In the nucleus, *at*-RA activates gene transcription by binding to retinoic acid receptors (RAR) and retinoid X receptors (RXR; Step 11; Giguère et al., 1987; Petkovich et al., 1987). The receptors form heterodimers (one RAR complexed with one RXR) and bind to DNA sequences, called RAR elements (RAREs) or RXR elements (RXREs), in the promoter region of the target genes (Chambon, 1996). The *13-cis* and *9-cis* RA isomers can also bind to RAR and RXR receptors but with lesser affinity than the *all-trans* isomer. This pathway describes the classical regulation of gene transcription through RA. However, RA signaling via other receptors, indirect pathways including intermediate transcription factors, or even more distant mechanisms, have also been described. So far, over 500 genes have been identified that are transcriptionally regulated by RA (Balmer & Blomhoff, 2002; Theodosiou et al., 2010).

Non-Genomic Signaling. Outside the nucleus, RA can effect protein translation either through binding to cytoplasmic RARs or independent of RA receptors (e.g. Aggarwal et al., 2006). In the example of regulating homeostatic synaptic plasticity (Step 13), un-liganded

RARα binds to mRNA encoding glutamate receptor A1 (GluR1; Step 14), resulting in repression of GluR1 translation from mRNA to protein. Upon binding of RA to RARα (Step 15), this translational repression is relieved. In the presence of fragile x mental retardation protein (FMRP) GluR1 mRNA is translated (Step 16) and the receptor protein inserted into the synaptic membrane (Step 17; Aoto et al., 2008; Park et al., 2021).

Catabolism. Given its high potency, a balance between synthesis and degradation is essential to maintain the correct levels of RA and ensure normal functioning. The principal enzymes responsible for endogenous RA clearance belong to the CYP26 family. RA regulates its own degradation (negative feedback control) by inducing CYP26 enzymes, which leads to reduced RA concentrations (Thatcher & Isoherranen, 2009).

Figure 1 A schematic of vitamin A metabolism in the cell.



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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CRABP1, 2, cellular retinoic acid–binding protein 1, 2; CRBP1, cellular retinol-binding protein 1; FMRP, fragile X mental retardation protein; GluR1, glutamate receptor A1; HSP, homeostatic synaptic plasticity; mRNA, messenger RNA; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; RBP4, retinol-binding protein 4; RDH10, retinol dehydrogenase 10; RRD, retinal reductase; STRA6, stimulated by retinoic acid 6; TTR, transthyretin.

1.2 The retinoid system in the neurobiology of depression and schizophrenia

Over the last three decades, research has focused increasingly on retinoids' role in the adult CNS. This was mainly prompted by the seminal work of Ann Goodman on retinoids and schizophrenia and the discovery that Accutane™ (isotretinoin, 13-*cis*-RA) – an oral retinoid medication for treatment of severe acne – was linked to increased risks for depression, suicide and psychosis (Bremner et al., 2012; Goodman, 1998). It is now known that RA is a highly potent regulator of essential neurobiological mechanisms including adult neurogenesis, neuroinflammation, dopaminergic, neuropeptide and neurotrophin signaling, neuronal and synaptic plasticity and hypothalamic neuroendocrine regulation (e.g. Aoto et al., 2008; X.-N. Chen et al., 2009; Chiang et al., 1998; Dziedzicka-Wasylewska & Solich, 2004; S.-H. Kim et al., 2023; Matsushita et al., 2011; Takahashi et al., 1999). Irregularities in these processes have been identified either as causal or contributive factors in schizophrenia and depression (Kahn & Sommer, 2015; Keshavan et al., 2015; Krishnan & Nestler, 2008). Both, schizophrenia and depression are multifactorial and multi-causal disorders where psychosocial, (epi-) genetic and neurobiological aspects interact to form complex etiological and progression patterns.

Depression is characterized by symptoms such as anhedonia, avolition, and depressed mood. Affected individuals experience a great degree of psychological strain and often function poorly within their social contexts making depression the world's second leading cause of disability (WHO, 2020). Schizophrenia, though less prevalent than depression, is associated with even greater disability which is in part due to the severity of symptoms. These can include disturbances in perception (delusions, hallucinations), mood (avolition, anhedonia), behavior (withdrawal, disorganized behavior), cognition (disorganized thought and speech) and movement (catatonia; DSM-V). The underlying neurobiological mechanisms are not fully understood; an overlap of neurobiological abnormalities between major psychiatric disorders, however, is undisputed. The active role of retinoids in cognitive function and brain physiology strongly suggests a link between retinoid signaling and psychiatric disorders. Evidence showing that alterations in RA-dependent mechanisms are linked to the neuropathophysiology of depression and schizophrenia is reviewed below.

1.2.1 Retinoic acid and monoamine neurotransmission

Disturbances in neurotransmitter systems can be seen in schizophrenia and depression. Commonly used antipsychotic and antidepressants target neurochemical systems such as the serotonin, dopamine or norepinephrine systems. RA has a variety of effects on all of these neu-

rochemical systems but in the context of schizophrenia and depression etiology, the dopamine system is of specific interest. Abnormal dopamine function in schizophrenia, and particularly in psychosis, is a well-established paradigm (Howes & Kapur, 2009). Pharmacologic interventions for treatment of disorders on the schizophrenia spectrum aim to reduce neurotransmission through the dopamine receptor D2, which bears a retinoic acid response element (RARE) in its promoter region and is thus transcriptionally controlled by RA (Samad et al., 1997). Similarly, antidepressants targeting different neurotransmitter systems all lead to a dose-dependent transcriptional up-regulation of the gene encoding the dopamine D2 receptor (Dziedzicka-Wasylewska & Solich, 2004). RA also transcriptionally activates the serotonin 1A receptor (Charest et al., 1993) and influences the mRNA expression of the norepinephrine transporter (Matsuoka et al., 1997).

1.2.2 Retinoic acid and HPA-axis activity

Stress is likely the most prominent and well-researched risk factor for psychiatric disorders. The hypothalamus-pituitary-adrenal (HPA) axis mediates the biological effects of stress and abnormal HPA activity plays a key role in the development of affective disorders (Barden, 2004). Stress-relevant information is processed by neurons containing corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus. The PVN receives major input from limbic areas like the amygdala and the hippocampus – areas that can be structurally and/or functionally impaired in depression and schizophrenia. In response to stimulation, CRH is released and acts on the anterior pituitary, which releases adrenocorticotropin (ACTH), which in turn triggers the release of cortisol from the adrenal glands. Cortisol has wide-ranging effects on several brain regions and closely interacts with the immune system (Sapolsky et al., 2000). In a negative feedback loop it self-regulates its own circulating levels by binding to glucocorticoid receptors to suppress hypothalamic CRH release. RA interacts with the HPA axis at several points. It regulates the expression of the CRH gene and ACTH receptors and influences the cortisol negative feedback loop via action on the glucocorticoid receptors (X.-N. Chen et al., 2009; Hu et al., 2013; Sesta et al., 2016). Metabolic enzymes relevant for RA synthesis as well as RA receptors are expressed in high density in the paraventricular zone of the hypothalamus (Meng et al., 2011; Shearer et al., 2010). Furthermore, Chen and colleagues (2009) showed that, compared to controls, the expression of RARα is increased in the PVN of patients with affective disorders, suggesting a link between alterations in the retinoid system and affective disorders. Altered HPA axis activity in schizophrenia is not discussed extensively outside the general diathesis-stress model but has been studied for its

interaction with the dopamine system, particularly in the neurodevelopmental context (Holtzman et al., 2013).

1.2.3 Retinoic acid and neuroinflammation

Given the vast systemic effects of cytokines and other inflammatory mediators on neuroendocrine function, neural plasticity and neurotransmitter metabolism, a chronic state of inflammation in the brain (primarily involving microglia, the brains innate immune cells) has been proposed as a contributing factor in the development of depressive symptoms (Kim et al., 2016). Inflammatory processes are an equally important factor in schizophrenia etiology. Alterations in inflammatory markers have been described (Na et al., 2014) and the use of anti-inflammatory agents has been proposed (Zhang et al., 2019). Potent anti-inflammatory effects of RA in the brain have repeatedly been demonstrated in experimental models. For example, RA downregulates microglia activity and attenuates both mRNA and downstream protein expression of pro-inflammatory mediators in activated rat microglia (Dheen et al., 2005; Hellmann-Regen et al., 2013). One proposed mechanism of action of these effects is the suppression of the inflammatory signaling pathway nuclear factor kappa B (NF-κB), which is central to the inflammatory response (Austenaa et al., 2009).

1.2.4 Retinoic acid, neurotrophin signaling and neurogenesis

Neurotrophins are proteins that support the survival, development and growth of neurons and are essential for a range of neuronal functions. As such, alterations in the neurotrophin system have been suggested to play a role in psychiatric disorders. Studies showed that neurotrophin expression in many brain areas and serum of patients with depression or schizophrenia is significantly different from healthy controls (Karege et al., 2002; Reinhart et al., 2015; Sen et al., 2008). Several antidepressive and antipsychotic drugs act on the neurotrophin system, mainly by altering signaling activity of the most prevalent neurotrophin in the adult brain – the brain-derived neurotrophic factor (BDNF; Casarotto et al., 2021; Castrén & Rantamäki, 2010; Pandya et al., 2013; Pedrini et al., 2011). The neurotrophin and retinoid systems are closely intertwined: RA is involved in neurotrophin signal transduction by way of regulating neurotrophin gene and receptor (tropomyosin receptor kinase-B, TrkB) expression, while RA synthesis can be induced by neurotrophins (Corcoran & Maden, 1999; Hernández-Pedro et al., 2008; Kaplan et al., 1993). Neurotrophins and RA support some of the same functions and work together to regulate neurogenesis in adult hippocampal cells (Jacobs et al., 2006; Takahashi et al., 1999). One study even showed improvements in some aspects of

hippocampal based learning and memory upon a course of oral isotretinoin therapy (Ormerod et al., 2012). Neurotrophin signaling and neurogenesis are processes supporting neuroplasticity, a key feature of neuronal systems to effect structural and functional changes in response to internal or external stimuli.

Compared to the adult brain, neuroplasticity in the developing brain is much greater. The neurodevelopmental model of schizophrenia assumes abnormal brain development – or altered developmental neuroplasticity – resulting in dysfunctional connectivity as a causal factor in the emergence of psychosis (Lerner et al., 2016). Given that RA is a potent developmental morphogen and the similarity between RA-dependent congenital anomalies and those seen in schizophrenia, retinoids are ascribed a prominent role in the neurodevelopmental context of schizophrenia.

1.2.5 Retinoic acid and synaptic plasticity

Neuroplasticity related adaptions occur i.a. at the synapses – the communication points between neurons. The modification of synaptic connections (synaptic plasticity) drives the modification of neural circuits. Given the fundamental importance of synaptic plasticity for information encoding, connectivity, structure and downstream cognitive and emotional function, disturbances in Hebbian and homeostatic synaptic plasticity are prominently discussed as a key factor in the pathophysiology of depression and schizophrenia (Cramer et al., 2011; Gao & Penzes, 2015; Keshavan et al., 2015; Vose & Stanton, 2016; Workman et al., 2018). Hebbian plasticity describes the changes at individual synapses known as long-term potentiation (LTP) and long-term depression (LTD) that typically occur during learning and memory formation. Homeostatic synaptic plasticity is a form of *metaplasticity* that acts to control LTP / LTD-related changes in synaptic excitability or inhibition by scaling synaptic strength within a neural network to maintain network stability (for a review on Hebbian and homeostatic synaptic plasticity see Yee et al., 2017). A line of highly interesting studies shows that RA plays a central role for homeostatic synaptic plasticity. Upon chronic synaptic inactivity, RA is synthesized locally and regulates compensatory synaptic transmission by effecting mRNA-translation of receptor proteins that mediate synaptic excitation or inhibition (Aoto et al., 2008; Arendt, Zhang, Ganesan, et al., 2015; Sarti et al., 2013). This process was recently shown to have antidepressive-like effects closely related to those of ketamine (Suzuki et al., 2021). Although RA is not directly involved in mediating Hebbian forms of plasticity, earlier studies showed a strong effect of RA on associative hippocampal learning (Chiang et al., 1998). More recent studies elegantly disentangle the functionally overlapping mechanisms, showing that

previous RA-dependent homeostatic regulation influences subsequent LTP and LTD (Arendt, Zhang, Jurado, et al., 2015; Hsu et al., 2019).

1.3 Rationale and aims

The evidence reviewed above provides possible mechanisms of retinoid involvement in the neurobiology of psychiatric disorders, suggesting that a dysregulation in retinoid homeostasis may be causal to the etiology or progression of the disorders. Notably, most of the insights stem from experimental preclinical studies. The aim of this dissertation project is to further illuminate the role of retinoids in schizophrenia and depression by assessing retinoid homeostasis in patients.

Only a few studies provide more direct evidence of altered retinoid homeostasis in depression. Postmortem studies in patients with depression revealed altered expression of retinoid transport, binding and signaling machinery in the hypothalamus and prefrontal cortex (X.-N. Chen et al., 2009; Qi et al., 2015). A recent study by Mulvey and Dougherty (2021) suggests that short nucleotide polymorphisms[2] (SNPs) associated with an increased risk for major depressive disorder share a transcriptional system that is regulated by RA. And lastly, clinical evidence of the depressogenic effects of exogenous RA, such as isotretinoin, closely links altered retinoid homeostasis to depression (Bremner et al., 2012). Yet, endogenous retinoid homeostasis in a clinical cohort has never been investigated. Thus, in a clinical observational trial, the RA-MDD study of this dissertation project assessed several retinoid homeostasis parameters in a sample of unmedicated patients with MDD and healthy controls. Based on the evidence reviewed above, we hypothesized that these parameters would differ between patients and controls.

Research into the role of retinoids in schizophrenia is more advanced which may in part be due to the relevance of RA for schizophrenia as a neurodevelopmental disorder. Notably, low maternal serum retinol in the second trimester is associated with significant risk for the development of schizophrenia spectrum disorders in the children (Bao et al., 2012). Based on proteomic and transcriptomic evidence of a disturbed retinoid system in schizophrenia (for a review see Reay & Cairns, 2020), and retinoids' vital role for normal brain functioning, the use of retinoids has been proposed for treatment of schizophrenia. Two clinical trials using the RXR agonist bexarotene as an adjunctive treatment showed good tolerability and improvement of symptoms (Lerner et al., 2008, 2013). As this suggest a therapeutic contribution of

[2] A genomic variant of a single DNA base pair.

the retinoid system, the second study of this dissertation project (RA-SZ) investigated if and how conventional antipsychotic medication modulates the retinoid system. It was hypothesized that clozapine may directly interact with retinoid homeostasis. In a mixed-method approach, the effects of clozapine on RA catabolism in murine peripheral tissue and brain, human brain and peripheral blood mononuclear cells (PBMC) were determined, and parameters of retinoid homeostasis were assessed in patients with and without clozapine medication and healthy controls.

2 General Methodology

Detailed descriptions of the methods employed in the studies of this dissertation project can be found in the methods section or supplemental material of the respective publications. The following paragraphs will provide a summary of the methods employed and contain brief explanations on the use of certain procedures.

Participants

Patients were recruited from our in- and outpatient units for participation in the clinical observational study on RA homeostasis in neuropsychiatric disorders (RAHND; ClinicalTrials.gov Identifier: NCT02439099) between 2015 and 2019. The study was approved by the local ethics committee (EA4/002/13). MDD patients (N=58) had a specialist-confirmed ICD-10 diagnosis of MDD and were currently not taking antidepressive medication. Patients were matched to healthy controls (N=58) on BMI, age, gender and smoking status. Patients included in the schizophrenia study had a specialist-confirmed DSM-V diagnosis of schizophrenia (SZ) for more than five years and were on stable medication with clozapine (N=10) or other antipsychotics (N=10) and were matched to healthy controls (N=10) on BMI, age, gender and smoking status.

Serum and PBMC isolation

For analysis of retinoid serum levels and isolation of peripheral blood mononuclear cells (PBMC), all participants provided a sample of 40ml peripheral venous blood at a routine blood draw upon admission. As RA is light sensitive, serum aliquots were kept in the dark or processed under dim yellow light and stored at -80°C until further analysis.

PBMC serve as a widely used cell model to study person-specific cell properties and dynamics such as metabolism of endogenous compounds or drugs (Rahmoune & Guest, 2017). According to previously published protocols (Regen et al., 2017), PBMCs were isolated from whole blood using FICOLL™ density gradient centrifugation.

Extraction of retinoids from serum

In order to measure retinoids by high-performance liquid chromatography (HPLC) they need to be extracted from the stabilizing protein matrix of the serum. To achieve this, liquid-liquid

extraction was used to separate different compounds based on their solubility. After the addition of acidified ethanol and hexan to each serum sample, vigorous vortex mixing and subsequent centrifugation, the sample separates into the organic phase, containing the retinoids, and the aequeous phase. The organic phase was carefully transferred to glass tubes and left to evaporate to dryness under a gentle stream of argon. Dried extracts were resuspended in HPLC eluent. An internal standard (acitretin) was added to each sample and standard solution to determine matrix effects and extraction efficiency.

In vitro metabolism assays in microsomal and synaptosomal fractions from human PBMC, human brain and murine tissue.

A classic model for studying metabolism of drugs and endogenous compounds are in vitro assays. For these assays, the tissue and PBMC are broken down into their subcellular elements to obtain only the microsomal (in PBMC) or synaptosomal (brain tissue) fractions, which contain the CYP metabolic enzymes. This is achieved by homogenization of the cells or tissue and various centrifugation steps. For the metabolism assays, a known quantity of protein (microsomal/synaptosomal fractions) is added to a vial together with the substrate and an enzymatic complex to start the reaction. The RA catabolism assays in the SZ study additionally contained a test compound (i.e., an antipsychotic drug). After incubation for one hour, the reaction is stopped and each sample is subjected to HPLC analysis. Subsequently, *at*-RA synthesis or degradation is quantified by comparing assays of metabolically active microsomal/synaptosomal fractions to inactive controls.

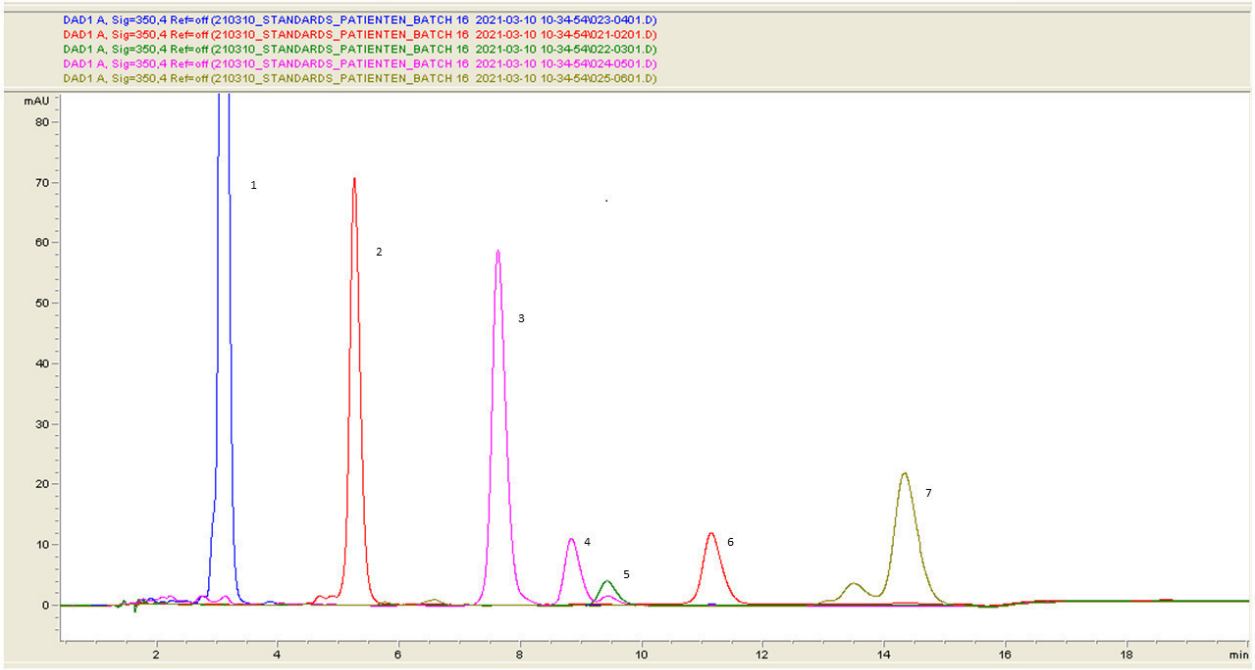
Human postmortem brain tissue from the superior temporal gyrus was obtained from the Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam). All five brain donors gave their written informed consent to brain autopsy and the use for research purposes. The Independent Review Board of the Vrije Universiteit in Amsterdam, Netherlands, granted original approval for all procedures including the redistribution of specimens. Additional approval for the use of the specimens for retinoid analyses was granted by the Charité ethics commission. The use of animal tissue in these experiments was approved by the German regulatory authorities (T 0268/15).

High-performance liquid chromatography

High-performance liquid chromatography is a highly specific method to identify and quantify different compounds within a substance. A sample is injected into the HPLC machine and is

carried to the separation column by an eluent (“mobile phase”). The column is filled with “packaging material”, called the stationary phase that interacts with the mobile phase and the sample. Depending on a compound’s individual interaction with the stationary phase, the time it takes to pass through the column differs for each compound. Thus, the compounds arrive at different times (retention time) at the end of the column where a detector registers time and amount of the compound. For serum analyses, a Phenomenex Synergi™ 4µm Hydro-RP 80A (250 mm x 4,6 mm) column was used at room temperature and the mobile phase consisted of H_20 + 0.1 % formic acid (eluent A) and acetonitrile + 0.1 % formic acid (eluent B). With a combination of isocratic and gradient elution and a flow rate of 1.7ml/min, excellent separation of ROL, RA isomers, oxidation products, and the synthetic retinoid acitretin that served as an internal standard for serum analyses, could be achieved. For retinoid analysis in the metabolism assays, isocratic elution of 15% eluent A: 85 % eluent B was used. The pharmacological assays of the schizophrenia study were analyzed using a Supelco Suplex® column (5 µm, 2.1 x 250 mm; Sigma Aldrich, Taufkirchen, Germany) with a flow rate of 0.65 ml/min and a mobile phase consisting of acetonitrile, 2% (w/v) ammonium acetate, methanol, 25 glacial acetic acid and n-butanol (69:16:10:3:2).

