Thyroid hormone modulation of early neocortical network development

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Gutachter: Prof. Dr. Thomas Voigt

Prof. Dr. Eckhard Friauf

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„Ich vermute, dass es vor allem für Laien schwierig ist,
sich vorzustellen, dass beim Erreichen oder
Nachvollziehen naturwissenschaftlicher Einsichten
auch von Genuss die Rede sein kann.“

Ernst Peter Fischer

Table of contents

Abbreviations

aCSF	artificial cerebrospinal fluid
APV	D-(-)-2-amino-5-phosphonopentanoic acid
Ara-C	Cytosine arabinoside
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BMI	(-)-bicuculline methiodide
$[Ca^{2+}]_i$	intracellular calcium concentration
CGE	caudal ganglionic eminence
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione disodium
CNS	central nervous system
Cort	corticosterone
DIV	days *in vitro*
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GABA	γ-aminobutyric butyric acid
$GABA_AR$	GABA type A receptor
GluR	glutamate receptor
HBSS	Hank´s balanced salt solution
KCC2	K^+-Cl^--cotransporter 2
NGF	nerve growth factor
NKCC	Na^+-K^+-Cl^--cotransporter
L-GABA	large GABAergic neurons
LR	linear regression
RT	room temperature
MGE	medial ganglionic eminence
MW-RST	Mann-Whitney Rank Sum Test
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
PTX	Picrotoxin
ROI	region of interest
SEM	standard error of the mean
S-GABA	small GABAergic neurons
T3	triiodothyronine
T4	thyroxine
TR	nuclear thyroid hormone receptor
TrkB	tropomyosin-related kinase B
TTX	Tetrodotoxin
VIP	vasoactive intestinal peptide

1. Abstracts

1.1 Abstract

GABAergic signalling plays a crucial role in the precise control of neuronal activity patterns. The early development of GABAergic neurons is regulated by intrinsic and extrinsic factors, including the thyroid hormone triiodothyronine (T3). Furthermore, GABAergic neurons develop in an intricate interrelationship with spontaneous network activity, which is a general feature of developing neuronal structures and plays a crucial role in the formation of neural networks by modulating survival and connectivity of neurons. The aim of this work was to study the impact of thyroid hormone T3 on early network development, especially on the development of GABAergic neurons and spontaneous network activity, in neuronal cultures prepared from embryonic rat cortex. The results show that T3 increases the overall density of glutamatergic and GABAergic synapses and enhances the frequency of early spontaneous network activity. In parallel, T3 affects neuronal development in a population specific manner, especially promoting somatic and axonal growth of large GABAergic interneurons and the density of small late born GABAergic neurons. In the presence of T3 large GABAergic interneurons form numerous GABAergic boutons and conspicuous axonal arborizations around cell bodies of non-GABAergic neurons. Interestingly, blocking neuronal activity by TTX or glutamate receptor blockers reveals a thyroid hormone-mediated component on somatic growth, but optimal growth of large GABAergic neurons is both activity and hormone-dependent. Additionally, the characteristic axonal innervation pattern could be prevented by the tropomyosin-related kinase B (TrkB) receptor inhibitor K252a, suggesting the involvement of brain-derived neurotrophic factor (BDNF) signalling in T3-dependent axonal growth. Further, T3 enhanced the expression of the potassium-chloride cotransporter 2 (KCC2), and accelerated the developmental shift from depolarizing to hyperpolarizing GABAergic signalling in non-GABAergic neurons. Finally, recovery experiments indicate that T3-deprivation during the first two weeks *in vitro* induced long-lasting deficits in the patterning of GABAergic axons, the density of small late born GABAergic neurons and the frequency of spontaneous network activity. This critical period parallels the phase of depolarizing GABAergic signalling as revealed by calcium imaging experiments. Taken together, the results indicate that T3 regulates the maturation of the cortical GABAergic system in a complex and subpopulation specific manner and thus contributes to the functional maturation of early cortical networks.

1.2 Zusammenfassung

Einleitung. GABAerge Interneurone sind ein wichtiger Bestandteil des cerebralen Cortex, da sie durch ihre hemmende Wirkung die Aktivität von erregenden Neuronen in präziser Weise kontrollieren können. Infolgedessen führen Störungen der Entwicklung und Funktion dieser GABAergen Neurone zu einer Reihe von neurologischen und psychiatrischen Störungen, wie z.B. Autismus, Epilepsie und Schizophrenie. Die Entwicklung von GABAergen Interneuronen wird sowohl durch intrinsische wie auch extrinsischen Faktoren beeinflusst. Ein wichtiger intrinsischer Faktor für die Ausbildung von neuronalen Netzwerken ist die sogenannte spontane Netzwerkaktivität, die während der frühen Netzwerkentwicklung das Überleben und die Verknüpfung von Neuronen steuert. Das Aufkommen spontaner Aktivität ist eine generelle Eigenschaft sich entwickelnder neuronaler Strukturen und ist gekennzeichnet durch synchrone Aktivität in einer großen Zahl von benachbarten Neuronen. Besonders interessant ist in diesem Zusammenhang, dass GABAerge Neurone und die Netzwerkaktivität sich während der Entwicklung gegenseitig beeinflussen, da die Reifung GABAerge Zellen einerseits aktivitäts-abhängig ist, andererseits GABAerge Transmission die Netzwerkaktivität durch eine vorübergehende erregende Wirkung auch fördert. Ein wichtiger extrinsischer Faktor für die Entwicklung GABAerger Interneurone ist das Schilddrüsenhormon Trijodthyronin (T3). Generell ist T3 ein wichtiger Entwicklungsfaktor für eine Vielzahl von Organen einschließlich des Gehirns. Dort steuert es die Migration und Differenzierung von Neuronen und deren Synaptogenese. Ein Mangel an T3 führt zu mentaler Retardierung und kann besonders während der späten embryonalen und frühen postnatalen Phase irreversible Schädigungen verursachen.

Ziel und Methoden. Das Ziel der vorliegenden Arbeit war es, die Einflüsse von T3 während der frühen Entwicklung von neuronalen Netzwerken zu untersuchen. Ein besonderes Augenmerk wurde dabei auf die voneinander abhängige Entwicklung von GABAergen Neuronen und spontaner Netzwerkaktivität gelegt. Die Ergebnisse sollen das grundlegende Verständnis der zugrunde liegenden Mechanismen verbessern, die zur Entwicklung von GABAergen Neuronen und damit der funktionellen Reifung des Cortexes beitragen. Die Versuche wurden an neuronalen Zellkulturen durchgeführt, die aus dem embryonalen Cortex von Ratten gewonnen wurden. Die Aktivität der Neurone wurde mit einem Calcium-sensitiven Farbstoff gemessen. Des Weiteren wurden die Zellkulturen immunzytochemisch gefärbt, um GABAerge Neurone sichtbar zu machen.

Ergebnisse. Die Ergebnisse zeigen, dass T3 sowohl die Frequenz der spontanen Netzwerkaktivität als auch die Zahl der glutamatergen und GABAergen Synapsen erhöht. Gleichzeitig beeinflusst T3 die Entwicklung von Neuronen in Zelltyp-spezifischer Weise, wobei die deutlichsten Veränderungen in der Zunahme in der Zellgröße und des Axonwachstums von sogenannten großen L-GABAergen Interneuronen und in der erhöhten Dichte von kleinen S-GABAergen Interneuronen gemessen wurde. Die L-GABAergen Neurone bilden nach Stimulation mit T3 eine Vielzahl von GABAergen Boutons und weitverzweigte Axone um die Zellkörper von nicht-GABAergen Neuronen aus. Interessanterweise erhöhte T3 auch die Zellgrößen wenn die neuronale Aktivität blockiert wurde, was einen direkten Einfluss von T3 auf das Neuronwachstum vermuten lässt. Allerdings wuchsen die GABAergen Zellen nur dann optimal, wenn sowohl das Hormon als auch neuronale Aktivität vorhanden waren. Die Ausbildung der typische Axonstruktur mit vielen Boutons und feinen Verästelungen durch T3 konnte außerdem mit einem Inhibitor des Neurotrophinrezeptors TrkB verhindert werden, was vermuten lässt, dass das Neurotrophin BDNF eine Rolle bei der Ausprägung der von T3 induzierten Axonstruktur spielt. Darüber hinaus erhöhte T3 die Expression des Kalium-Chlorid-Cotransporters KCC2, der für die hemmende Wirkung von GABA wichtig ist, und beschleunigte damit die für die Entwicklung typische Umkehrung von einer erregenden zu einer hemmenden Wirkung von GABAerger Transmission in nicht-GABAergen Neuronen. Abschließende Experimente haben gezeigt, dass die von T3-Mangel verursachten Entwicklungsstörungen nicht durch eine spätere Stimulation mit T3 nach der zweiten Kulturwoche korrigiert werden können, was darauf hinweist, dass T3-Mangel während der ersten zwei Kulturwochen dauerhafte oder zumindest lang anhaltende Defizite in der Axonausbildung L-GABAerger Neurone, der Dichte S-GABAerger Neurone und der Frequenz spontaner Netzwerkaktivität verursacht.

Diskussion. Zusammenfassend zeigen die Ergebnisse, dass T3 die Reifung des kortikalen GABAergen Systems auf komplexe und in einer Zelltyp-spezifischen Weise moduliert und damit zur funktionalen Reifung von jungen kortikalen Netzwerken beträgt. Besonders die Veränderungen der L-GABAergen Neurone könnten eine wichtige Rolle bei der Entstehung pathologischer Störungen bei T3-Mangel spielen. Da T3 die Netzwerkaktivität erhöht und diese wiederum die Reifung junger Netzwerke beeinflusst, könnte T3 hierdurch weitere indirekte Effekte auf Entwicklung von Neuronen ausüben. Die lang andauernden Veränderungen der Netzwerkstruktur verdeutlichen zudem die wichtige Bedeutung von T3 während der frühen Gehirnentwicklung.

2. Introduction

2.1 Network activity

2.1.1 Development of neuronal circuits

Proper function of the central nervous system (CNS) results from the establishment of specific neuronal networks with diverse neuronal phenotypes and precise synaptic connections between neurons during development. This network formation is achieved by a coordinated sequence of complex developmental events, which regulate neurogenesis, migration, differentiation and synaptogenesis (Zhang and Poo, 2001; Feller, 1999). The information for the organization of neural circuits relies on genetic programs and neuronal activity (Goodman and Shatz, 1993; Khazipov and Luhmann, 2006). Taking the presence and type of neuronal activity into account, roughly three phases can be distinguished in a simplified model of brain development: an early activity-independent phase, a phase characterized by the presence of spontaneous network activity and at least a phase of activity- and experience-dependent plasticity.

Thus, the initial developmental of neurons and the establishment of crude connectivity of neuronal networks, which precedes predominantly during embryonic development, are determined by genetic programs and occur independent of neuronal activity (Goodman and Shatz, 1993). During this phase the generation of neurons, the development of distinct neuronal phenotypes, the migration of neurons to a distinct position within a network, and the establishment of an initial set of connections take place (Blankenship and Feller, 2010). During the later activity-dependent phase, neural activity is thought to drive refinement of the initial crude connectivity (Goodman and Shatz, 1993; Katz and Shatz, 1996; Feller, 1999). As neurons start to form synaptic connections, neuronal circuits develop highly synchronized spontaneous network activity. In rats, this type of activity patterns emerges around birth and is most prominent during the first postnatal week (Ben-Ari et al, 2007). It is suggested that this early spontaneous activity is important for the initial wiring of neuronal structures and disappears upon maturation of sensory systems (Katz and Shatz, 1996). During further postnatal development the network is then modified in an experience-dependent manner (Khazipov and Luhmann, 2006). This final refinement of connections is set by experience-dependent activity from sensory stimuli. Thus, in contrast to intrinsic spontaneous activity the refinement is a result of interactions between the nervous system and the outside world.

2.1.2 Emergence of spontaneous network activity

Slow and synchronous spontaneous network activity is a characteristic feature of developing neuronal networks. It occurs transiently in many developing neural circuits, including the retina, the cochlea, the hypothalamus, the spinal cord, the cerebellum, the neocortex and the hippocampus, and is probably shared by all developing networks (Feller, 1999; O'Donovan, 1999; Ben-Ari, 2001; Ben-Ari et al, 2007; Blankenship and Feller, 2010). Interestingly, the patterns of spontaneous network activity and the mechanisms that generate it seem to be remarkably similar across various circuits (Blankenship and Feller, 2010). Spontaneous network activity emerges across large groups of neighbouring cells as neurons start to form synaptic connections and functional circuits develop. Early network activity is driven by glutamatergic and depolarizing GABAergic transmission and can be abolished by the sodium channel blocker tetrodotoxin (TTX) or by antagonists of chemical synaptic transmission (O´Donovan, 1999). In general, network activity involves correlated firing of large populations of neurons and consists of recurrent bursts of action potentials and concomitant increases of intracellular calcium concentrations, which last for up to hundreds of milliseconds and are separated by intervals up to a few minutes (Corlew et al., 2004; Moody and Bosma, 2005; Blankenship and Feller, 2010). Consequently, spontaneous activity can be examined by direct recording of the electrical activity or fluorescence imaging of the Ca^{2+} transients. As development proceeds, synchronous events with initially very low frequency develop to reach higher frequencies and maximal cellular participation, which includes nearly all neurons (Garaschuk et al., 2000; Opitz et al., 2002; Corlew et al., 2004).

2.1.3 Functions of network activity

Synchronous network activity plays an important role in the establishment of neuronal circuits and connections (Garaschuk et al., 2000; Voigt et al., 2005). As it emerges in a wide range of brain structures around birth, early network activity influences several developmental processes, which take place during early postnatal development, including neuronal migration and survival, differentiation, synaptogenesis, neurotransmitter specification and synaptic plasticity (Voigt et al., 1997; Voigt et al., 2005; Moody and Bosma, 2005; Khazipov and Luhmann, 2006). Especially the generation, migration and survival of GABAergic neurons might be affected by early network activity (de Lima et al., 2004, 2007, 2009). Electrical activity also induces changes in the global

properties of the neuron such as intrinsic excitability and patterns of gene expression and protein synthesis (Zhang and Poo, 2001) and thus network activity might also function as a synchronization factor during early development.

Furthermore, it is suggested that early spontaneous activity patterns guide the refinement of neural structures (Katz and Shatz, 1996). According to the Hebbian rule "neurons that fire together wire together", synchronous activity across large populations of cells is crucial for the proper wiring of developing neuronal networks (Cherubini et al., 2011). This activity-dependent but experience-independent form of synaptic plasticity might enable gross map formation, whereas map refinement takes place during the subsequent experience-dependent phases, e.g. 'critical periods' (Katz and Shatz, 1996; Hensch, 2004). Indeed, it has been shown that the emergence of spontaneous network activity promotes and synchronizes the activation of AMPA receptors and the formation of synaptic contacts (Liao et al., 2001; Voigt et al., 2005), enhances the survival of participating neurons (Voigt et al., 1997), and regulates the establishment of long-range corticocortical connections (Voigt et al., 2005). It is suggested that early spontaneous activity disappears upon maturation of sensory systems, when experience-dependent mechanisms further refine network connectivity (Katz and Shatz, 1996; Hensch, 2004).

