

Aus dem Institut für Anatomie II
der Heinrich-Heine-Universität in Düsseldorf
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**Heterogeneity of Astrocyte Density, Morphology and Connexins in the Mouse
Hippocampus**

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
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2026

Als Inauguraldissertation gedruckt mit Genehmigung der
Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez.:

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Für meine Eltern Norbert und Petra

Teile dieser Arbeit wurden veröffentlicht:

Uelwer A, Sivakumar M, Umirdinov K, Purath FFA, Oluma L, Anstötz M, von Gall C and Ali AAH (2026) Heterogeneity of astrocyte density, morphology and connexins in the mouse hippocampus. *Front. Neuroanat.* 20:1771439.
doi: 10.3389/fnana.2026.1771439 (Uelwer et al., 2026)

Zusammenfassung

Neben Neuronen rücken Gliazellen, insbesondere Astrozyten, zunehmend in den Fokus der Forschung. Sie erfüllen essenzielle Funktionen, darunter die Regulation der Blut-Hirn-Schranke, die Abfallbeseitigung über das glymphatische System, den Energiestoffwechsel sowie die Ionen- und Neurotransmitterhomöostase, und weisen entsprechend ihrer vielfältigen Funktionen eine ausgeprägte morphologische Heterogenität zwischen verschiedenen Hirnregionen auf. Astrozyten formen über Gap Junctions, die überwiegend durch die Connexine (Cx) Cx30 und Cx43 gebildet werden, ein eng gekoppeltes Netzwerk. Cx30 und Cx43 sind im Gehirn weit verbreitet und wesentlich für metabolische und elektrische Kopplungsprozesse astrozytärer Netzwerke. Im Hippocampus, einer zentralen Struktur kognitiver Prozesse, sind astrozytäre Cx an der adulten Neurogenese sowie an Lernen und Gedächtnisbildung beteiligt. Zudem tragen astrozytäre Cx zur Schlafregulation bei, zeigen eine tageszeitabhängige Expression und werden durch Hell-Dunkel-Bedingungen im Nucleus suprachiasmaticus des Hypothalamus, dem zirkadianen Taktgeber des Gehirns, moduliert. Es ist jedoch bisher wenig über regionale Unterschiede hinsichtlich der Astrozytenmorphologie und -zellichte sowie der räumlichen und zeitlichen Verteilung der Cx innerhalb des Hippocampus bekannt. Daher führten wir mittels Immunhistochemie eine umfassende Kartierung von Cx30 und Cx43 in den hippocampalen Regionen CA1, CA3 und Gyrus dentatus (GD) zwölf Wochen alter männlicher C57BL/6J-Mäuse durch. Zusätzlich analysierten wir die Immunreaktivität (Ir) von Cx30 und Cx43 in hippocampalen Unterregionen unter drei Lichtbedingungen: Standard-Photoperiode, konstante Dunkelheit und konstantes Licht. Dabei zeigten sich deutliche räumliche Unterschiede der Cx30- und Cx43-Ir, welche besonders im Stratum lacunosum-moleculare von CA1 und CA3 sowie in der subgranulären Zone des GD stark ausgeprägt war und positiv mit der astrozytären Dichte korrelierte. Die Astrozytenmorphologie variierte zwischen den Subregionen und zeigte die größte radiale Ausdehnung in den neuronalen Schichten (Pyramidenzell- und Körnerzellschichten). Unter Standard-Photoperiode war die Cx30- und Cx43-Ir während der Lichtphase und frühen Dunkelphase erhöht. Unter konstanter Dunkelheit zeigte sich insbesondere in CA1 und GD während der frühen (ehemaligen) Dunkelphase eine erhöhte Cx30- und Cx43-Ir, während sie unter konstantem Licht insgesamt reduziert war. Diese Befunde lassen darauf schließen, dass die hippocampalen Cx einer endogenen Rhythmizität unterliegen, die durch Licht moduliert wird. Die vorliegende Studie liefert somit eine detaillierte Charakterisierung der räumlichen und teilweise zeitlichen Verteilung astrozytärer Cx sowie der Astrozytenmorphologie in hippocampalen Regionen. Die Ergebnisse bilden eine Grundlage für weiterführende Untersuchungen zu Struktur-Funktions-Beziehungen im Kontext von Neuron-Glia-Interaktion und zur Rolle von Astrozyten bei der Modulation synaptischer Plastizität.

Summary

In addition to neurons, glial cells, especially astrocytes, have increasingly gained attention in recent years. Astrocytes fulfill essential functions, including regulation of the blood-brain barrier, waste clearance via the glymphatic system, energy metabolism, ion and neurotransmitter homeostasis and show pronounced morphological heterogeneity across different brain regions reflecting their diverse functional roles. Astrocytes form a robust, interconnected network via gap junctions formed by connexins (Cx), predominantly Cx30 and Cx43. Cx30 and Cx43 are widely distributed within the brain and are critical for a variety of astrocytic metabolic and electrical coupling functions. In the hippocampus, a key structure for cognitive processes, astrocytic Cx are involved in adult neurogenesis as well as memory formation and learning. Furthermore, astrocytic Cx contribute to sleep regulation, show time-of-day-dependent expression, and can be modulated by light/dark conditions within the brain's circadian rhythm generator, the suprachiasmatic nucleus of the hypothalamus. However, little is known about regional differences in astrocyte morphology and density, as well as the spatial and temporal distribution of Cx within the hippocampus. Therefore, we performed extensive mapping of Cx30 and Cx43 using immunohistochemistry in hippocampal CA1 and CA3 as well as in the dentate gyrus (DG) in twelve-week-old male C57BL/6J mice. We also compared the Cx30 and Cx43 immunoreaction (Ir) in hippocampal sublayers in mice kept under three different light conditions: standard photoperiod, constant darkness, and constant light. We found spatial differences in Cx30- and Cx43-Ir, which were particularly pronounced in the stratum lacunosum-moleculare of CA1 and CA3 and in the subgranular zone of DG and positively correlated with astrocytic density. Astrocytic morphology varied among the sublayers, showing the greatest radial extension in the neuronal layers (pyramidal and granule cell layers). Under standard photoperiod, Cx30 and Cx43 showed higher Ir during the light phase and the early dark phase. Under constant darkness, increased Cx30- and Cx43-Ir was observed particularly in CA1 and DG during the early (former) dark phase, while under constant light Cx30- and Cx43-Ir was generally reduced. These findings suggest that hippocampal Cx are subject to an endogenous rhythmicity that is modulated by light. Overall, the present study provides a detailed characterization of the spatial and partially temporal distribution of astrocytic connexins and morphology across hippocampal regions. These findings establish a basis for further investigations into structure-function relationships in the context of neuron-glia interactions and the role of astrocytes in the modulation of synaptic plasticity.

List of Abbreviations

ACTH	adrenocorticotrophic hormone
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARC	arcuate nucleus
ATP	adenosine triphosphate
A.U.	arbitrary units
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BMAL1	brain and muscle ARNT-like 1
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CK1	casein kinase 1
CLOCK	circadian locomotor output cycles kaput
CNS	central nervous system
CREB	cAMP response element-binding protein
CRH	corticotropin-releasing hormone
Cry/CRY	cryptochrome (gene/protein)
CT	circadian time
Cx	connexin(s)
DD	Dauerdunkel/constant darkness
DG	dentate gyrus
DKO	double knockout
DMH	dorsomedial hypothalamus
DNA	deoxyribonucleic acid
DT	disrupted time
EC	entorhinal cortex
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFAP⁺	GFAP-positive
GHT	geniculohypothalamic tract
Gja1	gap junction alpha-1 gene
GLAST	glutamate aspartate transporter
Gr	stratum granulare

GRP	gastrin-releasing peptide
GS	glutamine synthetase
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPT	hypothalamic-pituitary-thyroid
IGL	intergeniculate leaflet
ipRGCs	intrinsically photosensitive retinal ganglion cells
Ir	immunoreaction
kDa	kilodalton
LD	light-dark phase
LH	lateral hypothalamus
LL	constant light phase
LTP	long-term potentiation
lx	lux
MAPK	mitogen-activated protein kinase
Mol	stratum moleculare
mPFC	medial prefrontal cortex
MT1/2	melatonin receptor 1/2
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
Or	stratum oriens
PACAP	pituitary adenylate cyclase-activating polypeptide
PAK1	p21-activated kinase 1
Per/PER	period (gene/protein)
Pol	stratum polymorphe
POMC	pro-opiomelanocortin
Pyr	stratum pyramidale
PVN	paraventricular nucleus of the hypothalamus
µm	micrometer
Rad	stratum radiatum
REV-ERB	reverse strand of Erb
RHT	retinohypothalamic tract
ROR	retinoid-related orphan receptor
RORE	ROR response elements

SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SGZ	subgranular zone
SLM	stratum lacunosum-moleculare
VIP	vasoactive intestinal polypeptide
ZT	zeitgeber time
5xFAD	five familial Alzheimer's disease mutations
5-HT	5-hydroxytryptamine

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1 Introduction

1.1 Astrocytes

1.1.1 Definition, Classification, and General Characteristics

In addition to neurons, the mammalian brain contains at least an equally large population of supporting cells, the so-called glial cells (from the Greek *glia*, meaning “glue”), which were – among other scientists – first described by Rudolf Virchow in the mid-19th century (Jessen, 2004; Somjen, 1988). There are four different kind of glial cells in the central nervous system: the oligodendrocytes, which are primarily known for forming myelin sheaths, that increase nerve conduction velocity, microglia, which are mainly the macrophages of the central nervous system (CNS), ependymal cells, which line the ventricles, and astrocytes (Jessen, 2004; Verkhratsky et al., 2019).

Astrocytes (from the ancient Greek *ἄστρον* *ástron*, “star,” and *κύτος* *kýtos*, “cell”) are characterized by their star-shaped morphology and are commonly identified by the expression of the intermediate filament protein glial fibrillary acidic protein (GFAP), which forms the astrocytic cytoskeleton and contributes to the mechanical stability of the cell.

1.1.2 Morphological Subtypes

Two distinct astrocyte subtypes can be distinguished: Protoplasmic astrocytes are predominantly located in the gray matter and exhibit highly branched processes, which envelop synapses and blood vessels. In contrast, fibrillary (fibrous) astrocytes are primarily found in the white matter and have fewer branches, which form contacts with nodes of Ranvier as well as with blood vessels (Barres, 2008; Matyash & Kettenmann, 2010; Sofroniew & Vinters, 2010). This classification seems to be an oversimplification as astrocytes display morphological heterogeneity not only across different brain regions but also within the same region (Matyash & Kettenmann, 2010; Oberheim et al., 2012).

1.1.3 General Functions of Astrocytes in the CNS

In contrast to the initial assumption that glial cells primarily serve supportive roles, it is now well established that glial cells form a functional unit together with neurons and fulfill numerous essential tasks. In addition to their essential role in maintaining the blood–brain barrier, glial cells stabilize synapse formation, serve - as mentioned above - as electrical insulators through the formation of myelin sheaths, and perform crucial functions in maintaining extracellular homeostasis, especially for potassium and water

(Abbott, 2002; Jessen, 2004; Nagelhus & Ottersen, 2013; Simard et al., 2003). By modulating blood flow, astrocytes ensure an adequate supply of oxygen and energy in the form of glucose for the neurons (Carmignoto & Gomez-Gonzalo, 2010). Through the aquaporin-4 water channels in their end feet, astrocytes also regulate the perivascular clearance pathway, known as *glymphatic system*. This system enables the exchange of cerebrospinal and interstitial fluid and supports the removal of metabolic waste products, including amyloid-beta (Hablitz & Nedergaard, 2021; Iliff & Nedergaard, 2013). This waste disposal system appears to function particularly efficiently at night, suggesting the assumption that sleep deprivation is one of the pathomechanisms involved in the development of Alzheimer's disease (Nedergaard & Goldman, 2020).

Many studies even suggest that glial cells play a crucial role in neuronal survival. This is most clearly demonstrated in the peripheral nervous system. In disorders such as Charcot-Marie-Tooth disease, which are associated with mutations in genes encoding, for example, structural proteins of myelin, not only are Schwann cells rendered unstable, but axonal damage is also observed (Berger et al., 2002). Clinically, this manifests as progressive weakness and atrophy of muscles in the extremities (Morena et al., 2019).

In the developing brain, astrocytes promote neuronal migration and differentiation through the release of growth factors. For example, the secretion of thrombospondin plays a crucial role in synapse formation (Allen & Lyons, 2018; Christopherson et al., 2005).

In addition, astrocytes play a central role in maintaining neurotransmission homeostasis. By clearing neurotransmitters such as glutamate from the synaptic cleft and recycling them through the glutamate-glutamine-cycle, they prevent neuronal hyperexcitability (Danbolt, 2001; Hertz & Zielke, 2004). Furthermore, astrocytes regulate extracellular potassium levels, thereby stabilizing neuronal membrane potentials (Walz, 2000).

1.1.4 The Tripartite Synapse

Astrocytes form perisynaptic processes that establish close contacts with neurons, synapses, and blood vessels. An important concept in this context appears to be the notion of the *tripartite synapse*. The term emphasize that a synapse is not composed solely of a pre- and a postsynaptic membrane, but also includes an astrocyte, that actively modulates synaptic transmission (Araque et al., 1999; Ventura & Harris, 1999). Astrocytes not only modulate neuronal glutamate release, but also release glutamate themselves in a calcium-dependent manner, acting as active sources of gliotransmitters. Furthermore, they are critically involved in glutamate synthesis and clearance within the synaptic environment and are also essential for the synthesis of the neuroprotective molecule glutathione (Hertz

& Zielke, 2004). During ischemic events, not only is glutamate uptake in astrocytes reduced, but glutamate transporters may even work in reverse, releasing even more glutamate into the extracellular space and thereby contributing to the neurotoxic effect (Cellot & Ballerini, 2025).

1.1.5 Astrocyte Function in the Hippocampus

Astrocytes in the hippocampus play a key role in regulating synaptic transmission through gliotransmitter release and metabolic coupling, processes that support synaptic plasticity, learning and memory (Escalada et al., 2024). Furthermore, they seem to contribute significantly to stress-related anxiety-like behaviors (Li et al., 2024), particularly through glucocorticoid receptors in the ventral hippocampus (Lu et al., 2025).

1.1.6 Reactive Astrogliosis

Under some pathological conditions, astrocytes may undergo structural and functional changes, known as “reactive astrogliosis”. This includes an upregulation of GFAP (Sofroniew, 2009) and cell body and process hypertrophy (Wilhelmsson et al., 2006). Under conditions of severe or chronic damage, astrocytes may proliferate and, in some cases, even contribute to the development of a glial scar (Sofroniew & Vinters, 2010). Although this may inhibit axonal regeneration as a secondary effect, it primarily serves to limit the pathological process in case of ischemia, trauma, or inflammation within the CNS (Cieri & Ramos, 2025; Zhou et al., 2020). This process also appears to constitute a fundamental pathogenic mechanism in many neurodegenerative diseases such as Alzheimer’s disease (Kumar et al., 2023), amyotrophic lateral sclerosis (Vargas & Johnson, 2010), Parkinson’s disease (Glass et al., 2010) and multiple sclerosis (Mora & Chapouly, 2023).

1.2 Connexins and Gap Junctions

1.2.1 Structural Organization of Gap Junctions

Gap junctions are intercellular connections, that are fundamental for direct cell-to-cell communication by allowing the exchange of ions, metabolites and small signaling molecules (Goodenough & Paul, 2009). In chordates, including humans and mice, each hemichannel of a gap junction, named connexon, consists of six integral membrane proteins, the connexins (Cx) (Figure 1). Each connexin again possesses four transmembrane domains, two extracellular loops, and three cytoplasmic regions: the amino-terminal end, the carboxy-terminal end, and the cytoplasmic loop (Bruzzone et al.,

1996). Connexins are designated according to their approximate molecular weight: for example, Cx43 has a predicted size of approximately 43 kDa (Nielsen et al., 2012).

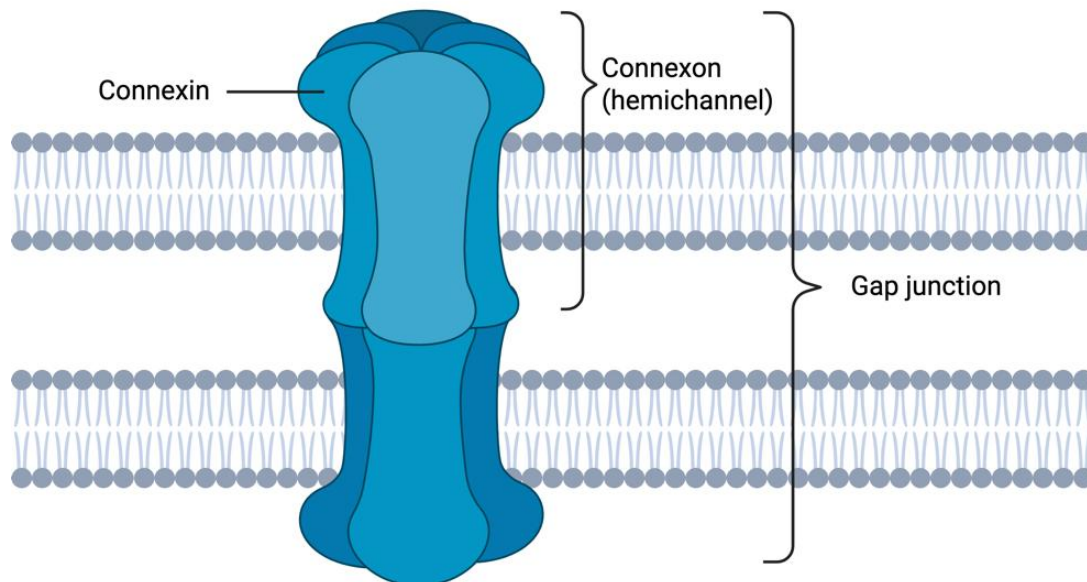


Fig. 1: Schematic illustration of a gap junction channel. Six connexin proteins together form a connexon or hemichannel. Two connexons from adjacent cells pair to create a gap junction, allowing direct intercellular communication through the exchange of ions and small molecules. Created in BioRender.com

1.2.2 Connexin Isoforms and Cellular Expression in the CNS

Connexins in the CNS are expressed by both neurons and glial cells, with specific isoforms restricted to particular cell types (Furshpan & Potter, 1959; Kuffler et al., 1966). At least twenty different Cx were found to be expressed in mice. For example, Cx36 is predominantly expressed in neurons, whereas oligodendrocytes express Cx29, Cx32, Cx45 and Cx47 (Condorelli et al., 2000; Vejar et al., 2019). Cx32 and Cx36 have been reported to be expressed in resting microglia, while activated microglia express more Cx43 (Caruso et al., 2023).