Figure 2 HPLC chromatogram of various retinoids.



Retinoid authentic standards in mobile phase using a Phenomenex Synergi™ 4µm Hydro-RP 80A column. [1] 4-*oxo*-RA 1µM; [2] acitretin 1µM; [3] 13-*cis*-RA 1µM; [4] 9-*cis*-RA 1µM; [5] *at*-RA 100nM; [6] ROL 1µM; [7] retinaldehyde 1µM.

Real-time PCR for relative expression of retinoid-relevant genes

mRNA was isolated from participants' blood according to manufacturer's instruction (Direct-zol DNA/RNA Miniprep Kit; Zymo Research, or PAXgene® tubes, PreAnalytiX GmbH). Absolut quantities of mRNA copies are expressed in ct (cycle threshold) values, where higher values correspond to smaller amounts of mRNA. Generally however, mRNA data is presented in relative values as the fold change of the gene of interest to an internal control (Schmittgen & Livak, 2008). For the RA-MDD study, individual data points are reported, where each target gene in each participant is expressed as the fold change ($2^{-\Delta Ct}$) from endogenous reference genes. For the RA-SZ study, data are expressed as fold change to endogenous reference genes and additionally as fold change of study to control group ($2^{-\Delta\Delta Ct}$). As these values show a strong log normal distribution, they were log-transformed for statistical analyses.

Statistical analyses

Group differences in retinoid serum levels, metabolism activity, and mRNA expression were analyzed using *t*-test (RA-MDD) or one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparisons test (RA-SZ). For determination of clozapine inhibition characteristics of RA catabolism, a 3- or 4-parameter non-linear regression was performed. P-values < 0.05 were considered statistically significant. IBM SPSS version 25 or GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) were used for numerical analyses. GraphPad was used for data visualization. Sample size and power calculations were performed with G*Power software version 3.1.9.2 (Erdfelder et al., 2009).

3 Summary of Results

This chapter gives a high-level summary of the main findings from the studies included in this dissertation project.

3.1 Study 1: Retinoid homeostasis in depression (RA-MDD)

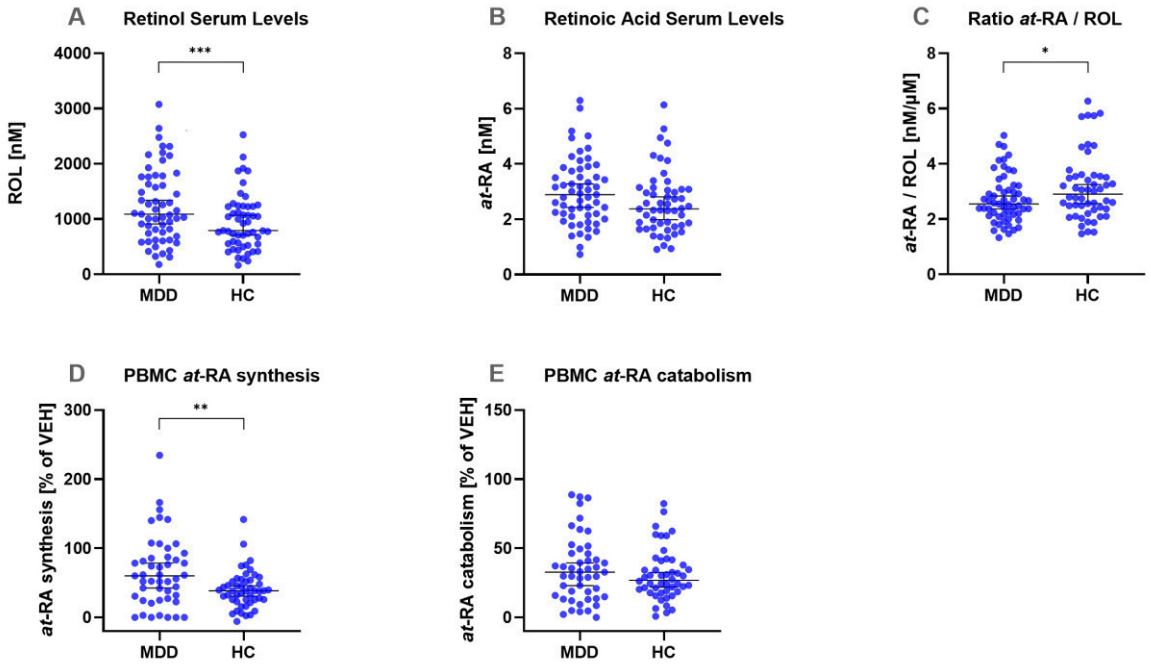
Otto LR, Clemens V, Üsekes B, Cosma NC, Regen F, Hellmann-Regen J. Retinoid homeostasis in major depressive disorder. *Translational Psychiatry*. 2023;13(67):1–7.

Increasing evidence from preclinical studies suggests a role of retinoids in the pathophysiology of depression. Retinoids, and specifically retinoic acid, potently regulate a range of essential neurobiological processes – such as neuroinflammation, neurogenesis and synaptic plasticity – that are found to be impaired in depression (Krishnan & Nestler, 2008). Only a few studies directly investigated the relationship between retinoids and depression. Most prominently, RA has been linked to depression in the context of psychiatric side effects of the oral retinoid Accutane™ (Bremner et al., 2012). Mulvey and Dougherty (2019) found that MDD-associated SNPs converge on a transcriptional system that is regulated by RA and postmortem studies in patients with mood disorders could show that mRNA expression of proteins relevant for retinoid transport, signaling and metabolism is altered (X.-N. Chen et al., 2009; Haybaeck et al., 2015; Qi et al., 2015). A hypothesis-free study found increased RARβ expression in patients with major depression compared to healthy controls (T. L. Huang et al., 2014).

The present study is the first to investigate retinoid homeostasis in a well-matched clinical sample of patients with MDD and healthy controls. Based on the literature, it was hypothesized that retinoid homeostasis parameters would be altered in patients compared to controls. As a disruption of homeostasis can occur at any one point of the transport, signaling or metabolism activities, several parameters that govern retinoid flux were assessed. To that aim, 116 patients and healthy controls were recruited as part of a clinical observational trial and serum levels of *at*-RA and its precursor ROL were determined. Additionally, the individual RA synthesis and metabolism activities were assessed and mRNA expression of RA homeostasis-relevant genes was quantified.

Results showed significantly higher ROL levels and *at*-RA synthesis activity in patients compared to controls. No group differences were observed in mRNA expression profiles of retinoid-relevant genes (Fig. 3).

Figure 3 Retinoid serum levels and metabolism in patients with major depression and healthy controls.

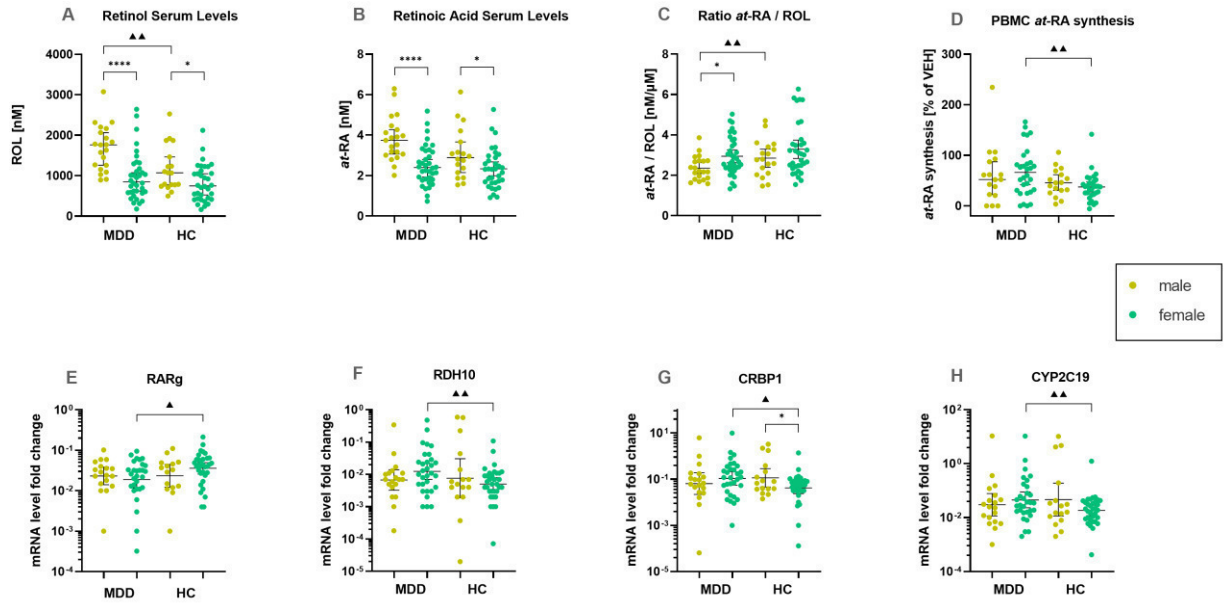


From Otto et al., 2023. Group differences between MDD patients and healthy controls (HC) in **A** ROL serum levels. **B** *at*-RA serum levels. **C** the ratio of *at*-RA to ROL. **D** *at*-RA synthesis activity in PBMC. **E** *at*-RA catabolism activity in PBMC. All error bars depict the median with 95%CI. *t*-tests were used for group comparisons.

Due to strong gender effects, secondary analyses were run and revealed that MDD-associated alterations of the retinoid system were different for men and women. Serum ROL levels were only increased in male MDD patients compared to male controls. ROL levels did not differ between female patients and controls (Fig. 4A). Conversely, increased *at*-RA synthesis activity was only found in female patients, showing no difference in activity between male patients and controls (Fig. 4D). Altered mRNA expression of RA homeostasis-relevant genes was also confined to female gender (Fig. 4E-H).

As hypothesized, several aspects of retinoid homeostasis differ between healthy controls and patients with MDD. Especially as patients did not take antidepressive medication, the observed changes in retinoid homeostasis can be interpreted as endogenous, MDD-associated compensatory (although insufficient) mechanisms. Interestingly, those mechanisms seem to differ between men and women. Future studies should take gender into account when investigating retinoid homeostasis in depression. Differences in psychological and biological aspects of depression are well-established (Mei et al., 2022; Schneider & Walther, 2020; Young & Korszun, 2010) and sex-specific retinoid dynamics are described in the preclinical literature (Arfaoui et al., 2013; Napoli, 2022).

Figure 4 Gender differences in retinoid homeostasis parameters in MDD patients and healthy controls.



From Otto et al., 2023. * marks significant within-group gender differences (i.e. male vs female in MDD or HC). ▲ marks significant group differences for within-gender comparisons (i.e. female MDD vs female HC) **A** retinol serum levels. **B** retinoic acid serum levels. **C** the ratio of *at*-RA to ROL. **D** *at*-RA synthesis activity in PBMC. **E-H** Gene transcription of retinoid pathway related genes in PBMC, fold change values presented as the $2^{-\Delta Ct}$. Error bars depict the median (A-D) or geometric mean (E-H) with 95% CI. *t*-tests were used for group comparisons.

3.2 Study 2: Antipsychotics, retinoids and schizophrenia (RA-SZ)

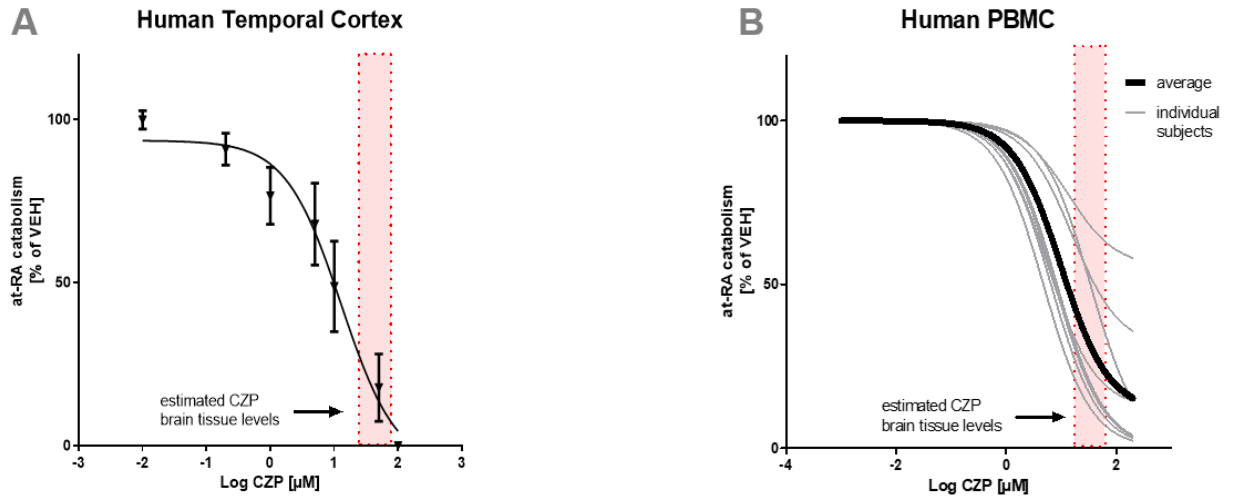
Regen F, Cosma NC, **Otto LR**, Clemens V, Saksone L, Gellrich J, et al. Clozapine modulates retinoid homeostasis in human brain and normalizes serum retinoic acid deficit in patients with schizophrenia. *Molecular Psychiatry*. 2021;26:5417–5428.

Even though retinoids in schizophrenia have received greater research interest than in depression, our study was the first to investigate a putative influence of the commonly used antipsychotic clozapine on the retinoid system. The underlying assumption to the research question was that some of clozapine's therapeutic effects may be mediated via the retinoid system. This was based on findings of disrupted retinoid signaling in schizophrenia (Feng et al., 2005; Goodman, 2005; Reay et al., 2020; Wan et al., 2006), clozapine's pleiotropic and strong antipsychotic effects, and the crucial role of retinoids for normal brain function (Wołoszynowska-Fraser et al., 2020).

In a three-pronged approach, this study investigated the relationship between clozapine and the retinoid system. In vitro pharmacological assays showed that clozapine, in fact, potently inhibits RA catabolism in murine tissues – including brain tissue. These findings could

be replicated in human brain tissue and PBMC (Fig. 5), and were complemented by clinical data that showed significantly increased *at*-RA serum levels in patients treated with clozapine compared to patients treated with antipsychotics other than clozapine.

Figure 5 Effects of clozapine on RA catabolism in human tissue.



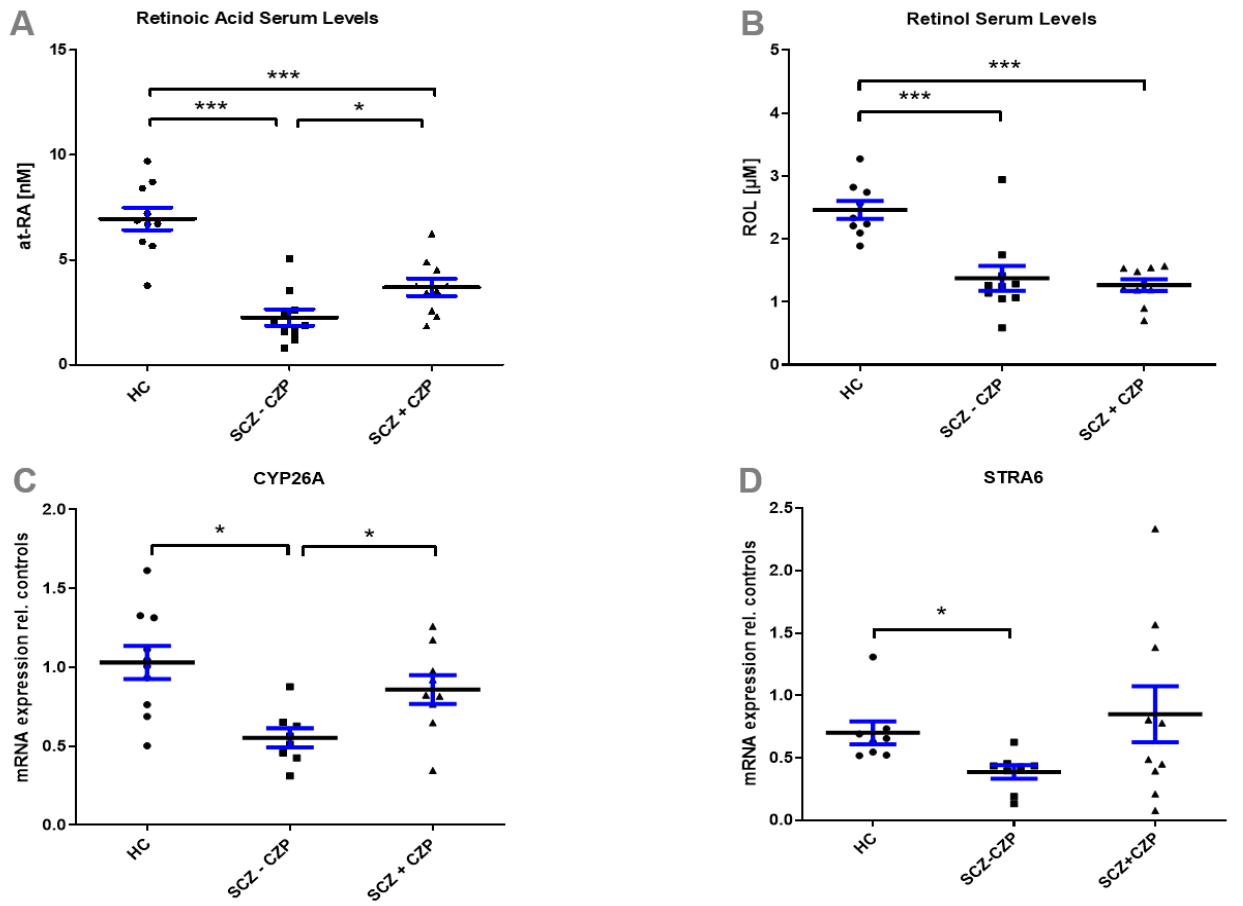
From Regen et al., 2021. Effects of clozapine on *at*-RA catabolism in **A** pooled postmortem human brain-derived synaptosomal fractions (IC50: 12.9 ± 1.56 µM). Values are given as mean and standard error of n ≥ 3 experiments. **B** Clozapine significantly blocks *at*-RA catabolism in microsomal fractions of human PBMC with an average IC50 value of 9.86 µM. The dotted rectangle demonstrates the brain tissue levels of clozapine during steady state.

at-RA serum levels for both patient groups were significantly lower than *at*-RA levels in healthy controls. mRNA profiles showed a reduced expression of RA-inducible STRA6 and the RA-inducible and RA-catabolizing enzyme CYP26A in the no-clozapine group. The mRNA patterns correspond with the *at*-RA serum level patterns, showing that lower *at*-RA levels are associated with reduced mRNA expression of certain *at*-RA-inducible genes (Fig. 6).

This study could show that clozapine can modulate the retinoid system and that aspects of retinoid homeostasis differ between schizophrenia patients with and without clozapine treatment and healthy controls. Although we cannot assert causality, the in vitro inhibition of RA catabolism by clozapine is mirrored in correspondingly higher *at*-RA serum levels in a clinical sample of patients receiving clozapine medication compared to patients not receiving clozapine. As this was a cross-sectional study, it was not designed to answer whether clozapine's therapeutic effects are mediated via the retinoid system. However, it provides strong associative evidence and future research should investigate the schizophrenia-clozapine-retinoid relation longitudinally. Furthermore, due to methodological challenges, the present study was unable to assess *at*-RA synthesis activity in PBMC. Exploring whether clozapine also has an influence on *at*-RA synthesis from ROL and whether synthesis activity in patient-derived

PBMC differs between patients with and without clozapine medication represents and interesting direction for future inquiry.

Figure 6 Retinoid homeostasis parameters in patients with schizophrenia and healthy controls.



From Regen et al., 2021. Group differences between healthy controls (HC), patients with schizophrenia and non-clozapine medication (SCZ – CZP), and patients with schizophrenia with clozapine medication (SZP + CZP) in **A** retinoic acid serum levels. **B** retinol serum levels. **C** mRNA expression of the RA catabolizing and RA-inducible enzyme CYP26A. **D** mRNA expression of the RA-inducible enzyme STRA6. One-way ANOVA with Newman-Keul's post-hoc test was used for group comparisons.

4 Discussion

As retinoids are broadly involved in regulating several essential neurobiological mechanisms, their involvement in psychiatric disorders – where these neurobiological mechanisms can be disturbed – is not surprising. The increased focus on RA functions in the CNS has helped to further elucidate the neurobiological underpinnings of health and disease. Notwithstanding the usual epistemological and translational gaps, three classes of evidence strongly support a role of retinoids in psychiatric disorders.

I. Insights from cell biology on molecular mechanisms identifying retinoids as central regulators of CNS development and function (e.g. Maden, 2002) and as a potential common denominator in molecular processes that are disturbed in psychiatric disorders – i.a. neuroinflammation, neurotrophic signaling, and neural and synaptic plasticity (Hu et al., 2020; e.g. Lane & Bailey, 2005; Reay & Cairns, 2020; Wołoszynowska-Fraser et al., 2020)

II. Experimental studies, where manipulation of the retinoid system is linked to physiological, functional and/or behavioral changes in animal models of psychiatric disorders (Hu et al., 2016; S.-H. Kim et al., 2023; Suzuki et al., 2021)

III. Direct evidence of altered retinoid parameters in neuropsychiatric disorders (X.-N. Chen et al., 2009; Goodman, 2005; Haybaeck et al., 2015; Reay et al., 2020; Wan et al., 2006)

This dissertation project mainly contributes to evidence class III by investigating retinoid parameters in two major psychiatric disorders with distinct symptomatic hallmarks but shared neurobiological disease pathways. Key findings from this dissertation include alterations in retinoid homeostasis in MDD patients and patients with schizophrenia compared to healthy controls as well as a strong inhibitory effect of the antipsychotic clozapine on *at*-RA catabolism. Clinical observations of altered retinoid serum levels were complemented by in vitro assessments of the individual functional RA metabolism and mRNA expression profiles of genes relevant to RA homeostasis. The following paragraphs will discuss the results in the context of the current literature and offer an outlook on potential applications of the findings.