2.1.4 Depolarizing GABA signalling during early development

γ-aminobutyric butyric acid (GABA) released by GABAergic interneurons is the main inhibitory neurotransmitter in the adult central nervous system (CNS) and exerts its fast actions by the activation of GABA type A receptors ($GABA_AR$). Because these receptors conduct Cl^- ions, the intracellular chloride concentration determines the strength and polarity of GABA-mediated neurotransmission (Ben-Ari et al., 2007; Kahle et al., 2008). In the presence of a high intracellular chloride concentration $GABA_A$ receptor activation results in chloride efflux and a depolarization of the cell membrane. In contrast, a low intracellular chloride concentration results in chloride influx and cell membrane hyperpolarisation. The intracellular chloride concentration of neurons in turn is regulated by the two cation chloride cotransporters sodium-potassium-chloride cotransporter 1 (NKCC1) and potassium-chloride cotransporter 2 (KCC2), which accumulate or extrude chloride respectively (Kahle et al., 2008; Blaesse et al., 2009). During early postnatal development a high expression of NKCC1 but low expression of KCC2 maintain a high intracellular chloride concentration and a depolarizing action of GABAergic signalling.

Later the developmental downregulation of NKCC1 and concomitant upregulation of KCC2 results in lower intracellular chloride concentration and a hyperpolarizing action of $GABA_A$ receptor opening (Fiumelli and Woodin, 2007).

Thus, the cells that will become inhibitory interneurons in adulthood are a primary source of depolarization during development (Ben-Ari et al, 2007; Blaesse et al., 2009). As GABAergic synapses are functionally active before glutamatergic ones, GABA is even the principal excitatory transmitter during early development that induces depolarization in the neonatal brain (Ben-Ari. 2001). Consequently, depolarizing GABA is crucial for the generation of spontaneous synchronous network activity in developing neuronal structures, as initially described in the developing hippocampus, and referred to as Giant Depolarizing Potentials (GDPs) (Ben-Ari et al, 1989). The depolarizing action of GABA and its contribution to spontaneous network activity have been confirmed in many neuronal structures (Blankenship and Feller, 2010) including developing cultured neuronal networks prepared from embryonic rat cortex (Voigt et al., 2001, 2005; Opitz et al., 2002; Baltz et al., 2010).

2.1.5 Network activity in cell culture

As in the neonatal cerebral cortex in vivo (Khazipov et al., 2004; Adelsberger et al., 2005) and acute slices (Garaschuk et al., 2000; Corlew et al., 2004; Allene et al, 2008), synchronized network activity also develops spontaneously in organotypic slice cultures (Gorba et al., 1999; McCabe et al., 2006; Johnson and Buanomano, 2007) and in networks formed by embryonic cortical neurons in culture (Muramoto et al., 1993; Opitz et al., 2002; Voigt et al., 2005). Interestingly, the mechanisms and the time course of activity development are quite similar to that observed in *in vivo* circuits.

In cultures from embryonic rat cortex network activity characterized by simultaneous transients of $[Ca^{2+}]_i$ involving many neurons first appeared at the end of the first week in vitro (Opitz et al., 2002). The slow rhythmic synchronous Ca^{2+} transients seen with imaging techniques reflect bursts of actions potentials recorded in single cells with the patch clamp technique (Opitz et al., 2002; Voigt et al., 2005). These spontaneous population events initially occur at a low frequency and are expressed only by a fraction of neurons. During the second week in culture the burst frequency increases and virtually all neurons become active and participate in synchronous network activity (Voigt et al., 1997; Opitz et al., 2002).

It has been shown that the synchronous calcium transients are dependent on depolarizing actions of GABAergic neurotransmission during the initial period of network activity between 8 and 12 DIV (Voigt et al., 2005). Consequently, the initiation of synchronous bursts can be blocked by GABA receptor antagonists until 12 DIV, but becomes independent from GABAergic signalling thereafter (Voigt et al., 2005; Klueva et al., 2008). During later development GABAergic signalling plays a modulatory role in the bursting patterns of neuronal networks, which starts with the depolarizing to hyperpolarizing switch in GABAergic signalling (Baltz et al., 2010). Electrical recordings on microelectrode arrays have shown that mature neuronal cultures exhibit temporally differentiated patterns of spontaneous network activity, which can be blocked by GABA receptor antagonists (Baltz et al., 2010).

2.2 GABAergic interneurons

2.2.1 Function of GABAergic neurons

Neuronal structures, and so the cerebral cortex, consist of neurons, which are remarkably heterogeneously in their phenotype, morphology, network position and function. In general, the neurons of the cerebral cortex can be divided into two main populations, glutamatergic pyramidal neurons and GABAergic interneurons, which release the neurotransmitter γ-aminobutyric acid (GABA). GABA binds to two types of receptors, the $GABA_A$ and $GABA_B$ receptor. Whereas the $GABA_B$ receptor is a G-protein coupled metabotropic receptor, the $GABA_A$ receptor is a ligand-gated ion channel highly permeable to Cl^- and bicarbonate (Ben-Ari et al., 2007). The net effect of the channel opening depends on the electrochemical gradient of these anions (cf. chapter 2.1.4), but in general $GABA_A$ receptor activation generates a membrane hyperpolarisation that act to reduce neuronal excitability (Ben-Ari et al., 2007).

Thus, GABA acting on Cl^- permeable $GABA_A$ receptors is the main inhibitory transmitter of the adult cortex and GABAergic signalling plays an important role in normal brain function by the precise control of neuronal activity (Markram et al., 2004; Buzsáki, 2006). GABAergic interneurons are believed to play important roles in balancing excitation and inhibition, controlling the timing of pyramidal cell firing, synchronizing network activity, and the generation of cortical rhythms (Markram et al., 2004; Bartos et al, 2007; Burkhalter, 2008). Interneurons regulate the spatio-temporal dynamics of neuronal network oscillations in a wide range of oscillation frequencies (Buzsáki, 2006), which are

thought to support brain function and are correlated to executive functions, learning and memory (Markram et al., 2004; Woo and Lu, 2006; Bartos et al, 2007). Thus it is not surprising that dysfunctions of GABAergic signalling are associated with pathological conditions, such as autism, mental retardation, epilepsies and schizophrenia (DeFelipe, 1999; Levitt et al., 2004; Lewis et al., 2005; Wonders and Anderson, 2006; Ben-Ari et al., 2007; Woo and Lu, 2006).

2.2.2 Diversity of GABAergic interneurons of the adult cortex

GABAergic interneurons make up 20 to 30 percent of the total neuronal population of neocortical areas and are remarkable heterogeneously in their form and functions (DeFelipe, 1997; Freund, 2003; Kawaguchi and Kubota, 1997; Somogyi and Klausberger, 2005; Markram et al., 2004). Several approaches have been used to classify the different subtypes of interneurons by estimating their morphology, their neurochemical composition and their firing properties (DeFelipe, 1997; Kawaguchi and Kubota, 1997; Freund, 2003; Markram et al., 2004). It has been shown that subgroups of interneurons differ in the morphology of their dendrites and axons, target different subdomains of postsynaptic cells, express different calcium-binding proteins, e.g. parvalbumin, calretinin and calbindin (Kawaguchi and Kubota, 1997), and show different firing properties, like fast spiking, regular spiking or irregular spiking (Freund, 2003; Somogyi and Klausberger, 2005). By targeting specific domains of pyramidal neurons, interneurons are able to control spatiotemporal aspects of neuronal activity. For example, soma-targeting interneurons can tightly control the output and synchronization of pyramidal cells, whereas dendrite-targeting interneurons are in an optimal position to modulate dendritic processing (Markram et al., 2004).

Based on the expression of different neurochemical markers, three main groups of cortical interneurons have been proposed in a simplified classification (Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997; Rudy et al., 2011). In brief, a predominant group of interneurons is characterized by the expression of the calcium binding protein parvalbumin. This group includes fast spiking basket cells and chandelier cells. These neurons typically have multipolar morphology and target the soma and proximal dendrites or the axon initial segment of pyramidal cells, respectively (Kawaguchi and Kubota, 1997; Freund, 2003; Markram et al., 2004). The second group includes interneurons expressing somatostatin and is typically associated with Martinotti cells.

These interneurons have ascending axons that arborize in layer I and make synapses with the apical dendrites of pyramidal cells. The last group has recently been suggested to be characterized by the expression of ionotropic serotonin transporter 5HT3a (Lee et al., 2010; Rudy et al., 2011). However, this group is more heterogeneous than the other groups and includes interneurons expressing the vasoactive intestinal peptide (VIP), neurons containing the calcium binding protein calretinin, reelin positive neurons of cortical layer I and also the so called neurogliaform cells (Kawaguchi and Kubota, 1997; Markram et al., 2004; Lee et al., 2010). These cells, with exception of neurogliaform cells, often have bipolar or bitufted morphologies, are predominantly located to the upper layers in the cortex and innervate dendritic domains of pyramidal cells (Rudy et al., 2011). Neurogliaform cells are a relative unique subpopulation of interneurons having multipolar morphology and a round axonal plexus composed of fine branches and forming electrical synapses (Kawaguchi and Kubota, 1997).

However, all these groups include several subtypes of interneurons that further differ in morphological and electrophysiological properties (DeFelipe, 1997; Kawaguchi and Kubota, 1997; Freund, 2003; Markram et al., 2004). The detailed classification of interneurons is still under investigation (Ascoli et al., 2008).

2.2.3 Origin and development of cortical GABAergic interneurons

The first GABAergic neurons of the mouse cerebral cortex are born as early as embryonic day 12 (Xu et al., 2004; Miyoshi et al., 2007; Batista-Brito and Fishell, 2009), but the period of GABAergic interneuron maturation extends towards late postnatal stages in rodents (Huang et al., 2007; Huang, 2009; Cossart, 2010). It is suggested that the maturation of GABAergic microcircuits results from the interplay between both intrinsic genetic programs and neuronal activity (Huang et al., 2007; Cossart, 2010). However, the exact factors and mechanisms regulating early interneuron development were unknown for long time, because the examination of GABAergic neurons is even more difficult during development than adulthood. As immature interneurons have not yet acquired their characteristic adult neurochemical and morpho-physiological features, it is difficult to identify and classify them (Cossart, 2010). Recently, the development of novel imaging techniques and genetic fate mapping strategies as well as the use of birth date studies and transplantation techniques opened new possibilities for the investigation of GABAergic neuron development.

Cortical interneurons are generated in the ventral telencephalon and migrate tangentially into the developing cortex (Wonders and Anderson, 2006). Recent studies have indicated that the morpho-physiological phenotype of interneurons is strongly predetermined by their embryonic origin (Butt et al., 2005). GABAergic interneurons of the cerebral cortex are generated predominantly in the medial ganglion eminence (MGE) and to a smaller degree in the caudal ganglion eminence (CGE) (Batista-Brito and Fishell, 2009; Vitalis and Rossier, 2011). The subpopulations originating from these two sites do not only express different neuronal transcription factors, but also end up in several distinct interneuron groups. The MGE gives rise to the parvalbumin- and somatostatin-containing neocortical interneurons (Xu et al., 2004; Miyoshi et al., 2007), which typically target the somatic and proximal dendritic domains of other neurons, while the CGE gives rise to the dendrite targeting calretinin- and VIP-expressing interneurons (Xu et al., 2004; Batista-Brito and Fishell, 2009; Vitalis and Rossier, 2011).

2.2.4 Activity-dependent maturation of GABAergic interneurons

The maturation of GABAergic neurons extends far into postnatal development. Thus, because of its early postnatal emergence, electrical activity is likely to modulate the late maturation of early-born interneurons as well as the development of late-born interneurons. Interneurons develop specific morphological phenotypes, with typical dendritic trees and axon targeting selected domains of postsynaptic neurons (Huang et al., 2007). The occurrence of this characteristic morphological features seems to be intrinsically determined (Huang et al., 2007), as the generation of characteristic axonal arbors and prominent GABAergic boutons proceeds in vivo (Karube et al., 2004) as well as in organotypic culture (Di Cristo et al., 2004; Chattopadhyaya et al., 2004). In contrast the refinement of GABAergic axons and their synapses is dependent on neuronal activity. Blocking neuronal activity with TTX reduces the number of GABAergic boutons in organotypic cortical cultures (Chattopadhyaya et al., 2004). The other way around, increasing neuronal activity by blocking inhibitory GABA signalling by the GABA receptor antagonist bicuculline increased the number of GAD65 immunoreactive terminals and the density of inhibitory synapses (Marty et al., 2000).

An important mediator of these activity-dependent effects is the neurotrophin brain-derived neurotrophic factor (BDNF). The expression and secretion of BDNF by projection neurons in the cerebral cortex is regulated by neuronal activity (Zafra et al., 1990;

Lessmann et al., 2003). And of course, interneurons have been demonstrated to express various neurotrophin receptors, including the BDNF receptor tropomyosin-related kinase B (TrkB) (Cellerino et al., 1996; Gorba and Wahle, 1999). BDNF effects on interneurons are manifold and strongly promote interneuron growth (Marty et al., 1996, 1997; Palizvan et al, 2004), neurochemical maturation (Jones et al., 1994; Altar et al., 1997; Huang et al., 1999; Patz et al., 2003, 2004), dendritic elongation and branching (Vicario-Abejon et al., 1998; Jin et al., 2003; Kohara et al., 2003) and synaptogenesis of GABAergic interneurons (Rutherford et al 1997; Seil and Drake-Baumann, 2000; Yamada et al., 2002; Palizvan et al, 2004). Furthermore, the strength and polarity of GABAergic signalling is dynamically modulated by neurotrophins through regulation of the postsynaptic KCC2 expression and thereby the reversal potential for chloride (Rivera et al., 2002; Aguado et al., 2003; Wardle and Poo, 2003).

Thus, because GABAergic neuron maturation is activity-dependent and in turn GABAergic signalling promotes network activity due to the depolarizing actions of GABA during development (see chapter 2.1.4), GABAergic neurons develop in close interrelationship with spontaneous network activity.

2.2.5 GABAergic neurons in cell culture

The development of cortical interneurons in cultures from embryonic rat neocortex has been extensively studied before (de Lima and Voigt, 1997; Voigt et al., 2001; de Lima et al., 2004). As mentioned above, the identity of cortical interneurons is predetermined by genetic factors. Thus interneurons develop morphological and physiological properties comparable to the *in vivo* situation when grown in organotypic slice cultures or dissociated neuron cultures (de Lima and Voigt, 1997; Voigt et al., 2001; Chattopadhyaya et al., 2004; Karube et al., 2004).