Cx30 and Cx43 are particularly predominant in astrocytes (Christian Giaume, 2013; Gosejacob et al., 2011). These two Cx show various expression levels in different brain regions (Batter et al., 1992; Nadarajah et al., 1996; Nagy et al., 1999).

1.2.3 Physiological and Pathophysiological Roles of Connexins in the CNS

Connexins, particularly Cx30 and Cx43, are essential for CNS functions such as potassium buffering (Wallraff et al., 2006), synaptic plasticity (Pannasch et al., 2011),

integrity of blood-brain barrier (Ezan et al., 2012), neuroprotection (Nakase et al., 2004) and neurogenesis (Kunze et al., 2009). Beyond these physiological roles, changes in Cx expression seems to play a major role in development of diseases. Huang et al. showed that aberrant expression of these Cx subtypes in the medial prefrontal cortex (mPFC) and hippocampus can affect neuronal activity in some brain regions and lead to depression-like behaviors (Huang et al., 2019).

1.2.4 Circadian Regulation of Connexin Expression

Several studies have demonstrated circadian regulation of connexin expression across different tissues. In the retina, for example, Cx36 transcript levels are higher during the subjective night phase compared to the subjective day phase under constant darkness (DD) (Katti et al., 2013), indicating an intrinsic circadian rhythm. Similarly, in the mouse bladder, Cx43 protein levels fluctuate with the rest-activity cycle: they are reduced during the inactive (sleep) phase, which is associated with an increased functional bladder capacity and consequently a decreased micturition frequency (Negoro et al., 2012). However, Cx30 and Cx43 in the suprachiasmatic nucleus (SCN) also exhibit time-of-day-dependent and circadian rhythmicity. Under light-dark (LD) conditions, protein expression of both connexins peaks during the light phase, corresponding to the inactive phase. Under constant darkness (DD), Cx43 shows a peak during the early subjective light phase (Ali et al., 2019). Together, these findings suggest that circadian modulation of connexins contributes to time-of-day-specific regulation of tissue function.

1.3 The Hippocampus

1.3.1 Structural Anatomy

The hippocampus (from the Greek *hippokampos*, meaning “seahorse”) is part of the limbic system and is located in the temporal lobe. The hippocampus can be subdivided into the dentate gyrus (DG), the *Cornu Ammonis* (CA1-3), and the subiculum. In addition, the hippocampus can be indistinctly subdivided into ventral and dorsal portions.

1.3.2 Circuit and Cellular Organization

The trisynaptic loop is a well-known model for signal pathways in the hippocampus. The major hippocampal signaling pathways are illustrated in Figure 2. The dentate gyrus gets information from the entorhinal cortex via the perforant path. The DG then uses the mossy fiber pathway to connect to the CA3 region, which projects to the CA1 region using the Schaffer Collateral pathway. CA1 is again connected to the entorhinal cortex (Basu &

Siegelbaum, 2015). While the trisynaptic loop provides a useful framework for hippocampal signal flow, recent studies reveal additional parallel and feedback pathways, highlighting a more complex circuitry (Lin et al., 2021; Sun et al., 2019; Xu et al., 2016). The CA2 region is located between CA3 and CA1. Although not part of the classical trisynaptic loop, the CA2 region represents an important parallel pathway within the hippocampus. It receives input from the entorhinal cortex and provides strong projections to CA1 and CA3, thereby modulating hippocampal output next to the classic trisynaptic circuitry (Bartasaghi & Gessi, 2004; Hitti & Siegelbaum, 2014; Robert et al., 2018). CA2, in this context, seems to have a critical role in maintaining the excitation-inhibition balance within the hippocampus (Boehringer et al., 2017).

The DG and CA regions are separated by the hippocampal fissure, containing the blood vessels for oxygen and nutritional supply. The main two arteries arise from the posterior cerebral artery (originating from the basilar artery) and the anterior choroidal artery (originating from the internal carotid artery) (Vockert et al., 2021).

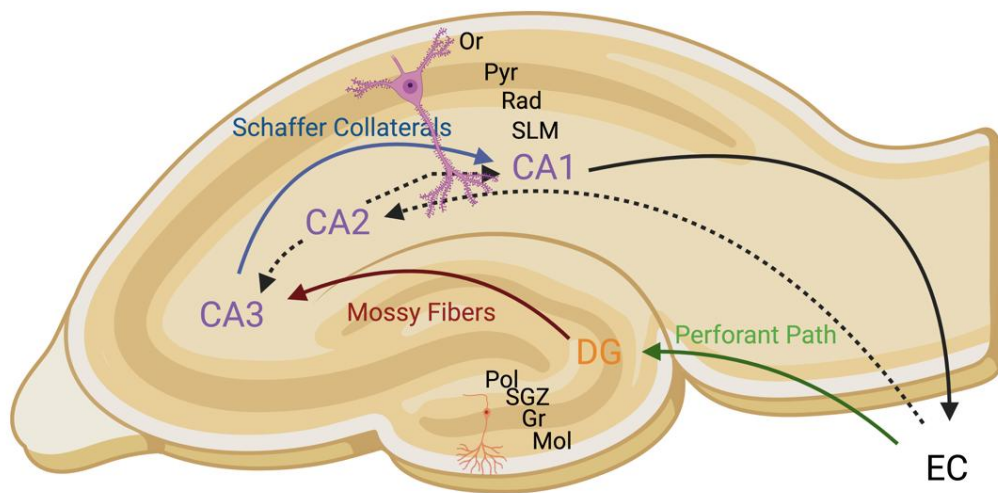


Fig. 2: Simplified schematic of the hippocampus showing the main hippocampal circuits. Input from the entorhinal cortex (EC) enters the dentate gyrus (DG) via the perforant path, is transmitted to CA3 through mossy fibers, and then to CA1 via Schaffer collaterals, forming the classical trisynaptic pathway. The CA2 region is positioned between CA3 and CA1 and modulate information flow within the hippocampus. Sublayers are abbreviated as followed: stratum oriens (Or), stratum pyramidalis (Pyr), stratum radiatum (Rad), and stratum lacunosum-moleculare (SLM) of CA1 and CA3, as well as stratum polymorphe (Pol), stratum moleculare (Mol), stratum granulare (Gr), and the subgranular zone (SGZ), defined as a two-nucleus-wide area along the inner border of Gr, of the DG. Created in BioRender.com.

At the histological level, the hippocampus forms the major part of the archicortex and is therefore a component of the allocortex. Unlike the neocortex, the hippocampus consists of only three layers. The middle layer contains the majority of the cell bodies of excitatory principal cells and is flanked by two cell-poor layers. In DG, the cell-rich layer is stratum granulosum (Gr) and contains the small granule cells, which are the principal excitatory neurons using glutamate as their neurotransmitter (Wheeler et al., 2015). Their dendrites reach into the stratum moleculare (Mol) and receive afferent input primarily from the entorhinal cortex, therefore containing the main part of the perforant path. The granule cell axons, known as mossy fibers, project into the stratum polymorphe (Pol), and form excitatory synapses (Soriano & Frotscher, 1994), mainly on CA3 pyramidal cells and local interneurons (Acsady et al., 1998). Additionally, many inhibitory interneurons are present in Gr and form inhibitory synapses to granule cells and their targets. Between the Gr and the Pol lies a narrow zone called the subgranular zone (SGZ), one of the few brain areas where adult neurogenesis occurs. In the SGZ, neural stem and progenitor cells proliferate, differentiate, and generate new granule cells. While developing from neuroprogenitors to neuroblasts, many of the new cells die of apoptosis and are phagocytosed by microglia (Sierra et al., 2010). In CA regions, the stratum pyramidale (Pyr) contains pyramidal neurons, which are larger than the granule neurons and exhibit the characteristic pyramidal morphology, forming the main cell-rich layer and building excitatory synapses to their targets. Their apical dendrites extend into the stratum lacunosum-moleculare (SLM) and the stratum radiatum (Rad), while their basal dendrites reach into the stratum oriens (Or). The latter layers receive inputs from the Schaffer collaterals and from subcortical brain regions such as septal nuclei. Projections from the entorhinal cortex and thalamus also terminate in the Or and in the SLM. In contrast, inputs originating from the brainstem predominantly target the proximal portion of the SLM (Ulrich Welsch, 2014; Valero & de la Prida, 2018).

Next to the excitatory cells, mainly the principal cells, there are up to 15% inhibitory interneurons abundant in the hippocampus with a large population being in Gr and Pyr, which use GABA as a neurotransmitter to regulate and synchronize neuronal activity (Pelkey et al., 2017).

Additionally, astrocytes are ubiquitously found in all regions of the hippocampus and contribute to homeostasis, glutamate uptake and potassium buffering. They are observed particularly in Or and Rad of CA1 and CA3 (Refaeli et al., 2021), however the detailed distribution in all regions of the hippocampus has not been described yet.

1.3.3 Functions in Cognition and Memory

The hippocampus is essential for declarative memory, meaning it helps remembering facts (semantic memory) and personal experiences (episodic memory) (Deshmukh & Knierim, 2012). It organizes information within a spatial and temporal context, allowing experiences to be stored as events that happened at a specific place and time (Howard & Eichenbaum, 2015). In this context the hippocampus plays an important role in context of *recollection*, which involves remembering details and context, while *familiarity* - the simple and vague feeling of knowing - relies more on surrounding medial temporal lobe regions (Opitz, 2014). Damage to the hippocampus typically leads to severe anterograde amnesia and difficulties forming new declarative memories (Deshmukh & Knierim, 2012). As part of the limbic system, the hippocampus participates in processing information about social interactions, helping encode and recall information about other individuals (Hitti & Siegelbaum, 2014). It also influences decisions what and when to eat by taking together multiple information about meal-related memories, external cues like food availability and internal signals such as hunger or hormonal status (Kanoski & Grill, 2017).

1.3.4 Role in Neurological and Psychiatric Disorders

The hippocampus is affected in many neurodegenerative diseases such as Alzheimer's disease, in which beta-amyloid-plaques deposition is associated with synaptic dysfunction and progressive structural degeneration, often beginning in the hippocampus, leading to memory loss and changing in behavior.

Next to epilepsy, several neurological and psychiatric disorders involve pathological alterations with the hippocampus (Ahmed et al., 2026). In major depressive disorder, reduced synaptic plasticity has been observed in the hippocampus as well as in other limbic and cortical regions (Fries et al., 2023). In chronic epilepsy, structural reorganization occurs in the form of "mossy fiber sprouting", where axons of granule cells in DG form recurrent excitatory synapses that contribute to hyperexcitability and promote seizure activity (Cavarsan et al., 2018). Moreover, hippocampal hyperactivity appears to play a central role in the pathophysiology of schizophrenia (Kraguljac et al., 2021).

Many studies of the last years have suggested a major role of hippocampal astrocytes in the pathogeneses of various neurodegenerative disorders (Pekny et al., 2016; Siracusa et al., 2019; Verkhratsky et al., 2010).

1.4 The Circadian System

Many organisms on our planet possess a so-called circadian rhythm - an internal biological clock. The term is derived from the Latin words *circa* (meaning “about”) and *dies* (meaning “day”) and refers to endogenous processes generated in a ca 24-hour rhythm. The SCN integrates environmental time cues, particularly the light-dark cycle, and synchronizes rhythmic physiological and behavioral processes through its function as the central circadian pacemaker (Figure 3). Circadian rhythms influence various behavioral and autonomic functions and are conserved across evolution from unicellular to multicellular organisms. In 2017, the Nobel Prize in Physiology or Medicine was awarded to chronobiologists Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discovery of two key genes that regulate this rhythm in the fruit fly (*Drosophila*) (Korf, 2016).

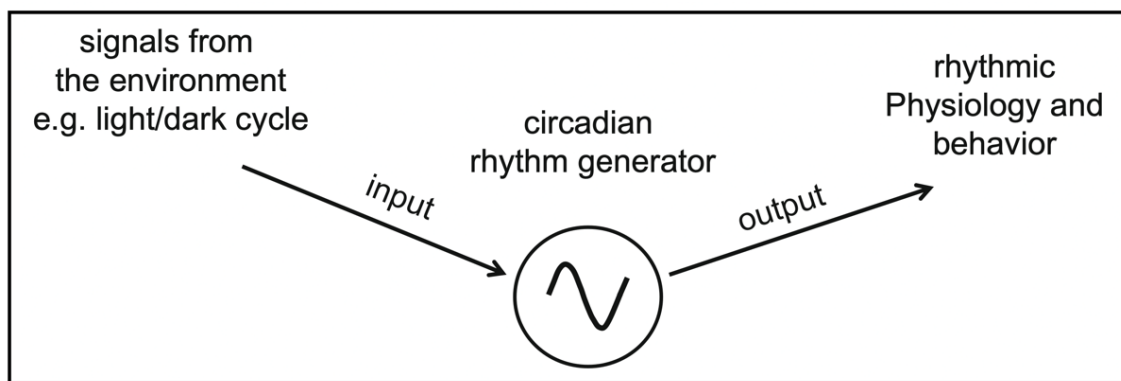


Fig. 3: Conceptual overview of the circadian system. The circadian rhythm generator produces an endogenous rhythm with a period length of approximately 24 h. Environmental signals, such as light, provide input to the circadian rhythm generator and synchronize the endogenous rhythm with external conditions. These environment cues are called *Zeitgebers*, the process of adjusting is named *entrainment*. The circadian rhythm generator then affects rhythmic physiology and behavior of an organism by output signals. Reproduced under license from the copyright holder (Korf, 2016).

1.4.1 Input to the Circadian System

The daily light-dark cycle constitutes the primary time giver, *Zeitgeber* in German, for the synchronization of circadian rhythms determining physiological and behavioral processes to the external environment, a process known as *entrainment*. In mammals, the light is received via the retina and transmitted to the brain. However, it was demonstrated that degeneration of classic retinal photoreceptors (rods and cones) in mice do not abolish the responses to changes of light and darkness (Ebihara & Tsuji, 1980; Foster et al., 1991).

While certain non-vertebrate species possess non-retinal light input pathways - such as the direct photic stimulation of photosensitive circadian pacemaker neurons by light penetrating neural tissue - in vertebrates, ocular enucleation results in a loss of circadian entrainment capacity (Lockley et al., 1997; Yamazaki et al., 1999). These findings suggested the existence of a third type of photoreceptors, next to rods and cones, which were identified within the retinal ganglion cells, and known as intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson, 2003).

Photoreceptors in the retina contain photopigments, named light-sensitive Opsins, which are molecularly characterized as heptahelical transmembrane proteins. The mouse retina contains different kinds of these photopigments: rhodopsin in rods, L- and S-Opsin in cones and melanopsin in ipRGCs, each with distinct spectral sensitivity maxima (Hattar et al., 2002). The axons of the ipRGCs transmit light information to the hypothalamic SCN via the retinohypothalamic tract (RHT) by releasing glutamate and pituitary adenylylate cyclase-activating polypeptide (PACAP) in the SCN. The glutamate activates NMDA- and AMPA-receptors in the SCN leading to a calcium influx and a phosphorylation of cAMP response element-binding protein (CREB). The activated CREB binds to calcium and cAMP response elements, which leads to the transcription of a clock gene, *Per1* (Korf, 2016; Welsh et al., 2010). This pathway forms the basis of circadian *entrainment*, i.e., the synchronization of the endogenous circadian rhythm with external environmental cues (Roenneberg et al., 2003). In contrast, the concept of *masking* refers to the direct influence of environmental factors on physiological processes and behavior, independent of the circadian clock. Nocturnal mammals, for example, exhibit increased locomotor activity during darkness while the light directly inhibits the locomotor activity (Korf, 2016; Shuboni et al., 2012).

Next to the RHT, the SCN also receives indirect input from the intergeniculate leaflet (IGL) via the geniculohypothalamic tract (GHT). This pathway uses neuropeptide Y (NPY) and gamma-aminobutyric acid (GABA) as neurotransmitters (Korf, 2016; Pickard, 1985). The IGL itself receives information from the RHT, suggesting a preprocessing of light information through the GHT. In addition to this photic pathway, the SCN also receives input from the raphe nuclei, which convey non-photoc signals by using serotonin, also known as 5-hydroxytryptamine (5-HT), as their neurotransmitter (Korf, 2016).

1.4.2 Suprachiasmatic Nucleus (SCN)

In mammals, the SCN serves as the central circadian pacemaker and is therefore also named *Masterclock*. The bilateral, paired nucleus is located in the anterior hypothalamus around the third ventricle above the optic chiasm, composed of approximately 10,000 tightly connected neurons in each SCN in addition to glial cells. While the neurons in the ventrolateral part (the *core*) mainly express vasoactive intestinal polypeptide (VIP), in the dorsomedial part (the *shell*) mostly arginine vasopressin (AVP) can be identified (Moore & Silver, 1998). Nearly all SCN neurons utilize GABA as a major neurotransmitter, which exerts both excitatory and inhibitory effects depending on the receptor context (Choi et al., 2008; Liu & Reppert, 2000; Moore & Speh, 1993). Other neuropeptides such as gastrin-releasing peptide (GRP), prokineticin 2, calretinin, neurotensin, and somatostatin play a subordinate role.

Numerous studies have demonstrated the critical role of the SCN in the generation of circadian rhythms as its lesioning or ablating in rodents leads to altered locomotor activity and drinking behaviors (Stephan & Zucker, 1972), changes in neurotransmitter receptors (Kafka et al., 1985) and disturbed estrous cycle (Mosko & Moore, 1978). Interestingly, the circadian rhythms in hamsters with disrupted rhythmicity could be restored through transplantation of SCN tissue (Lehman et al., 1987; Ralph et al., 1990).

1.4.3 Output of the Circadian System

Effective coordination between the central circadian clock and peripheral tissues requires systemic dissemination of signals generated by the SCN. In mammals, the information is either transmitted via neuronal projections or via releasing neurohormones (Korf & von Gall, 2006; Korf, 2016). The majority of the neuronal projections are to nuclei in the thalamus and hypothalamus, e.g. the paraventricular or the subparaventricular nuclei, but there are also efferences to sympathetic and parasympathetic neurons in the spinal cord or the brainstem as well as to intermediate neurons, that also receive information from other parts of the hypothalamic nuclei and forward them to other neurons (Kalsbeek et al., 2006; Korf & von Gall, 2006; Korf, 2016; Starnes & Jones, 2023).