4.1 Altered retinoid serum levels in schizophrenia and major depressive disorder

Retinoid serum levels in the context of neuropsychiatric disorders have thus far rarely been investigated. Before reviewing the findings it is important to note that focusing simply on the *direction* of change in certain peripheral retinoid biomarkers (i.e. higher or lower compared to controls) may be misguided at this point. Rather, in the context of findings from the RA-SZ (Regen et al., 2021) and RA-MDD (Otto et al., 2023) studies, it is suggested to speak of a retinoid dysregulation until the link is elucidated further by mechanistic longitudinal studies. Interpretations offered with regards to the meaning of the direction of change are merely a theoretical exploration.

Two studies showed that reduced RA serum levels are related to an increased risk for developing post-stroke depression (Duan et al., 2019; Yang et al., 2020). Significantly reduced ROL levels are also reported for children with autism and patients with Alzheimer's disease (Guo et al., 2018; Lopes Da Silva et al., 2014; Zhou & Li, 2018). Similar findings come from the somatic field where higher levels of retinoids are linked to a lower mortality risk in ischemic stroke and coronary artery disease and a lower risk for development of cancer and type 2 diabetes (Bushue & Wan, 2010; Han et al., 2021; Hu et al., 2016; Tu et al., 2019). In a 30-year prospective cohort study of nearly 30,000 men, overall and cause-specific mortality was linked to higher ROL serum levels (J. Huang et al., 2021).

Findings from the RA-SZ study of reduced *at*-RA serum levels in patients with schizophrenia compared to healthy controls fit nicely with the retinoid hypothesis of schizophrenia that suggests retinoid dysregulation as a causal factor in schizophrenia etiology (Goodman, 1998). Although the connection remains associative, these findings are especially intriguing in the context of the "hot topic" of homeostatic synaptic plasticity, where *at*-RA signaling plays a vital role, and that is suggested to be disturbed in schizophrenia (Aoto et al., 2008; Keshavan et al., 2015). A disturbed balance in neuronal and/or synaptic plasticity has a strong appeal as a factor in schizophrenia etiology as it considers both diminished and excessive plasticity which are roughly associated with the negative and positive symptoms of SZ, respectively (Forsyth & Lewis, 2017; Keshavan et al., 2015). Even though RA is a central regulator of neuronal and homeostatic synaptic plasticity (L. Chen et al., 2014; Maden, 2007), its role in schizophrenia-associated plasticity changes is far from clear and subject of elegantly designed future research.

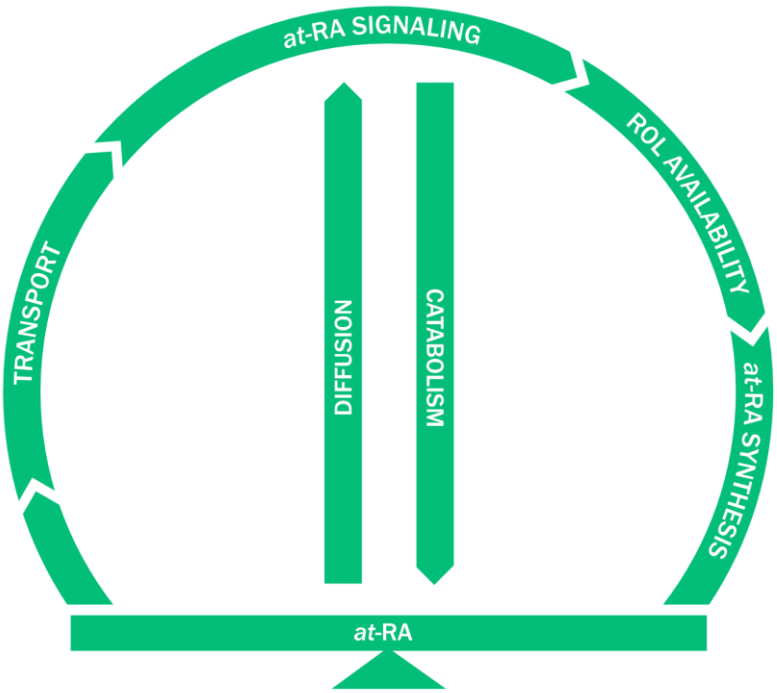
Interestingly, in contrast to findings for schizophrenia, retinoid levels in patients with MDD were higher than in healthy controls. Again, it is important to de-emphasize the direction of change as no evidence currently exists to substantiate any supposed meaning. However,

in light of the evidence reporting more beneficial health outcomes for higher endogenous retinoid levels, and the need of RA for normal functioning, the increased ROL levels in MDD patients can be interpreted as a compensatory (although insufficient) effort to meet the central demand of *at*-RA. This is further supported by the increased *at*-RA synthesis activity seen in the MDD cohort (Otto et al., 2023). Since patients did not currently take psychotropic or retinoid-altering medications, the significantly increased ROL levels are presumed to be the result of compensatory endogenous homeostatic adaptions to diminished central *at*-RA signaling.

An alternative interpretation of the results is to view increased ROL serum levels and *at*-RA synthesis activity not as compensatory but as pathological. Depressogenic effects of excess exogenous retinoids have been described (Bremner et al., 2012). It stands to reason that a dysregulated retinoid homeostasis resulting in higher retinoid levels may potentially have a pathophysiological contribution to MDD.

However, disentangling the intricate, and potentially person-specific relations of the parameters that govern retinoid homeostasis with each other as well as their interaction with other systems (i.e. immune system) is infinitely complex and neither study was designed to examine these interconnections. Furthermore, given the body's natural tendency for homeostatic regulation, it is difficult to separate "truly" pathological from compensatory mechanisms – especially as a disruption of homeostasis can occur at any one point and be internally regulated at many others (Figure 7). Drawing inferences from altered peripheral retinoid serum levels to potentially disturbed upstream RA-dependent mechanisms based on these findings would be premature. However, both studies (RA-MDD and RA-SZ) describe interesting associations that could be explored further.

Figure 7 Schematic of *all-trans* retinoic acid homeostasis.



The main factors influencing *at*-RA homeostasis and signaling are the availability of retinol as substrate, *at*-RA metabolism, and transport of *at*-RA to signaling sites within the cell, or in a paracrine manner via diffusion to neighboring cells. A dysbalance in any one of those factors may lead to altered *at*-RA availability and signaling but may also be compensated by the other factors within this system to maintain homeostatic balance.

4.2 The retinoid system as a biomarker and treatment target

The elucidation of neurobiological underpinnings in psychiatric disorders, in part, serves the purpose of identifying potential diagnostic biomarkers and molecular targets for developing improved treatments.

Based on findings from this dissertation and others, an all-purpose use of retinoids as diagnostic biomarkers for psychiatric disorders is not supported at this point. Instead, the results emphasize the importance of personalized or patient-specific approaches when considering the usefulness of retinoids as biomarkers. Findings from the RA-MDD and RA-SZ study on retinoid serum levels or metabolism activity show rather high interpersonal variability and MDD-associated alterations of retinoid homeostasis were gender-specific. Furthermore, a whole genome sequencing study revealed a specific risk of a rare RARβ gene variation only for the severe cognitive subtype of schizophrenia but not for the whole cohort (Reay et al., 2020).

Improvements of pharmacological agents for psychiatric disorders has focused more on decreasing side effects rather than on increasing efficacy which is in part due to a lack of new

molecular targets (Hyman, 2014). Targeting the retinoid system for treatment of psychiatric disorders has a strong appeal given RA's pleiotropic effects and involvement in many neurobiological processes that are altered in psychiatric disorders. Detracting from this appeal is the fact that RA is a ubiquitous molecule regulating numerous mechanisms also outside the CNS with diverse associations with several somatic disorders (Olsen & Blomhoff, 2020).

In two clinical trials, the synthetic RXR agonist bexarotene was successfully used as an add-on to antipsychotic medication in a cohort of patients with schizophrenia over the course of six weeks. All patients had been on a stable regimen of antipsychotic medication but showed significant clinical improvement once bexarotene was added to their regimen (Lerner et al., 2008, 2013). Cummings and colleagues (2016) as well as Endres et al. (2014) investigated the effects of bexarotene and acitretin (a synthetic retinoid) in patients with Alzheimer's disease. Acitretin showed a beneficial effect on the neurotrophic and neuroprotective protein APPα (Endres et al., 2014). The effects of bexarotene on amyloid burden differed depending on genetic risk factors (Cummings et al., 2016) – again emphasizing the role of a person-centric approach.

These studies deliver some encouraging evidence for the use of retinoids and RA receptor agonists as therapeutic agents in psychiatric and neurodegenerative disorders. However, two aspects merit consideration. First, although a mediation of the beneficial effects via the retinoid system is strongly suggested, retinoid homeostasis parameters were not assessed in neither of the studies. Secondly, other studies have found that systemic application of retinoids or retinoid receptor agonists is associated with an endogenous counter-regulation manifesting in the stark increase of RA catabolism and decreased synthesis, leading to a prolonged reduction of local RA signaling (Mihály et al., 2013). As such, a more indirect manipulation of the retinoid system, potentially by blocking RA catabolism, may be a more promising approach. So called retinoic acid metabolism blocking agents (RAMBAs) have successfully been employed in dermatology and oncology (Njar et al., 2006).

In an effort to extend these findings to schizophrenia treatment, the RA-SZ study thoroughly demonstrated that the antipsychotic medication clozapine strongly inhibits RA catabolism in murine brain, human temporal cortex specimens and human PBMC. Together with the clinical results of altered retinoid serum levels, these findings further support a role of the retinoid system in schizophrenia as they deliver first correlative evidence of potentially retinoid-mediated therapeutic drug effects. The effects of other psychotropic agents on RA catabolism were also examined. The stimulant modafinil, the SSRIs citalopram and sertraline or the anxiolytic diazepam did not block RA catabolism (Regen et al., 2021). Interestingly, a study

from 2015 could show that the SSRI fluoxetine inhibits RA catabolism, possibly by blocking CYP2C19 degradation of RA (Harvey & Preskorn, 2001; Hellmann-Regen et al., 2015). If a therapeutic effect of citalopram and sertraline via the retinoid system was to be assumed, data from the RA-SZ study suggest either a more indirect effect of these antidepressives on the retinoid system (e.g. via highly localized *at*-RA signaling for transcriptional upregulation of the dopamine D2 receptor) or manipulation of the system at a parameter other than RA catabolism. The data should be considered as preliminary and findings need to be replicated in larger samples with stringent multiple assessments of retinoid homeostasis parameters, therapeutic doses of the psychopharmacological agent, and clinical symptom severity over a course of several months. Nonetheless, this approach offers intriguing first insights into potential RA-mediated therapeutic effects of psychotropic drugs. Notably, the clozapine inhibitory effects on RA catabolism in human synaptosomes and PBMC microsomes also showed high interpersonal variability (Fig. 5), hinting at a potentially person-specific regulation of the retinoid system. Personalized approaches in psychiatry receive increasingly strong support given the large heterogeneity within disease entities, symptom overlaps between disorders, and the sometimes limited effectiveness of pharmacological treatments (e.g. Fournier et al., 2010).

Further advancement in mechanistic and pathophysiological understanding of retinoid signaling in psychiatric disorders is needed. Next to investigating RA homeostasis in psychiatric disorders and establishing the clinical relevance of a potential disruption, this also includes a better understanding of localized RA signaling, receptor specificity and genomic vs. non-genomic RA actions to fine-tune adverse and therapeutic effects of retinoid medication (e.g. Clark et al., 2020). Although generally well tolerated, the effects of agents like bexarotene and acitretin would need to be investigated over a longer period to establish their safe use given the previous reports on exogenous RA toxicity (Bremner, 2021; McCaffery et al., 2003). Insights into longer term use of systemic retinoids comes from the field of cancer therapeutics, where the use of retinoids is more established (e.g. Routhier A et al., 2010).

4.3 Strengths, limitations and future directions.

A strength of this dissertation project is the comprehensive, translational assessment of several aspects of retinoid homeostasis. To the best of our knowledge, both studies (RA-MDD and RA-SZ) were the first to report insights into the different parameters that govern retinoid homeostasis in clinical cohorts of patients with schizophrenia or major depressive disorder. Alt-

hough neither study was designed to assess the intricate interactions of the retinoid homeostasis parameters, they nonetheless offer compelling associative evidence of retinoid dysregulation in psychiatric disorders and can serve as a basis for future research on potential therapeutic applications.

Through the assessment of circulating serum retinoids, the individual functional aspects of retinoid metabolism and individual mRNA expression profiles of retinoid homeostasis related genes, this dissertation project offers a rare comprehensive picture of retinoid homeostasis in clinical cohorts. Given the inherent self-regulating properties of physiological homeostasis, assessing several homeostasis parameters at different levels presents a compelling method for capturing putative disruptions. The few other studies that investigate retinoid homeostasis in humans, primarily focus on serum retinoids but the complex and highly time and site-specific retinoid regulation warrants a broader and more representative assessment of retinoid homeostasis.

Several limitations of the studies of this dissertation project merit consideration when interpreting and extrapolating the results. Although both study cohorts were well matched on age, gender and smoking status, an important cofounding factor on retinoid homeostasis that was not assessed in either study is nutritional preference or eating habits. Furthermore, retinoid homeostasis was assessed peripherally. While there is a close interaction of the CNS with peripheral systems and well-established models that allow comparability from peripheral to central functions were employed (Guest et al., 2016), retinoid homeostasis is tightly controlled and highly site-specific, potentially rendering peripheral measures as too generic. However, increased local demand of *at*-RA requires transport of ROL from liver storage to target tissue and would be reflected in circulating ROL serum levels, as seen in patients with depression (Otto et al., 2023).

As remarked above, the cross-sectional design of the studies does not allow an inference of causality and cannot provide mechanistic insights into retinoid dysregulation in depression and schizophrenia. Longitudinal assessments and comparisons of remitted patients with non-remitted patients and healthy controls would deliver further intriguing insights. The small sample size of the schizophrenia cohort was the result of the large effect size of clozapine on RA metabolism, which was the primary outcome of the RA-SZ study. Clearly, for replication of the findings on retinoid serum levels and for investigation of the natural RA metabolism (in the absence of a drug), the sample would desirably be much larger. Originally, an investigation of un-medicated schizophrenia patients was planned analogously to the RA-MDD

cohort. Unfortunately, this was outside the time scope of this dissertation project as the recruitment of un-medicated schizophrenic patients proved difficult and the COVID-19 pandemic put a temporary halt to clinical research studies.

The investigation of retinoid homeostasis in un-medicated patients with major depression allowed a “clean” observation of natural retinoid homeostasis, unobstructed by the effects of psychotropic medication, delivering insights on a potential pathophysiological contribution of altered retinoid homeostasis. Further restrictions on the study sample like the absence of any major psychiatric or somatic comorbidities, however, limits the generalizability of the results to a subset of MDD patients. This is important to consider as depression frequently co-occurs with somatic or additional psychiatric disorders (Gold et al., 2020; Otte et al., 2016).

Very prominent topics at the intersection of retinoids and the pathophysiology of psychiatric disorders are the topics of neuroplasticity and especially metaplasticity. Antidepressive treatments like electroconvulsive therapy or ketamine target these mechanisms and the role of retinoids in neuronal plasticity and homeostatic synaptic plasticity (a form of metaplasticity) has repeatedly been demonstrated (L. Chen et al., 2014). Future research is likely to further explore the connection and leverage insights to design targeted and improved treatments which is especially intriguing in light of the high rate of insufficient response to current pharmacological treatment.

5 Conclusion

This dissertation offers insights into the endogenous retinoid homeostasis in patients with major depression and patients with schizophrenia. The comprehensive assessment of several homeostasis parameters revealed a dysregulation of retinoid homeostasis in patients compared to controls and the strong inhibition of retinoic acid catabolism by the antipsychotic medication clozapine. The studies of this dissertation project were the first to investigate retinoid homeostasis in MDD patients and the effect of clozapine on RA catabolism. This work provides a strong foundation on which the role of retinoic acid in the pathophysiology of depression and schizophrenia, as well as its value as a potential diagnostic biomarker and therapeutic target can be further investigated.

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Appendix

Original Publication RA-MDD Study

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ARTICLE OPEN

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Retinoid homeostasis in major depressive disorder

Lisa Rebecca Otto[1]✉, Vera Clemens[1], Berk Üsekes[1], Nicoleta Carmen Cosma[1], Francesca Regen[1] and Julian Hellmann-Regen[1]

The small, hormone-like molecule retinoic acid (RA) is a vital regulator in several neurobiological processes that are affected in depression. Next to its involvement in dopaminergic signal transduction, neuroinflammation, and neuroendocrine regulation, recent studies highlight the role of RA in homeostatic synaptic plasticity and its link to neuropsychiatric disorders. Furthermore, experimental studies and epidemiological evidence point to the dysregulation of retinoid homeostasis in depression. Based on this evidence, the present study investigated the putative link between retinoid homeostasis and depression in a cohort of 109 patients with major depressive disorder (MDD) and healthy controls. Retinoid homeostasis was defined by several parameters. Serum concentrations of the biologically most active Vitamin A metabolite, *all-trans* RA (*at*-RA), and its precursor retinol (ROL) were quantified and the individual in vitro *at*-RA synthesis and degradation activity was assessed in microsomes of peripheral blood-derived mononuclear cells (PBMC). Additionally, the mRNA expression of enzymes relevant to retinoid signaling, transport, and metabolism were assessed. Patients with MDD had significantly higher ROL serum levels and greater *at*-RA synthesis activity than healthy controls providing evidence of altered retinoid homeostasis in MDD. Furthermore, MDD-associated alterations in retinoid homeostasis differed between men and women. This study is the first to investigate peripheral retinoid homeostasis in a well-matched cohort of MDD patients and healthy controls, complementing a wealth of preclinical and epidemiological findings that point to a central role of the retinoid system in depression.

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INTRODUCTION

Depression is a complex and debilitating disorder affecting roughly 300 million people worldwide and exacting huge tolls on individuals and societies [1]. Given the multifactorial etiology and heterogeneous phenotypic expressions of the disorder, there is a vast number of concepts on depression pathophysiology. Alterations in major neurobiological processes such as monoamine neurotransmission, neuroendocrine regulation, inflammatory processes, and particularly multiple forms of cellular and synaptic plasticity have been described for depression [2, 3]. Accordingly, several targeted treatments have been developed, mainly targeting the level of monoaminergic and serotonergic neurotransmission. However, despite tremendous progress in elucidating neurobiological correlates, the final, individual, and possibly patient-specific molecular mechanisms and their reciprocal interactions are still incompletely understood [4, 5]. Modulating effects on neural plasticity at levels beyond direct neurotransmitter actions have recently been described for the hormone-like small molecule retinoic acid (RA) [6].

RA—the active metabolite of vitamin A (retinol, ROL)—is a highly potent neural regulator that acts in an auto- and paracrine manner and is involved in a range of brain physiological processes. While RA has previously mostly been considered in the context of embryonic and early postnatal development, its important role in the healthy functioning of the adult brain is now well established [7]. Studies investigating its molecular actions revealed a remarkable involvement of RA in essential neurobiological pathways that are also affected in neuropsychiatric disorders. For example, RA was shown to be a crucial component in the signal transduction pathways of dopaminergic and neuropeptide signaling, neuroinflammation, and hypothalamic neuroendocrine regulation [8–13]. Furthermore, several aspects of neuronal plasticity such as neurite outgrowth, neurogenesis, and Hebbian forms of synaptic plasticity rely on RA signaling [14–17]. Strikingly, RA also functions as a critical regulator during homeostatic synaptic plasticity which is a non-Hebbian form of plasticity and serves as a mechanism of metaplasticity to maintain network stability [6, 18–20].

Several lines of evidence link RA to depression. Most recently, Suzuki and colleagues [21] could show that the inactivity-dependent up-regulation of synaptic efficacy mediated through RA has a rapid, antidepressant-like effect in mice similar to the antidepressant effects of ketamine. A study by Mulvey and Dougherty [22] suggests that major depressive disorder (MDD) -associated functional single-nucleotide polymorphisms (SNP) share a transcription regulation system that is activated by retinoid transcription factors and thus highly responsive to regulation through RA. Post-mortem studies could show that RA signaling, transport, and metabolism machinery is expressed in the adult hippocampus, hypothalamus, and prefrontal cortex [23–26] and that mRNA expression profiles differ between patients with mood disorders and control subjects [27, 28]. Last but not least, a causal link between dysregulation in RA homeostasis and depression is suggested by findings on the depressogenic effects of exogenous RA—as often seen in dermatological treatment with systemic retinoids [29].

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Endogenous retinoid levels in neuropsychiatric disorders have thus far only been investigated by a few studies. Yang et al. [30], in their prospective study, identified reduced RA serum levels as a risk factor for the development of post-stroke depression at three months. Guo et al. [31] found reduced retinol levels in children with autism and meta-analytical evidence suggests RA dysregulation in Alzheimer's [32]. In our previous work, we identified reduced levels of RA and retinol as well as a dysregulation of retinoid homeostasis-associated genes in patients with schizophrenia [33].

The present study is the first to investigate endogenous retinoid homeostasis parameters in a cohort of well-defined patients with MDD and matched healthy controls. Based on previous evidence, we hypothesized that retinoid homeostasis would be altered in MDD patients as compared to controls. We assessed retinoid homeostasis through several parameters. Serum levels of the biologically most active RA isomer, *all-trans* retinoic acid (*at*-RA), and its precursor ROL were analyzed and the functional signaling activity of serum RA was assessed via reporter cell assays. The individual in vitro *at*-RA metabolism activity was assessed in peripheral blood-derived mononuclear cells (PBMC). Additionally, we assessed mRNA expression profiles of several RA homeostasis-related genes such as proteins involved in retinoid transport, signal transduction, and select enzymes essentially involved in local *at*-RA synthesis and degradation.