In neuronal cultures developing from dissociated embryonic rat cerebral cortices, two types of GABAergic neurons were described, named large L-GABAergic and small S-GABAergic neurons (de Lima and Voigt, 1997). These two subpopulations differ in their time of birth and morpho-physiological properties. Thus these neurons are suggested to be in vitro correlates of early and late born cortical interneurons in vivo.

Because immature GABAergic neurons do not express their typical adult phenotypes, the investigation of GABAergic neurons during early development is difficult. Therefore the subdivision of GABAergic neurons in two distinct groups that differ in their

time of birth and morphology provides a simplified but helpful approach to analyse the morphological and functional interneuron maturation in a population dependent manner, where these populations represent correlates of the distinct interneuron populations born in the medial and caudal ganglionic eminence.

L-GABAergic neurons. The L-GABAergic neurons are a very prominent type of GABAergic interneuron. They are postmitotic by the time of plating, develop a large cell body with a stellate dendritic tree, and form a dense network of long-range connections with their thick axons (Voigt et al., 2001). Their dendrites are densely innervated by presynaptic terminals and their axons contact the soma and proximal dendrites of numerous postsynaptic cells. In mature cultures, L-GABAergic neurons show a fast-spiking phenotype (Klueva et al., 2008), and express parvalbumin (de Lima et al., 2004; Klueva et al., 2008). Thus they show morphological and physiological properties similar to cortical parvalbumin neurons in situ (Voigt et al., 2001; de Lima et al., 2004; Klueva et al., 2008). L-GABAergic neurons belong to the lineage of cortical interneurons, which originate in the subcortical medial ganglionic eminence (MGE) and develop to form diverse types of GABAergic neurons, including parvalbumin-positive basket interneurons (Wonders and Anderson, 2006; Batista-Brito and Fishell, 2009). During network formation L-GABAergic neurons play an important role in the generation of early synchronous network activity (Voigt et al., 2001). Because of their high connectivity and the depolarizing actions of GABA during early development, these neurons are able to drive network oscillations even in the absence of glutamatergic neurotransmission (Voigt et al., 2001).

S-GABAergic neurons. S-GABAergic neurons are a second and numerically larger subpopulation of GABAergic neurons. These neurons are born later then the L-GABAergic neurons and are generated in vitro from precursors that do not produce GABA during the first few days in vitro (de Lima and Voigt, 1997). S-GABAergic neurons increase in their number at least until 12 DIV and are differentiated at around 14DIV. Then these neurons typically have small bipolar or multipolar cell bodies and very thin axons (de Lima and Voigt, 1997; de Lima et al., 2004). Because S-GABAergic neurons are double-labelled with calretinin (de Lima et al., 2004) they are thought to be correlates of calretinin/VIP interneurons that mainly populate the upper cortical layers in vivo (Kawaguchi and Kubota, 1997; Markram et al., 2004). In contrast to the population of parvalbumin-positive neurons this heterogeneous group of interneurons, including the calretinin-expressing neurons, is thought to originate from the caudal ganglionic eminence (Batista-Brito and

Fishell, 2009; Cossart, 2010). In culture these neurons strongly depend on glutamatergic activity during a critical time window, otherwise they are irreversibly eliminated from the network (de Lima et al., 2004).

2.3 Thyroid hormones

The thyroid hormones thyroxine (T4) and triiodothyronine (T3) are tyrosine-based hormones, which are primarily produced by the thyroid gland. Specific thyroid hormones receptors are abundantly expressed in all tissues, and thus thyroid hormones act on nearly every cell in the body (Hulbert, 2000). In general, thyroid hormones are responsible for regulation of cell metabolism and are essential for proper development and differentiation of nearly all cells. Thyroid hormones furthermore play a determining role in the early growth and development of most organs. Especially the brain of humans and other mammals has been identified as an important target of thyroid hormone actions for many years (Legrand, 1984; Dussault and Ruel, 1987). Thyroid hormones play an important role in the establishment of the cortical microarchitecture. Consequently, thyroid hormone deficiency in developing mammals, including humans, may lead to severe morphological and functional alterations of the CNS (Yen, 2001; Morreale de Escobar et al., 2004; Wallis et al., 2008).

2.3.1 Consequences of thyroid hormone deficiency

Defects in thyroid hormone signalling can be caused by impaired thyroid function, mutations in thyroid hormone receptors and transporters, and iodine deficiency, as iodine is an important compound in thyroid hormone synthesis (Bernal, 2009). The resulting hypothyroxinemia can occur at any stage of life and induces morphological and functional alterations of the CNS. However, the most devastating consequences of thyroid hormone deficiency take place during fetal and early postnatal development with poor growth and severe cognitive impairment (de Benoist et al., 2008). These deficits can be prevented by thyroid hormone replacement shortly after birth (Dussault and Ruel, 1987; Anderson, 2001). If not treated adequately, neonates exhibit general features of hypothyroidism as growth and mental retardation, tremor, spasticity and speech and language deficits (Morrela de Escobar et al., 2004; Williams, 2008). The most damaging disorders induced by thyroid hormone deficiency are irreversible mental retardation and cretinism (Delange, 2000; World Health Organization, 2004).

Also iodine deficiency caused by a low dietary supply of iodine causes thyroid hormone deficiency. The resulting damage increases with the extent of the deficiency, but also mild iodine deficiency causes subtle degrees of mental impairment that lead to poor school performance, reduced intellectual ability, and impaired work capacity (World Health Organization, 2004). It has been suggested that iodine deficiency is responsible for a mean intellectual quotient (IQ) loss of 10-15 points at the population level (Delange, 2000; de Benoist et al., 2008). Therefore iodine deficiency is a major public health problem for populations throughout the world, particularly for pregnant women and young children. Still, 266 million children and 2 billion people worldwide are at risk for iodine deficiency (de Benoist et al., 2008). Thus, iodine deficiency is the world's greatest single cause of preventable brain damage (World Health Organization, 2004).

Besides modulation of cognition and brain performance thyroid hormones have also been implicates in regulation of mood and behavior:

Depression. Thyroid hormone dysregulation has been linked to depression and thyroid hormone testing is a routine in psychiatric admission for depressive patients (Kirkegaard and Faber, 1998). Additionally, thyroid hormone application is a commonly used strategy to accelerate or augment a standard antidepressant therapy (Carvalho et al., 2009), although the mechanisms behind this effect are not clear. Recent evidence comes from animal studies showing that mice harbouring a mutant thyroid hormone receptor alpha 1 (TRalpha1) (Tinnikov et al., 2002) show depression-like behavior (Pilhatsch et al., 2010).

Anxiety. Mice with a point mutation in the TRalpha1 gene (Tinnikov et al., 2002) furthermore showed an anxious phenotype with reduced exploratory behavior and memory deficiencies, which could be improved following T3 administration (Venero et al., 2005, Wallis et al., 2008).

ADHD. Attention deficit and hyperactivity disorder has been diagnosed in the offspring of mothers exposed to mild-moderate iodine deficiency (Vermiglio et al., 2004). Accordingly, transgenic mice bearing a human mutant thyroid hormone receptor beta1 gene, a condition that also occurs in human resistance to thyroid hormones (RTH) (Weiss and Refetoff, 2000), display stereotypic behavioral features, hyperactivity and learning deficits (McDonald et al., 1998; Siesser et al., 2006).

2.3.2 Physiology

The thyroid hormones T4 and T3 are products of the thyroid gland. Whereas T4 is derived exclusively from synthesis and secretion by the thyroid gland, T3 is largely produced in peripheral tissues by enzymatic removal of an outer ring iodine atom from T4 (Hulbert, 2000; Patel et al., 2011). To fulfil its function in the brain, T4 first have to cross the blood-brain barrier by a specific transporter. After having entered the brain, T4 is taken up by astrocytes, where it is converted to T3 (Williams, 2008; Patel et al., 2011). This activation of T3 and also the inactivation of both thyroid hormones are done by specific enzymes, the iodothyronine deiodinases (Gereben et al., 2008). The type 2 deiodinase (D2) is expressed predominantly in astrocytes (Guadano-Ferraz et al., 1997) and catalyses the removal of an outer ring iodine atom from the 'prohormone' T4 to generate the more active product T3 (Hulbert, 2000; Patel et al., 2011). In contrast, the type 3 deiodinase (D3), which is located in neurons, inactivates T3 or prevents T4 being activated by catalysing removal of an inner ring iodine to generate the biological inactive diiodothyronine (T2) or reverse triiodothyronine (reverse T3, rT3) respectively (Hulbert, 2000; Williams, 2008; Patel et al., 2011). The generated T3 is released by astrocytes and taken up by neurons, presumably by active uptake via the specific transporter MCT8 (Williams, 2008; Patel et al., 2011). T3 then exerts its major actions directly in neurons and is finally degraded by D3 in neurons (Williams, 2008). Thus the control of local T3 levels in brain tissue takes place in functional units of astrocytes and neurons.

2.3.3 Signalling pathways

The mechanisms by which thyroid hormones exert their influence are complex and still not completely defined. In the classical view, thyroid hormones act via nuclear thyroid hormone receptors (TRs). There are two receptor types, TRalpha and TRbeta, both expressed in several isoforms (Forrest et al., 1990; Bradley et al., 1992). These thyroid hormones receptors are abundantly expressed in all tissues of the body (Hulbert, 2000). In the brain, TRalpha has been estimated to account for 70-80% of total TR expression and is widely expressed, whereas TRbeta shows more restricted expression patterns (Bradley et al., 1992). Because the physiological ligand of TRs is T3, it is considered to be the active form of thyroid hormones, whereas T4 might function as a prohormone (Williams, 2008).

Nuclear thyroid hormone receptors are ligand-modulated transcription factors that bind directly to the DNA at thyroid hormone response elements (TREs) located in gene

regulatory regions (Yen, 2001; Bernal, 2007; Bernal, 2009). Unlike most other nuclear receptor types, unliganded thyroid hormone receptors bind corepressor complexes with histone deacetylase activity (Yen, 2001; Bernal, 2007). Thereby they maintain a nonpermissive chromatin structure and inhibit gene transcription. By binding T3 these repressors are released and coactivator complexes are recruited to the DNA. These complexes induce chromatin opening by intrinsic histone acetyl transferase activity and enhance transcriptional activity (Yen, 2001; Bernal, 2007).

The repressing action of unliganded TRs exerts important physiological roles during the development but can also exhibit harmful activity (Hashimoto et al., 2001; Bernal et al., 2003; Bernal, 2007). In contrast to hypothyroid animals with unliganded receptors, mutant mice lacking all TRs do not display obvious signs of developmental abnormalities (Morte et al., 2002). Thus, the absence of the receptor is not equivalent to the absence of the hormone. This can be explained by the hypothesis that abnormal regulation of transcription by the unliganded receptor is responsible for the effects of profound hypothyroidism, rather than the direct missing of hormone (Bernal et al., 2003; Bernal, 2007). This hypothesis is supported by the observations that the expression of a dominant-negative point mutation in the TRalpha1 gene lead to the typical alterations of severe hypothyroidism (Tinnikov et al., 2002; Venero et al., 2005; Wallis et al., 2008; Bernal, 2009).

In addition to genomic actions, also extragenomic pathways of thyroid hormone action have been described (Davis et al., 2005; Cheng et al., 2010). Recently, it has been suggested that thyroid hormone receptors might also be located in the cytoplasma (Storey et al., 2006). There the TRs can interact with the regulatory p85 subunit of the phosphoinositide 3-kinase (PI3K) and regulate downstream targets such as mammalian target of rapamycin (mTOR) or Rac (Cao et al., 2005; Storey et al., 2006). Furthermore, it has been shown that T4 and, to a lesser extent, T3 bind to a membrane receptor formed by the integrin alphaVbeta3, which then activates the mitogen-activated protein kinase (MAPK) signalling cascade (Davis et al., 2005; Cheng et al., 2010). Finally, thyroid hormones have been suggested to regulate the kinetics of glutamatergic and GABAergic signalling potentially by direct non-genomic modulation of neurotransmitter receptors (Losi, et al., 2008; Puia and Losi, 2011). Additionally, thyroid hormones rapidly modulate protein phosphorylation (Sarkar et al., 2006) and cytoskeletal proteins (Zamoner et al., 2006). However, the mechanisms of non-genomic actions and their contribution to the physiological actions of thyroid hormones *in vivo* are still largely unknown.

2.3.4 Influence on brain development

It is suggested that thyroid hormones have no influence on very early developmental events, such as neurulation, neural fate induction and establishment of polarity, but regulate later processes, especially those related to early postnatal development, including myelination, differentiation, dendrite proliferation and synapse formation (Zoeller and Rovet, 2004; Bernal et al., 2003; Williams, 2008). Consequently, cellular correlates following T3 deprivation are damages in myelination, migration defects, altered cortical layering, and reduction of axonal or dendritic outgrowth, as it has been reported for neocortex, hippocampus and cerebellum (Bernal and Nunez, 1995; Anderson, 2001; Yen, 2001; Koibuchi et al., 2001; Morrelae de Escobar et al., 2004). Numerous studies pointed out the importance of thyroid hormones in the expression of presynaptic proteins involved in neurotransmitter release, including Synapsin (Hosoda et al., 2003), SNAP-25 (Zhang et al., 2008), Synaptophysin (Gong et al., 2010) and Synaptotagmin-1 (Wang et al., 2011). Accordingly, thyroid hormones have been shown to regulate the distribution of axonal projections (Li et al., 1995; Lucio et al., 1997), the number of dendritic spines (Ruiz-Marcos et al., 1980, 1982; Nimchinsky et al., 2002), and the differentiation of dendrites (Ipiña et al., 1987; Sala-Roca et al., 2008).

Recently it has been shown that disturbance of T3 signalling during early development led to profound defects in the maturation of the GABAergic system. Especially interneurons positive for the calcium binding protein parvalbumin are modulated by thyroid hormone signalling (Berbel et al., 1996; Venero et al., 2005; Gilbert et al., 2007; Wallis et al., 2008). It has been shown that hypothyroidism reduces the density of parvalbumin-positive neurons both in neocortex and hippocampus (Gilbert et al., 2007), as well as the density of parvalbumin-positive terminals and processes (Berbel et al., 1996). Additionally, reduced numbers of parvalbumin-immunoreactive interneurons have been reported in mice expressing a dominant-negative point mutation in the TRalpha1 gene without alteration of the total number of GABAergic neurons (Venero et al., 2005; Wallis et al., 2008).

2.3.5 Temoral aspects

The developmental timing is thought to be an important factor in thyroid hormone actions. In a simplified model, three stages of thyroid hormone dependent brain development can be distinguished (Williams, 2008): The first stage spans the time before

the onset of fetal thyroid hormone synthesis, which occurs at 16-20 weeks postconception in humans or by embryonic day E17.5-18 in the rat. During this period, thyroid hormone influences proliferation and migration of neurons in the cerebral cortex, hippocampus and medial ganglionic eminence. The second stage covers the perinatal and early postnatal period, when thyroid hormones modulate neurogenesis, neuron migration, axonal outgrowth, dendritic branching, synaptogenesis, and the initiation of glial cell differentiation and myelination. The third stage occurs in the later postnatal period, when thyroid hormones are critical for continuing maturation and plasticity.