The most common examples for neuroendocrine outputs of the SCN are the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic-pituitary-thyroid (HPT) axis. To provide a clearer understanding of the underlying regulatory circuits, the hypothalamic-pituitary-adrenal axis will be examined in greater detail in the following section. The SCN influences the activity of the dorsomedial hypothalamus (DMH) and the paraventricular nucleus of

hypothalamus (PVN) through a combination of peptidergic (e.g. AVP) and neurotransmitter-mediated pathways, thereby modulating the release of corticotropin-releasing hormone (CRH) (Kalsbeek et al., 2006). Among many stressors, that induce this release, there is evidence for a diurnal rhythm of this release due to regulation of the SCN (David-Nelson & Brodish, 1969). CRH is then delivered through the hypothalamo-hypophyseal portal circulation to the anterior lobe of the pituitary gland, where it stimulates the release of adrenocorticotrophic hormone (ACTH), which is a cleavage product from the hormone pro-opiomelanocortin (POMC). ACTH is then released into the bloodstream and causes the release of cortisol from the zona fasciculata - the middle layer of the adrenal cortex (Lim & Khoo, 2000). To prevent an excessive stress response, circulating cortisol binds to glucocorticoid receptors, e.g., in the hypothalamus, thereby inhibiting the HPA axis through a negative feedback mechanism (Tasker & Herman, 2011).

In addition to these neuroendocrine pathways, the SCN regulates circadian physiology through the control of pineal melatonin secretion. This process is mediated via a multisynaptic pathway, that involves the PVN, the intermediolateral column of the spinal cord and the superior cervical ganglion (SCG). Postganglionic sympathetic nerve fibers from the SCG directly innervate the pineal gland and release norepinephrine as their primary neurotransmitter (Korf, 2016). During the dark phase, norepinephrine release activates melatonin synthesis in the pinealocytes via adrenergic receptor signaling (Simonneaux & Ribelayga, 2003). Melatonin is then released into the bloodstream and the cerebrospinal fluid, where it acts as a systemic circadian signal by binding to melatonin receptors (MT1 and MT2), thereby triggering G protein-coupled signaling cascades in peripheral tissues (Dubocovich, 2007).

Interestingly, the SCN also appears to influence feeding behavior as well as hunger and satiety signals indirectly via efferent projections to the PVN, the arcuate nucleus (ARC), the DMH, and the lateral hypothalamus (LH) (la Fleur et al., 2024).

1.4.4 Intracellular Clock

Nearly every cell in mammals possesses such a molecular clock and is therefore capable of generating a rhythm independently of the SCN (Yi et al., 2022). In 1971, Konopka and Benzer identified a set of genes in *Drosophila melanogaster*, known as *period* genes, which proved to be essential components of the molecular clockwork. The molecular clock operates through a series of interconnected transcriptional-translational feedback loops.

The main feedback loop is composed of the transcription factors BMAL1 (brain and muscle ARNT-like 1) und CLOCK (circadian locomotor output cycles kaput), which heterodimerize in the nucleus and bind to promotor regions (Enhancer Box or E-Box), which results in transcription of *Per* (Period) and *Cry* (Cryptochrome) genes. The PER and CRY proteins are synthesized in the cytoplasm, where they gradually accumulate and show the highest concentration in the late subjective day or early subjective night (Hastings et al., 2007). With higher levels of PER and CRY proteins in the cytoplasm, more PER:CRY complexes translocate in the nucleus, where they inhibit their own transcription until they undergo degradation, maintaining a roughly 24-hours rhythm (Korf, 2016). A second loop to stabilize the oscillation of the molecular clock includes the CLOCK-BMAL1 heterodimers to promote the expression of the other transcription factors retinoid-related orphan receptor (ROR) alpha, beta or gamma and reverse strand of ERB (REV-ERB) alpha and beta, which themselves control the transcription of *Bmal1* via RORE (ROR response elements).

In this manner, many physiological processes are circadian regulated. For example, the expression of Toll-like receptor 9 and interleukin-6 - and thus, indirectly, the immune defense of an organism - depends on the BMAL1:CLOCK heterodimer (Curtis et al., 2014). But also the circadian changes in blood pressure and heart rate are directly influenced in this way (Curtis et al., 2007).

Although not yet fully understood, it is assumed that the SCN exerts a synchronizing function on the many peripheral clocks present in diverse body cells through e.g. the release of hormones via the pituitary gland (Kalsbeek et al., 2012).

Numerous recent studies have demonstrated a direct association between chronodisruption and therefore changes in expression of clock-controlled genes and a range of diseases, including metabolic syndrome, obesity, type 2 diabetes, cardiovascular diseases and various cancers (Schrader et al., 2024; Shafi & Knudsen, 2019; Sumova & Sladek, 2024).

term potentiation (LTP), and also a limited integration of information in a spatial and time-of-day context (Van der Zee et al., 2008). Specific deletion of *Bmal1* in mice results in altered astrocyte processes and changes in the cytoskeleton (Ali et al., 2020), as well as reduced expression of the astrocytic glutamate aspartate transporter (GLAST) (Korkmaz et al., 2025), supporting the conclusion that the molecular clockwork modulates astrocyte-neuron interactions. Consistent with this role in neural network function, these mice show impaired memory retrieval, while locomotor activity remains unaffected (Hasegawa et al., 2019). Interestingly, adult neurogenesis in the SGZ of DG seems to be highly influenced by the circadian system (Ali & von Gall, 2022), especially compromised by chronodisruption (Gibson et al., 2010).

As the primary external Zeitgeber, light critically shapes circadian organization and thereby influences cognition and affection. In mice, light seems to strongly influence the process of fear learning (Shan et al., 2015). While light in the daytime can help reducing symptoms of depression and bipolar disorders (Penders et al., 2016), it can also increase the risk of depressive symptoms, when applied right before bedtime (Bedrosian & Nelson, 2013). Exposure to constant light decreases the synaptic plasticity in the hippocampus and promotes neuroinflammation (Ketelauri et al., 2023; Schröder et al., 2023). Chronodisruption not only impairs the hippocampal function (Craig & McDonald, 2008; Fonken et al., 2012), it also leads to metabolic, cardiovascular and immune dysregulation (Korf et al., 2025).

1.6 Aim of the Thesis

Astrocytes in the brain exhibit region-specific heterogeneity, however, their spatial organization within hippocampal sublayers remains poorly understood. In this study, we analyzed astrocyte density and morphology together with the sublayer-specific distribution of Cx30 and Cx43 in the CA regions and DG using immunohistochemistry, providing correlations of astrocytic gap junction organization to the microvascular and neuronal environment.

In addition, the immunoreaction of Cx30 and Cx43 was examined in multiple times-of-day under different lighting conditions: standard photoperiod (LD), constant darkness (DD) and constant light (LL) to address the diurnal and circadian alteration as well as the effect of chronodisruption on astrocytic gap junctions.

All animal procedures were approved by the local government LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection; Germany; AZ: 84-02.04.2013.A358).

Our data will provide further insight into how lighting conditions and circadian integrity influence connexin expression and therefore astrocyte communication in the hippocampus, a key brain structure for learning, memory formation and spatial and temporal information integration.

2 Heterogeneity of Astrocyte Density, Morphology and Connexins in the Mouse Hippocampus, Uelwer A, Sivakumar M, Umirdinov K, Purath FFA, Oluma L, Anstötz M, von Gall C and Ali AAH, Front. Neuroanat. 20:1771439 (2026)

The following chapter presents a research article that forms an integral part of this doctoral thesis.



OPEN ACCESS

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RECEIVED 19 December 2025

REVISED 02 February 2026

ACCEPTED 12 February 2026

PUBLISHED 09 March 2026

CITATION

Uelwer A, Sivakumar M, Umirdinov K,
Purath FFA, Oluma L, Anstötz M,
von Gall C and Ali AAH (2026)
Heterogeneity of astrocyte density,
morphology and connexins
in the mouse hippocampus.
Front. Neuroanat. 20:1771439.
doi: 10.3389/fnana.2026.1771439

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Heterogeneity of astrocyte density, morphology and connexins in the mouse hippocampus

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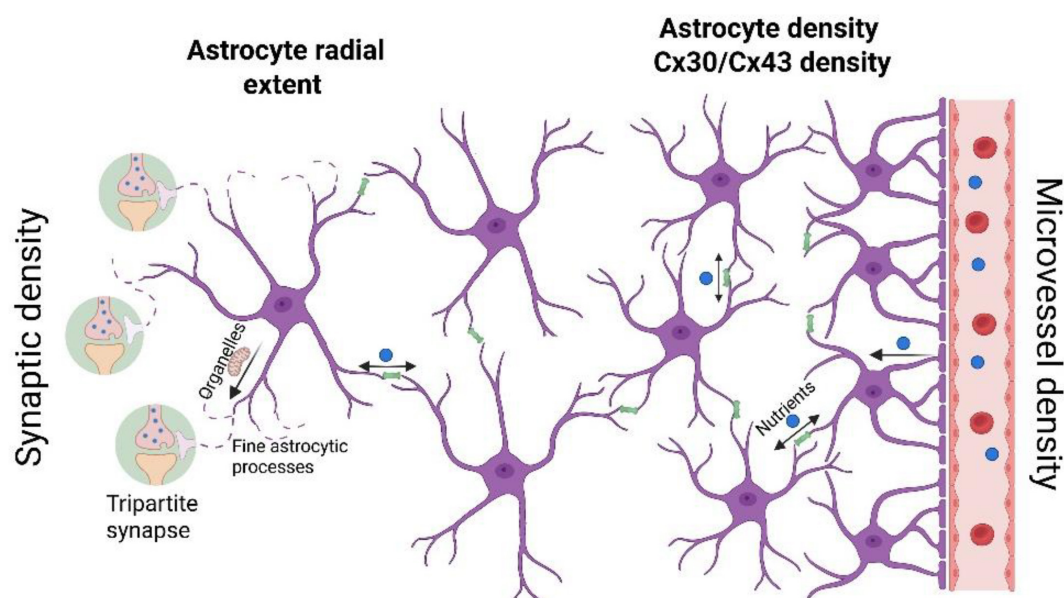
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The hippocampal formation is crucial for episodic learning and memory. In addition to neurons, astrocytes have also received increasing attention in recent years as essential components of brain networks by regulating the blood-brain barrier, eliminating waste products via the glymphatic system, supporting neuronal activity by providing energy supply and metabolic substrates, and regulating extracellular neurotransmitter levels. Astrocytes are heterogeneous and highly dynamic cells that respond to neuronal activity and dysfunction via morphological and functional changes. Astrocytic connexins (Cx) 30 and 43 form the molecular basis for gap junctions and hemichannels and are, thus, central to coupling, intercellular communication and network integration of astrocytes in the brain. However, little is known about the spatial heterogeneity of astrocyte density, morphology and Cx expression in the subregions and layers of the hippocampus. Therefore, in this study, we used immunohistochemistry to analyze the density and detailed morphological features of astrocytes and the spatial distribution of Cx30 and Cx43 in the layers of CA1, CA3 and dentate gyrus (DG). Astrocyte density correlated positively with the intensity of Cx30- and Cx43-immunoreaction (Ir). The stratum lacunosum moleculare (SLM) of CA1 and CA3 and the subgranular zone (SGZ) of DG showed the highest density of GFAP-positive (+) astrocytes and the strongest Cx30- and Cx43-Ir. The GFAP+ astrocytic processes had the largest radial extent in the pyramidal layer of CA1 and CA3 and in the granular layer of the DG. Our study provides a comprehensive anatomical and comparative mapping of astrocytic density, morphology and Cx distribution in the mouse hippocampus and provides an important basis for further studies on the dynamics of neuron-glia interaction under different physiological and pathological conditions.

KEYWORDS

astrocyte morphology, connexin, gap junctions, GFAP, spatial heterogeneity



GRAPHICAL ABSTRACT

Schematic illustration showing the spatial diversity of astrocytes where the highest density of GFAP+ astrocytes, the smallest radial extension of GFAP+ processes, and the highest density of Cx30 and Cx43 are found in the subregions where the most microvessels exist and, thus, are correlated to blood-brain barrier (BBB) function and higher metabolic activity of astrocytes in these layers. The largest radial extension of GFAP+ processes is found in subregions that have the highest synaptic density (Created by [Biorender.com](https://www.biorender.com)).

1 Introduction

Elucidating the complex hippocampal network is pivotal for understanding cognitive processes, especially in the context of neurological diseases such as Alzheimer's disease and temporal lobe epilepsy. The anatomy of the hippocampus in laboratory rodents such as mice and rats does not appear to differ significantly from that of primates and humans in terms of neural connections and layers (Zhao and Palomero-Gallagher, 2025; An et al., 2014).

The hippocampal formation is crucial for episodic learning and memory. It consists of the entorhinal cortex (EC), the subiculum (Sub), the dentate gyrus (DG) and the cornu ammonis (CA). The CA is divided into three distinct fields, CA1, CA2, and CA3, based on the cytoarchitecture of the pyramidal cells. In addition to preprocessed sensory information (Manns et al., 2007; Aronov et al., 2017), the hippocampal formation also integrates information about social interactions (Hitti and Siegelbaum, 2014; Lopez-Rojas et al., 2022), as well as aversive and appetitive stimuli (Skelin et al., 2019) and subcortical afferents (Amaral and Cowan, 1980; Vertes et al., 2004) that convey alertness, mood, and emotions. The neural connection between the EC and the hippocampus appears to be important for encoding the preprocessed sensory information (Manns et al., 2007; Aronov et al., 2017), in particular, in a spatial and temporal context (Moser et al., 2017; Wood et al., 2000; Manns et al., 2007; Eichenbaum, 2013; MacDonald et al., 2011; Kraus et al., 2015; Aronov et al., 2017), essential for navigation. The classical concept initially assumed a tri-synaptic connection, in which preprocessed sensory information from the EC reaches the DG via the perforant path and from there via the mossy fibers to the CA3 and from there via the Schaffer collaterals to the CA1, from where the output is transmitted via the Sub. This concept

has since been greatly expanded, and several parallel entorhinal-hippocampal loops are postulated, in which the medial EC (MEC) is more involved in the representation of spatial and temporal information, while the lateral EC (LEC) plays a greater role in the processing of object representations (Save and Sargolini, 2017; Moser et al., 2017) and social memory (Lopez-Rojas et al., 2022).

DG and CA have a trilaminar structure; a cell-rich layer with excitatory principal neurons, a polymorphic layer with the axons of the principal neurons, and a cell-poor layer with the dendrite trees of the principal neurons and afferent fibers. In the DG, these layers are referred to as granule cell layer (Gr), polymorphic layer (Pol) and molecular layer (Mol), the latter containing mainly the perforant path. Between Gr and Pol is a narrow layer of cells, the subgranular zone (SGZ), which is an important site of adult neurogenesis, where neuronal stem cells and progenitor cells proliferate, differentiate and produce new granule cells. In CA1, the first two layers are called the pyramidal cell layer and stratum oriens (Or), the latter of which also contains the basal dendrites of the pyramidal cells. According to their afferent connections, the cell-poor layer of the CA, in which the apical dendrites of the pyramidal cells are located, is divided into the proximal stratum radiatum (Rad) and the stratum lacunosum moleculare (SLM) adjacent to the hippocampal fissure (Zhao and Palomero-Gallagher, 2025; Valero and de la Prida, 2018). Or and Rad receive inputs from Schaffer collaterals as well as from subcortical telencephalic structures such as septal nuclei. Afferents from the entorhinal area and the thalamus also end in the Or and in the SLM. Afferents from the brainstem, on the other hand, end mainly in the proximal part of the SLM.

In addition to neurons, which have been the subject of intensive research for a long time, astrocytes have also been receiving increased attention in recent years as essential components of brain

networks. They significantly contribute to maintain brain function by regulating the blood-brain barrier, acting as a perivascular sheath for lymphatic drainage, and thus waste clearance, and playing a crucial role in neuron metabolism by supporting energy supply, providing metabolic substrates, and regulating the extracellular environment including the redox balance (Almeida et al., 2023) and the level of ions and neurotransmitters (Bélanger et al., 2011). The latter function also plays an important role in ensuring efficient synaptic transmission, as astrocytes take up and recycle neurotransmitters, which is why the unit consisting of neurons and astrocytes is also referred to as a tripartite synapse. In particular, the uptake of glutamate by astrocytes is of special importance, as this prevents neuronal excitotoxicity. In addition, astrocytes modulate neuronal activity by releasing gliotransmitters such as ATP, glutamate, and D-serine. Therefore, an intact astrocytic network is vital for neural homeostasis, plasticity and cognition. Within the hippocampus, astrocytes seem to regulate stress and anxiety-like behaviors (Li et al., 2024), via modulation of astrocytic glucocorticoid receptors (Lu et al., 2025). In addition, the astrocytic calcium activity is correlated with the affective state and plays an important role in anxiolytic behavior due to enhanced ATP release (Cho et al., 2022). Furthermore, astrocytic G-protein-coupled receptors critically regulate the working and spatial memory (Escalada et al., 2024). Accordingly, their dysfunction is associated with various neurological disorders, including Alzheimer's disease, temporal lobe epilepsy and anxiety-related disorders.

The complex and diverse functions of astrocytes are also reflected in their complex structure. They have a tree-like morphology with processes that envelop the blood vessels on the one hand and the synapses on the other. Astrocytes express connexons, hexameric protein complexes composed of six connexin (Cx) subunits, in particular Cx30 and Cx43 (Huang et al., 2021), that form hemichannels in the plasma membranes (Goodenough and Paul, 2009). Hemichannels allow the release of gliotransmitters such as glutamate, ATP, and aspartate into the extracellular space, thus facilitating communication between astrocytes and neighboring neurons (Ye et al., 2003; Xing et al., 2019). Two connexons from neighboring astrocytes form a gap junction that allows the passage of ions, small metabolites, and signaling molecules, resulting in electrical and metabolic coupling and, thus, a functional syncytium in the astrocytic network (Dossi et al., 2024; Kielian, 2008). Astrocytic Cx are essential for synaptic efficacy (Cheung et al., 2023; Pannasch et al., 2014), modulate neuronal network activity (Pannasch et al., 2011, 2014), contribute to neuroprotection, the integrity of blood-brain barrier (Ezan et al., 2012) and the glymphatic system (Cibelli et al., 2021) and might also be involved in adult neurogenesis (Talaverón et al., 2024). Our previous study shows that Cx30 and Cx43 play a role in the stability of circadian rhythms (Ali et al., 2019). In various neurodegenerative diseases and neuroinflammation, changes in astrocytic Cx can be observed, which in turn have an effect on progression by altering neuronal function and survival (Huang et al., 2021; Kielian, 2008). Astrocyte-specific Cx30 and Cx43 double knockout (Cx30/43 DKO) mice show changes in circadian rhythm stability (Ali et al., 2019), as well as deficits in sensorimotor performance and a complete lack of spatial learning and memory (Höslí et al., 2022). The latter indicates the special role of the astrocytic Cx for the hippocampal network. In fact, excitatory

synaptic transmission and long-term potentiation of CA1 neurons are significantly affected in Cx30/43 DKO mice (Höslí et al., 2022).