METHODS AND MATERIALS

Participants

Patients with MDD from our in- and outpatient units and healthy control subjects matched for age, BMI, and smoking status were recruited for the clinical observational study on RA homeostasis in neuropsychiatric disorders (RAHND; ClinicalTrials.gov Identifier: NCT02439099) between 2015 and 2019. All patients received standard care treatment, had a specialist-confirmed ICD 10 diagnosis of moderate or severe depression, and did not currently receive psychotropic medication. Participants who showed any clinical signs of acute inflammation were treated with retinoids or anti-inflammatory medication or had a diagnosed neurological, somatic, or additional severe psychiatric disorder were excluded. All participants were thoroughly informed and gave their written consent. The study was approved by the local ethics committee (EA4/002/13).

Blood samples

Samples of peripheral venous blood were obtained during a routine blood draw at admission for serum analysis of retinoid levels and isolation of PBMC. All samples were collected in a standardized manner before noon. There were no restrictions on prior meal intake. Serum aliquots were stored at −80 °C until further analysis. PBMC were isolated from heparinized blood by the FICOLL™ density gradient centrifugation, according to previously established protocols [34].

Extraction of retinoids from serum

Retinoids were extracted from sera using a liquid-liquid extraction protocol. To avoid photoisomerization, all experiments were carried out under dim red light. Serum samples were spiked with the synthetic retinoid *all-trans* acitretin as an internal standard (FC 1 µM) to assess recovery and account for inter-assay variability and to calculate final retinoid serum concentrations. Acidified ethanol and 2 vol hexane were added to 1 vol of serum. Samples were vortex mixed, shaken for 15 min, and then centrifuged at 4 °C, 1560×*g* for 5 min. The organic phase was carefully transferred into glass tubes and evaporated to dryness under a stream of argon. The same extraction procedures were applied to standard solutions for the determination of matrix effects. Dried extracts were reconstituted in HPLC eluent and centrifuged for 5 min at 21,000×*g* at 4 °C. An aliquot of 100 µl was injected into the HPLC system.

Preparation of crude microsomal fractions for RA metabolism assays

PBMC is a widely used model to study person-specific cell dynamics and is increasingly used as a source of biomarkers for diagnostics and prediction [35–37]. The crude microsomal fractions derived from the individual PBMC contain metabolic enzymes like CYPs that are involved in the turnover of drugs or endogenous substrates such as retinoids and are thus often used to study metabolic pathways. Metabolically active crude microsomal fractions were prepared in Dulbecco's phosphate buffered saline without Mg^{++} and Ca^{++} (pH 7.3; Gibco, Thermo Fischer) from participants' PBMC by homogenization and differential centrifugation steps according to previously published protocols [33]. Protein concentration was determined with the BCA method (Thermo Fischer, USA) and microsomal fractions were stored at −80 °C. To determine individual RA metabolic activity, in vitro assays containing the RA-metabolizing microsomal preparations from participants' cells were performed according to previously published protocols with minor adjustments [38]. In brief, heat-inactivated controls and metabolically active reactions contained microsomal preparations at a protein concentration of 250 µg/ml. For RA synthesis assays, ROL (FC 100 µM) and NADP+ were added to the reaction. *at*-RA (FC 2 µM) and NADPH Regeneration System (NRS; Promega, USA) were added for RA catabolism assays. After an incubation period of 60 min at 37 °C, the reaction was stopped by adding 4vol ice-cold methanol. Samples were then centrifuged at 21,000×*g* at 4 °C and subjected to HPLC retinoid analysis. *at*-RA synthesis or degradation was quantified by comparing metabolically active samples with heat-inactivated controls.

High-performance liquid chromatography

Retinoid analysis was performed on a reverse-phase Agilent 1100 HPLC system equipped with a binary pump, temperature-controlled column compartment, auto-sampler, and a high-sensitivity diode array detector (DAD). Separation was achieved using a Phenomenex Synergi™ 4 µm Hydro-RP 80 A (250 mm × 4.6 mm) column at room temperature. The mobile phase consisted of H_2O + 0.1% formic acid (eluent A) and acetonitrile + 0.1% formic acid (eluent B). For serum analysis, a combination of isocratic and gradient elution was used as described in Table 1 of the Supplement. At a constant flow rate of 1.7 ml/min, the total runtime was 24 min for one sample. This setup allowed for excellent resolution of retinal, ROL, RA isomers, and oxidation products (Supplementary Fig. 1). In a single HPLC run, several compounds could be clearly separated in serum, with a retention time of 5.1 min for the internal standard acitretin, 9.3 min for *at*-RA, and 10.9 min for ROL (Supplementary Fig. 2). All peaks and retention times were confirmed by authentic standards of the retinoid isomers (Sigmar Aldrich). For the quantification of retinoids in the metabolism assays, isocratic elution (15% A: 85 % B) was used with a total run time of 13 min per sample.

Real-time PCR for relative expression of retinoid-relevant genes

mRNA expression of relevant RA-homeostasis genes in PBMC was assessed by real-time PCR. RNA was extracted using Direct-zol DNA/RNA Miniprep Kit (Zymo Research) as per the manufacturer's instructions and the sample quality was checked afterward using Nanodrop Instrument (Thermo Fisher Scientific Inc., MA, USA). Total RNA was reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit™ (Thermo Fisher Scientific Inc., MA, USA) and stored at −20 °C until further measurement. Relative quantification (ΔCt) and melting curve analysis were both carried out using the StepOne™ Real-Time PCR System software. All primers were designed and checked for their quality using the Primer-BLAST software [39]. The sequences of primers are shown in Table 2 of the supplement. The expression fold change from the target to endogenous reference genes was calculated as $2^{-\Delta Ct}$ as suggested for presenting individual data points [40]. The values were log-transformed for statistical analysis.

Statistical analyses, power, and sample size calculations

Group differences were examined using chi-squared tests or independent samples *t*-tests depending on the level of measurement. Effect sizes are reported as Cohen's *d*. Linear regression was used for the analysis of the relationship between retinoid serum levels and reporter assay activity. Interaction effects were tested with a two-way ANOVA. Sample size and power calculations were performed expecting minimum group differences in serum RA levels based on previously published data comparing RA serum levels in different cohorts [41]. With an even sampling ratio, a power of 95%, and $\alpha = 0.01$, a total of 47 participants per group were needed to detect putative group differences. Data analyses were carried out with IBM SPSS version 25.

Table 1. Participant Characteristics.

		Total *N* = 116	MDD *N* = 58	HC *N* = 58
Sociodemographics				
Age (years)			38.8 (13.5)	36.7 (12.7)
Female *n* (%)		71 (61.2)	37 (63.8)	34 (58.6)
BMI			25.2 (5.3)	23.9 (3.1)
Current Smokers *n* (%)		43	22 (37.9)	21 (36.2)
Education *n* (%)[a]				
	Low	15 (19.2)	8 (22.2)	7 (16.7)
	Medium	21 (26.9)	14 (38.9)	7 (16.7)
	High	42 (53.8)	14 (38.9)	28 (66.7)
Clinical characteristics				
MADRS			23.8 (5.9)	NA
	Male[b]		17.3 (10.1)	
	Female		21.6 (9.7)	
BDI-II			28.7 (8.3)	NA
	Male		22 (12.2)	
	Female		25.5 (12.3)	
HDL cholesterol, mg/dl[a]			54.4 (18.7)	62.5 (16.4)
LDL cholesterol, mg/dl			105.2 (23.4)	98.7 (28.6)
Triglyceride, mg/dl			141.3 (85.3)	129.95 (78.5)
IL-6, ng/l			2.2 (1.4)	2.06 (1.4)
CRP, mg/l			2.7 (6.4)	1.4 (1.7)
Vitamin D3 25(OH), nmol/l[a]			62.3 (30.9)	76.8 (24.7)
Vitamin D3 1,25(OH), pmol/l			129.9 (55.95)	116.1 (34.97)

Values are expressed as mean (SD) unless otherwise indicated. Group differences were examined using chi-squared tests for categorical variables and *t*-tests for continuous variables.
[a]Significantly different from healthy controls.
[b]Significantly different from female.
NA not applicable, *BMI* body mass index, Education low: 10th grade, medium: secondary school, high: college/university. *MADRS* Montgomery–Åsberg Depression Rating Scale, *BDI* Beck Depression Inventory, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *IL-6* interleukin 6, *CRP* C-reactive protein.

GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA) was used for data visualization.

RESULTS

Participants

In total, 116 participants were recruited for the study. Seven participants (MDD = 6, HC = 1) had to be excluded due to emerging somatic or psychiatric disorders, or previously undocumented intake of psychotropic medication. Thus, 58 MDD patients and 58 healthy controls (61.2% female) were included in the study. The two groups did not differ in age, gender, or smoking status. Further demographic data and clinical characteristics are shown in Table 1. HDL cholesterol and Vitamin D3 25(OH) were higher in healthy controls compared to MDD patients. Triglyceride levels correlated significantly positively with *at*-RA ($r = 0.597$, $p = 0.000$) and ROL ($r = 0.532$, $p = 0.000$) levels while HDL levels showed a significant negative association with *at*-RA ($r = -0.377$, $p = 0.000$) and ROL levels ($r = -0.356$, $p = 0.001$).

Retinoid serum levels

at-RA and ROL serum levels were quantified by HPLC in 57 unmedicated clinically depressed patients and 52 healthy control participants. The ratios of *at*-RA to ROL were calculated. *at*-RA levels ranged from 0.73 nM to 6.3 nM with a mean of 2.8 nM (SD: ±1.17) and ROL levels ranged between 0.16 µM to 3.08 µM with an average of 1.1 µM (SD: ±0.61). Neither *at*-RA nor ROL serum levels showed a correlation with age, BMI, current smoking status, or level of education.

Altered retinol serum levels in patients with depression

ROL levels were significantly higher in patients as compared to healthy controls ($t = 2.63$, $p = 0.01$, $d = 0.5$). *at*-RA levels were also higher in patients but the group difference was not significant. Moreover, the individually calculated *at*-RA/ROL ratio was significantly reduced in the patient group ($t = -2.06$, $p = 0.042$, $d = -0.4$) (Fig. 1A–C).

Increased *at*-RA synthesis in patients with depression

To capture possible differences in retinoid metabolism, we assessed the individual in vitro *at*-RA synthesis and degradation activity in metabolically active PBMC-derived crude microsomal fractions. *at*-RA synthesis and degradation activity were assessed in standardized reactions normalized by heat-inactivated controls. The *at*-RA-synthesizing activity was significantly greater in the patient group than in the group of healthy controls ($t = 3.022$, $p = 0.003$, $d = 0.6$) (Fig. 1D). Composite scores of *ROL*atRA-synthesis* and *RA*atRA-catabolism* were built to obtain a measure of the individual homeostatic "in-" and "out"-flow, respectively. As expected, differences in group mean of retinoid serum levels and metabolism activity was also reflected in the individual composite scores, showing a significantly increased homeostatic "in-flow" in patients as compared to healthy controls ($t = 3.139$, $p = 0.002$, $d = 0.6$).

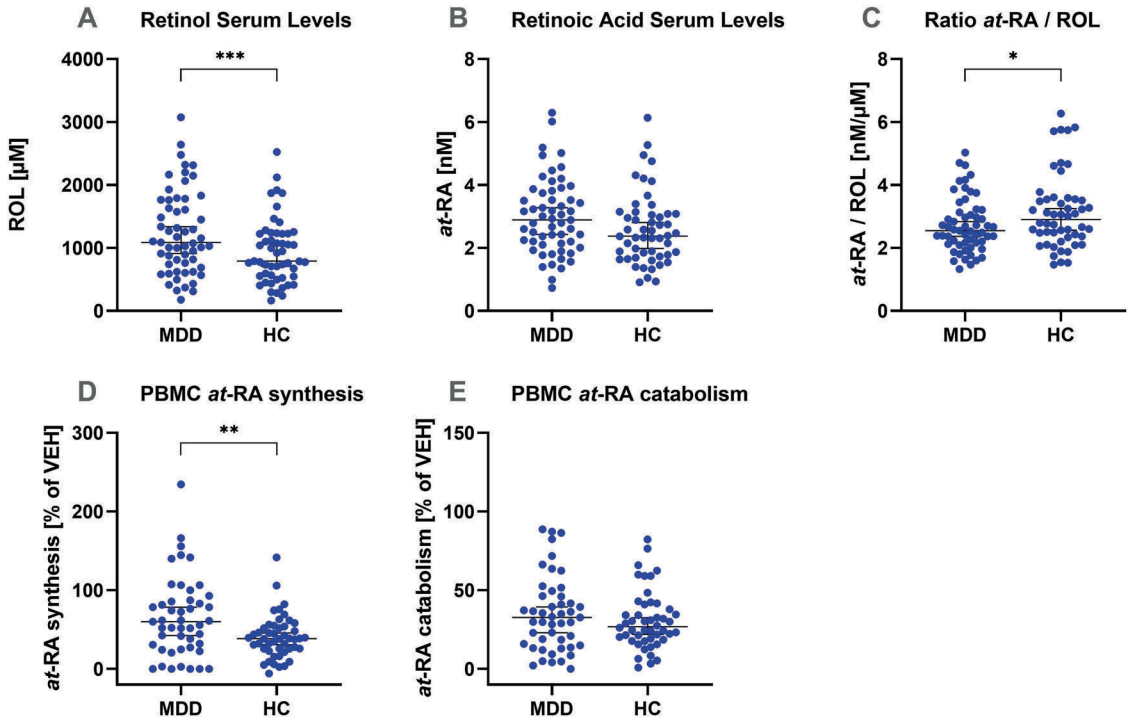

Fig. 1 Retinoid serum levels and metabolism in patients with major depressive disorder and healthy controls. Group differences between MDD patients and healthy controls (HC) in **A** ROL serum levels. **B** *at*-RA serum levels. **C** the ratio of *at*-RA to ROL. **D** *at*-RA synthesis activity in PBMC. **E** *at*-RA catabolism activity in PBMC. All error bars depict the median with 95%CI. *t*-tests were used for group comparisons.

Expression of retinoid-relevant genes

No overall group differences were found for any of the targets.

Gender differences in retinoid homeostasis parameters

In the patient group as well as in the control group, retinoid serum levels were significantly different in men and women. These findings together with evidence from the literature on gender differences in depression prompted us to examine group differences within gender for all retinoid homeostasis parameters. Gender differences within the group of healthy controls are also reported.

For serum ROL levels, we found significant group differences between patients and controls for the male gender only ($t = 2.915$, $p = 0.006$, $d = 0.9$) (Fig. 2A, B). ROL levels in women did not differ between groups. Interaction effects of group × gender were not significant.

Synthesis activity in female MDD patients was significantly higher than in female controls ($t = 3.072$, $p = 0.004$, $d = 0.8$) (Fig. 2D). No such difference was found for the male gender. No significant group or gender differences were observed for RA-catabolism activity.

Differences in mRNA expression of retinoid homeostasis-relevant genes between patients and healthy controls were found only for the female gender, showing higher expression of cellular retinol-binding protein 1 (CRBP1), retinol dehydrogenase 10 (RDH10) and CYP2C19 and reduced expression of retinoic acid receptor γ (RARg) in female patients compared to female controls (Fig. 2E–H).

Within the group of healthy controls, we found gender differences in mRNA expression of CRBP1 and serum levels of *at*-RA and ROL. CRBP1 was increased in men compared to women (Fig. 2G) and *at*-RA and ROL levels were significantly higher in men than in women (Fig. 2A, B).

DISCUSSION

In this well-matched cohort of unmedicated patients and healthy controls, we could show for the first time that important features of peripherally assessed retinoid homeostasis, which is a key player in regulating synaptic plasticity in the central nervous system, are altered in patients with MDD. Significantly increased ROL serum levels and *at*-RA synthesis activity in depressed patients highlights a possible pathophysiological contribution of altered retinoid homeostasis.

In our own previous work on retinoid homeostasis in patients with schizophrenia, we found reduced ROL and *at*-RA levels in patients compared to controls, as well as strong effects of the atypical antipsychotic clozapine on *at*-RA levels [33]. A study by Yang et al. [30] identified reduced RA serum levels as a risk factor for the development of post-stroke depression. Both studies report a dysbalance in retinoid serum levels or metabolism activity in psychiatric patients as compared to controls, and both studies suggest a protective role of endogenous RA. Further evidence of endogenous RA in disease stems from the somatic field. Similarly, higher *at*-RA serum levels are linked to more beneficial disease outcomes for cardiovascular disease, neurological syndromes, and cancer [42–45]. A recent prospective study of 29,104 men reports reduced overall and cause-specific mortality for participants with higher ROL serum levels during a 30-year follow-up [46]. In many of the disorders investigated, increased inflammation plays a central role in the onset and disease progression. The attenuating effect of RA in these cases can be explained by its strong anti-inflammatory properties [11, 47]. Many somatic disorders are closely associated with depression by way of shared biological, social, or psychological disease pathways [48], however, retinoid homeostasis in depression has thus far not been investigated.

Interestingly, in the present study, retinoid serum levels and *at*-RA synthesis activity were increased in patients compared to controls. Especially as patients did not currently receive psychotropic medication, the statistically significant differences between patients and controls are suggested to be the result of homeostatic adaptions of the endogenous retinoid signaling system in patients with MDD. Although the meaning, direction, and extent of such adaptive processes are certainly incompletely understood, there is

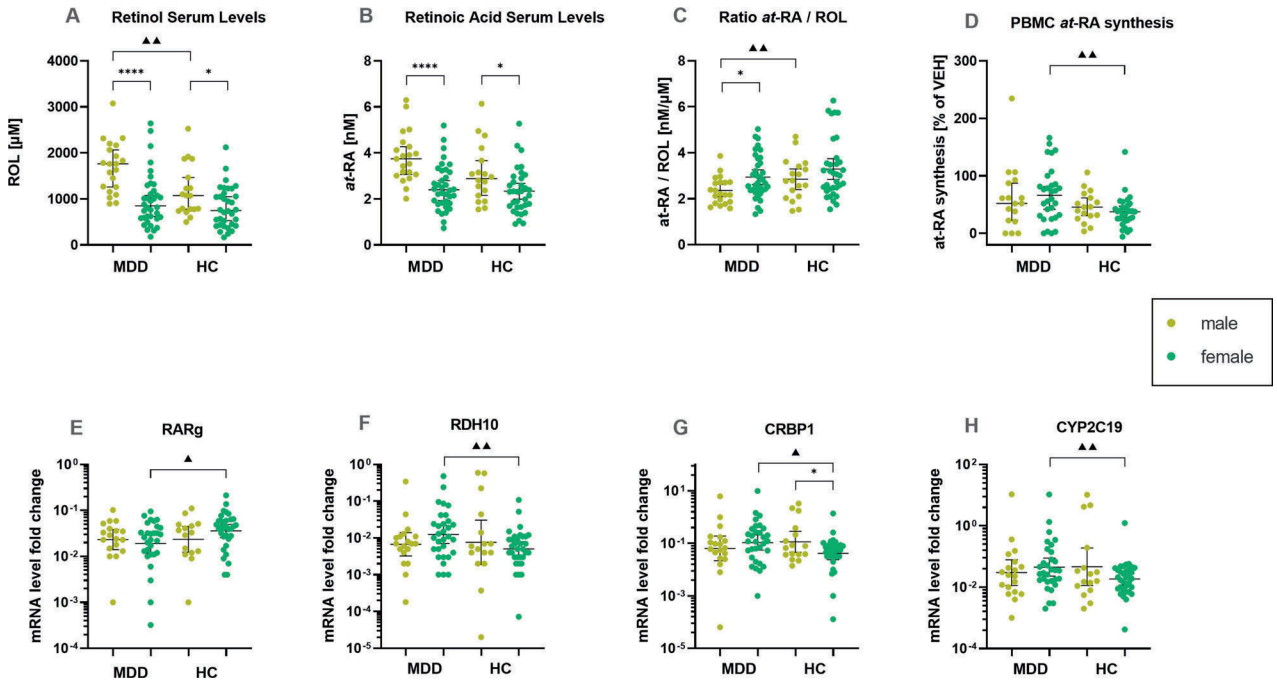

Fig. 2 Gender differences in retinoid homeostasis parameters. * marks significant within-group gender differences (i.e., male vs female in MDD or HC). ▲ marks significant group differences for within-gender comparisons (i.e., female MDD vs female HC) **A** ROL serum levels. **B** *at*-RA serum levels. **C** the ratio of *at*-RA to ROL. **D** *at*-RA synthesis activity in PBMC. **E**–**H** Gene transcription of retinoid pathway-related genes in PBMC, fold change values presented as the $2^{-\Delta Ct}$. Error bars depict the median (**A**–**D**) or geometric mean (**E**–**H**) with 95% CI. *t*-tests were used for group comparisons.

further evidence supporting the hypothesis of a compensatory, adaptive change within the homeostatically regulated system. *at*-RA is an important regulator and necessary for the normal functioning of a range of neurobiological processes that are implicated in depression [49]. Greater demand for *at*-RA would be reflected in increased peripheral bioavailability of its substrate ROL and increased *at*-RA synthesis activity as shown in our data (Fig. 1A, C). In the example of disturbed homeostatic synaptic plasticity, RA is produced upon chronic synaptic inactivity, to effect translational upregulation of postsynaptic glutamate receptors, leading to an upregulation of synaptic strength [6, 21]. Engaging the processes of homeostatic synaptic plasticity has also been proposed as a treatment option for MDD [3].

Throughout the analyses of the homeostasis parameters, we saw striking effects of gender. Within the group of healthy controls, *at*-RA and ROL levels were higher in men than in women, which is in line with previous findings for ROL [50]. The expression of CRBP1, which facilitates cellular uptake of serum ROL and delivers ROL intracellularly to select metabolic enzymes, was also increased in men compared to women. However, in vitro synthesis or catabolism activity did not differ between men and women. Based on our findings of gender differences in retinoid serum levels, and evidence suggesting that psychological and biological aspects of depression differ for men and women [51–53], we chose to also run within-gender comparisons of patients and controls in a secondary analysis. ROL serum levels were starkly increased in male depressed patients as compared to male controls while ROL serum levels did not differ between female patients and controls (Fig. 2A). Although other factors that we did not assess and control for might be at play, increased ROL serum levels might represent a male endophenotype of depression—or male-specific compensatory action.