Most of the thyroid hormone responsive genes identified so far are expressed and regulated by thyroid hormone during the early postnatal period (Oppenheimer and Schwartz, 1997; Bernal et al., 2003). Therefore, thyroid hormones have been suggested to accelerate and synchronize early neuronal maturation (Bernal et al., 2003). Based on the temporal patterns of thyroid hormone-dependent gene expression in the brain even a critical period of thyroid hormone sensitivity spanning the first 2-3 postnatal weeks in the rat has been suggested (Bernal et al., 2003).

Additionally, the developmental timing has also influence on the impact and reversibility of malformations induced by hypothyroidism (Anderson, 2001; Bernal et al., 2003). Deficiency of thyroid hormones during critical periods of development leads to profound and potentially irreversible defects of brain maturation. In both prenatal and early postnatal brain development a deficit of thyroid hormones causes severe neuronal damage leading to irreversible mental retardation and motor deficits (Yen, 2001; Morreale de Escobar et al., 2004; Bernal, 2009). In contrast, disorders following adult onset hypothyroidism are usually reversible with proper treatment (Bernal, 2009).

Interestingly, the critical period of thyroid hormone signalling largely parallels the time of early synchronous network activity. However, only rare evidence is available about the influence of thyroid hormone signalling on spontaneous neuronal activity. Sporadic reports suggest that thyroid hormones T4 and T3 enhance the frequency of spontaneous activity in neuronal cultures (Romijn et al., 1981; Hosoda et al., 2003). Thus it is feasible that thyroid hormones might regulate developmental processes, at least partly, by the modulation of spontaneous network activity.

2.4 Aims of the study

The main aim of the presented project was to study the effects of thyroid hormone T3 on the early development of cortical networks with an emphasis on GABAergic neurons. The emergence of spontaneous network activity is a general feature of developing neuronal structures and previous studies suggest that especially GABAergic neurons develop in a close interrelationship with the functional development of the network (Voigt et al., 2001; de Lima et al., 2004, 2007). As thyroid hormones have been shown to regulate the maturation of GABAergic neurons (see chapter 2.3.4) and as they have also been implicated in the regulation of neuronal activity (see chapter 2.3.5), it is feasible that they modulate the intricate development of GABAergic neurons and network activity.

The investigation of the thyroid hormone-mediated modulation of cortical network development involved the following steps:

- First, to study the influence of T3 on the development of spontaneous network activity.
- Second, to identify T3-mediated changes in the development of different neuronal subpopulations. Because GABAergic interneurons develop in a close interrelationship with the network activity, special attention was given to the morphological development of GABAergic interneurons.
- Third, to address the mechanisms and the direction of T3-mediated effects to identify which effects are directly mediated by T3 and whether T3-induced cellular alterations are activity-dependent.
- Fourth, to investigate the influence of T3 on the functional maturation of GABAergic signalling, e.g. the expression of the chloride transporters KCC2 and NKCC1.
- Fifth, as thyroid hormone actions might be restricted to limited periods, the sensitivity of GABAergic neurons and the network activity to T3 stimulation was addressed during different culture periods.

3. Materials and Methods

3.1 Animals

In the present work, Sprague Dawley rats *(Rattus norvegicus)* were used to prepare neuronal cultures. The rats were bred at the Institute of Physiology, Magdeburg. Handling with the animals was according to the rules of the Otto-von-Guericke University and the federal state Saxony-Anhalt.

3.2 Materials

The chemicals, solutions, antibodies, lab equipment and software used in this study are listed in the appendix (see chapter 7).

3.3 Cell culture

Neuronal cultures were prepared from embryonic rat cortex and cultured for 7-21 days in serum-free N2 medium in the presence of a surrounding glial feeding layer as described previously (de Lima and Voigt, 1997). All experimental procedures were approved by local government authorities.

3.3.1 Preparation of petri dishes

Cortical neurons were cultivated in the presence of a surrounding glia-feeding layer. This procedure required a special set-up of culture dishes, where neurons could grow in the central region separated from glia cells in the outer portion of the Petri dish. Each acid-cleaned glass cover slip was fitted to a 20-mm hole in the bottom of a 60-mm Petri dish and treated overnight with poly-D-lysine (0.1 mg/ml in borate buffer, pH 8.5, 36°C). Dishes were air dried, washed with sterile water and replenished with Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS, Invitrogen) until cell plating. To prevent the glial cells from spreading onto the cover slip, the central region was temporarily isolated by a plexiglas ring glued with silicon grease around the hole. The plastic ring was removed after neuron attachment to allow a free interchange of culture media between centrally cultured neurons and peripherally cultured feeder glia.

3.3.2 Astroglial cell cultures

Astroglial cultures were prepared from cerebral hemispheres of newborn Sprague-Dawley rats (postnatal day 0-3) as reported in detail (de Lima and Voigt, 1999). Briefly, hemispheres were dissected under sterile conditions, and meninges were carefully removed. The tissue was minced in Hank's medium (HBSS, Ca^{2+} and Mg^{2+} free, Invitrogen; 20mM HEPES, pH 7.4) and dissociated by trituration through G21 and G25 syringe needles. Cells were seeded at low densities in DMEM containing 10% FCS in tissue culture flasks (175 cm^2). The cells were maintained in a humidified 5%$C0_2$/95% air atmosphere at 36°C. Medium was replaced twice a week. Nonastroglial cells were removed from confluent 2-week-old cultures by shaking flasks overnight on a rotatory platform at 180 rpm (rotations per minute). Confluent astroglial cultures were used as feeder layers for pure neuronal cultures or for conditioning of freshly prepared serum-free medium. Purified astroglial cells were plated at a density of 300 cells/mm^2 in the outer portion of the Petri dish bottom five days before the neurons.

3.3.3 Neocortical neuron cultures

Neuronal cultures were prepared from cerebral cortices of embryonic Sprague-Dawley rats at embryonic day 16 (E16; day after insemination = E1). Rat embryos were removed by caesarean section from the anesthetized mother (4% chloral hydrate, 1 ml/100 g body weight). The mother was killed immediately after the removal of the embryos by an overdose of the anesthetic (10% chloral hydrate, 3 ml). The embryonic neocortical hemispheres were dissected under sterile conditions and rinsed in Hank's medium. Cells were taken from the dorsolateral parts of the telencephalic vesicles (excluding hippocampal and basal telencephalic anlagen). Meninges were removed, and cortical tissue was minced, rinsed in Hank's medium, and dissociated with a solution of 0.5% trypsin/EDTA (Boehringer, Mannheim, Germany) in Hank's medium for 25min at 36°C. Trypsin solution was then removed, and protease activity was blocked by trypsin inhibitor-DNAse solution (0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml DNAase I, 3 mg/ml bovine serum albumin in DMEM). The suspension was repeatedly passed through G21 and G25 syringe needles (3 times each). After centrifugation (10min, 1200rpm) cells were rinsed with Hank's medium, and the percentage of viable cells was determined by using propidium iodide.

Cells were seeded onto poly-D-lysine coated glass cover slips at a density of 300-500 cells/mm² in N2 medium (75% DMEM, 25% Ham's F12 and N2 supplements from Invitrogen). Cultures were treated at 6 DIV (days in vitro) with cytosine arabinoside (Ara-C) at a final concentration of 5 µM and a third of the medium volume was then changed after 24 hours. All cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 36°C. One third of the culture medium was refreshed once a week. To support neuronal survival in cultures that grew older then 14DIV purified astroglial cells were added at 9DIV to the neurons at a density of 300 cells/mm^2. The additional astroglia yielded a better cell survival and more consistent results in morphometric analysis during the third week *in vitro*.

3.3.4 Hormone application

Neurons were cultured for 14 days in serum-free N2 medium either in the presence or absence of triiodothyronine (T3, Sigma-Aldrich, St. Louis, MO). T3 was applied in our experiments at 0DIV (15nM) and a third of medium volume was refreshed at 7 DIV. This concentration range is in line with other in vitro studies showing physiological effects of T3 at concentrations between 5 and 30nM (Romijn et al., 1981; Potthoff and Dietzel, 1997; Hosoda et al., 2003; Hoffmann and Dietzel, 2004; Morte et al., 2010). Dose-response curves were obtained from experiments with T3 concentrations between 10^{-13} and 10^{-6} M (Fig. 6). Significant effects were already observed at concentrations as low as 12^{-12} M. Since higher T3 concentrations yielded more consistent results without noticeable side effects T3 was used in a concentration of 15nM throughout this study.

For reversal experiments all cultures were washed twice with prewarmed DMEM at 14DIV and the medium was replaced by fresh medium that was conditioned in astroglial flasks cultures for at least 24 hours and was either supplemented with T3 (15nM) or without T3 resulting in four experimental groups: a) chronically deprived cultures ($T3^{-}$), b) chronically T3-supplemented cultures ($T3^{+}$), c) cultures supplemented with T3 only during the third week *in vitro* ($T3^{-/+}$), d) and cultures supplemented during the first two weeks ($T3^{+/-}$).

3.3.5 Drugs application

In the initial experiments (sections 4.1.1-4.2.1, 4.2.5, 4.2.6), corticosterone (0.1µg/ml, Sigma) was added to the culture medium alone or in combination with T3 following the original protocol by Romijn et al (1981).

In the experimental groups the following drugs were added to the medium at various time points (see result section) and cultures were maintained until fixation time, with an additional drug application after 5 days (except BDNF and K252a): glutamate receptor (GluR) antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium, 10 µM; Tocris Cookson, Ellisville, MO) and APV (D-2-amino-5-phosphonopentanoic acid, 50 µM; Tocris), sodium channel blocker TTX (Tetrodotoxin, 1 µM, Alomone Labs, Jerusalem, Israel), recombinant human brain-derived neurotrophic factor (BDNF; 50 ng/mL; Pepro Tech, Rocky Hill, NJ), protein kinase inhibitor K252a (100 nM; Calbiochem by Merck KGaA, Darmstadt, Germany), KCC2 inhibitor VU0240551 (4 µM; Cat. sc-253834; Santa Cruz Biotechnology Inc., Santa Cruz, CA), $GABA_A$ receptor antagonists (-)-bicuculline methiodide (BMI; 20 µM; Sigma-Aldrich) and Picrotoxin (PTX; 10 µM; Tocris Cookson, Ellisville, MO).

In one series of experiments the thyroid hormone receptor antagonist 1-850 (Merck KGaA Darmstadt, Germany) (Schapira et al., 2003; Sui et al., 2008) was added at a concentration of 1µM to the culture medium at plating (0 DIV). For hormonal stimulation T3 (10ng/ml) was added 24h later. Drug application was repeated on 7 DIV and cultures were grown until 14 DIV.

3.4 Calcium-Imaging

3.4.1 Detection of calcium transients

Changes in neuronal intracellular calcium concentration ($[Ca^{2+}]_i$) were recorded as reported previously (Voigt et al., 2001; Opitz et al., 2002). Briefly, cell cultures were dye-loaded for 1 h in regular culture medium by adding fluo-3 pentacetoxy-methylester (Molecular Probes; purchased from MoBiTec, Goettingen, Germany) to a final concentration of 5 µM. Cultures were washed with Hepes-buffered artificial cerebrospinal fluid (aCSF; in mM: 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 0.75 $MgCl_2$, 1.25 NaH_2PO_4, 20 D-glucose, 15 Hepes/NaOH, pH 7.4) and allowed to equilibrate for 30 min. Recordings were performed at room temperature on an inverted microscope (Axiovert S100 TV, Zeiss, Oberkochen, Germany) equipped with a cooled charge-coupled device camera (CoolSNAP

ES; Roper Scientific, Ottobrunn, Germany). Sequences of fluorescence images were recorded for at least 120 seconds at 1 or 2 Hz from at least five randomly chosen fields per culture. A differential interference contrast (DIC) image of each field was acquired for later cell identification and in some experiments fields were marked with a diamond tool for identification of GABAergic neurons after post hoc GABA immunocytochemistry (see below).

3.4.2 Burst analysis

Images were processed with MetaMorph software (vers. 7.0, Molecular Devices, Sunnyvale, CA) as described in detail (Opitz et al., 2002). In each dish we recorded fluorescence images from five fields and determined the total number of neurons as well as the number of active neurons in each field. Total number of neurons in the field was assessed by examination of DIC images. Each cell was visually identified and marked as a region of interest (ROI). Additional ROIs were set above cell free area to determine the background noise. All ROIs were later superimposed on the fluorescence-images taken each second for 4 minutes to determine the fluorescence intensity over time. A change in $[Ca^{2+}]_i$ was considered significant when the absolute difference of gray values exceeded 5 times the standard deviation of background noise measured in cell free areas (Voigt et al., 2001; Opitz et al., 2002). Because 7.5% of cells can simultaneously be active when synaptic transmission was completely blocked (Opitz et al., 2002), synchronous bursts are defined as events in which at least 10% of all cells participate. Frequency of synchronous 'bursts' was calculated off-line with custom-made software written in MATLAB (vs. 7.5, MathWorks, Natick, MA) and Excel (Microsoft Corp., Richmond, WA).

3.4.3 Kinetic analysis

Kinetics of spontaneous calcium transients were analysed using Excel (Microsoft Corp., Richmond, WA) and the MiniAnalysis software (version 6.0.3, Synaptosoft, Decatur, GA). The fluorescence trace (F) of each individual cell was background corrected and normalized (dF/F0) according to the minimal fluorescence level during the sequence (F0). All single cell transients of the same field were averaged and considered as the averaged network activity (Cohen et al., 2008; Allene et al 2008; see also Fig. 26 B). The duration of averaged calcium transients (time between the event onset and the recovery to baseline level) was calculated semi-automatically using the MiniAnalysis software

(Synaptosoft). Because kinetics of calcium transients in the same field were rather homogeneous during the imaging session, the mean burst duration per field was also calculated and statistics were made on this data.

3.4.4 GABA shift experiments

To investigate the developmental changes in $GABA_A$ receptor mediated synaptic transmission the exposure to locally applied potassium and GABA agonist muscimol during calcium imaging was used (Owens et al., 1996; Ganguly et al., 2001; Baltz et al., 2010). Cultures were continuously perfused with an aCSF containing the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 2.5 µM; Tocris) and D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 12.5 µM; Tocris) to suppress spontaneous network activity. At defined time points during each recording session cells were locally perfused with short pulses of an aCSF containing either high potassium (KCl, 60 mM; Merck) to identify living neurons or the $GABA_A$ receptor agonist muscimol (200 µM; Sigma-Aldrich) using a multibarreled perfusion system (Baltz et al., 2010). The local application lasted 2 seconds resembling prolonged neuronal activation during population burst activity.