The glial fibrillary acidic protein (GFAP) is an intermediate filament (IF) type III protein, a structural marker that is found in the majority of CNS astrocytes (Jurga et al., 2021). The density and morphology of astrocytes in the cerebral cortex, including the hippocampus, shows layer-specific heterogeneity, which appears to be caused mainly by the different input signals (Viana et al., 2023; Karpf et al., 2022; Lanjakornsiripan et al., 2018). Cx also appear to exhibit region-specific differences in the brain (Nagy et al., 1999; Batter et al., 1992), which may be related to the activity level of surrounding neurons (Huang et al., 2019). However, little is known about the spatial heterogeneity of Cx in the sublayers of the hippocampus. Therefore, in this study, we analyzed the GFAP+ astrocyte density and the detailed morphological features as well as the spatial distribution of Cx30 and Cx43 in the sublayers of the CA and the DG using immunohistochemistry and sholl-based tracing. Our study contributes to detailed anatomical knowledge and better understanding of the heterogeneity of astrocyte morphology, density and subtypes as well as the formation of astrocytic gap junctions in relation to their neuronal environment, which could help probing the role of hippocampal astrocytes in learning, memory and anxiety modulation. This provides a basis for understanding the plasticity of astrocytes under physiological and pathological conditions.

2 Materials and methods

2.1 Animals and tissue processing

Adult male 12–15 weeks old C57BL/6J mice were housed in standard cages in temperature- and sound-proof cabinets (Beast master, Germany) with a light intensity of 600 lx during the light phase and 0 lx during the dark phase. Animal experiments were approved by the local government LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection; Germany; AZ: 84–02.04.2013.A358) and conform to European and international guidelines. Mice were deeply anesthetized using intraperitoneal injection of ketamine-xylazine mixture (100 mg/10 mg per kg, respectively). This was followed by transcardial perfusion with 0.9% NaCl, then 4% formalin. After post-fixation in 4% formalin for additional 24 h, the brains were cryoprotected in 20% then 30% sucrose until saturation. Parallel series of 30 µm thick coronal brain slices containing the dorsal hippocampus, bregma from −1.58 mm to −2.18 mm (Paxinos and Franklin, 2012), were prepared using a cryomicrotome (Reichert-Jung).

2.2 Immunohistochemistry

For each primary antibody, one of the parallel series was used and stained in a free-floating manner. Slices were washed using phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBST). To inhibit endogenous peroxidase activity, the slices were treated with 0.24% hydrogen peroxide (H₂O₂) for 30 min. Following this, the slices were washed and then incubated

with 5% normal goat serum in PBST for one hour to block nonspecific binding. Sections were incubated overnight at 4 °C with one of following primary antibodies: polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:3000, cat#: Z0334, DAKO, Denmark), polyclonal rabbit anti-Cx30 (1:1500, cat#: 71–2200, Thermo Fisher), polyclonal rabbit anti-Cx43 (1:250, cat#:71–0700, Thermo Fisher). Specificity of the antibodies against Cx30 and Cx43 has been previously validated using Cx30/43 DKO mice (Ali et al., 2019). The sections were then washed with PBST and incubated with biotin-conjugated goat anti-rabbit IgG (1:500, Vector Laboratories, BA-1000) for one hour. This was followed by washing and then incubation with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for one hour. Afterward, sections were rinsed in PBST followed by a 10-min incubation with 3,3'-diaminobenzidine (Sigma-Aldrich) and rinsed with PBS. The slices were mounted on slides, rinsed with water, dehydrated and coverslipped using Entellan (Merck Millipore).

2.3 Image acquisition and analysis

The following layers were analyzed in the CA1 and CA3: stratum oriens (Or), stratum pyramidale (Pyr), stratum radiatum (Rad), and stratum lacunosum moleculare (SLM). The following layers were analyzed in the DG: stratum polymorphe (Pol), molecular layer (Mol), stratum granulare (Gr), and the subgranular zone (SGZ) (Viana et al., 2023; Karpf et al., 2022; Paxinos and Franklin, 2012). The SGZ was defined as a two-nucleus-wide area along the inner border of Gr (Ali et al., 2015; Supplementary Figure 1).

Astrocytes were manually counted in a delineated area in each hippocampal subregion using ImageJ and astrocyte density was defined as the number of cells per mm². For the analysis of astrocyte morphology, three GFAP-stained astrocytes per layer were selected per mouse for further analysis based on the following inclusion criteria: clear boundaries, intact and no or few overlapping processes with neighboring astrocytes, as previously described (Ali et al., 2020). Due to the extensive overlap of the processes, we could not include the SGZ in the morphological analysis. Z-stacks of images were taken using the 100× objective lens in bright-field microscope (Nikon Eclipse E800). The GFAP-stained astrocytes were traced and three-dimensionally reconstructed by Neurolucida software (version 4.3.3, MicroBrightField Inc.). The following parameters were then further analyzed by Sholl analysis and averaged per mouse: cell body surface area, mean terminal distance of the endings from the cell body to quantify the mean radial extent, total length of the processes, total number of branches, total number of trees (indicated by number of first order processes), nodes/100 μm length, and total surface area of the processes.

For Cx30 and Cx43 analyses, three images per mouse were taken in bright-field microscope (BZ-9000, Keyence, Japan) with identical settings e.g., light intensity and exposure time for each antibody. For semi-quantitative analysis, the staining intensity of Cx30 and Cx43-immunoreaction (Ir) was measured in the delineated layers using ImageJ software (NIH). The background intensity, determined in a cell-free area within the corpus callosum, was subtracted. The resulting Ir intensity was averaged per mouse and expressed in arbitrary units (au). Normalization of Cx-Ir to

GFAP+ astrocyte density or area was not feasible, as staining sets for Cx and GFAP was performed using different cohorts of mice.

2.4 Statistical analysis

GraphPad Prism Software (GraphPad Software, Inc.) was used for statistical analysis. Normal distribution within groups was confirmed by using the D'Agostino and Pearson test. Data were presented as mean ± SEM. To assess differences between the subregions one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparison was used. To analyze the association between different parameters, the correlation coefficient (*r*) of pairs of variables from each hippocampal subregion was analyzed by Pearson Test. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Heterogeneity of GFAP+ astrocyte density in hippocampal layers

The density of GFAP+ astrocytes was heterogeneous across the layers of hippocampal regions (Figure 1A). The highest astrocyte density in CA1 was found in the SLM, whereas there were no statistical differences among Or, Pyr and Rad (Figures 1B, C). In CA3, the SLM showed the highest astrocyte density, whereas Pyr showed the lowest astrocyte density (Figures 1D, E). Within DG, the highest astrocyte density was found in SGZ, whereas the lowest astrocyte density was found in Gr (Figures 1F, G).

3.2 Heterogeneity of GFAP+ astrocyte morphology in hippocampal layers

Soma and processes of GFAP+ astrocytes showed morphological heterogeneity across the layers of CA1, CA3 and DG (Figure 2 and Supplementary Figure 2). In CA1 (Figures 2A, B) and CA3 (Figures 2F, G), the cell bodies were smallest in Pyr. The mean distance from the soma to the terminal point of the traced trees, as a measure for radial extent and total processes length, were largest in Pyr and shortest in SLM of both CA1 (Figures 2A, C, E) and CA3 (Figures 2F, H, J). The frequency of nodes/100 μm length, as a measure for the complexity of branching, was higher in Rad compared to Pyr and SLM in CA1 (Figures 2A, D) and to Or and SLM in CA3 (Figures 2F, I). In DG, the size of cell bodies was not different among Pol, Gr and Mol. However, in the Gr the GFAP+ processes showed the largest radial extent and the lowest complexity of branching (Figures 2K–O).

3.3 Heterogeneity of Cx30 and Cx43 immunoreaction in hippocampal layers

Cx30-Ir showed the highest intensity in SLM and the lowest intensity in Pyr and Rad in both CA1 (Figures 3A–C) and CA3 (Figures 3A, D, E). Cx30-Ir was more intense in Or compared

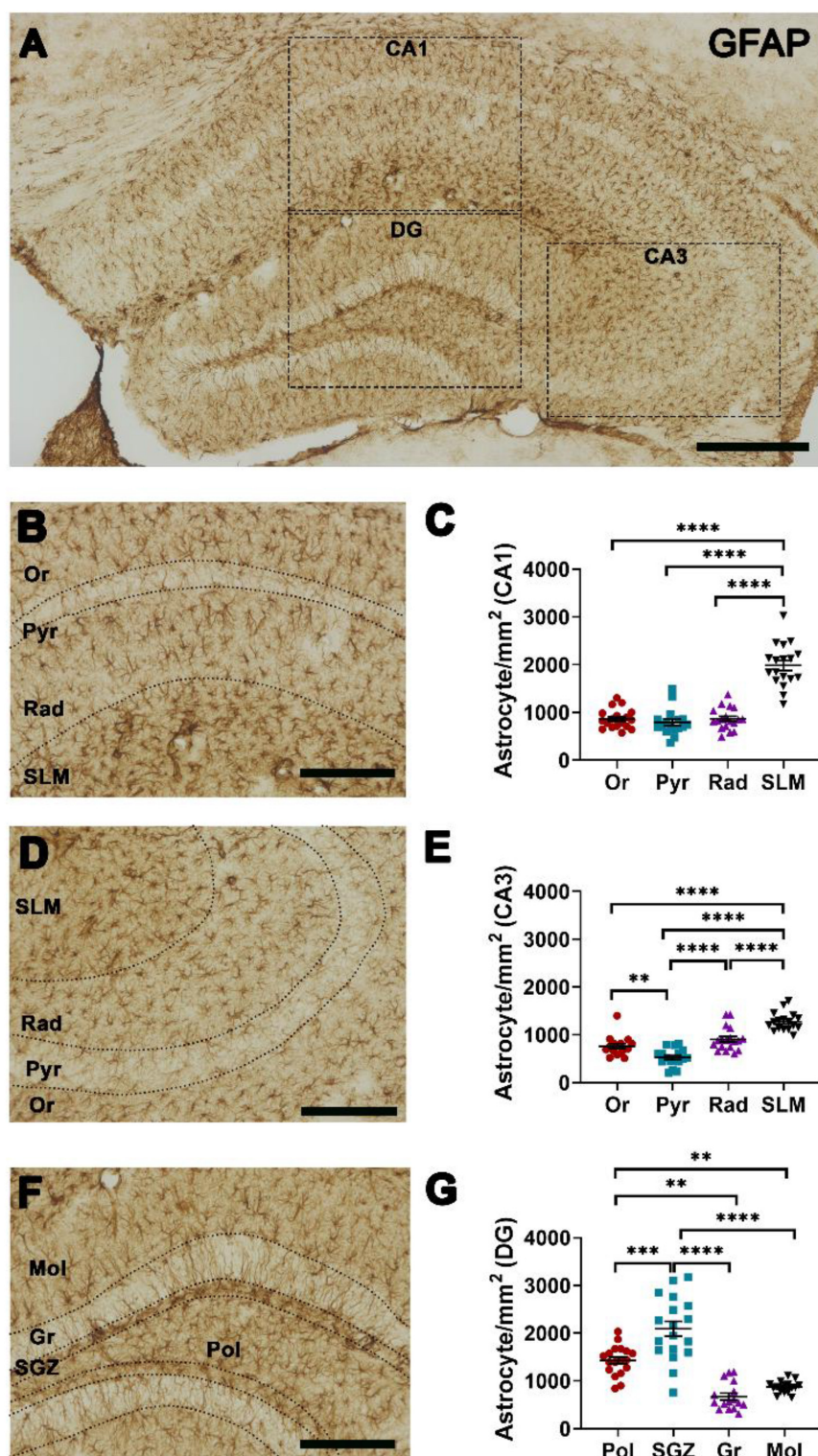


FIGURE 1

Heterogeneity in the density of GFAP-positive (+) astrocytes in the hippocampal layers. (A) Representative photomicrograph showing GFAP+ astrocytes in hippocampal CA1, CA3, dentate gyrus (DG) (insets), scale bar = 200 μ m. Representative photomicrograph of GFAP+ astrocytes at higher magnification in (B) CA1 and (D) CA3 subregions: Or, stratum oriens; Pyr, stratum pyramidale; Rad, stratum radiatum; SLM, stratum lacunosum moleculare, scale bar = 100 μ m. Quantification of GFAP+ astrocyte density in (C) CA1, (E) CA3. (F) Representative photomicrograph of GFAP+ astrocytes at higher magnification in DG subregions: Mol, stratum moleculare of DG; Gr, stratum granulare; SGZ, subgranular zone; Pol, stratum polymorphe, scale bar = 100 μ m. (G) Quantification of GFAP+ astrocytes in DG. One-wayANOVA followed by Tukey *post-hoc* tests; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 18$ mice.

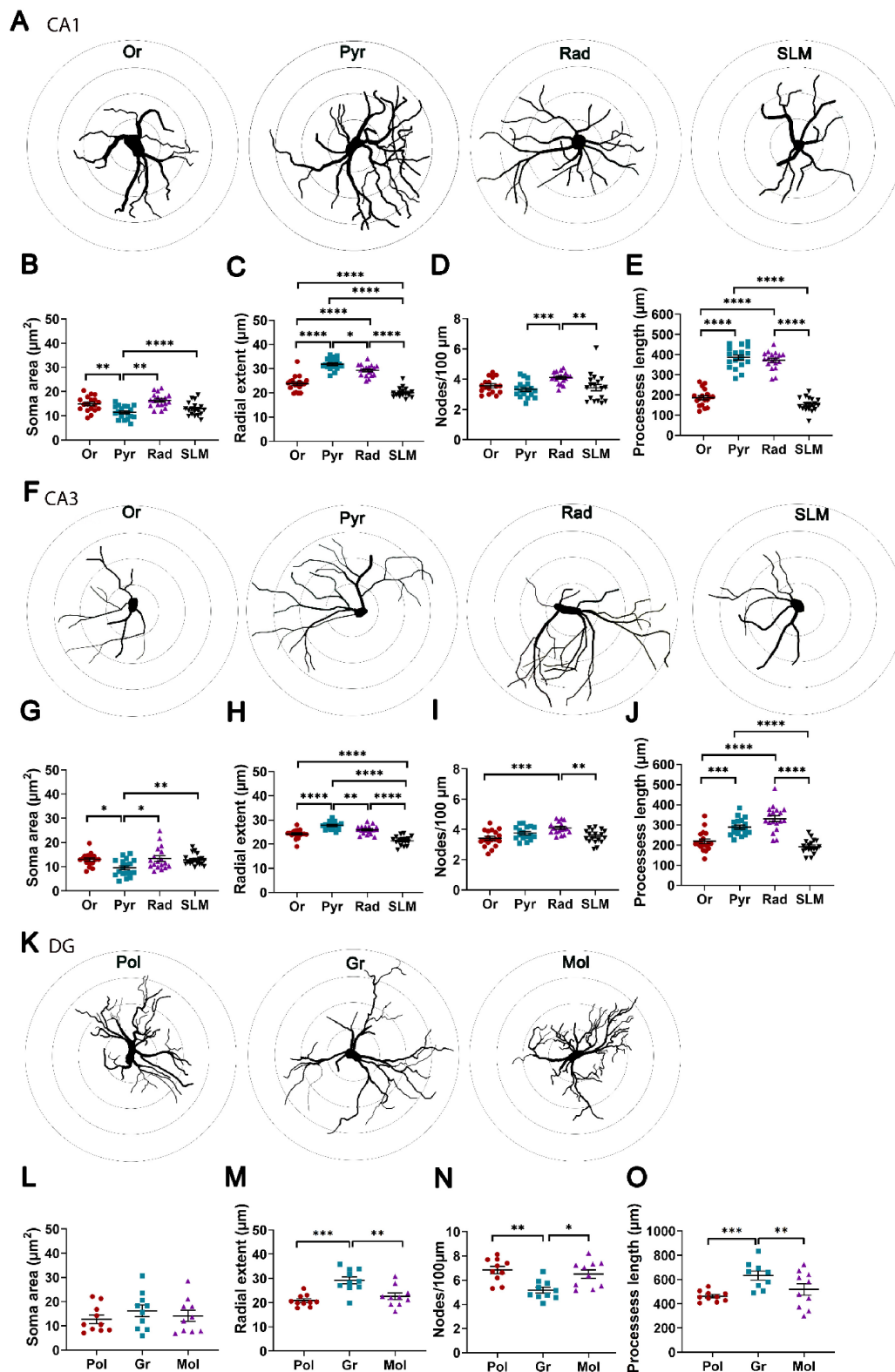


FIGURE 2

Heterogeneity in the morphology of GFAP-positive astrocytes in the hippocampal layers. (A) Representative Sholl-based tracing of astrocytes in CA1 layers in 10 μm segments from the soma center. (B–E) Quantification of astrocyte morphology variables in CA1. (F) Representative Sholl-based tracing of GFAP-positive astrocytes in CA3 layers. (G–J) Quantification of astrocyte morphology variables in CA3. (K) Representative Sholl-based tracing of GFAP-positive astrocytes in DG layers. (L–O) Quantification of astrocyte morphology variables in DG. Gr, stratum granulare; Mol, stratum moleculare of DG; Or, stratum oriens; Pol, stratum polymorphe; Pyr, stratum pyramidale; Rad, stratum radiatum; SGZ, subgranular zone; SLM, stratum lacunosum moleculare of CA1 and CA3. One-way ANOVA followed by Tukey *post-hoc* tests; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ using. $n = 10$ in DG and $n = 18$ mice in CA1 and CA3.

to Pyr in both CA1 (Figures 3A–C) and CA3 (Figures 3A, D, E). In DG, Cx30-Ir showed the highest intensity in SGZ and the lowest intensity in Gr, and lower intensity in Mol compared to SGZ (Figures 3A, F, G).

Cx43-Ir showed the highest intensity in SLM and the lowest intensity in Rad of both CA1 (Figures 4A–C) and CA3 (Figures 4A–E). In Pyr of both CA1 (Figures 4A–C) and CA3 (Figures 4A, D, E), Cx43-Ir was less intense than in SLM but more intense than in Rad. In DG, Cx43-Ir showed the highest intensity in Mol and the lowest intensity in Gr. Cx43-Ir intensity was significantly lower in Pol than in Mol (Figures 4A, F, G).