The in vitro metabolism assays showed greater *at*-RA synthesis in female MDD patients than in female healthy controls (Fig. 2D). Though circumstantial and warranting further investigation, this dynamic is partially mirrored across levels of analysis in our data on mRNA expression patterns of genes relevant to RA synthesis (Fig. 2E–H). CRBP1, RDH10—one of the enzymes involved in the first of the two-step oxidation from ROL to *at*-RA—and one of the RA catabolizing enzymes, CYP2C19, were increased in female MDD patients compared to female controls, suggesting increased turnover of both *at*-RA and its substrate ROL. While CYP2C19 activity in *at*-RA breakdown is relatively low, it is interesting to note that it is also involved in the pharmacokinetics of psychotropic drugs. Fluoxetine, a major serotonin reuptake inhibitor (SSRI), potently blocks RA catabolism by inhibiting CYP2C19 activity [54, 55]. Gender differences in the pharmacokinetics and antidepressant response to SSRIs have been described [56, 57].

Taken together, we see different manifestations of altered retinoid homeostasis for men and women. While ROL serum levels were affected in male patients, retinoid metabolism was altered for female MDD patients. Gender differences and sex-specific retinoid dynamics in adult humans are scarcely discussed in the literature. Some support for gender differences in retinoid homeostasis comes from preclinical studies showing sex-specific expression patterns of retinoid receptors, heterogeneous retinol distribution across the brain areas of males and females, and strong effects of sex hormones on RA activities [58–60].

Based on previous studies on the central role of the retinoid system for depression, we sought to elucidate a putative dysregulation of retinoid homeostasis in MDD by systematically assessing various aspects of retinoid homeostasis in unmedicated patients and healthy controls. A dysbalance in homeostasis may become apparent at any one point of the transport, binding, and metabolism activities. By assessing several parameters that govern retinoid flux and determining *at*-RA availability, we obtained a broad picture of peripheral retinoid homeostasis. As psychotropic medication can influence RA metabolism [33] and the retinoid signaling system is also linked to other psychiatric, neurological, and somatic disorders, we selected patients who did not currently take any psychotropic medication and did not have major psychiatric, neurological, or somatic

comorbidities. This allowed us to investigate the MDD—retinoid associations in a "raw state". However, the MDD population without somatic or psychiatric comorbidities only represents a part of the MDD spectrum, possibly limiting the generalizability of our findings to a subgroup of MDD patients. For some group comparisons, the differences reported are rather small, yet statistically significant. Whether they are of clinical relevance remains to be investigated as there is currently no definition of minimally clinically important differences for these retinoid parameters in an MDD cohort. However, as this is an exploratory study, we chose to report even small differences between groups, as they may help to clarify the hypothesis of the involvement of retinoids in depression. Furthermore, the cohort was not originally powered to investigate gender differences. The gender distribution in this study mirrors the gender distribution of MDD in the general population, however, the sample size of male participants may have been too small to detect subtle group differences in mRNA expression or metabolism activity. Taking gender into account, future studies should assess retinoid homeostasis longitudinally to investigate the effects of treatment and compare homeostasis parameters in remitted patients to healthy controls. In addition, exploring the relationship between cholesterol and the retinoid system in depression might be an interesting avenue for future research. Given that RA is involved in lipid metabolism [61, 62], our results on significantly lower HDL levels in MDD patients, and the strong correlation of triglyceride and HDL levels with retinoid serum levels provide preliminary insights that should be evaluated further.

In conclusion, next to group differences in ROL serum levels and at-RA synthesis activity, our data suggest that MDD-associated alterations in retinoid homeostasis differ between men and women. Relations among the different homeostasis parameters as well as their relation to depression pathophysiology are highly intricate. Our study was not designed to mechanistically assess these relations. Instead, we cross-sectionally assessed several homeostasis parameters which can serve as a demonstration that investigating retinoid signaling as a putative pathophysiological mechanism of depression might be worthwhile for achieving a better understanding of the disease's underlying neuropathophysiology.

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AUTHOR CONTRIBUTIONS

LRO: data acquisition, data preparation, statistical analyses, interpretation of data, and writing of the first and final drafts of the paper. VC: data acquisition, interpretation of results, critical revision of the first draft of the paper, approval of the final version of the manuscript. BÜ: analyses and interpretation of PCR data, critical revision of the first draft of the paper, approval of the final version of the paper. NCC: data acquisition, critical revision of the first draft of the paper, approval of the final version of the paper. FR: interpretation of data, critical revision of the first draft of the paper, approval of the final version of the paper. JHR: conception and study design, interpretation of data, critical revision of the first draft of the paper, approval of the final version of the paper.

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The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplement

This supplement provides information on the additional assessment of retinoic acid signaling activity via reporter cell bioassay. The results were nicely aligned with the HPLC measurement of serum. As a matter of completeness, methods and results are described here.

Furthermore, Table 1 gives details on HPLC mobile phase, Table 2 lists the PCR sequence primers and Table 3 shows all mRNA expression profiles of the retinoid homeostasis-relevant genes. Figures 1 shows a HPLC chromatograph of retinoid authentic standards and Figure 2 shows separation of retinoids in human serum.

Retinoic Acid Reporter Cell Bioassay

To functionally assess the overall RA signaling activity, as a complementary measure to HPLC, we performed a cell-based reporter bioassay with participants' sera. To that aim, SIL15 RARE reporter cells developed by Wagner and colleagues were cultured according to previous protocols (1) and seeded in a 96-well plate at a density of $5*10^4$ cells per well. Medium containing either 10% participant's serum or fetal calf serum containing various concentrations of retinoic acid (0-100 nM) was added to the cells and left to incubate over night. Cells were subsequently lysed and added to a solution containing Fluorescein-Di-3-D-Galactopyranoside (FDG) as a substrate of β-galactosidase. Kinetic measurements for fluorescein fluorescence, which are proportional to the amount of induced enzyme in the lysates, were performed and functional RA signaling was calculated using the lysates from cells treated with known standards.

at-RA serum levels showed a strong relation to RA signaling activity in the reporter cell bioassay *($F = 7.919$, $p = 0.006$, $R^2 = 0.097$)*. The signaling activity was specific to RA, as ROL serum levels did not correlate with signaling activity. Accordingly, as group differences in *at*-RA concentration were not significant in the serum analyses, no group differences were seen for RA signaling activity. No gender differences were found for functional RA signaling.

29 **Table 1 Composition of HPLC mobile phase for retinoid analysis in serum**

Time (min)	A (%)	B (%)
0-15.5	15	85
16	0	100
20	0	100
21	15	85

Solvent A: water + 0.1 % formic acid
Solvent B: acetonitrile + 0.1 % formic acid

30

31

32 **Table 2 Primer Sequence (5' > 3')**

18S	GTG TTG AGG AAA GCA GAC ATT G	/56-FAM/TGC AGA ATC/ZEN/CAC GCC AGT ACA AGA/3IABkFQ/	GGT CTT CAC GGA GCT TGT T
ACTB	CGA GGA CTT TGA TTG CAC ATT G	/5HEX/TT GTT ACA G/ZEN/G AAG TCC CTT GCC ATC C/3IABkFQ/	ACT GGG CCA TTC TCC TTA GA
GUSB	GAC AGT GGG CTG GTG AAT TA	/56-FAM/AC CTG TTC A/ZEN/A GTT GGA AGT GCG TCT /3IABkFQ/	CAT TCG CCA CGA CTT TGT TT
GAPDH	ACA TCG CTC AGA CAC CAT G	/5HEX/AA GGT CGG A/ZEN/G TCA ACG GAT TTG GTC /3IABkFQ/	TGT AGT TGA GGT CAA TGA AGG G
RARA	ACA CTA CGA ACA ACA GCT C	TCC ACA GTC TTA ATG ATG CAC	
RARB	TGA TGG AGT TGG GTG GAC TT	CGG ACT CGC AGT GTA GAA AT	
RARG	GGT TTG GGA GAA AAT GTG TCG	GAG TCG CTC CTT ATT GGT GG	
RXRG	CAT GAA GAG GG AAG CTG TG	CCA CTG GTA GCA CAT TCT G	
RDH10	TAG CCG ATG TTA CTG TCC CT	GGG AAC ATT AGC ACA CAC CA	
ALDH1A1	GTG GCA AGA AAT TTC CTG TC	TCA ACA TCC TCC TTA TCT CCT	
ALDH1A2	GTT ACA ATG CCT TAA ATG CCC	AGC CAA ATT CTC CCA TTT CTC	
ALDH1A3	TAC AAC GCC CTC TAT GCA C	CAA AGC GTA TTC ACC TAG TTC TC	
CRBP1	TGG CAT CAC TTC CAG CAT TAT T	TAA ACT GAC CCT TGA CTG AGC	
CYP26A1	GAA GAG TAA GGG TTT ACT TTG C	CCC GAT GTA TTT AAG TTG TTC C'	
CYP26B1	TGG TGG AGA TGG AAT GTG GC	TTG CTC CCC GTC AGA AGA AC	
CYP26C1	ACT GGT TAG TTC AGG GCT C	TGA ACA CTG TCC CAT AGC G	
LRAT	GTT CCT TAT CCG TCT CAT TCC C	CAG GAT GTT AGC TCC GTA GG	
STRA6	AAC TGC CGA GAC CAC ACA AC	ACG ACA TTC TCT GGC CCT TC	
CRABP1	GGC TTG CTC CTA CTT TCA GG	TAG GGA TAC AAG AGG CAC CA	

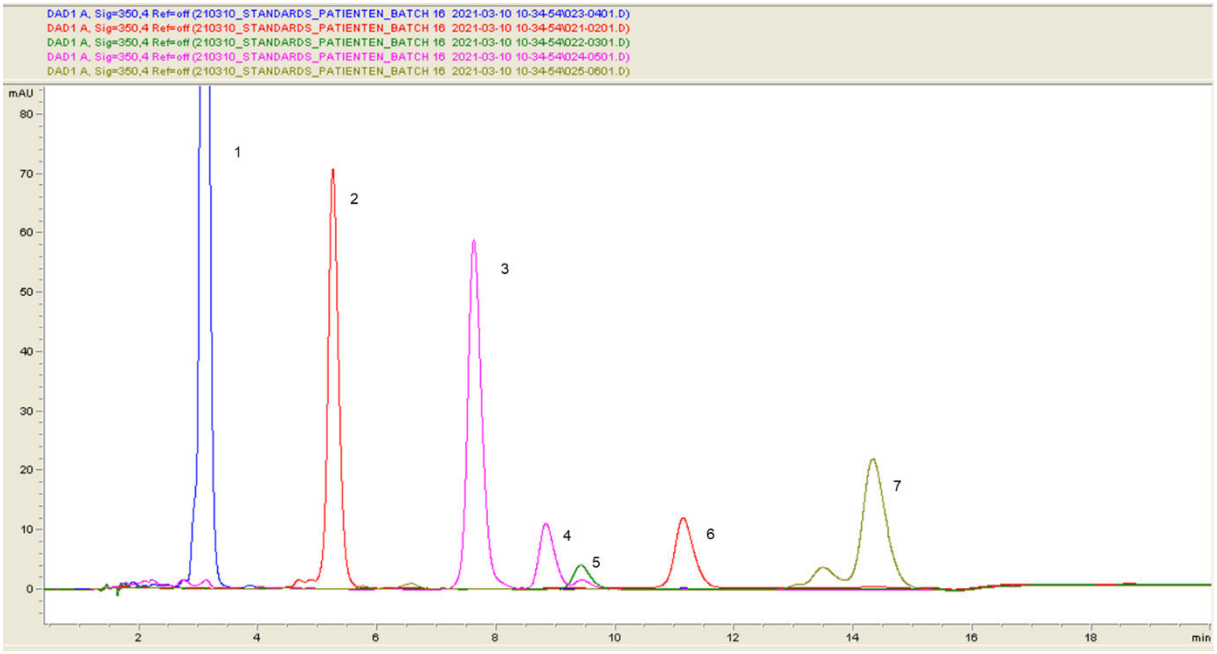
CRABP2	CAC CAC AGA GAT TAA CTT CAA GG	TTC ACC AGG CTC TTA CAG G	
CYP1A1	CTG AGG TCC TGA TAA GCA C	AAC TTC TCA TTC AGG TCC TG	
RDH5 (RDH1)	CTG TAG GTC ACT TGG GCT CC	TAA CGT GCA TCT CCA CCC AC	
RAI	TGC CGT AGT AAT CCA CCC CA	AAC GGG TTT TCC TCC CAA CC	
CYP2C8	GCA GTT ACC AAA GGG ATT GTT T	/56-FAM/TG AAG AAT G/Zen/C TAG CCC ATC TGG CT/3IABkFQ/	GAG TTG CAG GTG ATA GCA GAT
CYP2C9	TCC CTT CCC TGA AGA TCT AGT G	/56-FAM/TC GAC CTC C/Zen/A TTA CGG AGA GTT TCC T/3IABkFQ/	GCA ACT GTT ACA GAG TAT GGA GAA
CYP2C19	TGT CTG AAG AAG CAC AGA TGG	/56-FAM/TT TGG ACC A/Zen/G AGG AAA GAG AGC TGC /3IABkFQ/	GGT CAG AAG AAG CAT CAC AGA TA

Table 3 mRNA expression of genes relevant to retinoid homeostasis

	MDD				**HC**			
	male		**female**		**male**		**female**	
	Mean	N	Mean	N	Mean	N	Mean	N
RARa	0,3712	19	0,5128	31	0,4751	16	0,4175	33
RARb	0,2512	19	0,4324	31	0,8416	16	0,0974	33
RARg	0,0323	18	0,0302	28	0,0363	15	0,0502	33
RXRg	0,0174	19	0,0265	31	0,0349	16	0,0067	33
RDH5	0,2886	18	0,2919	30	0,2365	16	0,0696	33
RDH10	0,0265	19	0,0423	31	0,0931	16	0,0107	33
ALDH1A1	0,0337	19	0,0229	31	0,0323	16	0,0158	32
ALDH1A2	0,0013	17	0,0015	25	0,0011	13	0,0011	33
ALDH1A3	0,0006	17	0,0019	25	0,0005	13	0,0003	27
CRBP1	0,4611	19	0,5504	31	0,5223	16	0,1019	33
CRABP1	0,0115	19	0,0137	31	0,0185	16	0,0047	32
CRABP2	0,0041	18	0,0044	29	0,0036	13	0,0042	33
LRAT	0,0762	19	0,1057	31	0,0428	16	0,0209	33
STRA6	0,3160	19	0,4076	31	0,6586	16	0,1213	33
RAI	0,0618	19	0,0101	31	0,0640	15	0,0127	33
CYP1A1	0,0006	14	0,0021	25	0,0041	16	0,0009	31
CYP2C8	0,6814	19	0,2797	31	1,5095	16	0,3343	33
CYP2C9	0,7689	19	0,2673	31	1,2473	16	0,0425	33
CYP2C19	0,6105	19	0,4626	30	1,2288	16	0,0600	33
CYP26A1	0,3624	19	0,4047	31	0,5633	16	0,1084	33
CYP26B1	0,5735	19	0,7735	31	0,5824	16	0,2395	33
CYP26C1	0,0040	19	0,0046	31	0,0066	16	0,0014	33

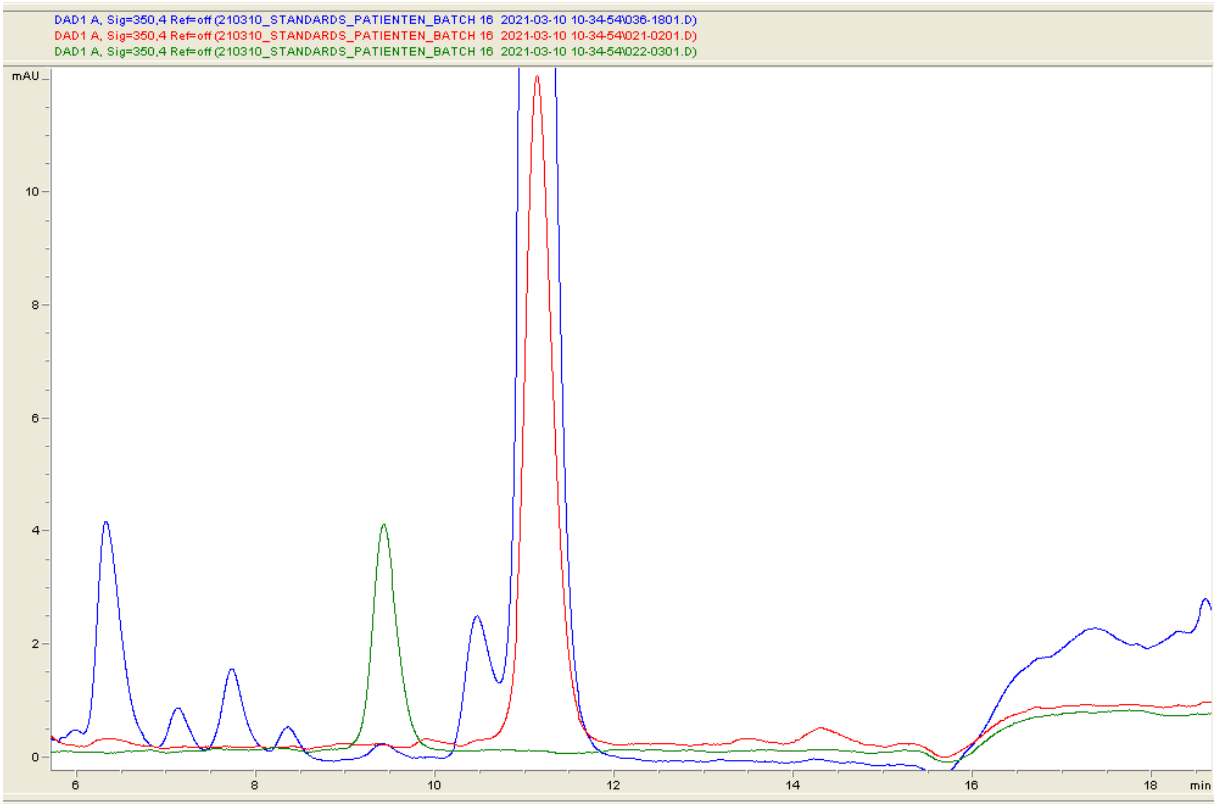
Values are expressed as fold change from the target to endogenous reference genes.

Fig. 1 Separation of various retinoids by HPLC



Retinoid authentic standards in running buffer. [1] 4-*oxo*-RA 1µM; [2] acitretin 1µM; [3] 13-*cis*-RA 1µM; [4] 9-*cis*-RA 1µM; [5] *at*-RA 100nM; [6] ROL 1µM; [7] RAL 1µM

Fig. 2 Detection of retinoids in serum



Chromatograph of a serum sample (blue line), *at*-RA 100nM authentic standard in running buffer (green line) and ROL 1µM authentic standard in running buffer (red line).

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Original Publication RA-SZ Study

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ARTICLE

Clozapine modulates retinoid homeostasis in human brain and normalizes serum retinoic acid deficit in patients with schizophrenia

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Abstract

The atypical antipsychotic clozapine is one of the most potent drugs of its class, yet its precise mechanisms of action remain insufficiently understood. Recent evidence points toward the involvement of endogenous retinoic acid (RA) signaling in the pathophysiology of schizophrenia. Here we investigated whether clozapine may modulate RA-signaling. Effects of clozapine on the catabolism of *all-trans* RA (*at*-RA), the biologically most active metabolite of Vitamin A, were assessed in murine and human brain tissue and peripheral blood-derived mononuclear cells (PBMC). In patients with schizophrenia with and without clozapine treatment and matched healthy controls, *at*-RA serum levels and blood mRNA expression of retinoid-related genes in PBMCs were quantified. Clozapine and its metabolites potently inhibited RA catabolism at clinically relevant concentrations. In PBMC-derived microsomes, we found a large interindividual variability of the sensitivity toward the effects of clozapine. Furthermore, *at*-RA and retinol serum levels were significantly lower in patients with schizophrenia compared with matched healthy controls. Patients treated with clozapine exhibited significantly higher *at*-RA serum levels compared with patients treated with other antipsychotics, while retinol levels did not differ between treatment groups. Similarly, in patients without clozapine treatment, mRNA expression of RA-inducible targets CYP26A and STRA6, as well as at-RA/retinol ratio, were significantly reduced. In contrast, clozapine-treated patients did not differ from healthy controls in this regard. Our findings provide the first evidence for altered peripheral retinoid homeostasis in schizophrenia and suggest modulation of RA catabolism as a novel mechanism of action of clozapine, which may be useful in future antipsychotic drug development.

Introduction

The atypical antipsychotic clozapine remains the first choice in treatment-resistant schizophrenia (SZ) [1–6]. Despite its potential to cause agranulocytosis/granulocytopenia and metabolic side effects, it exhibits extraordinary antipsychotic properties with negligible risk of extrapyramidal side effects [7]. Clozapine is known for its moderate binding affinity to various neurotransmitter receptors while exhibiting a low affinity to the canonical D2 receptor [8]. These direct neurotransmitter-associated effects cannot sufficiently explain the remarkable efficacy of clozapine in treatment-refractory SZ. This implies that other non-neurotransmitter-associated effects may be expected to underlie its outstanding antipsychotic properties [9–12]. Moreover, both clozapine and its two major metabolites, clozapine-N-oxide (CNO) and N-desmethylclozapine (NDC), exhibit neuroprotective and anti-inflammatory properties [13, 14].

Accumulating evidence suggests that disruption of synaptic functions, triggered at different stages during life, constitutes the neurobiological basis of adaptation deficits in the brain circuitry of patients with SZ [15]. These processes are regulated by paracrine retinoid signaling [16–18]. Several lines of evidence directly point toward disturbed retinoid signaling in schizophrenia [19–25]. All-trans retinoic acid (*at*-RA), the most active metabolite of vitamin A is locally produced and tightly regulated in brain tissue through

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cytochrome P450- (CYP450) mediated degradation into polar metabolites [26]. Retinoid signaling plays pivotal roles during embryonic development and neuronal differentiation [27, 28]. In the adult brain, RA gradients between synthesizing and degrading sites of brain tissue also control several aspects of neuronal plasticity, including long term potentiation and depression (LTP/LTD) [29, 30], neuritogenesis [31], and the process of metaplasticity [16, 17]. Metaplasticity is required for scaling synaptic strength within a neuronal network in a homeostatic manner and appears to be disturbed in SZ [18, 32–34], possibly due to dysregulated RA signaling in these patients [35]. In addition, evidence from hypothesis-free transcriptomic/proteomic analyses and genome-wide association data point toward altered retinoid signaling in the pathogenesis of SZ [21, 23, 36–38]. Finally, dopaminergic pathways are under direct transcriptional control of retinoids [39]. Based on these associations, first retinoid-targeting clinical trials using bexarotene, a retinoid-X-receptor (RXR)-directed retinoid, show promising results in patients with SZ [40–42].