3.4.5 Analysis of stimulation induced calcium transients

Images were processed off-line using MetaMorph software (Molecular Devices) and fluorescence data were processed with Excel (Microsoft Corp.). For each cell we calculated the background-corrected baseline fluorescence and the standard deviation of baseline fluctuations during a 5-s-period before local stimulant application. To detect stimulation induced changes in intracellular calcium concentration the maximal emitted fluorescence in response to local stimulation was detected. A cell was considered responsive to muscimol or KCl when the change in fluorescence exceeds five times the standard deviation of baseline fluctuations. The fraction of muscimol responsive cells was calculated per field as percentage of KCl responsive cells.

3.5 Morphometric analysis

3.5.1 GABA-Immunocytochemistry

GABA-immunocytochemistry was used for identification and morphometric analysis of the neuronal populations. GABA-containing neurons were labelled with a monoclonal mouse anti-GABA antibody (Szabat et al., 1992) as described previously (de Lima and Voigt, 1997; Voigt et al., 2001; de Lima et al., 2007). Cultures were fixed 30 min at 36°C by adding 70% glutaraldehyde to the culture medium to make a final concentration of 3.5%. Cultures were then thoroughly washed in Tris/metabisulfite solution (0.85% sodium metabisulfite in 0.05M Tris buffer; pH 7.5). To reduce aldehyde-induced autofluorescence and unspecific antibody binding cultures were treated with ethanolamine (1M in Tris/metabisulfite solution) for 20 min at RT, and preincubated for 3 hours at room temperature (3% bovine serum albumin, 10% normal goat serum, 0.6% Triton X-100 in Tris/metabisulfite solution). After washing, cultures were incubated with monoclonal mouse anti-GABA antibody (1:200; clone 5A9; Chemicon, Temecula, CA) in Tris/Metabisulfite solution containing 3% bovine serum albumin, 10% normal goat serum, 0.6% Triton X-100 overnight at room temperature. After the primary antibody incubation all washes were made with phosphate buffered saline (0.1M, pH 7.4). Primary antibodies were labelled with secondary goat anti-mouse antibody (1:200; Convance Inc, Princenton, NJ) and mouse peroxidase-anti-peroxidase (1:200, Sternberger, Baltimore, MD) in PBS containing 10% normal goat serum, 2% bovine serum albumine, 5% Sucrose and 0.3% Triton X-100 for 2 hours at room temperature. Antibody-peroxidase complexes were made visible by 0.01% 3, 3' diaminobenzidine tetrahydrochloride, 0.004% H_2O_2, 1% Nickel ammonium sulfate, 50mM Imidazole in 50mM Tris-HCl saline buffer. After the final PBS wash, coverslips were dehydrated in an ethanol series, cleared in two changes of xylene, and mounted over clean slides with Fluoromount (Serva, Heidelberg, Germany).

3.5.2 Population analysis

Analysis was focused on the following neuronal subpopulations: 1) large GABAergic interneurons (L-GABA), 2) late-born small mainly fusiform GABAergic interneurons (S-GABA) and 3) non-GABAergic neurons. In each experiment the cell density and the cell body size of GABAergic and non-GABAergic neurons were quantified in sets of treated cultures compared with a set of age-matched control cultures. Cell counts were made in 10 fields per coverslip with the aid of a grid in the microscope eye piece.

Fields were chosen at regularly spaced points over the coverslip surface and countings were made using different magnifications depending on the cell density (Field size range: 125 µm x 125 µm to 625 µm x 625 µm).

3.5.3 Single cell morphometry

The quantification of soma area was based on high magnification individual drawings of the cell bodies (40x objective for L-GABAergic neurons and 100x objective for S-GABAergic and non-GABAergic neurons; no more than 5 cells were drawn in each of randomly chosen fields). Drawings of cell bodies and dendrites were made with an upright microscope (Zeiss, Standard WL) equipped with a Camera lucida. Drawings of cell body profiles were scanned and a digitalized version was analysed with MetaMorph software. Similarly, drawings of dendritic profiles were made for L-GABAergic cells using a 40x objective followed by digitalization and analysis with MetaMorph software.

3.5.4 GABA bouton density

To quantify the local GABAergic synaptic innervation in low density cultures, micrographs of fields containing small groups of non-GABAergic neurons were taken with a CCD camera (Spot slider, Diagnostic Instruments, Sterling Heights, MI) with a 100x oil immersion lens (Axiophot, Zeiss, Oberkochen, Germany). In each field (120 µm x 90 µm) the total number of neurons and the total number boutons formed by GABAergic axons was counted and expressed as fraction of boutons per neuron. Counts were made in 20 fields per cover slip.

3.5.5 Morphology of GABAergic axons

To quantify the number of GABAergic boutons and the local axonal ramifications formed by GABAergic axons, micrographs of randomly chosen fields were taken with a charge-coupled device camera (Spot slider, Diagnostic Instruments, Sterling Heights, MI) with a 40x objective on an upright microscope (Axiophot; Zeiss). In each image a central region of interest (120 µm x 90 µm) was selected and the total number of neurons, the total number of GABAergic boutons and the total number of branches formed by GABAergic axons was counted. Additionally the total length of GABAergic axons was measured in each field using MetaMorph software (Molecular Devices). Counts and measurements were made in 10 fields per cover slip.

3.6 Analysis of synaptic densities

3.6.1 Synapse immunocytochemistry

For immunocytochemical synaptic staining cell cultures were fixed for 30 min at 36°C in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4) containing 0.12M glucose, followed by chilled methanol (-20°C) for 10 min at 4°C. After washing in phosphate-buffered saline (PBS, 0.01M, pH 7.4), cultures were incubated in 0.25% Triton X-100, 10% goat serum, 5% sucrose, 2% BSA in PBS for 10 min at room temperature. Cell cultures were stained for presynaptic marker synapsin and one of the postsynaptic proteins PanShank or GluR2 to detect glutamatergic synapses and for synapsin and gephyrin to detect GABAergic synapses. The double staining was performed sequentially by first incubating with polyclonal rabbit anti-Synapsin1/2 (1:500, Cat.No. 106 002, Synaptic Systems, Göttingen, Germany) overnight at 4°C followed by goat anti-mouse Cy2 (1:400, Dianova, Hamburg, Germany) for 2 h at room temperature. After a short fixation (30 min, 4% paraformaldehyde, 36°C) cultures were incubated with monoclonal mouse anti-PanShank (1:100, clone N23B/49, NeuroMab, Davis, CA), monoclonal mouse anti-GluR2 (1:100, clone L21/32, NeuroMab) or monoclonal mouse anti-gephyrin (1:1000, Cat. No. 147 011, Synaptic Systems, Göttingen, Germany) overnight at 4°C followed by goat anti-rabbit Cy3 (1:400, Dianova, Hamburg, Germany) for 2 h at room temperature. Cell cultures were rinsed thoroughly with PBS between every incubation step. After a final wash in PBS, coverslips were dehydrated in an ethanol series, cleared in two changes of xylene, and mounted on clean slides with Fluoromount (Serva, Heidelberg, Germany).

3.6.2 Analysis of synaptic stainings

For quantitative evaluation of the synaptic staining at least two coverslips were analysanalysed per experimental condition in each of at least two experiments. Two consecutive micrographs for the two antibody labels were taken at five randomly chosen fields per culture dish with a CCD camera (Spot slider, Diagnostic instruments, Sterling Heights, MI) and a 100x oil immersion lens (Axiophot, Zeiss, Oberkochen, Germany). The bandwidth of filter sets used to visualize Cy3 and Cy2 did not overlap. The pairs of micrographs were brought into register using the MetaMorph software (vers. 6.0, Universal Imaging Corp., West Chester, PA) and five randomly chosen fields measuring 10 µm x 10 µm were selected in each pair of photographs. In each field the amount of all

synapsin/GluR2, synapsin/PanShank or synapsin/gephyrin co-localized clusters was determined and expressed as percentage of all synapsin positive clusters.

3.7 Western blotting

3.7.1 Specificity of antibodies used in western blots

For recognition of NKCC1 we used the well characterized and commonly used monoclonal antibody T4 (Lytle et al., 1995). The hybridoma culture supernatant containing the monoclonal mouse T4 antibody, developed by Dr. Christian Lytle and Dr. Bliss Forbush III, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The antibody specificity for NKCC detection has been characterized extensively (Lytle et al., 1995, Zhang et al., 2006). The T4 antibody was developed against the last 310 residues of the carboxy terminus of the human colonic NKCC1, but is not NKCC-isoform specific and recognizes both NKCC1 and NKCC2. However, in neocortical neurons the T4 antibody is suggested to exclusively recognize NKCC1 (Sun and Murali, 1999) since the expression of NKCC2 is not detected in the brain (Payne and Forbush, 1994; Payne et al., 2003). Following the manufacturer's instructions the T4 antibody was used at a concentration of 330ng/ml (1:100) for Western blots. The T4 antibody gave two bands, one averaged ~145 kDa and a possible dimer at ~250 kDA. In all blots the T4 antibody recognized several nonspecific bands at lower molecular weights as reported elsewhere (Zhang et al., 2006).

For analysis of KCC2 expression we used an affinity purified IgG polyclonal rabbit anti-KCC2 (4 µg/ml; Cat. KCC21-A; Alpha Diagnostic International Inc, San Antonio, TX) directed against an 18 amino acid peptide of rat KCC2 located on the cytoplasmic N-terminus (Nakanishi et al., 2007). Specificity of the antibody has been tested elsewhere by eliminating staining in Western blots by preabsorbtion with the immunizing peptide for KCC2 (Chee et al., 2006). We tested the expression of KCC2 also exemplary with monoclonal mouse anti-KCC2 antibody (1-10 µg/ml; Clone N1/12; UC Davis/NIH NeuroMab Facility, Davis, CA). This antibody is directed against amino acid residues 932-1043 of the intracellular carboxyl terminus of KCC2 and recognition of this sequence has been established elsewhere (Williams et al., 1999; Gulyas et al., 2001; Munoz et al., 2007).

In our experiments both antibodies give rise to identical staining pattern of KCC2 protein on Western blots.

Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with monoclonal mouse anti-GAPDH (1:1000; clone 1D4; Covance, Princeton, NJ) and has been used as a loading control for western blotting experiments.

3.7.2 Blotting procedure

Neuronal cultures were rinsed in phosphate-buffered saline (PBS), and homogenized in ice-cold RIPA lysis buffer (150 mM NaCl; 1% Igepal; 0.5% Sodium deoxycholate (Doc); 0.1% sodium dodecyl sulfate (SDS); in 50 mM TrisHCl, pH 8.0) supplemented with 4% commercial protease inhibitor mixture (Complete; Roche diagnostics GmbH, Mannheim, Germany) and 0,5% phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). Samples of at least five sister cultures were pooled per age and experimental group. Debris was pelleted by centrifugation at 13000rpm for 30 min. Supernatant was denaturated at 95°C for 5min, and the protein concentration of the supernatant was determined using BCA Protein Assay Kit (Pierce by Thermo Fischer Scientific Inc., Waltham, MA, USA). Equal amounts of proteins (20µg) were seperated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean Tetra Cell; Bio-Rad Laboratories Inc., Hercules, CA) and transferred onto nitrocellulose membrane (Optitran BA-S 83; Whatman, Maidstone, United Kingdom, distributed by Whatman, Dassel, Germany) using semi-dry method (EBU-4000; C.B.S. Scientific, Del Mar, CA, USA). Membranes were incubated in blocking solution (5% milk in 0.1M PBS, 1% GNS, 0.1% Tween) for at least 30min at room temperature, washed one time in 0.1M PBS + 0.1% Tween (PBST) and then probed overnight at 4°C with primary antibodies at dilutions indicated above (in 1% milk, 1% GNS, 0.1% Tween in 0.1M PBS). Membranes were washed thoroughly with PBST, incubated for 2 hours at room temperature with horseradish peroxidase linked secondary antibodies polyclonal goat anti-mouse immunoglobulins/HRP (1:10000; Cat. P0447; Dako, Glostrup, Denmark) or polyclonal goat anti-rabbit immunoglobulins/HRP (1:2000; Cat. P0448; Dako) in 0.1M PBS, 1% milk, 1% GNS, 0.1% Tween, and washed again extensively with PBST. Molecular weight markers (PageRuler Unstained Protein Ladder; Fermentas by Thermo Fischer Scientific Inc.) linked to Precision Protein StrepTactin-HRP conjugate (1:10000; Cat. 161-0380; Bio-Rad Laboratories Inc., Hercules, CA) were used to identify molecular

weight of stained protein bands. Detection using enhanced chemiluminescence (ECL) substrate SuperSignal West Dura (Pierce) was carried out with GeneGnome5 (Syngene by Synoptics Ldt, Cambridge, UK), and the intensity of the bands was quantified using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). Intensity values were corrected by corresponding GAPDH levels and normalized to the mean intensity of $T3^{-}$ samples of 7DIV old cultures.

3.8 Statistics

All statistical tests were performed with SigmaStat software (version 2.03, SPSS Inc., Chicago, IL). Differences between T3-treated and T3-deprived cultures were tested using unpaired Student's t-test or Mann-Whitney Rank Sum Test (MW-RST). For comparison of multiple culturing conditions differences were initially tested with one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks followed by *ad hoc* pair wise tests using t-test or Mann-Whitney Rank Sum Test. The proportions of double-labelled synapses in different conditions were compared with the Chi-square test. Cause and effect relationship of axonal parameters was tested using linear regression (LR). The local cell density was set as the independent variable and axonal parameters as dependent variables and the *p*-value and the coefficient of determination (r^2) were calculated. Data are presented as means ± standard error of the mean (SEM). A *p*-value of <0.05 was considered to be statistically significant and alpha value was set to 0.05. Asterisks in graphs show the level of statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

4. Results

4.1 Thyroid hormone modulation of network activity and neuronal populations

In general, embryonic neurons grown in dissociated culture start to develop neuronal processes quickly after plating and form first synapses after 3-4 days *in vitro* (de Lima et al., 1997). With increasing connectivity cultured cortical networks spontaneously develop synchronized rhythmic activity (Muramoto et al., 1993; de Lima et al., 1997; Voigt et al., 2005). This recurrent activity characterized by simultaneous transients of $[Ca^{2+}]_i$ emerges normally at the beginning of the second week in culture (Opitz et al., 2002). During further development the frequency of these spontaneous bursts of activity increases and virtually all neurons become synchronously active (Voigt et al., 1997; Opitz et al., 2002; Baltz et al., 2010). Whereas young cultures show almost large and stereotypic bursts, mature cultures develop highly diverse burst patterns containing network events with variable neuronal participation, spike numbers and durations (Baltz et al., 2010).