3.4 Correlation between Cx30, Cx43, GFAP+ astrocyte density and morphology

Both Cx30- and Cx43-Ir correlated positively with the GFAP+ astrocyte density in the different hippocampal layers (Figures 5A, B). The SLM of CA1 and CA3 showed the highest density of GFAP+ astrocytes and the most intense Cx30- (Figure 5A) and Cx43-Ir (Figure 5B). In contrast, the intensity of Cx30- and Cx43-Ir correlated negatively with radial extent of GFAP+ astrocytes (Figures 5C, D). The Pyr and Rad of CA1 and Gr of DG showed the highest radial extent and the lowest Cx30- (Figure 5C) and Cx43-Ir (Figure 5D). Likewise, density and radial extent of GFAP+ astrocytes correlated negatively (Figure 5E). Neither the intensity of Cx30- and Cx43-Ir nor the density of GFAP+ astrocytes correlated significantly with any of the other morphological parameters (Supplementary Figures 3, 4).

4 Discussion

The understanding of the heterogeneity of astrocytes in different brain regions and cortical layers is of great interest, as it contributes decisively to elucidate the multiple roles of astrocytes in supporting neurons in terms of nutrition supply and synaptic plasticity under physiological and pathological conditions (Matyash and Kettenmann, 2010; Zhang and Barres, 2010; Jinno, 2011; Keller et al., 2018; Li et al., 2023; Viana et al., 2023; Karpf et al., 2022). In this study, we provide the first comprehensive and comparative analysis of the spatial distribution of astrocytic density, morphology, and the major astrocytic gap junction proteins Cx30 and Cx43 in the subregions and layers of the mouse hippocampus. Our data show an association between the intensity of the astrocytic gap junction proteins Cx30 and Cx43 and GFAP+ astrocytes and an association between higher astrocyte density and lower radial extent of the GFAP+ processes in hippocampal subregions and layers.

The density of GFAP+ astrocytes was highest in the SLM of the CA1 and CA3, which is consistent with an earlier study showing the highest density of GFAP+ astrocytes in the SLM of the mouse hippocampus (Shimada et al., 1992). In addition, the SLM has the abundance of microvessels (Shimada et al., 1992) and the highest glucose uptake (Shimada et al., 1989) in the mouse hippocampus. These data suggest that in the SLM, a dense network of astrocytes interacting with the dense capillary network is primarily involved in

the uptake of metabolites such as glucose (Shimada et al., 1992). The astrocytes in the SLM have comparatively short GFAP+ processes and the lowest radial extent, which is consistent with the study by Viana (Viana et al., 2023) and also with the assumption by Shimada et al. (1992) that the astrocytes in contact with the capillary network tend to have shorter processes. Importantly, the intensity of Cx30- and Cx43-Ir was also highest in the SLM of the CA1 and CA3. Astrocytes are metabolically coupled to each other via Cx30 and Cx43, but also to neurons, in order to provide them with essential metabolites such as glucose and lactate (Mayorquin et al., 2018). Astrocytes also interact with endothelial cells via Cx43 to regulate the permeability of the blood-brain barrier and thus glucose homeostasis (De Bock et al., 2011, 2022; Zhao et al., 2018). However, additional testing of astrocytic gap junction coupling remains to be performed in future studies to confirm functional heterogeneity across the hippocampal layers. Taken together, these data suggest that the uptake and distribution of nutrients from the capillary network in the SLM occurs via the Cx-coupled network of astrocytes that have a small radial extent.

In the DG, there is a similar correlation between the density of astrocytes and microvessels (Shimada et al., 1992), which are significantly higher in Pol and Mol than in Gr (Shimada et al., 1992). Similarly, we found a higher density of GFAP+ astrocytes, a smaller radial extent of GFAP+ processes and a higher intensity of Cx30- and Cx43-Ir in Pol and Mol than in Gr. These data suggest that Cx30 and Cx43 participate in uptake and distribution of nutrients from the capillary network in the Pol and Mol via the Cx-coupled network of small radially extended astrocytes. However, in contrast to previous studies on astrocyte heterogeneity in the mouse hippocampus (Viana et al., 2023; Shimada et al., 1992), we included the SGZ, one of the main neurogenic niches. Here we found the highest density of GFAP+ astrocytes, the highest intensity of Cx30-Ir and a high intensity of Cx43-Ir. In addition to regular astrocytes, GFAP+ cells in SGZ also include radial glial-like neural stem cells that continuously proliferate and produce progenitor cells that differentiate into mature granular neurons that are integrated into hippocampal circuits, thus playing a crucial role in maintaining hippocampal structural and functional plasticity (Ming and Song, 2011). Cx30/43-DKO mice show significant decrease in proliferation of radial glia-like neural stem cells in the SGZ and number of granular neurons, indicating the important role of this Cx in adult neurogenesis (Kunze et al., 2009). In particular, channel-dependent functions of Cx43 are essential for adult neurogenesis in the SGZ (Zhang et al., 2018). Remarkably, the blood vessel density is higher within the SGZ compared to other DG layers (Sun et al., 2015). The blood vessels in the SGZ represent an integral part of the neurogenic niche in the SGZ, providing not only oxygen and nutrients but also hormones and neurotrophic factors that promote self-renewal, proliferation and differentiation of stem cells (Karakatsani et al., 2023). Astrocytes might act as key regulators of blood vessels in the SGZ by modulating blood flow, contributing to vascular stability and growth, and facilitating communication between the vascular system and neural stem cells, thereby preserving the neurogenic niche (Karakatsani et al., 2023). In the other large neuronal stem cell niche, the subventricular zone (SVZ), there is a direct interaction between neural stem cells and endothelial cells, in which Cx43, expressed by both cell types, plays an essential role. The endothelial cells appear to modulate neural stem cell proliferation in a Cx43-dependent

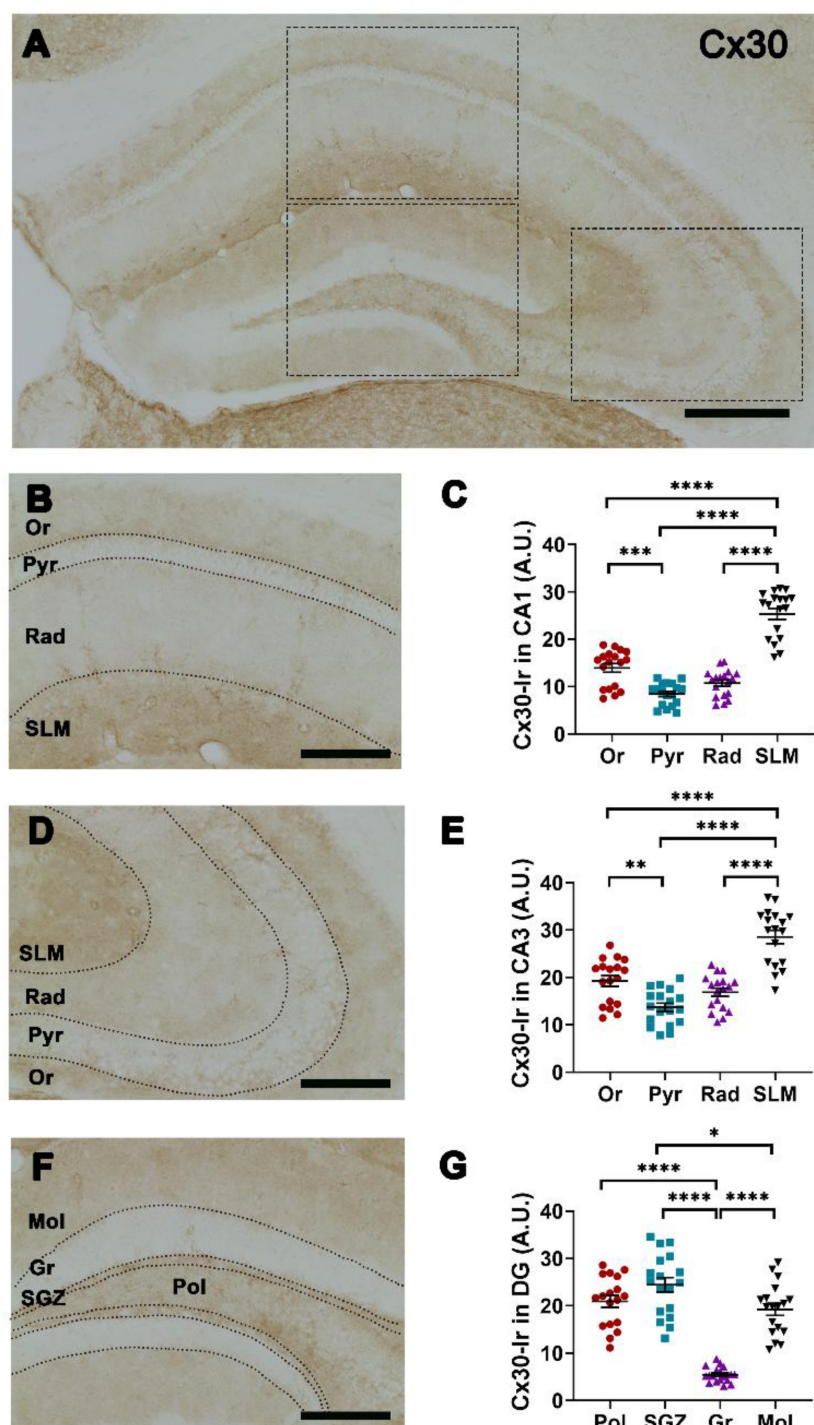


FIGURE 3

Spatial distribution of connexin 30 (Cx30) in hippocampal layers. (A) Representative photomicrograph showing Cx30-immunoreaction (Ir) in hippocampal CA1, CA3, dentate gyrus (DG) (insets), scale bar = 200 μ m. Representative photomicrograph of Cx30-Ir at higher magnification in (B) CA1 and (D) CA3 subregions: Or, stratum oriens; Pyr, stratum pyramidale; Rad, stratum radiatum; SLM, stratum lacunosum moleculare, scale bar = 100 μ m. Quantification of Cx30-Ir in (C) CA1, (E) CA3. (F) Representative photomicrograph of Cx30-Ir at higher magnification in DG subregions: Mol, stratum moleculare of DG; Gr, stratum granulare; SGZ, subgranular zone; Pol, stratum polymorphe, scale bar = 100 μ m. (G) Quantification of Cx30-Ir in DG. One-way-ANOVA followed by Tukey *post-hoc* tests; * p < 0.05; ** p < 0.01; *** p < 0.001, **** p < 0.0001. n = 18 mice.

channel-independent manner (Genet et al., 2023). Moreover, the astrocytes in the SGZ show the strongest expression of the two main glutamate receptors and the highest glutamate transporter current (Karpf et al., 2022). Taken together these data suggest that Cx30

and Cx43 participate in uptake and distribution of nutrients and signaling molecules from the capillary network in the SGZ via the Cx-coupled network of astrocytes and radial glial-like neural stem cells.

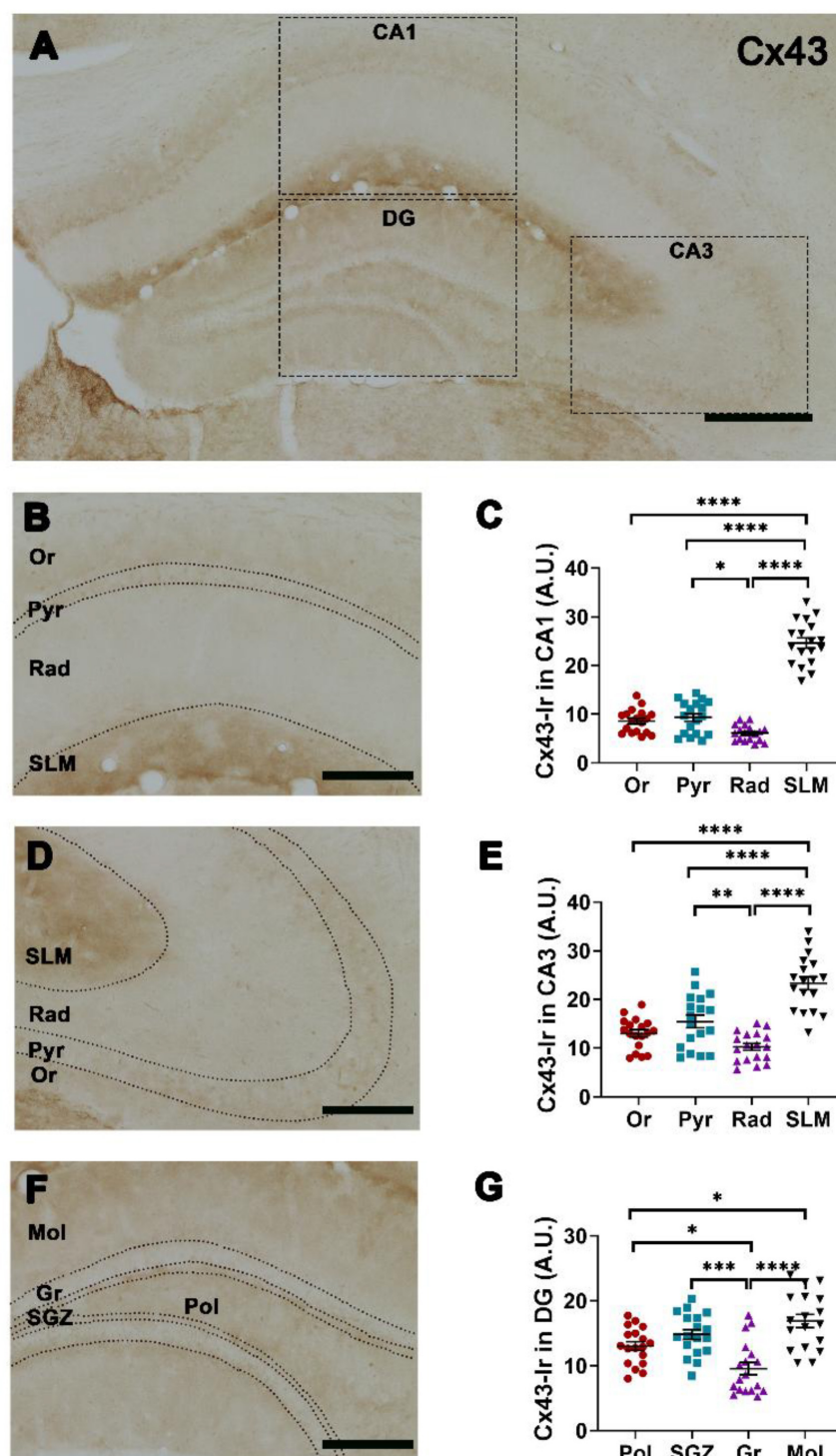


FIGURE 4

Spatial distribution of connexin 43 (Cx43) in hippocampal layers. (A) Representative photomicrograph showing Cx43-immunoreaction (Ir) in hippocampal CA1, CA3, dentate gyrus (DG) (insets), scale bar = 200 μm . Representative photomicrograph of Cx43-Ir at higher magnification in (B) CA1 and (D) CA3 subregions: Or, stratum oriens; Pyr, stratum pyramidale; Rad, stratum radiatum; SLM, stratum lacunosum moleculare, scale bar = 100 μm . Quantification of Cx43-Ir in (C) CA1, (E) CA3. (F) Representative photomicrograph of Cx43-Ir at higher magnification in DG subregions: Mol, stratum moleculare of DG; Gr, stratum granulare; SGZ, subgranular zone; Pol, stratum polymorphe, scale bar = 100 μm . (G) Quantification of Cx43-Ir in DG. One-way ANOVA followed by Tukey *post-hoc* tests; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. $n = 18$ mice.

Astrocytes Cx also play a crucial role in hippocampal neuron communication and plasticity (Hösli et al., 2022). In the mouse CA1, Rad has the highest density of total and excitatory synaptic

input, while SLM has a higher density of inhibitory input than Rad and Or (Santuy et al., 2020). A study in the human CA1, which in contrast to the aforementioned study also examined the

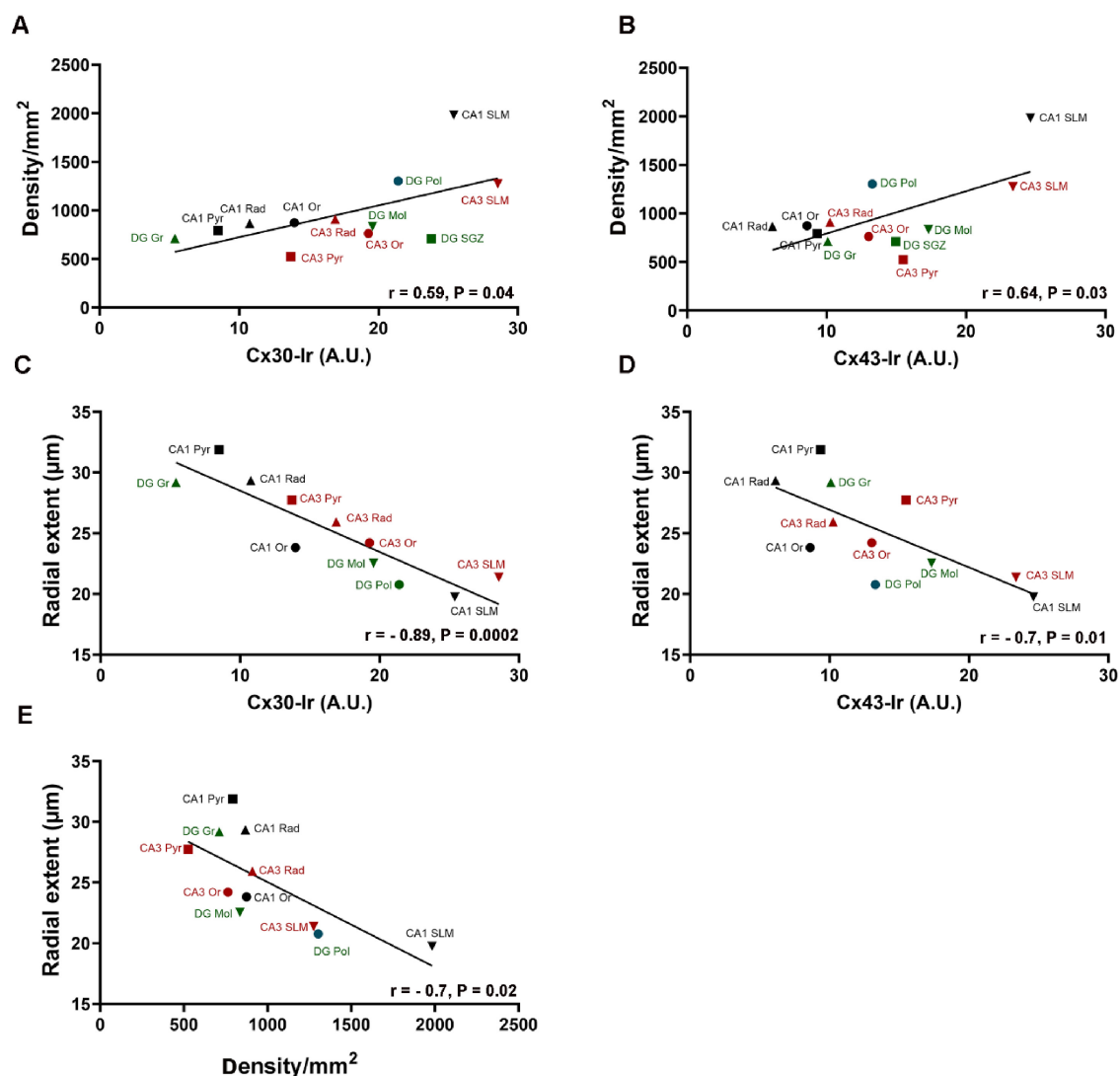


FIGURE 5

Correlation between connexins (Cx) and density and radial extent of GFAP positive (+) astrocytes in hippocampal layers. Scatterplots showing the relationship between density of GFAP+ astrocytes and (A) Cx30 and (B) Cx43, between the radial extent of GFAP+ astrocytes and (C) Cx30 and (D) Cx43 (x-axis) and between (E) astrocytic radial extent and astrocytic density in hippocampal subregions and layers. A linear regression line has been added to illustrate the associations. Gr, stratum granulare; DG, stratum moleculare of DG; Or, stratum oriens; Pol, stratum polymorphe; Pyr, stratum pyramidale; Rad, stratum radiatum; SGZ, subgranular zone; SLM, stratum lacunosum moleculare of CA1 and CA3. r , Pearson's correlation coefficient; $p < 0.05$ significant correlation; $n = 18$ mice.