Based on evidence for dysregulated retinoid homeostasis in SZ, we hypothesized that some of the pleiotropic actions of clozapine might be mediated via direct effects on RA homeostasis. We hypothesized that clozapine might modulate RA homeostasis via direct interactions with local brain *at*-RA catabolism, which is highly prevalent in the human brain and the most relevant regulatory step in brain *at*-RA signaling [43–45]. To identify possible effects of clozapine and its metabolites on retinoid homeostasis, we used a previously established methodology to assess tissue-specific catabolism of RA in murine tissues, human postmortem brain tissue and human peripheral blood-derived mononuclear cells (PBMC) in vitro [46–48]. Furthermore, we assessed various RA homeostasis-related parameters in an observatory clinical study of patients with SZ with and without clozapine medication and matched healthy controls.

Materials, patients, and methods

Materials

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise stated.

Cell culture and tissue preparation

Serum and PBMC isolation

For a detailed description of serum and PBMC isolation, please refer to the Supplement S1. In brief, whole blood was collected in the respective vacuum-extraction tubes and prepared according to the manufacturer's instructions. Aliquots were stored at −80 °C. For PBMC isolation, heparinized blood was extracted by FICOLL™ density gradient centrifugation, following previously published protocols [49].

Preparation of crude microsomal and synaptosomal fractions for RA metabolism assays

Human postmortem brain tissue from the superior temporal gyrus of five healthy donors was obtained from the Netherlands Brain Bank (NBB, Netherlands Institute for Neuroscience, Amsterdam). All donors gave written informed consent for brain autopsy and for the use of specimens for research purposes. Mouse tissue was derived from C57/BL6, P0-P3, male and female animals. Animals were sacrificed by decapitation before tissue preparation. All experiments including animals were registered and approved by German regulatory authorities (T 0268/15) and approved by a local institutional review board.

CYP450-containing, metabolically active crude microsomal and synaptosomal fractions from brain tissues and isolated PBMCs were prepared according to previously published protocols with minor modifications [48, 50]. For a detailed description of the procedures, please refer to the Supplementary S1. In brief, tissues or cells from healthy donors (Supplementary Table 1) were homogenized and metabolically active fractions were prepared by differential centrifugation steps. Metabolically active crude synaptosomal/microsomal fractions were stored at −80 °C, protein concentrations were determined by the BCA method (Thermo Fisher, USA).

RA catabolism assay

In vitro assays to quantify RA metabolism were performed as previously published [51, 52]. A more detailed description of the methods is available in Supplementary S1. In brief, samples contained metabolically active enzyme preparations, the test compound (drug) at the desired final concentration, RA (1 µM), NADPH (800 µg/ml) and assay buffer. Heat-inactivated controls were incubated at 95 °C for 15 min. Reactions were allowed to incubate for 60 min at 37 °C and were stopped by the addition of ice-cold methanol. Subsequently, all samples were centrifuged at 21,000 × *g*, 4 °C and subjected to retinoid analysis. All steps were carried out under dim, yellow light. RA metabolic activity was calculated by comparing RA degradation in metabolically active samples with heat-inactivated controls.

Participants

Healthy donors and SZ patients within the clinical observational study on RA homeostasis in neuropsychiatric disorders

(RAHND; ClinicalTrials.gov Identifier: NCT02439099) were included. The local ethics committee approved the study (EA4/002/13). All patients had a clinical DSM-5 diagnosis of SZ more than five years prior to inclusion were treated in our clinic as in- or outpatients and were on a stable medication with clozapine ($N = 10$) or other antipsychotics ($N = 10$; Supplementary Table 2). All participants were matched for age, weight, BMI, and smoking status, including matched healthy controls ($N = 10$; Table 1). Participants arrived at the laboratory between 8 and 12 a.m. after an overnight fast for blood collection. Each participant provided a total volume of 40 ml of peripheral venous blood.

Serum extraction of retinoids

The extraction of retinoids from human sera was performed by a liquid–liquid extraction procedure using the synthetic retinoid acitretin as an internal standard to assess recovery and account for inter- and intra-assay variability. Liquid–liquid extraction was performed by spiking fractions of 1 ml of serum with internal standard (Acitretin) dissolved in DMSO, then adding 1 vol of acidified ethanol containing 3% (v/v) orthophosphoric acid to 1 vol of patient serum. Samples were vortexed for 1 min and 2 vol of hexane was added. Samples were vigorously vortexed for 15 min and centrifuged at 1560 × *g* at 4 °C for 5 min. The supernatant was evaporated to dryness under a gentle stream of Argon. Samples were resuspended in 1 ml of HPLC running buffer.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was performed as previously described [52] and specified in detail in Supplementary S1. In brief, RA isomers and RA degradation products were quantified using an Agilent 1100-series HPLC system equipped with a Supelco Suplex® column (5 µm, 2.1 × 250 mm; for pharmacological assays) or a Phenomenex Synergi RP 4 µm 80 A column (for serum analyses) and a 1260-series diode-array detector with UV detection at 340 nm for *at*-RA detection and 320 nm for retinol (ROL) detection. Peaks were identified by authentic standards and purity was routinely checked by online spectral analysis.

Table 1 Participant characteristics.

	Schizophrenic patients		Healthy controls
	Clozapine	Other medication	
	$N = 10$	$N = 10$	$N = 10$
Age (years ± SD)	42.2 ± 11.1	42.5 ± 13.9	42.2 ± 11.3
Male/female (*n*)	5/5	5/5	5/5
BMI ± SD	25.2 ± 5.0	27.3 ± 5.1	25.9 ± 3.9
Current smokers (*n*)	6	6	6
PANSS total score ± SD[a]	72.5 ± 21.2	86.6 ± 18.1	NA
Years of medication[a]	15.3 ± 9.9	4.5 ± 3.9	NA

BMI Body Mass Index, *PANSS* Positive and Negative Syndrome Scale, *NA* not applicable.

[a]Significantly different from healthy controls.

Real-time PCR

Blood was collected in PAXgene® tubes (PreAnalytiX GmbH) and RNA was extracted following the manufacturer instructions. Total RNA was then reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit™ (Thermo Fisher Scientific Inc., MA, USA). All primers were designed and checked for their quality using the Primer-BLAST software [53]. Primer sequences and further details on qPCR analyses are available in the Supplementary S1.

Statistical analyses

Numerical analyses were performed using GraphPad statistical software version 5.04 (GraphPad Software, La Jolla, USA). For inhibition characteristics of RA catabolism by clozapine, 3- or 4-parameter nonlinear regression analysis was performed using GraphPad Prism. Values for maximum inhibition and half-maximal inhibitory concentrations (IC50) were calculated based on the regression analysis. Differences between groups were investigated by Student's *t* test, one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparisons test or Kruskal–Wallis test with Dunn's multiple comparisons test when appropriate. P values < 0.05 were considered statistically significant. Values are given as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Clozapine blocks RA catabolism in murine whole brain

In mouse whole brain-derived synaptosomal fractions, clozapine strikingly blocks RA catabolism, starting at concentrations as low as 1 µM when assessed by either reduction of *at*-RA levels or synthesis of the *at*-RA degradation product 4-oxo-RA (Fig. 1a, b).

The representative chromatograms (Fig. 1a) demonstrate low to absent RA catabolism in heat-inactivated samples and substantial RA degradation in active samples by means of reduced *at*-RA peaks and increased RA metabolites. Upon addition of clozapine at 5 or 50 µM, *at*-RA peaks in active synaptosomes increase, while peaks of the RA metabolite 4-oxo-RA decrease in a concentration-dependent

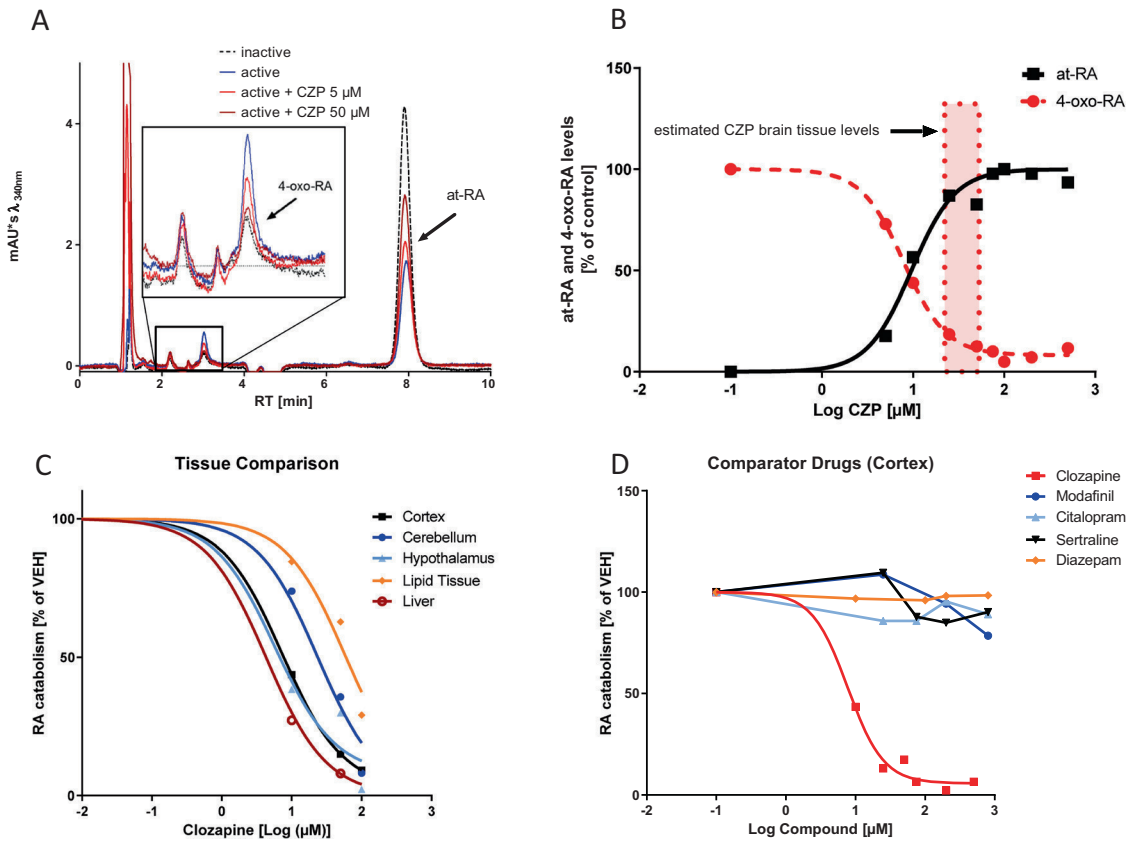

Fig. 1 Clozapine blocks RA degradation in murine tissues. a HPLC Chromatograms of *at*-RA catabolism assays from representative samples containing heat-inactivated (blue line) or active crude synaptosomal fractions from mouse brain exposed to *at*-RA at 1 µM and treated either with vehicle (black, dashed line) or clozapine at 5 and 50 µM (red lines). **b** Levels of *at*-RA (solid line) and its polar metabolite 4-OXO-RA (dashed line) following in vitro incubation (1 h, 37 °C) with murine brain-derived synaptosomes and *at*-RA 1 µM starting concentration in the presence of various clozapine concentrations. Values are calculated relative to heat-inactivated and vehicle-treated controls. Clozapine significantly inhibits *at*-RA degradation in a dose-dependent manner, blocking on average > 80% of *at*-RA catabolism in microsomal preparations, as calculated by 4-parameter nonlinear regression analysis. The dotted rectangle demonstrates the brain tissue levels of clozapine reached during steady state. **c** *at*-RA catabolism was measured by quantifying the total *at*-RA turnover in clozapine-treated murine tissue samples relative to vehicle-treated samples. Different murine brain regions and other tissues exhibit differential sensitivity toward clozapine. **d** Various other psychotropic drugs were assessed in the same pharmacological assays, including the stimulant modafinil, the two antidepressants citalopram and sertraline and the benzodiazepine diazepam. Neither of the tested drugs exhibited inhibitory effects comparable to clozapine. All assays were performed at least in duplicates using pooled synaptosomes/microsomes from $N = 3$ animals.

manner, indicating that clozapine potently blocks RA catabolism.

Figure 1b further demonstrates the strong inhibitory effect of various clozapine concentrations on RA degradation when measured either by quantification of *at*-RA concentration or by measuring the increase in the concentration of RA metabolites (4-oxo-RA). The half-maximal inhibitory concentrations (IC50 value) for clozapine were 7.9 µM as determined by measuring the reduction in *at*-RA and 7.8 µM as determined by measuring the synthesis of 4-oxo-RA.

Local brain levels of clozapine that are expected to be reached when clozapine is given at therapeutically relevant doses [54] are indicated in the figures by means of rectangles labeled "brain tissue levels". These levels were calculated based on observations by Wilk et al. and particularly Baldessarini et al., who measured both serum and brain clozapine levels in rats at steady state after intraperitoneal administration [54, 55]. While serum levels in rats reached levels comparable to human serum concentrations, lipophilic clozapine is preferentially distributed to brain tissue resulting on average in 24-fold higher brain concentrations compared with serum levels [54]. In humans, serum clozapine concentrations between 350 and 600 ng/ml are considered to be associated with an optimal therapeutic response [56]. Therefore, brain tissue levels of 8400–14400 ng/ml, which equals 25.7–44.05 µM, are expected.

The increase in *at*-RA and the decrease in the synthesis of 4-oxo-RA in response to clozapine exposure were significantly correlated (Pearson's $r = 0.990$, $P < 0.001$),

suggesting that measuring either the decrease of *at*-RA or the increase of 4-oxo-RA may be adequate to assess RA catabolism.

Clozapine blocks RA catabolism in various murine tissues

As we were interested in local differences of the effects of clozapine on RA catabolism and because of the known effects of clozapine on energy homeostasis with weight gain as one of the prominent side effects of the drug, we also assessed the impact of clozapine on RA catabolism in murine cerebellum, hypothalamus and in non-neuronal tissues (liver tissue and lipid tissue from visceral fat; Fig. 1c). Clozapine exhibited a differential impact on RA catabolism with most pronounced effects in liver (IC50 = 4.44 µM), cortex (IC50 = 7.30 µM) and hypothalamus (IC50 = 5.76 µM) and less pronounced effects in cerebellum (IC50 = 54.12 µM) and in lipid tissue (IC50 = 699.5 µM).

Impact of other psychotropic drugs on cerebral RA catabolism

In order to compare the observed effects of clozapine on RA catabolism with other psychotropic drugs, we next tested the effects of the antidepressants citalopram and sertraline, the benzodiazepine diazepam and the stimulant modafinil on RA catabolism, using equally high concentrations, including concentrations above therapeutically relevant tissue levels (see Supplementary Table 3 for estimated brain concentrations) [57–61]. Neither of the tested compounds affected the degradation of RA in mouse cortex-derived synaptosomes to the extent of clozapine (Fig. 1d).

Effects of clozapine on human tissues

Next, we analyzed the impact of clozapine on RA catabolism in human PBMC-derived microsomes from eight healthy donors (Fig. 2; for participant characteristics see Supplementary Table 1). Clozapine strikingly inhibited RA catabolism in all samples (Fig. 2a), yet the degree of inhibition varied considerably between subjects. All subjects exhibited IC50 values (mean 12.5 µM, ±9.2) within or even below the estimated brain tissue concentrations of clozapine (Fig. 2a, b). The sensitivity of RA catabolism toward inhibition by clozapine (IC50) also differed markedly between subjects (Fig. 2b). While five out of eight subjects exhibited high sensitivity with IC50 values well below the calculated mean of 12.5 µM, there were three subjects with IC50 values markedly above the mean of 12.5 µM (Fig. 2b). For five out of eight subjects, inhibition was predicted to result in 100% inhibition of RA catabolism (Fig. 2c).

Finally, we assessed RA catabolism in pooled metabolically active synaptosomes from human postmortem brain tissue of five healthy donors (Fig. 2d). Pooled synaptosomes, all prepared from the superior temporal gyrus of the donors, exhibited strong RA catabolic activity. *At*-RA degradation was also strikingly affected by clozapine with an IC50 value of 12.9 µM (Fig. 2d), which is almost identical to the average IC50 value observed for human PBMCs (Fig. 2a) and comparable to murine brain (Fig. 1a).

Clozapine metabolites N-Desmethylclozapine and Clozapine-N-oxide also affect *at*-RA catabolism

The two major metabolites, "pharmacologically inert" CNO and NDC, both exhibit neuroprotective and anti-inflammatory properties via unknown mechanisms, starting at concentrations as low as 0.01 µM for CNO and 1 µM for NDC [13]. Interestingly, both metabolites exhibit RA catabolism-blocking properties in murine cortex, pooled human PBMCs and human cortex (Fig. 3). Significant inhibition occurs at low micromolar concentrations that are expected to be reached in clinical practice. The strongest inhibition of RA metabolism was seen for both NDC (IC50 = 0.40 µM) and CNO (IC50 = 2.9 µM) in pooled human PBMC-derived microsomes (Fig. 3c, g).

Effects of clozapine on serum *at*-RA levels and PBMC mRNA transcripts of RA-related genes in patients with schizophrenia

To assess clinical relevance for the observed effects of clozapine on RA catabolism, we measured for the first time *at*-RA concentration in the serum of patients with SZ who had been on clozapine medication for more than five years (clozapine group), matched patients with SZ taking antipsychotics others than clozapine (no-clozapine group, Supplementary Table 2), and matched healthy controls (Table 1).

We found significantly reduced serum levels of *at*-RA in patients with SZ compared with matched healthy controls ($P < 0.0001$; Fig. 4a). In patients with SZ, *at*-RA serum levels were lowest in the no-clozapine group of patients with SZ (2.2 ± 1.2 nM) and significantly higher in the clozapine group (3.7 ± 1.3 nM; $P < 0.05$). Both treatment groups exhibited lower *at*-RA levels when compared with healthy controls (7.1 ± 1.8 nM; $P < 0.001$).

ROL levels were also significantly reduced in patients with SZ of the no-clozapine group (1.37 ± 0.19 µM, $P < 0.001$) and the clozapine group (1.27 ± 0.09 µM, $P < 0.001$) compared with healthy controls (2.46 ± 0.43), yet without the effect of medication (Fig. 4b).

Finally, the ratio of *at*-RA to ROL was calculated for each subject (Fig. 4c). Interestingly, ratios were significantly reduced only in the no-clozapine group (1.64 ± 0.16 nM/µM)

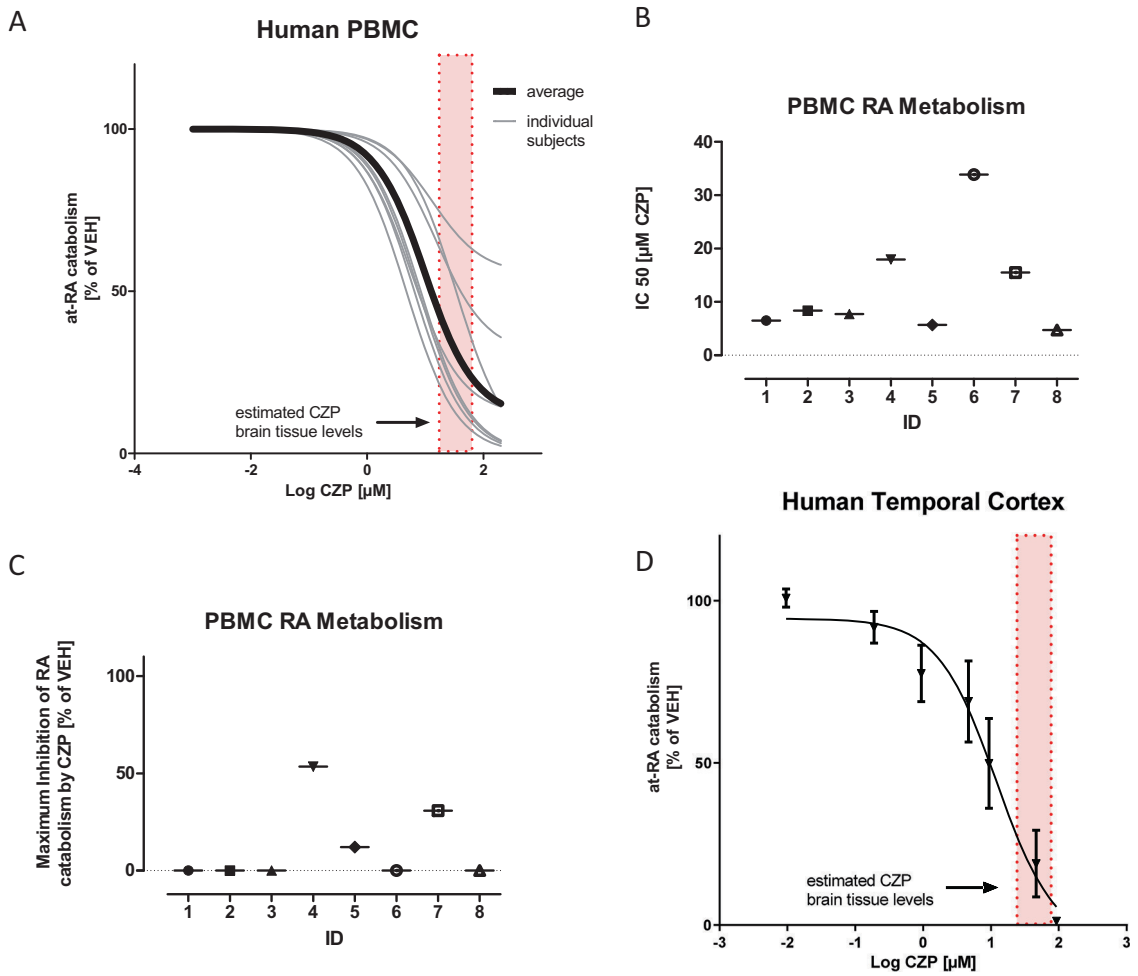

Fig. 2 Clozapine blocks *at*-RA degradation in human tissues. **a** Effects of clozapine on *at*-RA degradation in PBMC-derived microsomal preparations were assessed using cells from 8 healthy donors. Clozapine significantly blocked *at*-RA turnover in all subjects (lightweight curves) with an average IC50 value of 9.86 µM (dark bold curve). **b** IC50 values and **c** maximum inhibitability, as calculated by 4-parameter nonlinear regression, exhibited significant variability between the 8 subjects. **d** Clozapine also significantly blocks *at*-RA degradation in pooled human brain-derived synaptosomal preparations derived from postmortem tissue from 5 healthy subjects (IC50: 12.9 ± 1.56 µM). The dotted rectangle demonstrates the brain tissue levels of clozapine during steady state. Values are given as mean and standard error of $n \geq 3$ experiments.

when compared with healthy controls (2.88 ± 0.28 nM/µM; $P < 0.001$) and subjects from the clozapine group (2.88 ± 0.20 nM/µM; $P < 0.001$). Ratios in the clozapine group did not differ from healthy controls.