4.1.1 T3 modulation of spontaneous network activity

To evaluate the effects of the T3-supplemented medium, sets of two weeks old neocortical cultures (13-14DIV) were examined with Fluo-3 calcium imaging (Fig. 1). The chronic addition of triiodothyronine (T3) to the growth medium, beginning with the time of plating, dramatically increased the frequency of spontaneous bursts of activity (Fig 1 B, D, E; 1.63 ± 0.16 bursts/min in T3-deprived cultures [mean ± SEM, n = 20 fields from 4 cultures/3 preparations] vs. 5.97 ± 0.68 bursts/min in T3-treated cultures [mean ± SEM, n = 15 fields from 3 cultures/3 preparations], $p < 0.001$, MW-RST). The 14-day-old T3-treated cultures also showed a more irregular burst pattern than T3-deprived cultures (Fig. 1 A-D). Additionally to large bursts, in which most cells were synchronously active, hormone-treated cultures showed an excess of smaller bursts, in which only 20 to 40% of all neurons participate (Median = 26.303, n = 390 events). In contrast, in hormone-deprived cultures larger bursts containing 80 to 100% active cells were more frequent (Median = 91.045, n = 73 events, 4 cultures from 4 preparations, $p < 0.001$, MW-RST). Total active cells and maximal attendance per synchronous event were not altered by T3 treatment. Thus, T3-treated cultures show both an increase in events and a more mature pattern of synchronous activity.

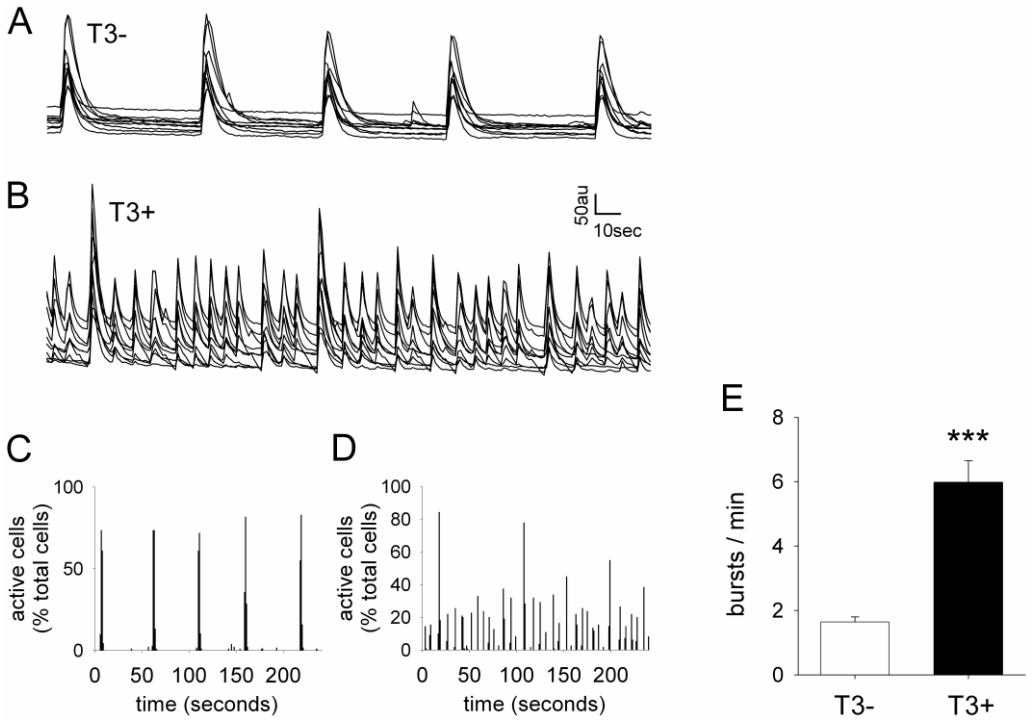

Figure 1: T3 increases frequency of in spontaneous synchronous network activity.
A-B. Traces show the intracellular calcium signal of individual neurons in representative fields from 14DIV old cultures grown either in the absence (A) or presence (B) of triiodothyronine. **C-D.** Activity histograms of neurons from the same fields as shown in A and B, respectively. **E.** Number of synchronous bursts is dramatically enhanced in two-week-old cultures (13-14DIV) treated with T3. Besides changes in burst frequency, T3-stimulated cultures also offered a more irregular burst pattern than T3-deprived cultures. T3-treated cultures show more bursts with 20-40% participation than T3-deprived cultures, which show more larger bursts containing 80-100% participating neurons (A-D).

4.1.2 T3 modulation of GABAergic and glutamatergic synapses

The development of spontaneous network activity in neuronal cell cultures is usually paralleled by a rapid increase in the number of synaptic contacts and the activation of AMPA receptors in the synaptic network (Muramoto et al., 1993; Voigt et al., 2005). Therefore it was tested whether the T3 mediated increase in spontaneous network activity in this experimental setup is also correlated to changes in synaptic density. The relative numbers of functional glutamatergic and GABAergic synapses were quantified by double labelling pre- and postsynaptic components (Fig. 2). Glutamatergic synapses were identified by co-localization of Synapsin/PanShank or Synapsin/GluR2 and GABAergic synapses were identified by co-localization of Synapsin/Gephyrin (Gundelfinger and tom Dieck, 2000; Yu et al., 2007).

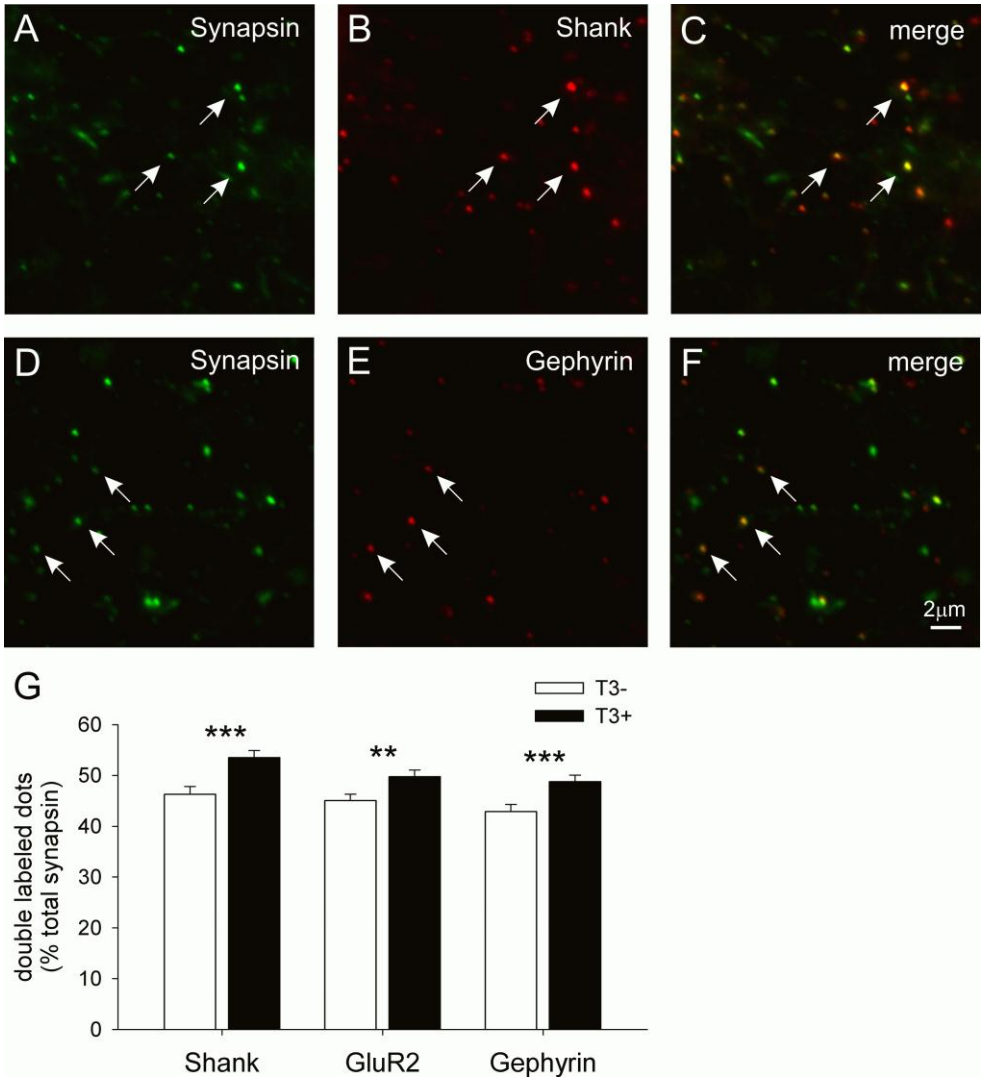

Figure 2: T3 increases the density of glutamatergic and GABAergic synapses.
A-C. Fluorescence images of 14-day-old cultures show Synapsin/PanShank double-labeled glutamatergic boutons. **D-F.** Images of 14-day-old cultures show synapsin/gephyrin double labeled GABAergic synapses. **G.** T3 treatment increased the percentage of Synapsin/GluR2, Synapsin/PanShank and Synapsin/Gephyrin double labeled clusters in 14-day-old cultures (Glutamatergic synapses: data from 6-7 cultures, 2-3 preparations; GABAergic synapses: data from 6 cultures, 2 preparations).

Colocalized pre- and postsynaptic markers in cortical cell cultures showed an even distribution of synaptic contacts (Fig. 2 A-F; see also Voigt et al., 2005). The percentage of double-labelled clusters compared with the total number of synapsin dots was used to quantify synaptic connectivity (Fig. 2 G). Hormone addition to the culture medium increased the percentage of Synapsin/GluR2 (49.73 ± 1.33 % in $T3^+$ vs. 45.06 ± 1.25 % in $T3^-$; mean ± SEM, N = 150 fields, P <0.01, Chi-square test), Synapsin/PanShank (53.46 ± 1.46 % in $T3^+$ vs. 46.25 ± 1.54 % in $T3^-$; mean ± SEM, N = 175 fields, P <0.001, Chi-

square test) and Synapsin/Gephyrin (48.74 ± 1.29 % in $T3^+$ vs. 42.87 ± 1.41 % in $T3^-$; mean ± SEM, N = 150 fields, P <0.001, Chi-square test) double-labelled clusters in 14 day-old cultures. These results suggest an increased growth of glutamatergic and GABAergic synapses in T3-treated cultures.

4.1.3 Impact of T3 enrichment on neuronal morphology

The next question was if the functional changes in network activity are correlated to morphological alterations of neurons. Especially evaluating the maturation of GABAergic interneurons is of special interest, because they develop in a close interrelationship with the spontaneous network activity (Voigt et al., 2001; de Lima et al., 2004, 2007). In neuronal networks developing from dissociated embryonic rat cerebral cortices, two types of GABAergic neurons were described (chapter 2.2.5; see also de Lima and Voigt, 1997; Voigt et al., 2001; de Lima et al., 2004). A prominent type of GABAergic neuron, the L-GABAergic neuron, develops a large cell body, stellate dendritic tree and form long range connections with thick axons (Fig. 3 A, B, arrow). S-GABAergic neurons, a second and numerically larger subpopulation of GABAergic neurons, are later born, have smaller fusiform to bipolar or multipolar cell bodies and very thin axons (Fig. 3 A, B, arrow heads).

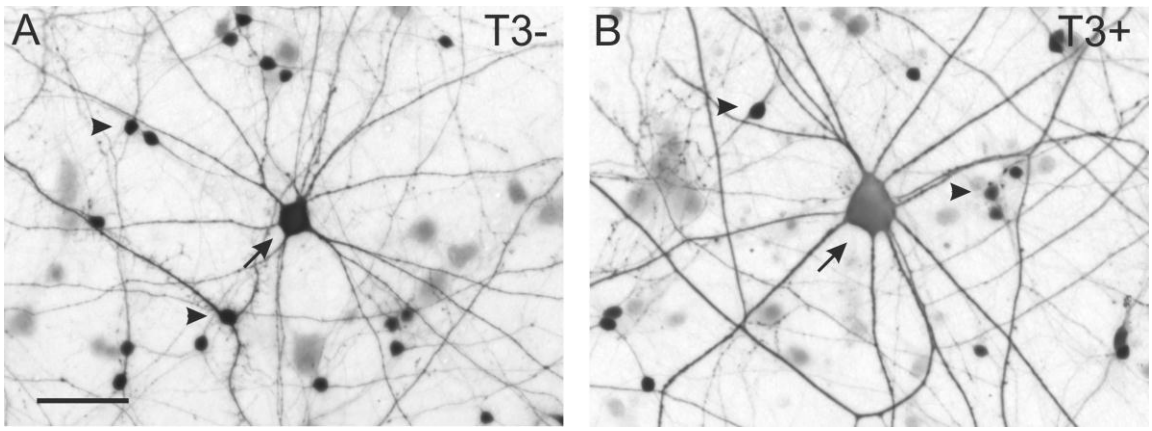

Figure 3: Identification of neuronal subpopulations in neuronal cultures.
A-B. Images show GABA immunostained 14-day-old cultures grown in the absence (A) or presence (B) of T3. Each image shows a large (L-)GABAergic neuron (arrow) among smaller (S-)GABAergic neurons (arrowheads) and unstained non-GABAergic neurons. Note that large L-GABAergic neurons are hypertrophic in the T3-enriched cultures compared with T3-deprived cultures. Scale bar = 50 µm.

To evaluate the impact of T3 supplement on their morphological maturation, the density and the soma sizes of L-GABAergic interneurons, S-GABAergic interneurons and, for complementation, non-GABAergic neurons were assessed in 14-day-old GABA-immunostained cultures (Fig. 4).

The relative proportion of all GABAergic neurons is increased in T3-treated cultures (Fig. 4 G; 13.1 ± 0.81 % in $T3^+$ compared with 7.99 ± 0.60 % in $T3^-$; $p < 0.001$, MW-RST). The relative increase is due to a simultaneous decrease of the non-GABAergic population (Fig. 4 C; 674.83 ± 26.68 cells/mm² in $T3^+$ compared with 1059.03 ± 43.45 cells/mm² in $T3^-$; $n = 100$ and $n = 110$ fields respectively, $p < 0.001$, MW-RST). The density of L-GABAergic neurons remained constant between T3-treated and T3-deprived cultures (Fig. 4 A; 5.30 ± 0.44 cells/mm² in $T3^+$ compared with 5.60 ± 0.39 cells/mm² in $T3^-$, $n = 100$ and $n = 110$ fields respectively, $p = 0.386$, MW-RST), but S-GABAergic neurons' density increased significantly (Fig. 4 B; 86.18 ± 5.28 cells/mm² in $T3^+$ compared with 68.52 ± 4.46 cells/mm² in $T3^-$; $n = 100$ and $n = 110$ fields, respectively, $p = 0.01$, MW-RST; data in figure 4 were obtained from 10 T3-deprived and 11 T3-treated cultures from five preparations).