Pyr, showed the highest density of total and excitatory synaptic contacts in the Pyr, followed by Rad and then Or and SLM, while SLM also had the highest density of inhibitory synapses (Montero-Crespo et al., 2020). Since we observe the highest density of GFAP+ astrocytes and the most intense Cx30 and Cx43-Ir in the SLM, there is therefore no association with the density of excitatory synapses, but if anything, with the density of inhibitory synapses. Although some studies suggest that Cx30 and Cx43 are more strongly associated with excitatory than inhibitory synaptic transmission (Pannasch et al., 2014), other reports suggest that astrocytes are active modulators of both excitatory and inhibitory synapses in the mouse hippocampus via Cx (Hardy et al., 2021), and that astrocyte networks have a physiologically optimized size to adequately regulate neuronal functions (Hardy et al., 2023). However, it is likely that the Cx-mediated morphological changes of the fine astrocyte processes, that envelop the synapses and thus

modulate synaptic strength and plasticity, play a greater role in excitatory synapses than in inhibitory synapses (Pannasch et al., 2014). Although GFAP staining is not suitable for visualizing these fine synapse-associated astrocytic processes, it can be used to show the backbone structure of the processes and, thus, the morphological complexity (Viana et al., 2023). Moreover, GFAP is a component of the intermediate filament cytoskeleton, which not only ensures the structural integrity and spatial organization of the fine astrocyte processes but is also involved in the transport of organelles (Creighton et al., 2019; Jones et al., 2018; Pajares et al., 2023). Regarding the morphology of the GFAP+ processes in CA1 and CA3, we report that Rad and Pyr showed the highest radial extent, the highest number of total and first order processes (indicated by number of trees), and the longest processes, indicating the most complex and far-reaching morphology in these two layers, which have the highest density of excitatory synapses

(Santuy et al., 2020; Montero-Crespo et al., 2020). Our findings largely align with those of Viana et al., 2023, which also show the highest complexity of the GFAP+ processes in the Rad of CA1, but did not investigate the Pyr or CA3. Therefore, we assume that the large radial extension of GFAP+ astrocytes in the CA1 and CA3 layers with high synapse density is related to the transport of organelles such as mitochondria and endoplasmic reticulum, which are essential for maintaining ion and neurotransmitter homeostasis.

Importantly, the astrocytes are able to modulate their morphology to build slightly overlapping individual territories that adapt available space, which known as “tiling,” resulting in an inverse relationship between the astrocyte density and their extent (domain size) (Grosche et al., 2013; Barber et al., 2021). Among all hippocampal layers investigated in this study, the astrocyte density was positively correlated with the intensity of Cx30- and Cx43-Ir and negatively correlated with the radial extent of GFAP+ processes. Consistently, intensities of Cx30- and Cx43-Ir were negatively correlated with the radial extent of GFAP+ processes. This suggests an inverse relationship between the number of astrocytes/astrocytic gap junctions and the size of the area covered by their backbone within the hippocampal layers. Noteworthy, the negative correlation between Cx-Ir may be an indirect reflection of the negative correlation between astrocytic density and their radial extent.

Our study leads to two major conclusions regarding the diversity of astrocytes in the subregions and layers of the hippocampus. (1) The highest density of GFAP+ astrocytes, the smallest radial extension of GFAP+ processes, and the highest density of Cx30 and Cx43 are found in the layers where, according to the literature, the most microvessels are located. This includes the SLM and also SGZ, which, as a neurogenic niche, exhibits a high metabolic rate. We therefore suspect a connection between these astrocytic properties and the uptake of nutrients from the capillaries. (2) The largest radial extension of GFAP+ processes is found in the CA layers, which are known to have the highest synapse density. We suspect that this large backbone, on the one hand, serves as a scaffold for the fine processes and, on the other hand, for the transport of cell organelles into synaptic areas. Future studies should test these proposed relationships and investigate the dynamic changes in these astrocytic properties under physiological and pathological conditions.

4.1 Limitations

In this study, we used GFAP as an astrocyte marker, which also detects neural stem/progenitor cells and specialized glia and has also been used in relevant studies (e.g., Kunze et al., 2009; Shimada et al., 1992; Viana et al., 2023), ensuring good comparability. However, we cannot rule out the possibility that we have not detected all subpopulations of astrocytes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: <https://researchdata.hhu.de/handle/entry/199>.

Ethics statement

The animal study was approved by the LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection; Germany; AZ: 84Ů02.04.2013.A358). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AU: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. MS: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – review & editing. KU: Data curation, Methodology, Software, Writing – review & editing. FP: Formal analysis, Writing – review & editing. LO: Data curation, Formal analysis, Writing – review & editing. MA: Software, Validation, Visualization, Writing – review & editing. CG: Conceptualization, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declared that financial support was received for this work and/or its publication. This publication has been supported by Heinrich Heine University Düsseldorf, Germany (number: ULBD-26-36304).

Acknowledgments

We thank Hanna Bellert, Angelika Hallenberger, Ursula Lammersen, Ralf Fassbender for excellent technical assistance. We also thank Martin Holub for assistance with Neurolucida software.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnana.2026.1771439/full#supplementary-material>

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SUPPLEMENTARY FIGURE 1

Representative photomicrograph showing GFAP-immunoreactive (Ir) astrocytes in hippocampal regions. Analysis involved stratum oriens (Or), stratum pyramidale (Pyr), stratum radiatum (Rad), and stratum lacunosum moleculare (SLM) of CA1 and CA3, as well as stratum polymorphe (Pol), stratum moleculare (Mol), stratum granulare (Gr), and the subgranular zone (SGZ), defined as a two-nucleus-wide area along the inner border of Gr, of the DG. Scale bar = 200 μ m.

SUPPLEMENTARY FIGURE 2

Heterogeneity of astrocytic morphology in mouse in the hippocampal layers. Quantification of astrocyte morphology in (A–C) in CA1, (D–F) in CA3 and (G–I) in DG. Gr, stratum granulare; Mol, stratum moleculare of DG; Or, stratum oriens; Pol, stratum polymorphe; Pyr, stratum pyramidale; Rad, stratum radiatum; SGZ, subgranular zone; SLM, stratum moleculare of CA1 and CA3. One-way ANOVA followed by Tukey *post-hoc* tests. $n = 10$ in DG and $n = 18$ mice in CA1 and CA3. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

SUPPLEMENTARY FIGURE 3

Scatterplots showing the relationship between astrocytic Cx30 and Cx43 (x-axis) with different morphological parameters of astrocytes (y-axis) in hippocampal subregions. A simple linear regression line has been added to illustrate the correlation. Gr, stratum granulare; Mol, stratum moleculare of DG; Or, stratum oriens; Pol, stratum polymorphe; Pyr, stratum pyramidale; Rad, stratum radiatum; SGZ, subgranular zone; SLM, stratum moleculare of CA1 and CA3. (r) indicates the correlation coefficient using Pearson correlation test. $p < 0.05$ indicates a significant correlation.

SUPPLEMENTARY FIGURE 4

Scatterplots showing the relationship between astrocyte density/mm² (x-axis) with different morphological parameters of astrocytes (y-axis) in hippocampal subregions. A simple linear regression line has been added to illustrate the correlation. Gr, stratum granulare; Mol, stratum moleculare of DG; Or, stratum oriens; Pol, stratum polymorphe; Pyr, stratum pyramidale; Rad, stratum radiatum; SGZ, subgranular zone; SLM, stratum moleculare of CA1 and CA3. (r) indicates the correlation coefficient using Pearson correlation test. $p < 0.05$ indicates a significant correlation.

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3 Additional Methods

3.1 Animals

Additional cohorts of adult male 12-15 weeks old C57BL/6J mice were housed in standard cages in temperature- and sound-proof cabinets (Beast master, Germany) with a light intensity of 600 lx during the light phase and 0 lx during the dark phase. All mice were granted unrestricted access to food and water ad libitum. Mice were left for two weeks for acclimatization to their environment before starting the experiment.

Mice were divided into three groups. One group (n=18, also used in Uelwer et al., 2026) was kept under normal photoperiod (12h light/12h darkness, 12:12 LD) with lights on at 6:00 am (Zeitgeber Time (ZT)00) and off at 6:00 pm (ZT12). Animals were sacrificed at six time points with 4 h-interval (during light phase: ZT02, ZT06, ZT10 and during the dark phase: ZT14, ZT18 and ZT22); n = 3 animals at each time point.

Another group (n=18) was kept under constant darkness (DD) for at least 38 hours prior to their euthanization at the circadian time points (CT, CT00 = lights on in the former light phase, 6 am, CT02, CT06, CT10, CT14, CT18 and CT22); n = 3 animals at each time point.

The third group (n=18) was kept in constant light (LL) for two weeks and scarified at disrupted time points (DT, DT00 = lights on in the former light phase, 6 am); n = 3 animals at each time point.

All procedures were performed at the animal facility of the Faculty of Medicine at Heinrich-Heine-University in Düsseldorf, Germany. Animal experiments were approved by the local government LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection; Germany; AZ: 84–02.04.2013.A358) and conform to European and international guidelines.

3.2 Tissue Processing, Immunohistochemistry, Image Acquisition and Analysis

Tissue processing, immunohistochemistry, image acquisition, and analysis were performed exactly as described in the paper. All experimental procedures strictly followed the methodology and protocols outlined in the referenced study.

3.3 Statistical Analysis

GraphPad Prism Software (GraphPad Software, Inc.) was used for statistical analysis. Error bars represent the standard error of the mean (SEM).

To assess heterogeneity among subregions as well as effect of time-of-day, the normality was tested using D'Agostino & Pearson or Kolmogorov-Smirnov test. The parametric test One-way ANOVA followed by Tukey's posthoc test for multiple comparison was used for the data sets that passed the normality test. For the datasets that failed the normality test, the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test were used.

Two-way ANOVA followed by Tukey's posthoc test for multiple comparison was used to analyze the effect of different light conditions in hippocampal subregions. A *p*-value of less than 0.05 was considered to indicate statistical significance.

4 Additional Results

4.1 Time-of-Day-Dependent Rhythms of Astrocytic Gap Junctions under Standard Photoperiod (LD) in the Mouse Hippocampal Layers

In CA1 and CA3, the Cx30 immunoreaction (Ir) was higher during the light (ZT02, ZT06, ZT10) and the early dark phase (ZT14) as compared to the later dark phase (ZT18 and ZT22) in all sublayers. In the DG, the Cx30-Ir was significant higher during the light and the early dark phase compared to the later dark phase in Mol, SGZ and Pol. The Cx30-Ir in Gr was generally low and did not show any significant differences between the time points (Figure 5A-E).

In CA1 and CA3, the Cx43-Ir in Or showed a peak in the mid-light phase (ZT06) compared to later dark phase (ZT18, ZT22). In Pyr and Mol, Cx43-Ir showed constantly higher levels during the light and the early dark phase (ZT02, ZT06, ZT10 and ZT14) as compared to the later dark phase (ZT18 and ZT22), while in stratum radiatum there were no significant differences among the examined time points. In DG, the Cx43-Ir in Mol was higher during the light and early dark phase compared to the later dark phase. In Gr, the Cx43-Ir showed a peak at ZT02, while it was significantly increased at ZT10 and ZT14 compared to ZT18 in SGZ. Cx43-Ir did not show any significant time-of-day differences in Pol (Figure 5F-J).

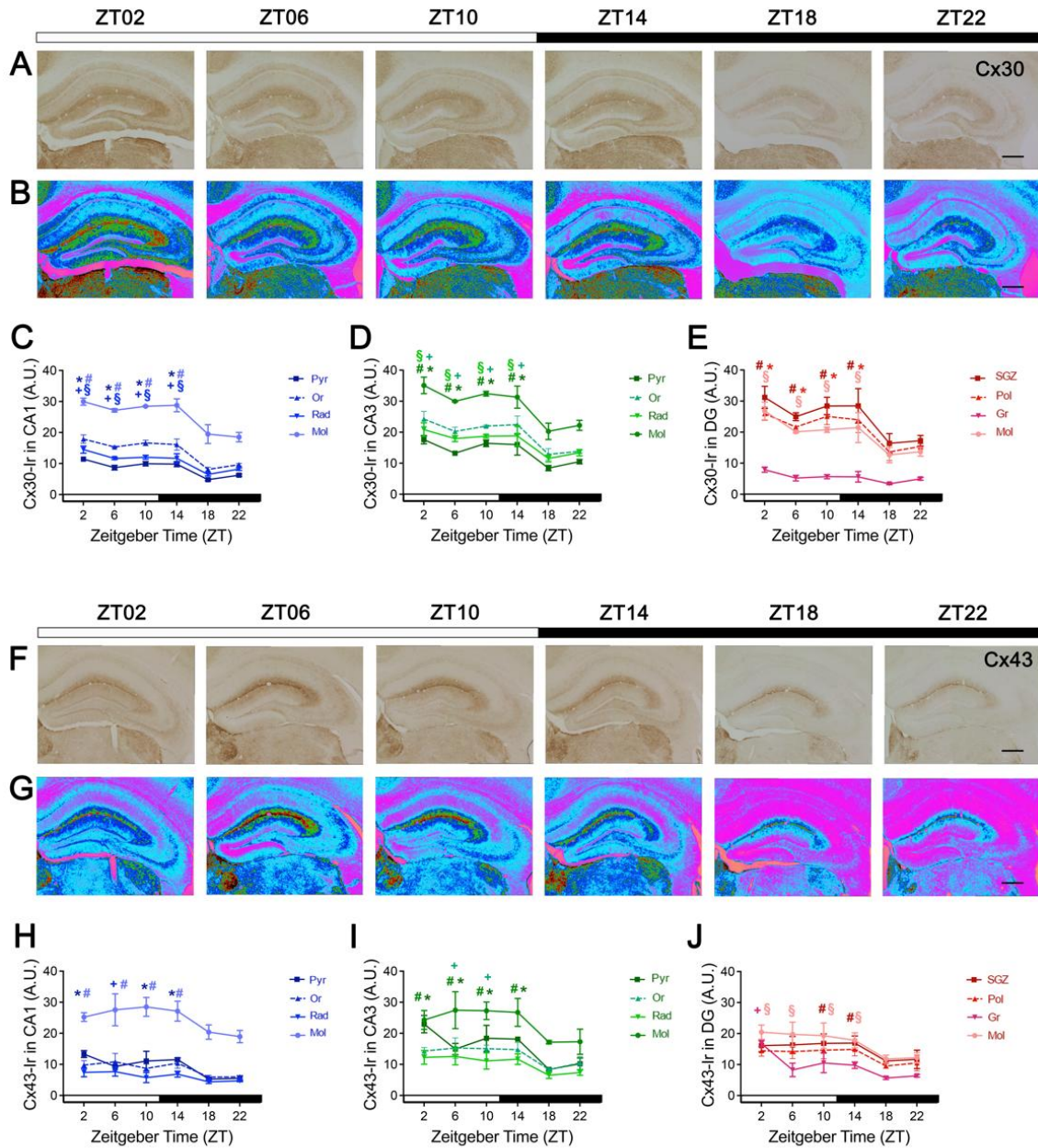


Fig. 5: Time-of-day-dependent rhythms of astrocytic gap junctions under standard photoperiod (LD) in the mouse hippocampal layers. (A)+(F) representative microphotographs showing the immunoreaction of Cx30 (A) and Cx43 (F) in mouse hippocampus under LD. The white bar indicates light phase, while the black bar indicates the dark phase. Zeitgeber time (ZT)02 indicates 2 hours after lights on, ZT14 indicates 2 hours after lights off. (B)+(G) Representative pseudocolor images of Cx30 (B) and Cx43 (G) for qualitative analysis with different colors depending on the level of immunoreaction (yellow = absent, magenta = very weak, light blue = weak, dark blue = moderate, green = strong, red = very strong). Quantification of Cx30 and Cx43 immunoreaction (Ir) as arbitrary units (A.U.) of grey values above background in (C)+(H) CA1, (D)+(I) CA3 and (E)+(J) DG. Blue and green (+) in stratum oriens, (*) in stratum pyramidale, (§) in stratum radiatum, (#) in stratum moleculare indicates significant time-of-day-dependent differences in CA1 and CA3, respectively ($p < 0.05$). Red (+) in Stratum granulare, (*) in Stratum polymorphe, (§) in Stratum moleculare, (#) in subgranular zone indicates significant time-of-day-dependent differences in DG ($p < 0.05$). $n = 18$ mice, 3 mice per each time point. Scale bar = 200 μ m.