PBMC mRNA transcript levels of the RA catabolic enzyme CYP26A (Fig. 4d) and the RA-inducible protein STRA6 (Fig. 4f) both exhibited a similar profile as observed for the *at*-RA/ROL ratio (Fig. 4c). Here, we found significantly lower CYP26A levels in the no-clozapine group of patients with SZ (0.55 ± 0.17) compared with healthy controls (1.0 ± 0.33; $P < 0.05$) or subjects treated with clozapine (0.85 ± 0.27; $P < 0.05$), while there was no difference between clozapine-treated patients and healthy controls. In contrast, there were no main effects of group for the RA-synthesizing enzyme aldehyde dehydrogenase 1A2 (ALDH1A2; Fig. 4e).

We also assessed mRNA expression of the retinoic acid receptor (RAR) and RXR α, β and γ isotypes (Fig. 5). While RAR-γ was not detectable, there were no significant main effects of group for any of the receptors. Finally, mRNA expression of non-specific RA-degrading cytochrome isoforms CYP1A2, 2D6, and 3A4 were quantified in PBMCs, exhibiting significantly reduced expression of CYP2D6 and 3A4 in clozapine-treated subjects (Fig. 4f–h).

Discussion

We demonstrated for the first time that clozapine and its major metabolites specifically inhibit *at*-RA catabolism in a dose-dependent and clinically relevant manner. Furthermore, we provide clinical evidence on significantly reduced

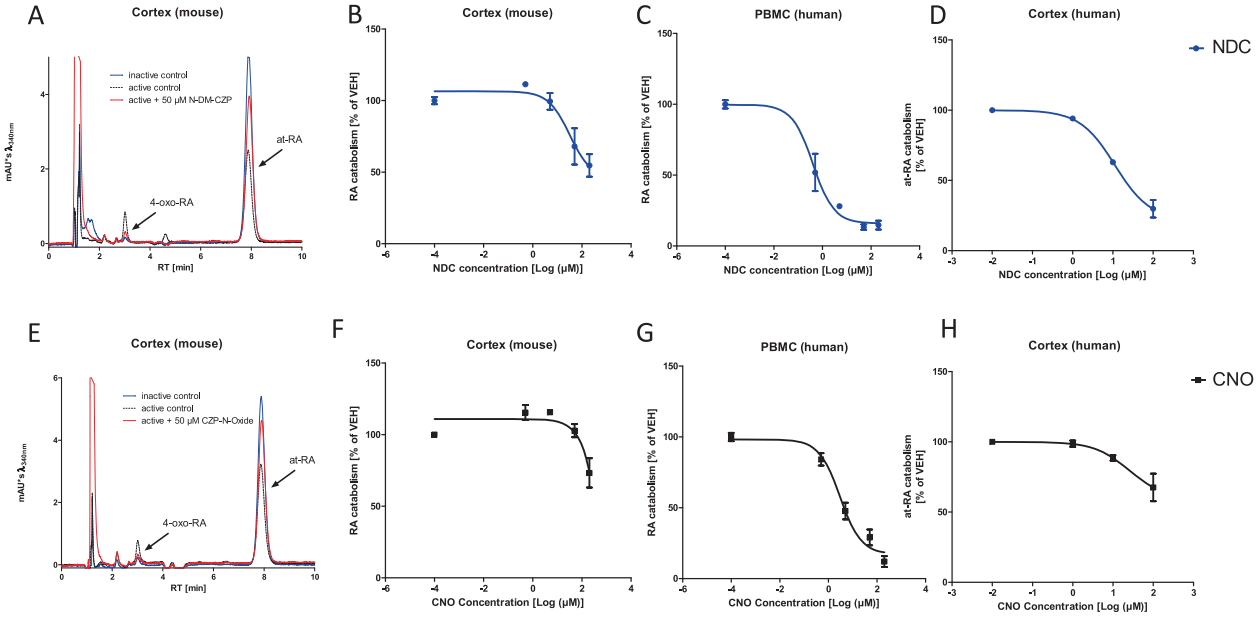

Fig. 3 Impact of Clozapine Metabolites on *at*-RA Catabolism. **a** Representative chromatographs of *at*-RA catabolism in murine cortex demonstrate the impact of N-Desmethylclozapine (NDC) on *at*-RA degradation. NDC significantly blocks *at*-RA catabolism in synaptosomes derived from mouse cortex (**b**), human PBMCs (**c**) and human cortex (**d**). Clozapine-n-oxide (CNO) also exhibits significant, yet less pronounced inhibition in murine cortex (**e**, **f**), human PBMCs (**g**) and human cortex (**h**) with IC50 values within the expected brain tissue levels according to 4-paramter nonlinear regression analysis. All experiments were performed at least in duplicates and values are given as mean and standard error.

retinoid serum levels in patients with schizophrenia and on clozapine positively modulating RA homeostasis in clozapine-treated patients.

Our findings are in line with the "retinoid hypothesis of schizophrenia" [35], which is based on several fundamental findings. First, endogenous retinoids are crucially involved in maintaining neuronal homeostasis by acting as potent endogenous neuroprotective compounds and regulators of inflammation, particularly microglial activation [62, 63]. Moreover, retinoid signaling is increasingly recognized as a key mediator in metaplasticity [16], which is suggested to be disturbed in SZ [34, 64].

In line with the hypothesis of deranged metaplastic processes during the course of illness, several other lines of evidence have also suggested dysregulated retinoid homeostasis in the pathophysiology of schizophrenia [40]. While early studies have clearly identified retinoid dysregulation at the transcriptome level in patients with SZ [37], there is also more recent genome-wide proof for retinoid dysregulation in SZ [21]. Moreover, evidence from retinoid-based treatments supports the hypothesis that specific retinoid-targeting interventions may serve as novel treatment options [41, 42].

When we think about retinoid-based interventions to treat e.g. patients with SZ, it is important to consider that the use of exogenous retinoids may be somewhat compromised by homeostatic adaptations within the locally controlled retinoid signaling network (e.g., induction of local degradation, downregulation of endogenous synthesis) [65]. To overcome this problem, it may be an option to enhance retinoid signaling by means of blocking RA degradation, which is a pivotal step in controlling local RA levels [28, 65, 66]. We previously identified this mechanism for pleiotropic minocycline [46, 48, 67], a tetracycline antibiotic and modulator of microglial activity discussed for use in SZ [68–70]. Out of all antipsychotics available, clozapine is still considered the most effective drug in treatment-refractory SZ. Nevertheless, its precise mechanism of action remains unclear. Based on our findings for minocycline and the fact that catabolism of clozapine involves RA-degrading CYP450 enzymes, we hypothesized clozapine to functionally block endogenous RA catabolism. Further support for this hypothesis comes from a partial overlap of the side effects of clozapine and RA, which include dyslipidemia and impaired bone marrow functions, particularly reduced white blood-cell counts [71, 72].

To assess the effects of clozapine on RA degradation, we functionally measured *at*-RA catabolism in various target tissues in the presence of various clozapine doses. While effects on RA catabolism were expected, the extent to which low, physiological clozapine concentrations blocked *at*-RA degradation was rather unexpected, and so were our findings on NDC and CNO, which equally affect *at*-RA degradation, thereby possibly explaining their pleiotropic effects [13].

Using RA-metabolizing microsomes of donor-derived PBMCs, we provide a potentially valuable tool to quantify a

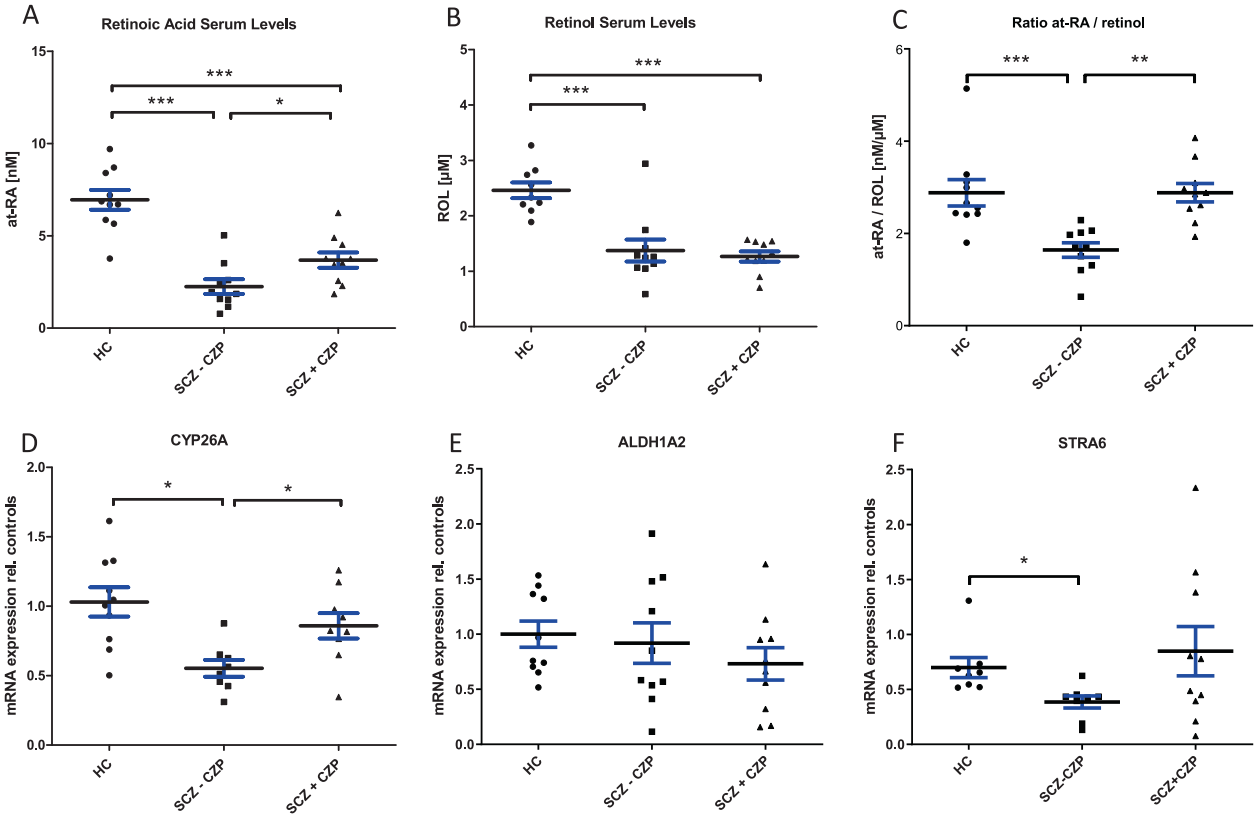

Fig. 4 Retinoid Homeostasis in Patients with Schizophrenia with and without Clozapine. a Retinoic acid serum levels were measured in healthy controls (HC; $N = 10$), patients with schizophrenia receiving antipsychotic medication other than clozapine (SCZ − CZP; $N = 10$) and patients with schizophrenia currently receiving clozapine (SCZ + CZP; $N = 10$). Serum *at*-RA levels were significantly reduced in patients with schizophrenia compared with controls regardless of medication (***$P < 0.0001$, one-way ANOVA with Newman–Keul's post-hoc test). *at*-RA levels in clozapine-treated patients were significantly increased compared with no-clozapine patients (*$P < 0.05$, one-way ANOVA with Newman–Keul's post-hoc test). **b** Serum retinol (ROL) levels were significantly reduced in patients with schizophrenia from either medication group compared with HC (***$P < 0.0001$). **c** The *at*-RA to ROL ratio was significantly reduced only in the SCZ − CZP group. **d** Whole blood mRNA expression of *at*-RA-catabolizing and *at*-RA-inducible CYP26A was also strikingly lowered only in SCZ − CZP subjects while there were no differences between HC and SCZ + CZP (*$P < 0.05$, one-way ANOVA with Newman–Keul's post-hoc test). **e**, **f** mRNA levels of the RA-synthesizing enzyme ALDH1A2 did not differ between groups, while mRNA levels of RA-inducible gene STRA6 were significantly decreased in SCZ − CZP compared with healthy controls (*$P < 0.05$, one-way ANOVA with Newman–Keul's post-hoc test).

patient-specific response of retinoid homeostasis to clozapine. While our study cannot directly answer the question of whether enhanced retinoid signaling may causally underlie clozapine's antipsychotic mode of action, our approaches are suitable to correlate the individual retinoid response to clozapine with clinical response in a longitudinal approach, which will be investigated prospectively in future clinical trials.

Retinoid dysregulation in SZ is a convincing theory; however, evidence on functionally altered retinoid signaling in SZ remains associative [19, 21, 25, 35]. We, therefore, assumed alterations in peripheral serum levels of *at*-RA to reflect an overall disturbed retinoid signaling.

Based on our finding that clozapine strikingly affects local *at*-RA degradation we established a three-group design including patients with SZ on an established drug regimen with clozapine (>5 years continuous exposure), patients with SZ taking other antipsychotic drugs and healthy controls, all matched for age, gender, BMI and smoking status. Using a highly sensitive HPLC-based method [50, 73] we were able to identify significantly reduced *at*-RA as well as ROL serum levels, reduced *at*-RA/ROL ratios as well as reduced expression of the RA-inducible and RA-metabolizing CYP26A and the RA-inducible protein stimulated by retinoic acid 6 (STRA6) in schizophrenia patients without clozapine treatment (Fig. 4). Furthermore, confirming our in vitro data, both *at*-RA serum levels, *at*-RA/ROL ratios, and CYP26-levels were increased in patients on clozapine compared with patients on other antipsychotics. Interestingly, PBMC mRNA expression of the RA-synthesizing enzyme ALDH1A2, for which genetic and epigenetic associations with SZ were

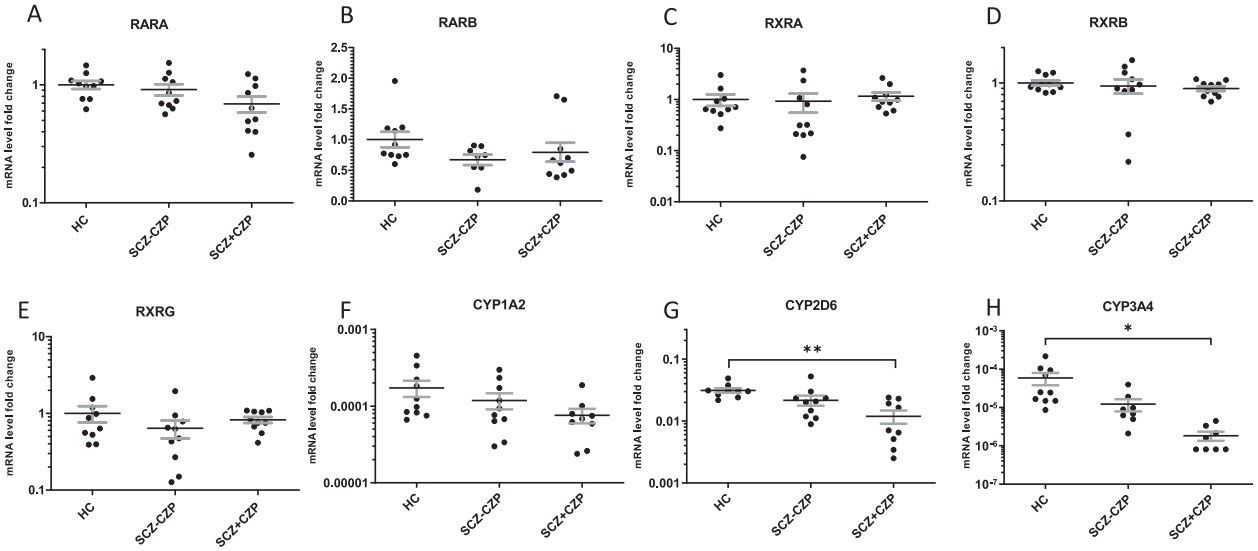

Fig. 5 mRNA Expression of RA Receptors and clozapine-metabolizing CYP450 Isozymes. mRNA transcripts of the main retinoid receptors RARA (**a**), RARB (**b**), RXRA (**c**), RXRB (**d**) RXRG (**e**) and clozapine- as well as *at*-RA metabolizing CYP1A2 (**f**), CYP2D6 (**g**) and CYP3A4 (**h**) were detectable in whole blood-derived mRNA. While there were no main effects of group on any of the five receptors (one-way ANOVA), the pattern of RA-inducible RARB is similar to the patterns of STRA6 and CYP26A. Interestingly, both CYP2D6 and CYP3A4 mRNA levels were strikingly decreased in SCZ + CZP patients compared with healthy controls (*$P < 0.05$, **$P < 0.001$, one-way ANOVA with Newman–Keul's post-hoc test).

demonstrated, did not differ between groups [74, 75]. While the expression of RA-inducible RARB, for which associations with schizophrenia have been demonstrated [21, 25] did not differ significantly between groups (Fig. 5b), the tendency toward reduced expression in SCZ-CZP is similar to the pattern observed for CYP26A and STRA6 (Fig. 4). Rather unexpected were findings on reduced CYP2D6 and CYP3A4. On the one hand, reduced expression of non-specific RA-degrading cytochromes may provide a mechanism for increased at-RA serum levels in clozapine-treated subjects. On the other hand, recent in vitro evidence demonstrates the downregulation of CYP2D6 mRNA upon stimulation with retinoids [76], which suggests that reduced levels of CYP2D6 in clozapine patients may also be a consequence of increased RA-signaling. The results must, however, be interpreted with caution, particularly because little is known about the role and functional implications of altered blood-cell-based mRNA expression of CYP450 isozymes.

Limitations of our study include the cross-sectional design and the comparably small sample size of the clinical part. While we were able to show significant inhibition of *at*-RA catabolism at a functional level in various tissues and patient-specific cells, our methodological approach cannot reveal the relative contribution of the different CYP isoforms to the observed effects, which putatively varies between tissues and individual subjects.

While the small sample size was a result of the large effect size of 1 µM clozapine in PBMC-derived microsomes (for details see Supplementary S1), the cross-sectional design cannot provide evidence for a role of clozapine's RA-modulating effects in its antipsychotic mechanism. Due to methodological limitations, endogenous at-RA metabolites were not detectable in patient-derived samples. Thus, we cannot definitely attribute the alterations to either increased catabolism or decreased anabolism in SZ patients. Despite our careful control for age, weight, and smoking status, confounding effects of eating habits or nutritional preferences on at-RA homeostasis that were not assessed in the present design cannot be definitely ruled out. On the other hand, the large effect sizes that we observed in both our in vitro studies and the patient-specific biological endpoints strongly suggest a clinical relevance of our findings.

Moreover, human brain samples served as generic human brain tissue and were not available from multiple regions. Thus, our present study was not able to resolve the observed effects in a region-specific manner, which should undoubtedly be pursued in future studies. Finally, while our assays were able to detect effects of the test compounds on gross RA catabolism in the respective tissues, we were not able to discriminate the degree to which specific CYP450 isoforms may have contributed to the observed effects. Major challenges are the unspecific effects of pharmacological inhibition [66]. Such an in-depth analysis of which subjects, and via which isozymes, clozapine may specifically block RA catabolism should be the subject of future studies.

In conclusion, we have identified clozapine to strikingly impact RA catabolism in human adult brain tissue and

PBMCs, suggesting a retinoid-related mechanism of action of both clozapine and its two major metabolites. Moreover, we identified dysregulation of retinoid homeostasis in patients with SZ at several levels and we revealed potential effects of clozapine on the latter, suggesting normalization of the deficits as part of clozapine's pleiotropic actions. Our strategy to quantify the impact of clozapine on RA homeostasis parameters in patient-derived material could serve as a versatile and simple tool to assess the patient-specific retinoidergic response to clozapine, which might correlate with later treatment response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplement S1

The following supplemental information provides further details on the materials used and the methods performed in the present study, particularly on study participants, retinoid analyses, cell culture techniques, qPCR primer sequences and on the preparation of metabolically active synaptosomes / microsomes.

Methods and Materials

Participants

Healthy donors and schizophrenia patients within the clinical observational study on RA homeostasis in neuropsychiatric disorders (RAHND; ClinicalTrials.gov Identifier: NCT02439099) were included. The local ethics committee approved the study (EA4/002/13). All patients had a clinical DSM-V diagnosis of schizophrenia (SZ) more than 5 years prior to inclusion, were treated in our clinic as in- or outpatients and were on a stable medication with clozapine (N = 10) or another antipsychotic (N = 10; supplementary table 2). All participants were matched for age, weight, BMI and smoking-status, including matched healthy controls (N = 10; table 1). Participants arrived at the laboratory between 8 and 12 a.m. after an overnight fast for blood collection. Each participant provided a total volume of 40 ml of peripheral venous blood. In order to estimate the required minimum sample size for the clinical population, the following assumptions were made: Average serum levels of clozapine readily reach > 1 µM under steady state conditions (Hiemke et al., 2018). Thus, effects of 1 µM clozapine on *at*-RA catabolism in human PBMC-derived microsomes was taken as reference to estimate a minimum effect size (Fig. 2 A). Based on the average inhibitory effects of 1 µM clozapine (84.9 % +/- 5.66) vs. vehicle (100 % +/- 10.3) in PBMC-derived microsomes from n = 8 healthy donors, an effect size of d = 1.817 was calculated using the software G*power Version 3.1.9.2. Based on a two-tailed t-test with a power of 95 %, an allocation ratio of 1, and an alpha error probability of 5 %, the required sample size per group was calculated as n = 9.