The sizes of neuronal somata in GABAergic and non-GABAergic subpopulations provide an additional measure of the overall growth and differentiation of these neurons in culture (Fig. 4 D-F, H). The comparison of T3-treated cultures with T3-deprived cultures showed a large increase in the soma size of L-GABAergic cells (Fig. 4 D; 311.51 ± 7.53 μm² in $T3^+$ vs. 207.78 ± 6.03 μm² in $T3^-$; $n = 210$ neurons for each set, $p < 0.001$, MW-RST). In contrast, S-GABAergic cells showed a slight decrease in cell size (Fig. 4 E; 46.31 ± 0.51 μm² in $T3^+$ vs. 47.83 ± 0.56 μm² in $T3^-$; $n = 244$ and $n = 273$ neurons respectively, $p = 0.028$, MW-RST). Also an increase but to a much lesser extent was measured in case of non-GABAergic neurons (Fig. 4 F; 119.14 ± 3.02 μm² in $T3^+$ vs. 108.45 ± 2.46 μm² in $T3^-$; $n = 335$ and $n = 404$ neurons respectively, $p = 0.001$, MW-RST). Note that the density of L-GABA was not changed (Fig. 4 A) suggesting that the soma size increase is due to cell growth and not caused by elimination of smaller cells. However, soma size alterations of subpopulations of S-GABAergic neurons and non-GABAergic neurons could be consequence of alterations of population density, i.e., the elimination of smaller non-GABAergic neurons (Fig. 4 C) or the preservation of more immature and smaller S-GABAergic neurons (Fig. 4 B).

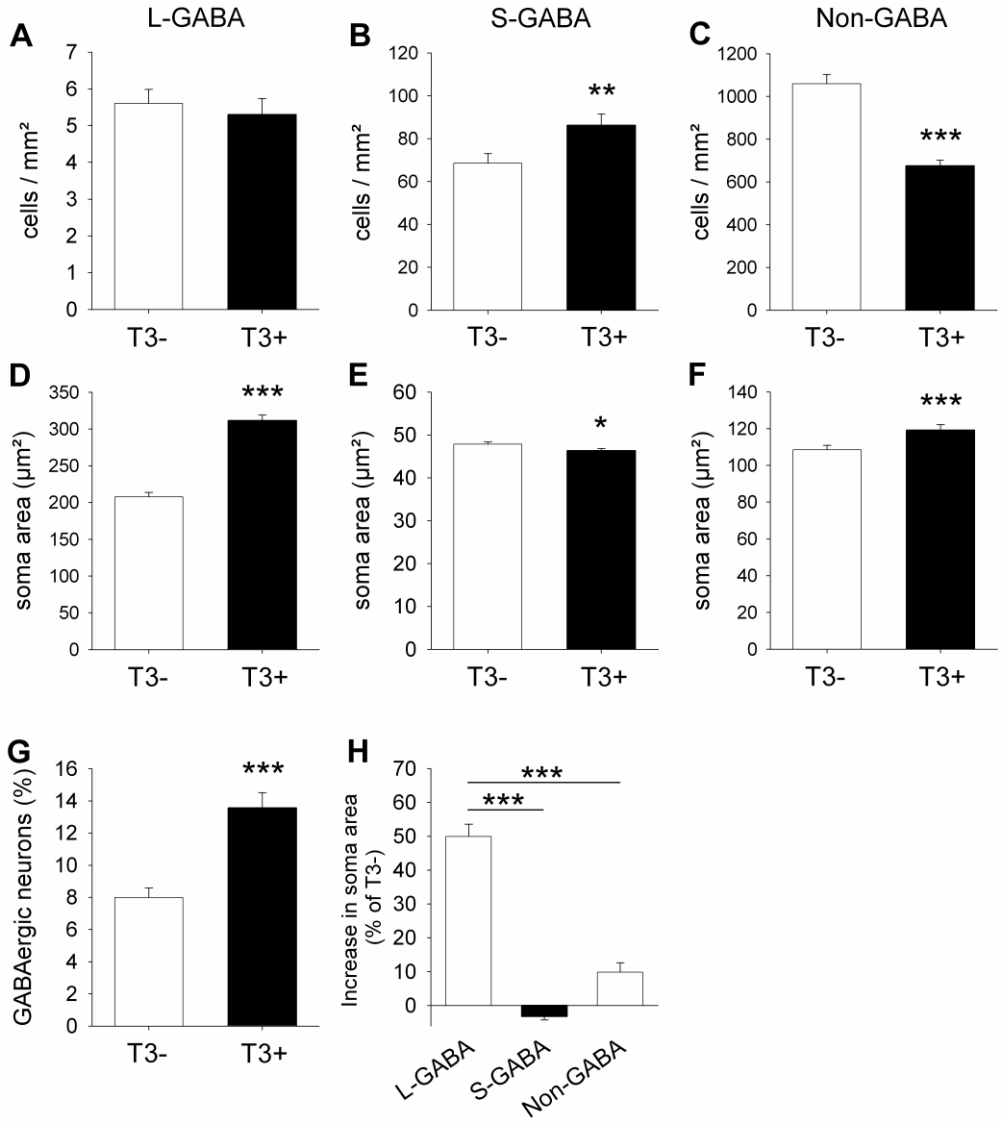

Figure 4: Influence of T3 on the density and morphology of neuronal subpopulations.
A-B. The quantification of neuronal density shows no significant change of L-GABAergic neurons density (A) and a small increase in S-GABAergic neurons density (B) in T3-treated cultures. In contrast, the density of non-GABAergic neurons decreased in T3-treated cultures (C). **D-F.** The soma size of L-GABAergic neurons increased significantly in T3-treated cultures (D). The other subpopulations showed smaller relative changes of soma size (H): The size of S-GABAergic somata decreased slightly (**E**), and non-GABAergic neurons' size showed a small increase (F). **G**. As a consequence of T3 treatment, the relative proportion of the total GABAergic neurons increased significantly. **A-H.** Data from 2-3 cultures per experimental set from each of five preparations. *n* =160, 7DIV; *n* = 170, 14DIV; 2-3 cultures from each of three preparations.

Thus, T3 modulation specifically alters the density of the neuronal subpopulations, resulting in an increase of the relative density of GABAergic neurons. Moreover, notable growth of L-GABAergic neurons occurs in the presence of T3 modulation. As shown before, these cellular alterations are paralleled by an increase in the frequency of

spontaneous network activity and the overall density of functional glutamatergic and GABAergic synapses.

4.2 Mechanisms of T3-mediated effects

4.2.1 Triiodothyronine actions on GABAergic neurons and network activity

Since in the original publication by Romijn et al.(1981) the culture medium of the experiments above contained in addition to the thyroid hormone T3 also corticosterone. Because corticosterone was suggested to affect growth and spontaneous activity in cultures (Romijn et al., 1981) and alter cortical circuits' development (Stone et al., 2001; Leret et al., 2007; Lupien et al., 2009), a control experiment was performed to look for the effects of this hormone separately, and for possible synergistic actions with T3. Hormones were added to the culture medium, each alone or in combination, during the second week in vitro and synchronous network activity and soma sizes of L-GABAergic neurons were measured (Table 1, Fig. 5). The addition of T3 to the culture medium, alone or in combination with corticosterone, significantly increased the burst frequency and soma area of L-GABAergic cells (Fig. 5 C-F). Cultures treated with corticosterone alone neither showed significant increase in burst frequency nor soma enlargement of L-GABAergic cells (Fig. 5 B, E, F). No synergistic effects were apparent, when corticosterone was applied together with T3 (Fig. 5 D, E, F). Thus the results from this set of experiments suggest that hormone-mediated increases in burst frequency and growth of L-GABAergic neurons were mediated by T3. This result concurs with previous studies showing that T3 influence neuronal electrical activity in cortical cultures (Romijn et al., 1981; Hosoda et al., 2003). Whereas corticosterone can rapidly modulate excitatory (Karst and Joels, 2005; Olijslagers et al., 2008) and inhibitory postsynaptic currents (Maggio and Segal, 2009), no changes were observed in the slow early network activity after chronic corticosterone treatment in 14DIV old cultures. This does not exclude, however, that corticosterone has other, more transient effects, either in other aspects or in later stages of network development not considered here.

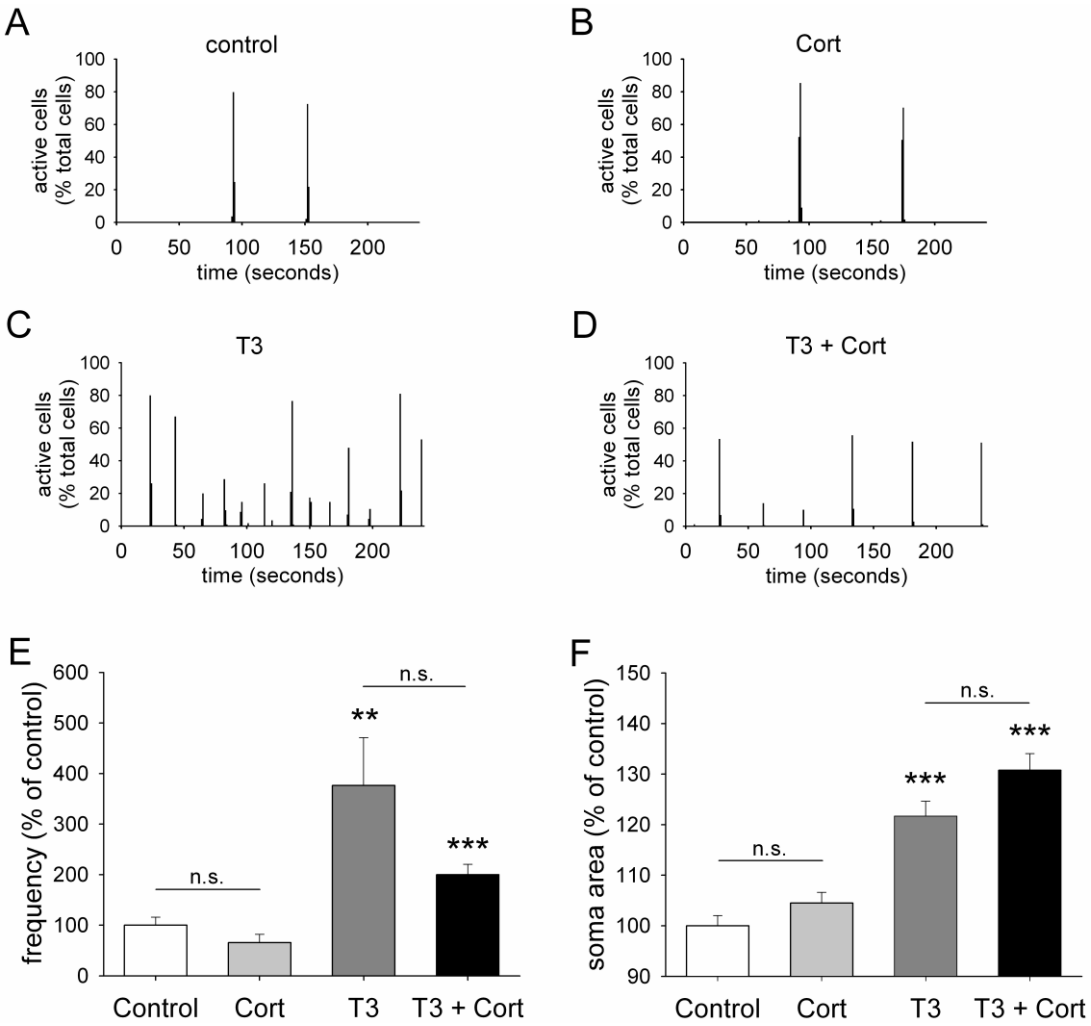

Figure 5: T3, but not corticosterone, modulates burst frequency and neuron size.
A-D. Time histograms show the network activity profiles of neurons from representative 14-day-old cultures either without hormones (A), in the presence of corticosterone (B), T3 (C) or both hormones (D). **E.** Increase in spontaneous network activity could be observed after culturing neurons in triiodothyronine alone or in combination with corticosterone. In contrast, treatment with corticosterone alone does not affect burst frequency. **F**. T3 treatment alone increased the soma area of L-GABAergic neurons. Graphs in E and F show normalized burst frequency and soma size relative to the mean of hormone-deprived cultures (see also Table 1).

Table 1: T3 and corticosterone effects on network burst frequency and soma size of L-GABAergic neurons.

	Burst frequency (bursts/min)			L-GABA size (µm²)		
	Mean±SEM	N[#]	*P*	Mean±SEM	N[§]	*P*
Deprived	0.45±0.07	10		164±3	120	
T3	1.69±0.42	9	0.002	200±5	100	<0.001
Cort	0.30±0.07	11	0.154	172±4	120	0.141
T3 + Cort	0.90±0.09	10	<0.001	219±4	120	<0.001

N*, number of recorded fields from 2 cultures, 2 preparations; N°, number of neurons from 5-6 cultures, 2 preparations respectively. *P* indicates result of t-test (burst frequencies) or MW-RST (soma sizes) compared with hormone-deprived cultures. Cort, corticosterone.

4.2.2 Thyroid hormone concentrations under experimental conditions

In line with other in vitro studies showing physiological effects of T3 at concentrations between 5 and 30nM (Romijn et al., 1981; Potthoff and Dietzel, 1997; Hosoda et al., 2003; Hoffmann and Dietzel, 2004; Morte et al., 2010), the concentration of T3 applied in the experiments described so far was 15nM. Compared with the plasma concentration of T3 in vivo, and disregarding any degradation during the culture time, the dose used in the cultures is apparently in a supraphysiological level. In young rats, total T3 serum concentrations of ~1 nM and free T3 of 1 to 6 pM have been reported (Hulbert, 2000). However, the cerebral cortex derives most of its T3 from local deiodination of T4 (total plasma concentration 60 nM in young rodents). Moreover cells can regulate their T3 content by means of active transport (Hulbert, 2000). Thus, defining the exact concentration of active T3 acting locally on cortical neurons is difficult. Although cell cultures mimic the intact cerebral cortex with respect to the metabolism of thyroid hormones and the regulatory mechanisms that control T3 levels, the more vigorous metabolism in vitro can rapidly degrade T3, dramatically reducing the initial dose after 24 hours (Leonard and Larsen, 1985).

The aim of the next experiment was to test if the observed alterations in both soma sizes of GABAergic neurons and frequency of spontaneous network activity by T3 are dose-dependent and if they are detectable at concentrations of T3 comparable to the physiological plasma concentration. Cultures were treated with different concentrations of T3 (range 10^{-13} to 10^{-8} M) for 14DIV (Table 2 A). Compared with T3-deprived cultures, the network activity (Fig. 6 A) and the soma area (Fig. 6 B) significantly increased in cultures with T3 concentrations above 10^{-12}M, and statistical significance increased with increasing concentrations. In one additional set of experiments, the effects of T3 from 10^{-12} up to 10^{-6} M were tested. As before, with T3 concentrations above 10^{-10} M no further increase or decrease in burst frequency and soma area of L-GABAergic cells was observed (Table 2 B). These results show that concentrations of T3 comparable with free plasma T3 concentration (10^{-12} M) are sufficient to elicit changes in early network activity and GABAergic neurons growth. Maximal changes were seen with concentrations $>10^{-10}$ M. Since higher T3 concentrations yielded more consistent results without noticeable side effects T3 was also used in the following experiments in the initial concentration of 15nM.