4.2 Effect of Constant Darkness (DD) on Astrocytic Gap Junction Rhythms in the Mouse Hippocampal Layers

Under DD, the Cx30-Ir-level in CA1 stratum oriens, stratum radiatum and stratum moleculare showed a peak at the early subjective night (CT14) compared to the subjective day (CT02 and CT06), while in stratum pyramidale, it was also significant higher at CT14 compared to CT02. The Cx30-Ir did not show any significant time-of-day-dependent differences in any of the CA3 subregions. In DG stratum moleculare, subgranular zone and stratum polymorphe, the Cx30-Ir was significant higher during the early subjective night compared to the subjective day. Stratum granulare did not show any significant time-of-day differences in Cx30-Ir (Figure 6A-E).

The Cx43-Ir showed a significantly higher level at the early subjective night (CT14) in CA1 stratum pyramidale compared to the late subjective night (CT22) and in stratum moleculare during early and mid-subjective night (CT14, CT18) compared to subjective day and the late subjective night (CT22). In stratum oriens and stratum radiatum of CA1, no significant time-of-day-dependent differences in Cx43-Ir were found. The Cx43-Ir did not show any significant differences between the time points in any of CA3 subregion either (Figure 6F, G, I). In DG, stratum moleculare showed a significantly higher Cx43-Ir at mid-subjective night (CT18) compared to late subjective night (CT22). Stratum polymorphe showed a peak of Cx43-Ir at the early subjective night compared to the subjective day (CT06 and CT10) and the later subjective night (CT22). The immunoreaction of Cx43 in Stratum granulare was significant higher during the early subjective night compared to the later subjective night (Figure 6F, G, J).

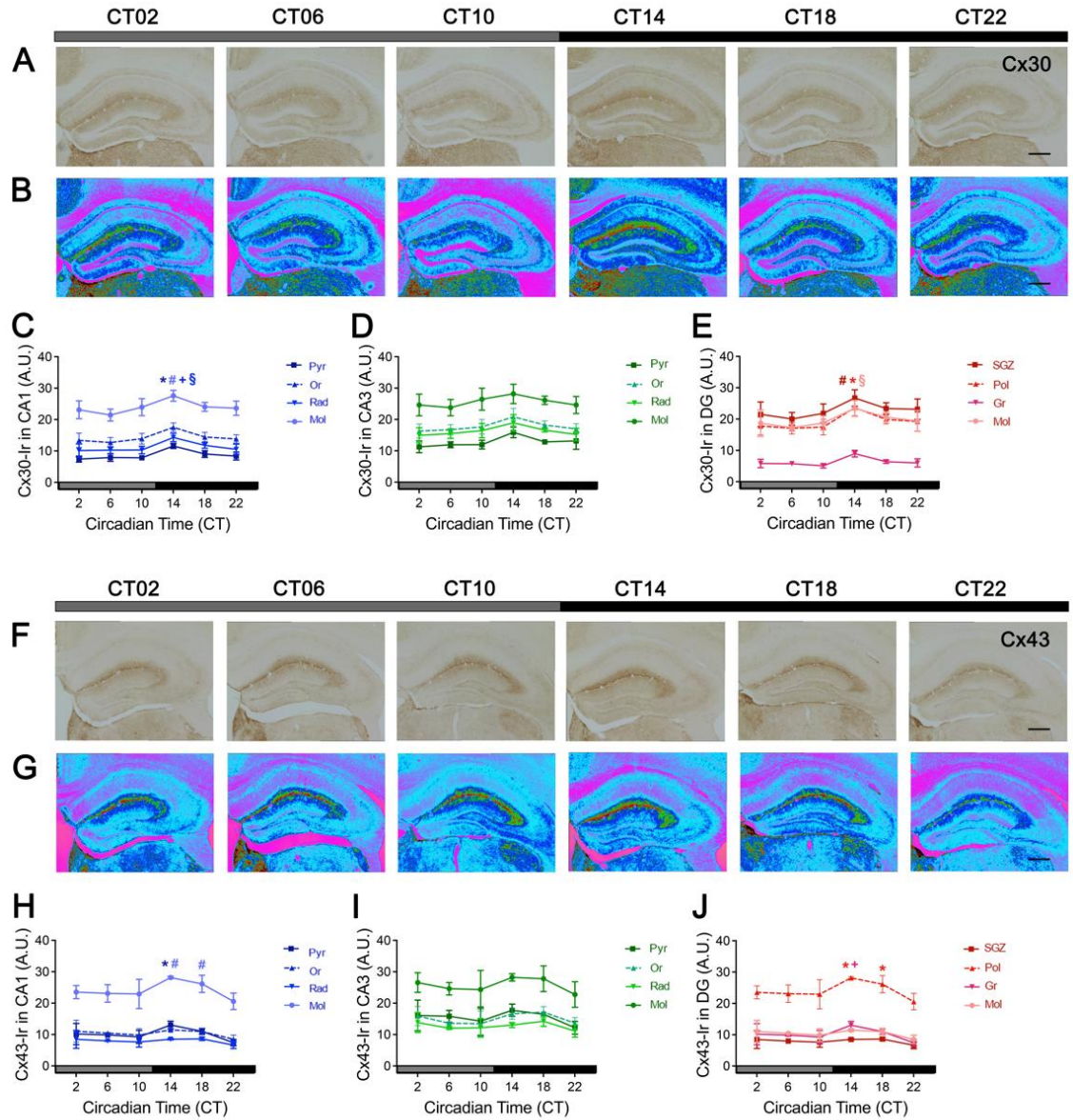


Fig. 6: Effect of constant darkness (DD) on astrocytic gap junction rhythms in the mouse hippocampal layers. (A) and (F) representative microphotographs showing the immunoreaction of Cx30 (A) and Cx43 (F) in mouse hippocampus under DD. The black and the grey bar indicates the dark phase. (B) and (G) representative pseudocolor images of Cx30 (B) and Cx43 (G) for qualitative analysis with different colors depending on the level of immunoreaction (yellow = absent, magenta = very weak, light blue = weak, dark blue = moderate, green = strong, red = very strong). Quantification of Cx30 and Cx43 immunoreaction (Ir) as arbitrary units (A.U.) of grey values above background in CA1 (C) + (H), CA3 (D) + (I) and DG (E) + (J). Blue and green (+) in stratum oriens, (*) in stratum pyramidale, (\$) in stratum radiatum, (#) in stratum moleculare indicates significant time-of-day-dependent differences in CA1 and CA3, respectively ($p < 0.05$). Red (+) in Stratum granulare, (*) in Stratum polymorphe, (\$) in Stratum moleculare, (#) in subgranular zone indicates significant time-of-day-dependent differences in DG ($p < 0.05$). n = 18 mice, 3 mice per each time point. Scale bar = 200μm.

4.3 Effect of Constant Light (LL) on Astrocytic Gap Junction Rhythms in the Mouse Hippocampal Layers

Under LL, the Cx30-Ir in CA1 stratum oriens and stratum moleculare showed a significant greater level during the late former night phase compared to the former day phase, while CA1 stratum pyramidale and stratum radiatum did not show any time-of-day-dependent significant difference (Figure 7A, B, C).

The Cx30-Ir did not show any significant time-of-day-dependent differences in any of the CA3 subregions (Figure 7D).

In DG stratum polymorphe and stratum moleculare, the Cx30-Ir was higher during the late former night phase compared to the former day phase. Stratum granulare and subgranular zone did not show any significant time-of-day-dependent differences in Cx30-Ir (Figure 7E).

In CA1 stratum oriens and stratum moleculare, the Cx43-Ir was significantly higher in the former night phase compared to the former day phase, while for the Cx43-Ir in stratum pyramidale and stratum radiatum, no significant differences could be observed (Figure 7F, G, H). In CA3 stratum pyramidale and stratum moleculare, the Cx43-Ir showed an increase in the former night phase. In addition, the Cx43-Ir in stratum moleculare showed another peak in the mid former day. The Cx43-Ir in Stratum oriens and Stratum radiatum of CA3 did not show any significant differences between the investigated time points (Figure 7I). In stratum granulare, subgranular zone and stratum polymorphe of DG, the Cx43-Ir was significantly greater in the former night phase compared to the former day phase, while in Stratum moleculare no significant time-of-day-dependent variation of Cx43-Ir was observed (Figure 7J).

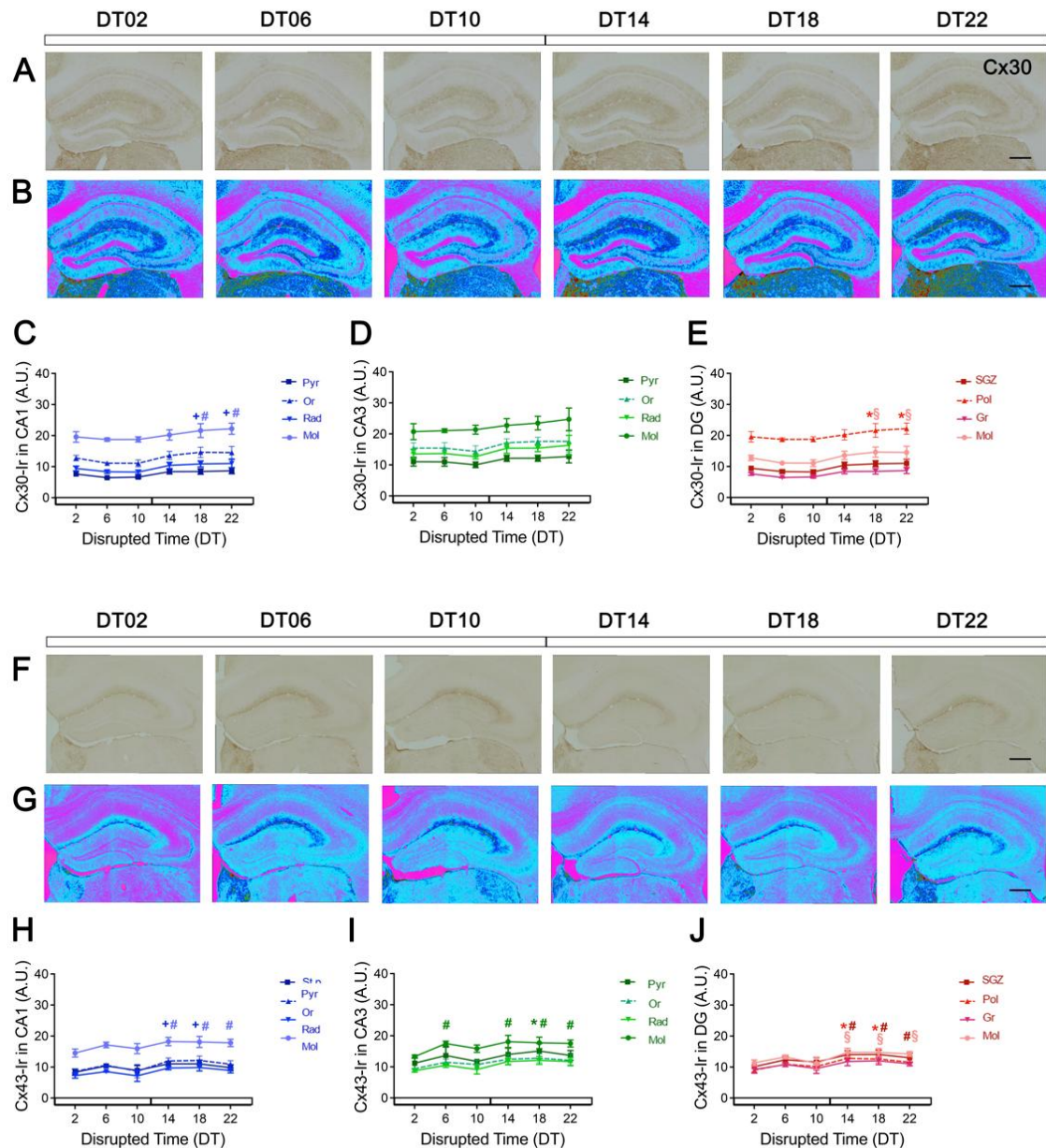


Fig. 7: Effect of constant light (LL) on astrocytic gap junction rhythms in the mouse hippocampal layers. (A) and (F) representative microphotographs showing the immunoreaction of Cx30 (A) and Cx43 (F) in mouse hippocampus under LL. The white bar indicates light phase. (B) and (G) representative pseudocolor images of Cx30 (B) and Cx43 (G) for qualitative analysis with different colors depending on the level of immunoreaction (yellow = absent, magenta = very weak, light blue = weak, dark blue = moderate, green = strong, red = very strong). Quantification of Cx30 and Cx43 immunoreaction (Ir) as arbitrary units (A.U.) of grey values above background in CA1 (C) + (H), CA3 (D) + (I) and DG (E) + (J). Blue and green (+) in stratum oriens, (*) in stratum pyramidale, (§) in stratum radiatum, (#) in stratum moleculare indicates significant time-of-day-dependent differences in CA1 and CA3, respectively ($p < 0.05$). Red (+) in Stratum granulare, (*) in Stratum polymorphe, (§) in Stratum moleculare, (#) in subgranular zone indicates significant time-of-day-dependent differences in DG ($p < 0.05$). $n = 18$ mice, 3 mice per each time point. Scale bar = 200 μ m.

5 Discussion

In this study, we provide the first detailed evidence for spatial heterogeneity of the main astrocytic gap junction proteins Cx30 and Cx43, as well as astrocytic morphology, among the subregions of the mouse hippocampus. Our findings indicate that the abundance of the astrocytic gap junction proteins Cx30 and Cx43 correlates with the density of GFAP-positive (GFAP⁺) astrocytes, and that increased astrocyte density in hippocampal subregions and layers is associated with a reduced radial spread of GFAP⁺ processes. In addition, we found that Cx30 and Cx43 show layer-specific time-of-day variations in the mouse hippocampus, with generally higher immunoreactivity during the light and early dark phase under standard light-dark conditions. Under constant darkness, partly endogenous rhythmic peaks during the subjective night persist in some layers, whereas constant light attenuates these temporal patterns.

5.1 Heterogeneity of Astrocytic Density, Morphology and Connexins in the Mouse Hippocampus

Astrocytes represent a heterogeneous population of cells that vary in functional and morphological properties across brain subregions (Matyash & Kettenmann, 2010). Rather than serving solely as passive support cells, astrocytes actively participate in synaptic transmission, neuronal metabolism, and network homeostasis (Xin & Bonci, 2018). This functional diversity is particularly evident in laminated brain structures such as the hippocampus, where neuronal density, synaptic organization, and activity patterns differ markedly between subregions and layers.

In the present study, we provide detailed evidence for a pronounced spatial heterogeneity of the major astrocytic gap junction proteins Cx30 and Cx43, as well as astrocytic morphology, across hippocampal subregions and layers in the mouse. Our findings extend previous observations of region-dependent astrocytic diversity and suggest that astrocytic properties are tightly linked to the cellular and functional organization of hippocampal circuitry.

5.1.1 Astrocytic Gap Junction Networks

Astrocytes form extensive intercellular glial networks through gap junctions, which allow the diffusion of ions, second messengers, and metabolites between neighboring cells. Gap junctions enable astrocytes to actively shape the neurotransmission and the neuronal activity, for example via regulation of energy substrate delivery to neurons or spatial

buffering of extracellular potassium (Giaume & Theis, 2010; Xin & Bonci, 2018). Among the different connexins expressed in the brain, Cx30 and Cx43 represent the predominant astrocytic gap junction proteins.

5.1.2 Regional and Layer-Specific Distribution of Astrocytic Connexins in the Hippocampus

Previous studies have demonstrated that astrocytes display a heterogeneity of gap junction and astrocytic coupling across brain regions. For instance, astrocytes isolated from rat hypothalamus contain four-fold greater expression of Cx43 and functional intercellular channels, compared to striatal astrocytes (Batter et al., 1992). Moreover, the expression of the gap junction alpha-1 gene (*Gja1*), encoding Cx43, is higher in the hypothalamus and lower in the cerebellum than in the cerebral cortex and even varies among cortical sublayers (Boisvert et al., 2018; Nadarajah et al., 1996). Further, different degrees of astrocytic coupling between CA1 and CA3 within the rat hippocampus, as well as across the layers in DG, have been reported (D'Ambrosio et al., 1998; Karpf et al., 2022). Houades et al. also demonstrated the region-specific differences in Cx43-expression in juvenile brains. Moreover, Cx43 appears to be the predominant Cx in juvenile brains, whereas Cx30 is more present in mature astrocytes (Houades et al., 2006; Kunzelmann et al., 1999). Interestingly, although their expression patterns overlap, the functions of Cx30 and Cx43 may differ among brain regions (Griemsmann et al., 2015; Kunzelmann et al., 1999).

In line with these observations, we observed a heterogeneity of Cx30- and Cx43-Ir among the hippocampal layers. Both connexins showed the highest abundance in Mol of CA1, CA3 and DG and the lowest in the layers containing neuronal cell bodies including Pyr of CA1 and CA3, as well as Gr of DG. The heterogeneous distribution of Cx30 and Cx43 observed in the present study likely reflects region-specific functional requirements.

5.1.3 Connexin Expression and Synapse Type in Hippocampal Subregions

Astrocytes play a critical role in neuronal communication and synaptic plasticity. In the CA1 region, Pyr contains the highest density of excitatory synapses, followed by Rad and Or, whereas the SLM exhibits a higher proportion of inhibitory synapses (Montero-Crespo et al., 2020). Mol and SLM are relatively cell-free layers and are mainly composed of dendrites of granule neurons in DG and pyramidal neurons in CA1 and CA3, respectively, where they establish numerous excitatory synapses with entorhinal afferents. Thus, extensive astrocytic networks may be required for regulation of the glutamatergic

neurotransmission extant in this area. In our study, both Cx30 and Cx43 immunoreactivity, as well as astrocytic density, were highest in SLM, suggesting a potential correlation with inhibitory synapses. However, the literature on this topic is inconclusive with some studies reporting opposite trends (Hardy et al., 2021; Pannasch et al., 2014). For instance, Pannasch et al. proposed that connexins may be more involved in the regulation of excitatory synapses (Pannasch et al., 2014). These findings suggest that the role of connexins may differ between regions, depending on the specific functions of astrocytes in each part of the hippocampus.

5.1.4 Connexin Expression in Astrocytes Near the Hippocampal Vasculature

Previous studies have reported higher concentrations of Cx30 and Cx43 in perivascular regions, which may explain their stronger immunoreactivity observed in the Mol surrounding the hippocampal fissure, where the main hippocampal vasculature is located (Johnson, 2023; Kunzelmann et al., 1999). Our results further show that astrocytes in SLM possess the shortest processes and the lowest radial extent, suggesting that astrocytes in close contact to capillaries tend to exhibit shorter processes. Immunoreactivity of Cx30 and Cx43 was also highest in SLM of CA1 and CA3, indicating a potential higher density of synaptic interactions between astrocytes and neurons, as well as among astrocytes themselves. Together, these findings support the hypothesis that connexins play an important role in the formation and maintenance of the blood-brain barrier and may contribute to the regulation of glucose and lactate uptake required for neuronal metabolism.