Cell culture and tissue preparation

Serum and PBMC Isolation

For serum isolation, blood was collected from serum collection tubes (Type SST Advance™, BD Biosciences, Germany). Serum was prepared according to the manufacturer's instructions. Additionally, all samples were either kept in the dark or processed under dim yellow light during the whole procedure. Aliquots were stored at -80 °C. For PBMC isolation, blood was collected from healthy donors (supplementary table 1) using heparinized vacuum-extraction tubes (BD Biosciences) and extracted by FICOLL™ density gradient centrifugation, following previously published protocols (Regen et al., 2017). In brief, samples of heparinized whole blood were carefully layered on top of density medium. After centrifugation, cells were extracted from the interphase, resuspended in PBS, washed twice in PBS and resuspended in RPMI 1640 Medium + GlutaMAX(Biochrom, Germany), containing 25% heat-inactivated fetal calf serum (Biochrom), 1 % Penicillin/Streptomycin (10,000 U / 10 mg per ml; Biochrom, Germany) for cryopreservation.

Tissue Preparation for RA Catabolism Assays

All experiments including animals were registered and approved by German regulatory authorities (T 0268/15). Murine tissues were isolated from newborn C57/BL6-mice (P0-P3) after sacrification by decapitation. Organs were rapidly removed, tissues were prepared and brain regions were dissected using a stereo-microscope (Carl Zeiss, Germany) in ice-cold cell culture media. Samples were weighed and snap-frozen using liquid nitrogen. All samples were stored at -80 °C until further analysis.

Human post-mortem brain tissue from the superior temporal gyrus of 5 healthy donors was obtained from the Netherlands Brain Bank (NBB, Netherlands Institute for Neuroscience, Amsterdam). All donors gave written informed consent for brain autopsy and for the use of specimens for research purposes. All samples and associated data were anonymized to

maintain anonymity of the donors. All procedures on collection and (re-)distribution of the tissue samples were positively evaluated by the Independent Review Board of the VU University (Amsterdam, Netherlands). The experimental procedures on the specimens, including the assessment of retinoid homeostasis, were additionally positively evaluated by the local Charité Ethics Commission. Frozen human brain tissue samples were processed in analogy to murine brain samples.

Preparation of Crude Microsomal and Synaptosomal Fractions

CYP450-containing, metabolically active crude microsomal and synaptosomal fractions from brain tissues and PBMCs were prepared according to previously published protocols with minor modifications (Regen et al., 2016a, Regen et al., 2015). In brief, for preparation of PBMCs, a total of 5 * 10^7 cells were subjected to 50 µl of assay buffer (30mM sodium phosphate buffer, pH 7,4), sonicated for 30 s, centrifuged (10 min. at 1,000 * g at 4 °C), and supernatant was saved. Pellets were resuspended in 50 µl of assay buffer, sonicated and centrifuged again, the resulting supernatants that contained metabolically active, crude synaptosomal / microsomal fractions was then stored at -80 °C while remaining pellets were discarded. For tissue preparations, tissues were resuspended in a 9-fold volume of assay buffer to prepare a 10% homogenate (w/v) by homogenization using a 15 ml glass Teflon homogenizer. Murine tissue preparations were always pooled from three different animals. Following initial centrifugation at 1,000 * g for 10 min. at 4°C, the supernatants were again centrifuged at 100,000 * g for 45 min. at 4°C. The resulting pellets, which contained the metabolically active crude synaptosomal / microsomal fractions were resuspended in assay buffer. Protein concentrations were determined by the BCA method (Thermo Fisher, USA).

RA catabolism assay

In-vitro assays to quantify RA catabolism were performed as previously published (Hellmann-Regen et al., 2015, Regen et al., 2016b). In brief, samples containing metabolically active enzyme preparations were diluted to yield comparable final protein concentrations (100 - 500 µg/ml). Assays were performed in a total reaction volume of 60 µl. Each reaction contained the test compound (drug), *at*-RA (0.1 µM), NADPH (800 µg/ml), metabolically active microsomal / synaptosomal preparations and assay buffer. Reactions were set up by pipetting a range of concentrations of the test drug or vehicle (PBS) to the enzyme-containing samples and allowed to pre-incubate for 30 min. on ice. Heat-inactivated controls were inactivated at 95 °C for 15 min. and also placed on ice. To start the reactions, both NADPH and *at*-RA were added simultaneously, tubes were vortexed and placed in a shaking water-bath incubator for 60 min at 37 °C. After 60 min, reactions were stopped by the addition of 4vol (240 µl) of ice cold methanol. Subsequently, all samples were centrifuged at 21.000 x g, 4° C and subjected to retinoid analysis. All steps were carried out under dim, yellow light. Normalization of *at*-RA metabolic activity was achieved by comparing *at*-RA degradation in metabolically active samples with heat-inactivated controls. Maximum catabolic activity was defined as the reduction in at-RA concentration that occurred over the course of the reaction in metabolically active samples that were treated with vehicle only instead of the compound. Catabolic activity in compound-treated samples was then expressed as a percentage of the maximum catabolic activity. Metabolic activity in heat-inactivated controls was virtually absent and at-RA levels in heat-inactivated controls did not differ over the course of incubation.

at-RA catabolism was initially assessed in whole brain-derived synaptosomal fractions from mouse brains (postnatal day 0-3) in the presence of clozapine at various concentrations covering the clinically relevant range that is reached in murine brain tissue. The samples consisted of pooled synaptosomal fractions from n=3 animals and exhibited strong *at*-RA metabolic activity. In subsequent experiments, various concentrations of clozapine were tested in *at*-RA catabolism assays using pooled murine cortex-derived synaptosomal preparations (n= 3-6 animals).

Serum Extraction of Retinoids

Extraction of retinoids from human sera was performed by a liquid-liquid extraction procedure using the synthetic retinoid acitretin as an internal standard to assess recovery and account for inter- and intra-assay variability. All steps were performed under dim, red light and using glass vials. Liquid-liquid extraction was performed by first spiking fractions of 1 ml of serum with internal standard solved in DMSO to yield a final concentration of 1 µM, then adding 1 vol of acidified ethanol containing 3 % (v/v) orthophosphoric acid to 1 Vol of patient serum. Samples were vortexed for 1 min. and 2 vol of hexane were added. Samples were vigorously vortexted for 15 minutes and centrifuged at 1560 * g at 4 °C for 5 min. The supernatant was completely removed and evaporated to dryness at room temperature under a gentle stream of dry Argon. Samples were subsequently resuspended in 1 ml of HPLC running buffer.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was performed as previously described (Regen et al., 2016b). RA isomers and RA degradation products were quantified using an Agilent 1100-series HPLC system with a binary pump, isocratic elution at a flow rate of 0.65 ml / min and UV detection using a diode array detector (1260-Series) at 340 nm. The system was further equipped with a temperature-controlled column department and a programmable, temperature controlled autosampler. A Supelco Suplex® column (5 µm, 2.1 x 250 mm; Sigma-Aldrich, Taufkirchen, Germany) was used for separation of the cis- and trans- isomers of RA from pharmacological assays and a Phenomenex Synergi RP 4 µm 80A column was used for separation of endogenous retinoids isolated from serum. The mobile phase for the pharmacological assays consisted of acetonitrile, 2% (w/v) ammonium acetate, methanol, glacial acetic acid and n-butanol (69:16:10:3:2; v/v). Elution was performed isocratically for the retinoid quantifications from the pharmacological assays at a flow rate of 0.65 ml/min within a total analysis time of 12 min. For retinoids isolated from serum, a gradient elution was

performed using mobile phase (A) composed of H2O + 0.1 % formic acid : acetonitrile (15:85 v/v) and a second mobile phase (B) composed of 100 % acetonitrile. Gradient elution was performed (A : B) 0-13 min.: 15:85, 13-14 min.: 0:100, 14-18 min.: 0:100, 18-19 min.: 15:85, runs were terminated after 25 min. All compounds were verified by authentic standards. Peak purity was monitored by online spectral analysis.

Real-Time PCR

Blood was collected in PAXgene® tubes (PreAnalytiX GmbH) and RNA was extracted in accordance with the manufacturer instructions. Total RNA was then reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit™ (Thermo Fisher Scientific Inc., MA, USA) and until further measurement all cDNA's were then stored in -20°C. q PCR was performed with LightCycler™ 480 SYBR Green I Master (Roche, Mannheim, Germany) using 500 nM standard primer concentrations, following the recommended instrument protocol from manufacturer's instruction in Applied BiosystemsStepOne™ Real-Time PCR System (CA, USA) to quantify the expression levels. Melting curve analysis was performed to identify specific products with 1.2°C increment from 65 to 95°C. Relative quantity (delta Ct) and melting curve analysis were carried out using the StepOne™ Real-Time PCR System software. All primers were designed and checked for their quality using the Primer-BLAST software (Ye et al., 2012). Sequence (5' > 3') as follows retinoic acid receptor alpha (RARA) (F: ACACTACGAACAACAGCTC, R: TCCACAGTCTTAATGATGCAC), retinoic acid receptor beta (RARB) (F:TGATGGAGTTGGGTGGACTT, R: CGGACTCGCAGTGTAGAAAT), retinoid X receptor alpha (RXRA) (F: GGGCATGAGTTAGTCGCAGA, R: CTGACGGGGTTCATAGGTGA), retinoid X receptor beta (RXRB) (F: CAGAAGCTCAGGCAAACAC, R: TAAGGTCTTTGCGGATGGT), retinoid X receptor G (RXRG) (F: CATGAAGAGGGAAGCTGTG, R: CCACTGGTAGCACATTCTG), aldehyde dehydrogenase 1A1 (ALDH1A2) (F: GTTACAATGCCTTAAATGCCC, R: AGCCAAATTCTCCCATTTCTC), cytochrome P450 26A (CYP26A) (F:

GAAGAGTAAGGGTTTACTTTGC, R: CCCGATGTATTTAAGTTGTTCC), stimulated by retinoic acid receptor-6 (STRA6) (F: AACTGCCGAGACCACACAAC , R: ACGACATTCTCTGGCCCTTC), Cytochrome P450 1A2 (CYP1A2) (F: ATGTGAGCAAGGAGGCTAAGG, R: CATCTCATCGCTACTCTCAGGG), Cytochrome P450 2D6 (CYP2D6) (F: GTGATTCATGAGGTGCAGC, R: GAGTGTCGTTCCCTTAGGG), Cytochrome P450 3A4 (CYP3A4) (F: CACCCCCAGTTAGCACCATTA, R: CCCACGCCAACAGTGATTA). Geometric means of two housekeeping genes, GAPDH and RFLP0, were used for data normalization.

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Supplementary Table 1. Characteristics of Healthy Donors for PBMC-derived Microsomes

	Healthy Donors
	N = 8
Age (years ± SD)	33 ± 7.7
Male / Female (n)	3/5
BMI ± SD	20.90 ± 2.16
Current smokers (n)	0

Abbreviations: BMI, Body Mass Index

Supplementary Table 2. Medication

	Clozapine group	Other antipsychotics
Matched Pairs (Nr.)	Clozapine + Comedication (mg per day)	Antipsychotic (mg per day)
1	Clozapine 375, Escitalopram 10	Olanzapine 7.5, Sertraline 100
2	Clozapine 350, Venlafaxin 225, Paliperidone 3	Paliperidone 3, Biperiden 4, Pipamperone 40
3	Clozapine 600, Valproate 2000, Haloperidol 2	Risperidone 3
4	Clozapine 400	Paliperidone 6, Trazodone 50, Duloxetine 60
5	Clozapine 550, Sertraline 75, Lamotrigine 100	Aripiprazole 15, Promethazine 50, Trazodone 50
6	Clozapine 300, Sertraline 100, Pregabalin 200	Aripiprazole 15
7	Clozapine 50	Paliperidone 3
8	Clozapine 350, Risperidone 5	Risperidone 2, Paliperidone 6, Pregabalin 100, Pipamperone 40
9	Clozapine 75, Risperidon 2	Paliperidone 6, Sertraline 100
10	Clozapine 550	Paliperidone 6, Aripiprazole 30, Venlafaxine 262.5

Supplementary Table 3. Local Brain Concentrations

Estimation of Brain Tissue Concentrations

Drug	Serum Levels (Therapeutic Range)	Expected Brain Levels	Reference
Sertralin	10 - 150 µg/l	0.72–5.2 mean 2.52 [mg/kg] postmortem	Nedahl et al., 2018
Citalopram	50-110 µg/l	0.052–4.30 mean 1.46 [mg/kg] postmortem	Nedahl et al., 2018
Modafinil	17.3 ± 3.13 mg/ml	n.d.	Wuo-Silva et al., 2016
Diazepam	100 - 2500 µg/l	0.12 mg/kg postmortem	2016. L. Skov, K.M.D. Holm, et al.

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Deutsche Zusammenfassung

Die vorliegende Dissertationsschrift untersucht die Rolle von Vitamin A bei Patient:innen mit unipolarer depressiven Störung, sowie bei Patient:innen mit Schizophrenie. Im Gegensatz zu vielen somatischen Erkrankungen bleiben einige Aspekte der Pathophysiologie und den zugrunde liegenden molekularen Mechanismen bei psychiatrischen Erkrankungen schwer fassbar. Eine Fülle von Forschungsarbeiten beschreibt die Retinsäure – der biologisch aktivste Vitamin A Metabolit, welcher die physiologischen Effekte von Vitamin A vermittelt – als ein hochpotentes Molekül, das an der Regulierung von wesentlichen neurobiologischen Mechanismen zentral beteiligt ist und daher möglicherweise eine Rolle bei psychiatrischen Erkrankungen spielt. Es zeigt sich eine bemerkenswerte Überschneidung zwischen Retinsäure-regulierten Mechanismen und den typischerweise auftretenden, erkrankungsassoziierten Veränderungen dieser Mechanismen bei psychiatrischen Erkrankungen – einschließlich Veränderungen in der synaptischen Plastizität, Neurotransmission und Neuroinflammation. Entsprechend wird eine Störung der Retinoidhomöostase mit dem Auftreten von Depression und Schizophrenie in Verbindung gebracht. Eine umfassende Untersuchung der Retinoidhomöostase bei Patient:innen mit Depression wurde bisher jedoch nicht durchgeführt. Die Rolle von Retinoiden bei Schizophrenie wurde bereits etwas umfassender beschrieben. Ob und wie jedoch häufig verwendete Antipsychotika einen Einfluss auf das Retinoidsystem haben, ist bislang nicht untersucht.

In zwei klinischen Beobachtungsstudien befasst sich die vorliegende Dissertationsschrift mit verschiedenen Aspekten der peripheren Retinoidhomöostase bei Patient:innen und gesunden Kontrollpersonen. In der RA-MDD-Studie, die eine Kohorte von 109 Patient:innen und Kontrollen umfasste, wurden Retinoid Serumspiegel, die individuelle Retinsäure Stoffwechselaktivität und mRNA Expressionsprofile von Retinoidhomöostase-relevanten Genen bestimmt. In der RA-SZ-Studie wurden die Auswirkungen des hochwirksamen und häufig eingesetzten Antipsychotikums Clozapin auf den Retinäurekatabolismus untersucht. Retinoidspiegel und mRNA Expressionsprofile wurden bei Patient:innen mit Schizophrenie, die eine Clozapin Medikation erhielten, bei Patienten ohne Clozapin Medikation und bei gesunden Kontrollen bestimmt (N = 24). Zusätzlich wurde die Wirkung von Clozapin auf den RA-Katabolismus in murinem und menschlichem Gewebe, einschließlich Gehirngewebe, untersucht.

Bei Patient:innen mit Depression waren die Retinol Serumspiegel, sowie die Retinsäure Syntheseaktivität signifikant höher als bei Kontrollpersonen. Interessanterweise zeigte sich ein starker geschlechtsspezifischer Aspekt der Depressions-assoziierten Veränderungen im

Retinoidsystem. So waren die Retinolspiegel nur zwischen männlichen Patienten und Kontrollpersonen signifikant unterschiedlich, während die Retinsäuresynthese sich nur zwischen weiblichen Patientinnen und Kontrollpersonen unterschied. Ergebnisse der RA-SZ Studie zeigten, dass Clozapin den Retinsäurekatabolismus stark hemmt. Dies spiegelt sich wider in entsprechend höheren Retinsäure Serumspiegeln bei Patient:innen, die Clozapine einnehmen, im Vergleich zu Patient:innen ohne Clozapin Medikation. Im Vergleich zu gesunden Kontrollen waren die Retinsäure Serumspiegel bei Patient:innen signifikant niedriger.

Die vorliegende Dissertation liefert deutliche Hinweise auf eine veränderte Retinoidhomöostase bei Depression und Schizophrenie. Die hemmende Wirkung von Clozapin auf den Retinsäurekatabolismus, sowie die entsprechend höheren Retinsäurespiegel, legen nahe, dass Clozapin möglicherweise über die Regulierung des Retinoidsystems wirkt. Die umfassenden Erhebungen verschiedener Parameter der Retinoidhomöostase bei Patient:innen und Kontrollpersonen liefern wertvolle, neue Erkenntnisse in den untersuchten Kohorten. Ein kausaler Zusammenhang zwischen Retinoidsignaling und Depression oder Schizophrenie lässt sich aus diesen Ergebnissen jedoch nicht ableiten. Nichtsdestotrotz stellen die Ergebnisse dieser Dissertation eine solide Grundlage dar, auf der (i) der mutmaßliche pathophysiologische Beitrag einer gestörten Retinoidhomöostase bei Depressionen und (ii) die potenzielle Vermittlung therapeutischer Wirkungen von Clozapin über das Retinoidsystem weiter untersucht werden können.

Eidesstattliche Versicherung

Hiermit erkläre ich, die vorliegende Dissertation selbstständig verfasst und ohne unerlaubte Hilfe angefertigt zu haben. Alle Hilfsmittel, die verwendet wurden, habe ich angegeben. Die Dissertation ist in keinem früheren Promotionsverfahren angenommen oder abgelehnt worden.

Berlin, 12. April 2023
Lisa R. Otto

Anteilserklärung

Erklärung gemäß § 7 Abs. 3 Satz 4 der Promotionsordnung über den Eigenanteil an den veröffentlichten oder zur Veröffentlichung vorgesehenen eingereichten wissenschaftlichen Schriften im Rahmen meiner publikationsbasierten Arbeit

I. Name, Vorname: Otto, Lisa Rebecca

Institut: Fachbereich Erziehungswissenschaft und Psychologie

Promotionsfach: Psychologie

Titel: Master of Science

II. **Nummerierte Aufstellung der eingereichten Schriften (Titel, Autoren, wo und wann veröffentlicht bzw. eingereicht):**

1. **Otto LR**, Clemens V, Üsekes B, Cosma NC, Regen F, Hellmann-Regen J. Retinoid homeostasis in major depressive disorder. *Translational Psychiatry*. 2023;13(67):1–7.
2. Regen F, Cosma NC, **Otto LR**, Clemens V, Saksone L, Gellrich J, et al. Clozapine modulates retinoid homeostasis in human brain and normalizes serum retinoic acid deficit in patients with schizophrenia. *Molecular Psychiatry*. 2021;26:5417–5428.

III. **Darlegung des eigenen Anteils an diesen Schriften**:

zu II. 1.: Studienkoordination und Rekrutierung (maßgeblich), Methodenentwicklung (überwiegend), Versuchsdesign (maßgeblich), Datenerhebung – klinische Daten und Laboranalysen (überwiegend), Datenauswertung und –darstellung (vollständig), Interpretation der Ergebnisse (überwiegend), Erstellen des Manuskriptes (vollständig), Überarbeitung des Manuskriptes im Rahmen des Peer-Review Prozesses (vollständig)

zu II. 2.: Studienrekrutierung (in Teilen), Methodenentwicklung (maßgeblich), Versuchsdesign (in Teilen), Datenerhebung – Laboranalysen (maßgeblich), Interpretation der Ergebnisse (in Teilen), Erstellen des Manuskriptes (in Teilen)

Publikationsliste

Peer-reviewed Journals

1. **Otto LR**, Clemens V, Üsekes B, Cosma NC, Regen F, Hellmann-Regen J. Retinoid homeostasis in major depressive disorder. *Translational Psychiatry*. 2023;13(1).

2. Regen F, Cosma N-C, **Otto LR**, Clemens V, Saksone L, Gellrich J, et al. Clozapine modulates retinoid homeostasis in human brain and normalizes serum retinoic acid deficit in patients with schizophrenia. *Molecular Psychiatry*. 2021;26(9):5417–28.

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4. **Otto LR**, Sin NL, Almeida DM, Sloan RP. Trait emotion regulation strategies and diurnal cortisol profiles in healthy adults. *Health Psychology*. 2018;37(3):301–5.

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1. Rogge, A. A., Fischer, F., **Otto, L.**, & Rose, M. (2022). Empirische Erfassung patient* innenberichteter Merkmale: PROMs und PREMs. *AINS-Anästhesiologie· Intensivmedizin· Notfallmedizin· Schmerztherapie*, 57(02), 150-155.

2. Long, P., Ritschl, V., **Otto, L.**, Rogge, A., Koppert, L., Huberts, A., & Stamm, T. (2022). Systematic literature review examining the mere-measurement effect of patient reported measures: the interaction of emotional valence and frequency of exposure as an independent factor in patient change. *medRxiv*, 2022-03.
3. Kirchberger VH; Fischer K;Rollwage A; **Otto L**; Rose M. Machbarkeitsstudie indikationsübergreifendes Patient-Reported Outcome Measurement –Digitalisierung nutzen für eine patientenzentrierte Gesundheitsversorgung (DigiPROM). *Bericht Bundesgesundheitsministerium,* ZMVI1-2519FEP400, 2021.

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