5.1.5 Connexin Expression in SGZ and Its Potential Role in Adult Hippocampal Neurogenesis

Our data show a high density of GFAP⁺-astrocytes and a strong immunoreactivity for Cx30 and Cx43 in SGZ of DG. In addition to astrocytes, the SGZ contains radial glial-like neural stem cells that also express GFAP and play a fundamental role in adult neurogenesis. These cells proliferate and generate progenitor cells that subsequently differentiate into granule neurons (Ming & Song, 2011). Interestingly, Cx30/Cx43 double-knockout (DKO) mice exhibit reduced proliferation of radial glia-like neural stem cells in SGZ, resulting in a decreased number of granule neurons (Kunze et al., 2009). These findings highlight the contribution of connexins to adult neurogenesis in the hippocampus.

The SGZ is also the layer of DG with the highest vascular density (Sun et al., 2015). Blood vessels in this region supply oxygen and nutrients to resident cells and appear to regulate adult neurogenesis through the release of hormones and neurotrophic factors (Karakatsani et al., 2023). Astrocytes, as mentioned above, are essential for maintaining the blood-brain barrier and actively regulate blood flow, vascular stability and angiogenesis. This suggests that astrocytes play a crucial role in maintaining the neurogenic niche within the SGZ.

5.1.6 Morphological Heterogeneity of Astrocytes Across Hippocampal Layers

Beyond molecular differences, we also observed a morphological heterogeneity of astrocytes across hippocampal layers. Astrocytes located in Rad and Pyr of CA1 and CA3 showed a more complex branching pattern compared to those in Or or Mol of CA1 and CA3.

These observations are consistent with previous studies demonstrating layer-specific astrocytic morphologies within the hippocampus, with the most complex arbor found in the Rad of CA1 and displaying layer-specific features within the DG (Karpf et al., 2022; Viana et al., 2023). Similar principles have been described in the neocortex, where astrocytic morphology, such as arborization, cell orientation, territorial volume, and glial synaptic ensheathment, depends on neuronal layering and synaptic density (Lanjakornsiripan et al., 2018). Astrocytes seem to be more complex in layers that are rich in synaptic contacts. It is well known that astrocytes with their processes ensheath synapses in order to regulate neurotransmitter uptake, maintain ion homeostasis and enable synaptic plasticity. This could lead to the assumption that astrocytic morphology is influenced by local synaptic and neuronal activity levels.

Thus, the morphological heterogeneity may be related to the neuronal organization, evolutionary spatial restrictions, as well as degree of structural interactions with synapses in addition to synaptic density and neuronal activity levels (Viana et al., 2023). Taken together, the heterogeneity of astrocytic connexins immunoreactivity and morphology may be correlated with differences in the cellular and functional organization among hippocampal layers, however, further research is needed to elucidate the region-specific structure-function relationship.

5.1.7 Role of GFAP in Astrocytic Structural Organization

In the present study, we used GFAP as a marker to identify astrocytes. As an intermediate filament protein, GFAP is not ideally suited to visualize the fine astrocytic processes that

directly interact with synapses. Nevertheless, it can be used to represent the main structural framework of astrocytic processes and therefore it serves as a useful marker to describe the overall morphology of astrocytes (Viana et al., 2023). GFAP contributes to cytoskeletal organization and may support the intracellular transport of organelles such as mitochondria and the endoplasmic reticulum (Creighton et al., 2019; Jones et al., 2018). In our analysis, astrocytes located in the Rad and Pyr of CA1 and CA3 showed the most complex arborization patterns. These layers also contain a high density of excitatory synapses. This observation suggests that the increased structural complexity of astrocytes in these regions may support the transport and distribution of organelles required to maintain cellular and synaptic homeostasis.

5.1.8 Correlation Between Astrocyte Density, Connexin Expression, and Astrocytic Morphology

Our results show a positive correlation between the density of GFAP⁺ astrocytes and the immunoreactivity of Cx30 and Cx43 across hippocampal layers. In regions with higher astrocyte density, the connexin immunoreactivity was also greater. This could lead to the assumption that the size and the connectivity of the astrocytic network grow with the cell density.

Interestingly, increased astrocyte density was associated with a reduced radial spread of GFAP⁺ processes. In this context, the concept of "domain" could be useful for understanding this correlation. It describes the fact that each astrocyte establishes contact with neighboring cells via its processes within its own "territory", which overlaps only minimally with the territory of adjacent astrocytes (Ogata & Kosaka, 2002; Refaeli et al., 2021). In regions with high astrocyte density, individual astrocytes occupy smaller territories, resulting in a more compact morphology with reduced radial extent. Refaeli et al. have already demonstrated that the size of the domains differs across the individual layers of CA1. Our findings on the different astrocyte morphology in the layers of CA1, as well as in CA3 and the DG, further support the notion that astrocyte organization is closely linked to the neuronal architecture.

5.1.9 Astrocytic Connexins and Regional Vulnerability

Cx30 and Cx43 as the main astrocytic connexins play a fundamental role in the function of astrocytes and neurons.

Mice with an astrocyte-specific deletion of Cx43 combined with a global loss of Cx30 develop a widespread pathology of the white matter. In contrast, the gray matter is comparatively less affected. Notably, within the hippocampus, astrocytes in the CA1

region exhibit pronounced cellular edema, leading to a vacuolation of CA1 region (Lutz et al., 2009). This regional vulnerability highlights the critical role of astrocytic coupling for maintaining tissue integrity, particularly in CA1.

Some diseases also show the region-specific response in changes in the hippocampus: Nairuz et al. investigated astrocytic responses in 5xFAD mice, a mouse model with five distinct human mutations, specific for Alzheimer's diseases. They demonstrated that different hippocampal subregions exhibit different astrocytic responses and neuronal activity patterns in association with beta-amyloid plaque deposition. Especially CA1 and CA3 appeared highly vulnerable to amyloid plaque deposition, as evidenced by increased astrocyte density and elevated GFAP expression as markers for pronounced reactive astrogliosis (Nairuz et al., 2024).

These findings show that astrocytic heterogeneity is not only a characteristic of physiological organization but also plays a critical role in region-specific pathological responses.

5.1.10 Implications for Epilepsy and Network Excitability

Although astrocytes, through their homeostatic functions such as potassium buffering and glutamate clearance, provide a certain degree of protection against epileptogenic signals, their interconnection through gap junctions appears to play a critical role in the propagation of pathological excitatory activity under certain circumstances. In particular, the hippocampus appears to be a highly vulnerable region in the context of epilepsy (Coulter & Steinhauser, 2015; Onodera et al., 2021; Tian et al., 2005; Wallraff et al., 2006). The layer- and region-specific differences in connexin expression observed in this study may therefore explain the differential susceptibility to epileptic activity within hippocampal subregions.

5.2 Circadian and Time-of-Day-Dependent Heterogeneity of Connexins

5.2.1 Circadian Regulation of Astrocytic Functions

Astrocytes rhythmically express circadian clock genes, including *Bmal1* (Barca-Mayo et al., 2017), *Per1-2* (Prolo et al., 2005), *Clock*, and *Cry1-2* (Marpegan et al., 2011), which regulate the rhythmic astrocytic functions such as ATP release (Marpegan et al., 2011). Furthermore, the astrocytic markers GFAP (Lavialle et al., 2001; Lavialle & Serviere, 1993) and Ezrin (Lavialle et al., 2011) exhibit a time-of-day-dependent oscillation in the

rodent SCN. Our earlier study showed fluctuation of astrocytic gap junctions Cx30 and Cx43 in mouse SCN, with a peak during the light and early dark phase, indicating that their expression may be driven by the light-dark cycle (Ali et al., 2019). Interestingly, Bmal1 regulates the Cx43-glutamate signaling in astrocytes (Xue et al., 2022), which indicates that gap junctions can be regulated directly by the molecular clock. On the other hand, the autonomous astrocytic clock is a key player in circadian regulation of rhythmic locomotor, cognitive functions and sleep/wake cycles (Hastings et al., 2023). Besides, mice with double deletion of Cx30 and Cx43 in astrocytes (DKO), although being able to entrain their spontaneous locomotor activity to a regular light-dark cycle, their re-entrainment after jet lag was affected, indicating that astrocytic gap junctions play a role in the circadian functions (Ali et al., 2019). In sum, these data suggest a bidirectional relationship between the astrocyte networks and the circadian system. In agreement with this notion, we observed time-of-day-dependent changes in the Cx30 and Cx43 in mouse hippocampus, consistent with our previous findings in SCN (Ali et al., 2019), in most of DG, CA1 and CA3 layers and particularly within the molecular layer.

5.2.2 Daily Fluctuations of Connexins in Different Tissues

Importantly, the daily fluctuation across the day has been observed in connexins such as Cx40 and Cx43 in mouse heart (Tong & Cui, 2016), Cx43 in the urinary bladder (Negoro et al., 2012), as well as Cx36 in the retina (Katti et al., 2013), suggesting a common mechanism of regulation, probably the phosphorylation state, which is modulated by the circadian system (Zhang et al., 2023; Zhang et al., 2015).

5.2.3 Light-Dependent Input to the Hippocampus

Although the hippocampus is traditionally considered for its cognitive function, it plays a role in the visual system (Turk-Browne, 2019). The hippocampus receives light information from the retina via different pathways, including direct input to CA1 pyramidal layer (Lo & Anderson, 2011) and indirect input via SCN-dependent (Morin et al., 1994; Watts et al., 1987), or SCN-independent pathways (Cui et al., 2013; Fernandez et al., 2018). Interestingly, the SCN regulates GABA signaling to the septum, which, in turn, modulates septal cholinergic input to the hippocampus (Ruby et al., 2008). Other inputs from the visual cortex deliver the visual information to the hippocampus (Furtak et al., 2007). In addition, evidence shows that light modulates the hippocampal synaptic strength (Zheng et al., 2020) and affects mood as well as cognitive functions in mice (Fernandez et al., 2018) and in human (Castilla, 2023; Samani, 2011) via regulation of

brain-derived neurotrophic factor (BDNF) levels (Soler et al., 2018) and p21-activated kinase 1 (PAK1) (Shan et al., 2015).

5.2.4 Molecular Mechanisms Regulating Astrocytic Connexins

The underlying mechanism of modulating the astrocytic connexins by light is still questionable. Importantly, there is a link between the astrocytic glutamate transporter-1 and glutamine synthetase (GS), which are enhanced during the daytime (Leone et al., 2015), and Cx43 in hippocampus (Li et al., 2018). Furthermore, the mitogen-activated protein kinase (MAPK), a crucial modulator of hippocampal neuronal activity and synaptic plasticity, shows diurnal and circadian oscillation (Eckel-Mahan, 2012). MAPK regulates Cx43 phosphorylation (Nimlamool et al., 2015), which may be correlated with increased Cx43 hemichannels (Giaume et al., 2013; Zhang et al., 2023).

5.2.5 Astrocyte-Neuron Interaction and Purinergic Signaling

Time-of-day changes of neuronal activity within hippocampus have been reported. For instance, hippocampal neurons show higher LTP (Chaudhury et al., 2005) and reduced surface expression of NMDA receptors during the dark phase (McCauley et al., 2020). In addition, a previous study showed a diurnal variation in AMPA receptor, Glutamate receptor 1, and in synaptopodin, which regulates the structural synaptic plasticity (Schröder et al., 2023). These alterations of hippocampal neuronal activity may be correlated with fluctuation of astrocytic function. On the other hand, the connexins hemichannels regulate the astrocyte-mediated adenosine triphosphate (ATP) release and, thus, modulate the synaptic transmission to match energy demand (Dale et al., 2023; Lezmy, 2023), suggesting bidirectional regulation between astrocytes and neuronal activity. Interestingly, the extracellular ATP concentration is negatively correlated to the neuronal activity (Lezmy, 2023). Consistently, extracellular ATP shows diurnal changes in rat hippocampus that peaks during the light phase (Dworak et al., 2010). We also showed earlier that the purinergic P2 receptors, which are predominantly activated by ATP and its analogues, show spatial and temporal heterogeneity in the hippocampal sublayers (Lommen et al., 2021).

5.2.6 Possible Role of Corticosterone

Another potential mechanism that may account for temporal oscillation of Cx30 and Cx43 may be related to the fluctuation of corticosterone levels. Corticosterone is high during the early dark/activity phase and induces a modulatory effect on the hippocampal LTP (McCauley et al., 2020) and thus may indirectly affect the astrocytic gap junctions.

Moreover, the activity of hippocampal Cx43 may be directly influenced by corticosterone (Birnie et al., 2023; Quesseveur et al., 2015).

5.2.7 Effects of Constant Light Conditions

Next, we examined the effect of constant conditions on spatial heterogeneity of astrocytic gap junctions in the mouse hippocampal layers. Under constant conditions, we found that the heterogeneity of Cx30- and Cx43-Ir across the layers was not abolished, although Cx43 showed decreased heterogeneity across DG layers under constant light. This may indicate a region-specific effect of chronodisruption on astrocytic gap junctions.

Importantly, exposure to constant conditions mainly reduced the overall Cx30- and Cx43-Ir, with constant light having a stronger effect, reminiscent of previous observations in SCN (Ali et al., 2019). The Mol was strongly affected by constant light in all hippocampal subregions, suggesting its higher vulnerability to chronodisruption. The Cx43-Ir in DG was affected by both constant conditions. Ketelaoui et al. showed that exposure to LL for two weeks leads to mild glial activation and increased cleaved caspase 1, suggesting an assembly of “ready to start” inflammasome that turns the brain more vulnerable to other negative stimuli (Ketelaoui et al., 2023).

Under constant conditions, the time-of-day-dependent changes of Cx30 and Cx43 were damped in CA1 and DG, while nearly abolished in CA3. Under DD, the highest levels of Cx30 and Cx43-Ir were observed during the early subjective dark phase, suggesting a correlation between astrocytic gap junctions in CA1 and DG and the beginning of locomotor activity during this phase in mice. Under LL, the rhythm was more attenuated with the highest levels of Cx30 and Cx43-Ir shifted to the late subjective dark phase, indicating that chronodisruption affects the rhythmic expression of glial connexins. Previous data from SCN showed that under constant conditions, the fluctuation of Cx30 was entirely degraded, indicating that it is strongly dependent on the light-dark cycle, whereas fluctuation of Cx43 only persisted in constant darkness (Ali et al., 2019). This may point to regulation of Cx30 and Cx43 by light, while Cx43 is regulated by the circadian clock in the SCN, whereas both Cx30 and Cx43 are regulated by light and circadian clock in the hippocampus.

5.3 Conclusion

This study provides the first detailed evidence for spatial heterogeneity of the astrocytic gap junction proteins Cx30 and Cx43, as well as astrocyte morphology, across hippocampal subregions and layers, showing that higher astrocyte density correlates with increased connexin expression but reduced radial process spread. Cx30 and Cx43 also exhibit time-of-day-dependent fluctuations driven by both light-dark cycles and circadian mechanisms. These findings indicate that hippocampal connexins are subject to endogenous rhythmicity that is modulated by light. Chronodisruption, such as constant light or darkness, dampens connexin expression, highlighting the importance of light and circadian cues for regulating astrocytic networks and their potential role in neuron-glia interactions.

This study provides a detailed comparative mapping of the spatial and temporal patterns of astrocytic gap junctions, along with the spatial organization of astrocytic morphology across hippocampal subregions and layers, and examines the effect of chronodisruption on connexin expression. This could yield an important basis for further studies to elucidate the dynamics of structure-function relationships in context of neuron-glia interaction for modulating synaptic plasticity via astrocytes.

5.4 Limitations

Two limitations of our study should be acknowledged. First, GFAP is just one possible marker for astrocytes. It is predominantly expressed in reactive astrocytes and therefore may underestimate the astrocytes and their arborization under physiological conditions. Further studies using other astrocytic markers such as S100B are needed.

Second, connexin immunoreactivity does not necessarily reflect functional gap junction coupling. Functional assays, such as dye coupling, would be required to directly demonstrate intercellular connectivity.

5.5 Outlook

In summary, the present study demonstrates that astrocytes in the hippocampus show heterogeneity in connexin expression, density, and morphology across subregions and layers. These differences correlate at least partially with the functional organization of hippocampal circuits. Our findings support the concept that astrocytes are not solely supportive cells, but highly structured, region-specific partners in neuronal networks.

Understanding astrocytic heterogeneity and its possible impact on neurons and their function is essential for understanding both physiological brain function and the mechanisms underlying neurological diseases. Future studies integrating molecular, morphological, and functional approaches will be necessary to improve our understanding of the structure–function relationships of astrocytic networks in the hippocampus. In addition, it would be of interest to investigate the astrocyte density, connexins, and morphology in younger mice to determine whether the findings described here are an effect of synaptic plasticity.

6 References

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Danksagung

Mein größter Dank gilt meiner Doktormutter, Frau Prof. Charlotte von Gall, mein Promotionsvorhaben an ihrem Institut durchführen zu dürfen. Sowohl ihre Vorlesungen in der Neuroanatomie als auch ihre Tätigkeit als Tischdozentin haben meine Begeisterung für die Anatomie maßgeblich geprägt. Durch meine Arbeit als Studentische Hilfskraft und schließlich als Doktorandin erhielt ich die Möglichkeit, wissenschaftliches Arbeiten zu erlernen und weiterzuentwickeln.

Ohne die Unterstützung von Frau Dr. Amira Ali wäre dieses Projekt nicht möglich gewesen. Sowohl bei fachlichen Fragen rund um das Promotionsvorhaben als auch bei persönlichen Anliegen hatte sie stets ein offenes Ohr und stand mir jederzeit mit wertvollen Ratschlägen zur Seite.

Mein herzlicher Dank gilt zudem dem gesamten labortechnischen Team, insbesondere Angelika Hallenberger, Ulla Lammersen, Hanna Bellert und Ralf Faßbender, für die geduldige Anleitung sowie die tatkräftige Unterstützung während der Durchführung der Experimente.

Ebenso danke ich den wissenschaftlichen Mitarbeiterinnen und Mitarbeitern des Instituts für den stetigen fachlichen Austausch, das konstruktive Feedback und die zahlreichen hilfreichen Anregungen.

Darüber hinaus gilt mein besonderer Dank meinen Eltern Norbert und Petra Uelwer, meinem Bruder Dr. Tobias Uelwer sowie meinem Freund Malte Roeling, die geduldig auf den Abschluss dieser Arbeit gewartet und mich in meinem bisherigen Leben stets unterstützt haben. Ohne euch wäre vieles nicht möglich gewesen.