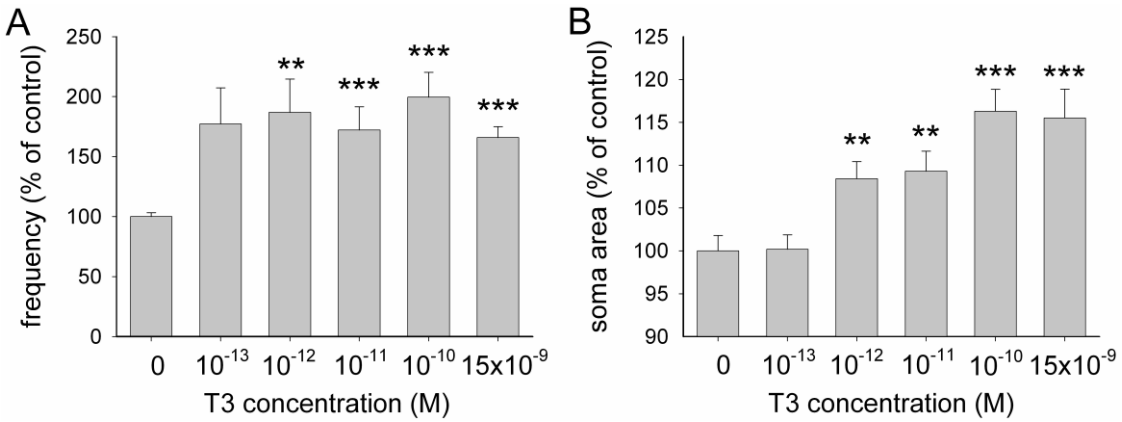

Figure 6: Dose-response curve of T3 actions on burst frequency and GABAergic neurons size.
A, B. Cultures were treated with different concentrations of T3 for 14 days (0-14DIV). A significant increase in burst frequency (A) and soma area of L-GABAergic neurons (B) was evident already at a concentration as low as 10^{-12} M T3. Graphs show normalized burst frequency and soma size relative to the mean of hormone-deprived cultures.

Table 2: Dose response curve of T3-mediated effects.

	Normalized burst frequency			Normalized L-GABA size		
	Mean ± SEM	N#	*P*	Mean ± SEM	N§	*P*
A						
0 M	100±3	15		100±2	120	
10^{-13} M	177±30	15	0.051	100±2	120	0.939
10^{-12} M	186±28	15	0.004	108±2	119	0.002
10^{-11} M	172±19	15	<0.001	109±2	124	0.006
10^{-10} M	200±21	15	<0.001	116±3	122	<0.001
$15x10^{-9}$ M	166±9	15	<0.001	115±3	80	<0.001
B						
0 M	100±11	17		100±2	92	
10^{-12} M	301±52	17	<0.001	108±3	90	0.028
10^{-9} M	388±99	17	<0.001	118±3	90	<0.001
$3x10^{-8}$ M	402±44	17	<0.001	117±4	87	<0.001
10^{-6} M	375±66	17	<0.001	114±3	89	<0.001

N#, number of recorded fields from (A) 3 cultures, 2 preparations and (B) 2 cultures, 1 preparation; N§, number of neurons from (A) 2-6 cultures, 2 preparations and (B) 3 cultures, 1 preparation. *P* indicates comparison against T3-deprived cultures (0M, MW-RST).

4.2.3 No acute effects of T3 on network activity

Beside its role in gene regulation, T3 shows fast non-genomic actions and is hypothesized to interact with transmitter receptors (Farwell et al., 2006; Zamoner et al., 2006; Losi et al., 2008). Therefore it was probed whether T3 changes spontaneous network activity when applied acutely (Fig. 7 A). The acute effect of T3 on burst activity was tested

in cultures grown in either hormone-free medium (Fig. 7 B, white bars) or hormone-supplemented medium for 14-21 days (Fig. 7 B, grey bars; 20 fields, 5 fields from each of 4 cultures, 2 preparations). Before the addition of T3 five fields were imaged in hormone-free ringer solution (Fig. 7 B, open bars). These recordings showed the already described significant increase in burst frequency after long time treatment with T3 compared with T3-deprived cultures. No significant change in burst frequency was observed after acute addition of T3 (10 ng/ml; Fig. 7 B, dashed bars; T3-deprived cultures in T3-free ringer solution: 4.5 ± 0.41 burst/min, in the presence of acute T3: 4.04 ± 0.35 bursts /min, p = 0.395, t-test; T3-treated culture in T3-free ringer solution: 14.31 ± 1.69 bursts /min, in the presence of T3: 11.54 ± 1.4 bursts /min, p = 0.208, MW-RST, mean ± SEM). This set of experiments demonstrate that T3 significantly increases burst frequency when applied over a period of days, but has no effect when applied for minutes.

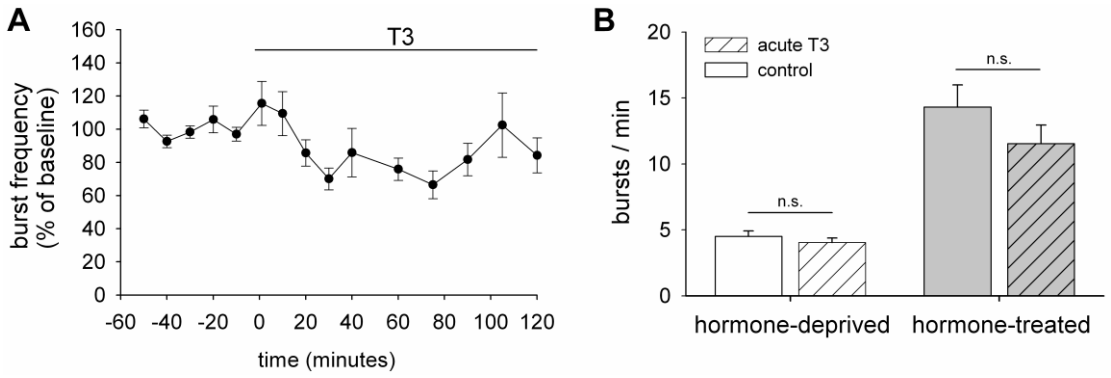

Figure 7: No acute burst frequency modulation after T3 stimulation.
A. Time histogram showing the mean burst frequency of recorded fields before and during the application of T3 to the recording solution. **B.** Burst frequency was not altered by acute presence of T3 in the imaging solution. White bars (left side): cultures were grown in T3-free medium; grey bars (right side): cultures were grown in T3-supplemented medium. Imaging was done in T3-free ringer solution (open bars) and repeated after the addition of T3 to the recording solution (dashed bars). Recordings were made in 14 to 21-day-old cultures; n = 20 fields from 4 cultures, 2 preparations.

4.2.4 Blockade of nuclear receptor signalling by TR antagonist 1-850

The lack of short-term effects of T3 concurs with the idea of a slow, nuclear receptor mediated action. To test this, cultures were treated chronically with the thyroid hormone receptor (TR) antagonist 1-850 (1µM) in the absence or presence of T3 (Fig. 8). As expected, T3 significantly increased the burst frequency (Fig. 8 A, $T3^-$: 2.22 ± 0.20 bursts/min; $T3^+$: 7.86 ± 0.67 bursts/min, mean ± SEM, n = 20 fields, 4 cultures, 2

preparations, $p < 0.001$, MW-RST) and the soma size of L-GABAergic interneurons (Fig. 8 B, $T3^-$: 325.56 ± 6.16 µm^2, $T3^+$: 386.70 ± 7.15 µm^2, mean ± SEM, n = 120 neurons, 6 cultures, 2 preparations, $p < 0.001$, t-test). Addition of T3 receptor antagonist 1-850 significantly diminished the T3-mediated increase in burst frequency (Fig. 8 A, $T3^+$/1-850: 3.24 ± 0.32 bursts/min, $p = 0.01$, t-test, compared with $T3^-$) and prevented the increase in soma size of L-GABAergic neurons (Fig. 8 B, $T3^+$/1-850: 332.84 ± 6.38 µm^2, $p = 0.413$, t-test, compared with $T3^-$). In hormone-deprived cultures T3 receptor antagonist 1-850 only slightly decreased both network activity and soma size (Fig. 8 A, $T3^-$/1-850: 1.75 ± 0.11 bursts/min, $p < 0.041$, t-test, compared with $T3^-$; Fig. 8 B, $T3^-$/1-850: 294.04 ± 5.33 µm^2, $p < 0.001$, t-test, compared with $T3^-$). These results corroborate that T3-induced alterations in burst frequency and neuron morphology are, at least in part, mediated by nuclear thyroid hormone receptors.

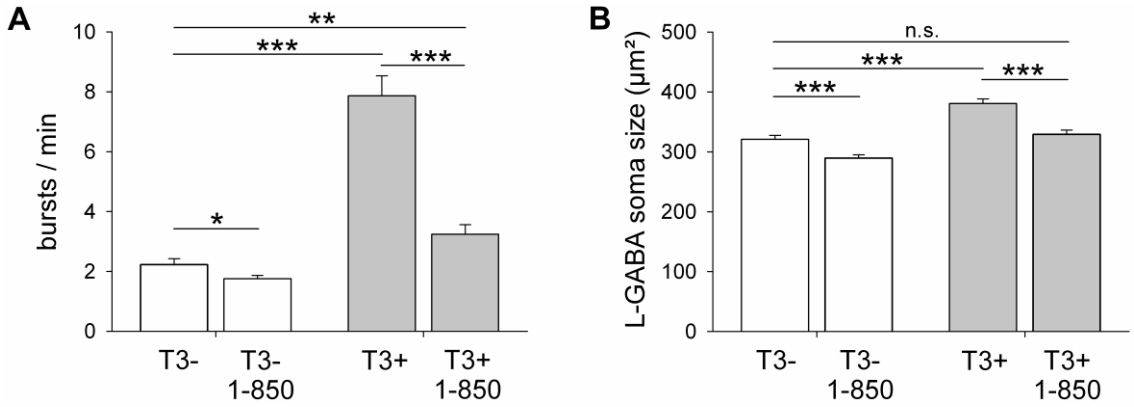

Figure 8: Modulation of T3-mediated actions by thyroid hormone receptor antagonist 1-850.
A, B. Chronic treatment with the thyroid hormone receptor antagonist 1-850 significantly diminished the T3-mediated increase in burst frequency (A) and prevented the increase in L-GABAergic soma size (B).

4.2.5 Sequence of T3-mediated soma enlargement and burst frequency increase

The initial experiments have shown that synchronous network activity enhancement parallels the increased growth of L-GABAergic neurons by thyroid hormone at 14DIV (chapters 4.1.1, 4.1.3). The next question was if hormonal modulation of L-GABAergic interneurons' growth occurs before spontaneous network activity develops. This is relevant because L-GABAergic interneurons play a role in the emergence of spontaneous network activity (Voigt et al., 2001). On the other hand, the maturation of GABAergic neurons is activity-dependent (Patz et al., 2004; Jin et al., 2003; Chattopadhyaya et al., 2004; de Lima

et al., 2004). So it is also possible that GABAergic maturation is not the cause but rather the effect of activity enhancement by T3 stimulation.

Hormone treatment during the first week did not result in an increase of spontaneous activity, suggesting that the regulation of activity was mainly due to the presence of T3 during the second week (Fig. 9 A; $T3^-$ at 7DIV: 0.86 ± 0.23 bursts/min, mean ± SEM, n = 14 fields; $T3^+$ at 7DIV: 0.93 ± 0.20 burst/min, n = 19 fields, $p < 0.804$, t-test; $T3^-$ at 14DIV: 1.93 ± 0.19 bursts/min, n = 15 fields; $T3^+$ at 14DIV: 3.73 ± 0.39, n = 13 fields, $p < 0.001$, t-test; 3-4 cultures, 3 preparations per experimental set and time point). But already in 7-day-old T3-treated cultures the mean soma size of L-GABAergic neurons was larger compared to age matched T3-deprived cultures (Fig. 9 B; 153.71 ± 2.39 μm^2 versus 139.18 ± 2.39 μm^2 respectively; mean ± SEM, n = 160 neurons per set, $p <$ 0.001, t-test). As expected, T3-treated sister cultures (cultures derived from the same preparations; 7-8 cultures, 3 preparations for each condition) allowed to grow until 14 DIV showed a much larger increase in soma size (291.73 ± 7.81 μm^2 in $T3^+$ compared with 181.29 ± 6.23 μm^2 in $T3^-$; mean ± SEM, p = 170 neurons per set, $p < 0.001$, MW-RST). Thus, the difference of L-GABAergic soma size between treated and untreated cultures increased significantly from 10% after one week treatment to more than 61% after two weeks treatment ($p < 0.001$, MW-RST; Fig. 9 B). Apparently this increase in soma size precedes any increase in burst frequency.

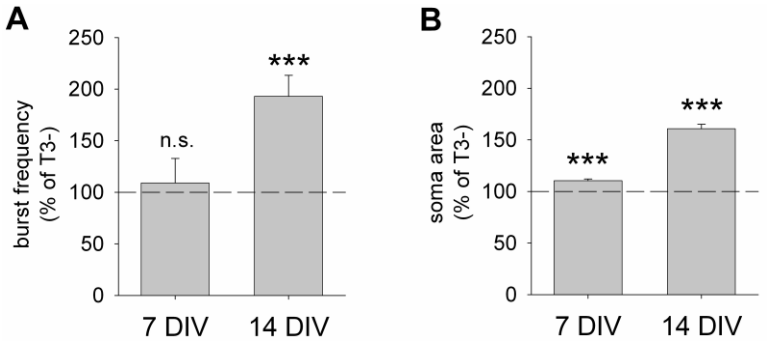

Figure 9: Sequence of T3-mediated neuronal growth and burst frequency modulation.
A. T3 treatment during the first week in vitro did not change the frequency of spontaneous activity, in contrast with the significant increase in burst frequency after two weeks of treatment. **B**. Measures of L-GABAergic neurons sizes in 7-day-old cultures reveal a small but significant increase in T3-treated cultures. After two weeks in vitro, L-GABA neurons in T3-treated cultures were considerably larger than in T3-deprived cultures. Asterisks show significance compared with T3-deprived cultures.

4.2.6 Activity-dependence of L-GABAergic neurons growth

To test if the morphological alterations of L-GABAergic and non-GABAergic neurons are independent from the T3-mediated increase of network activity, it was probed whether antagonists to receptors and channels mediating network activity interfere with the morphological effects of T3 treatment. Sodium channel blocker TTX or glutamate receptor (GluR) blockers APV and CNQX were added to the culture medium during the second week in vitro to silence neuronal activity (7-14DIV; Fig. 10, Table 3) (Opitz et al., 2002; de Lima et al., 2004).

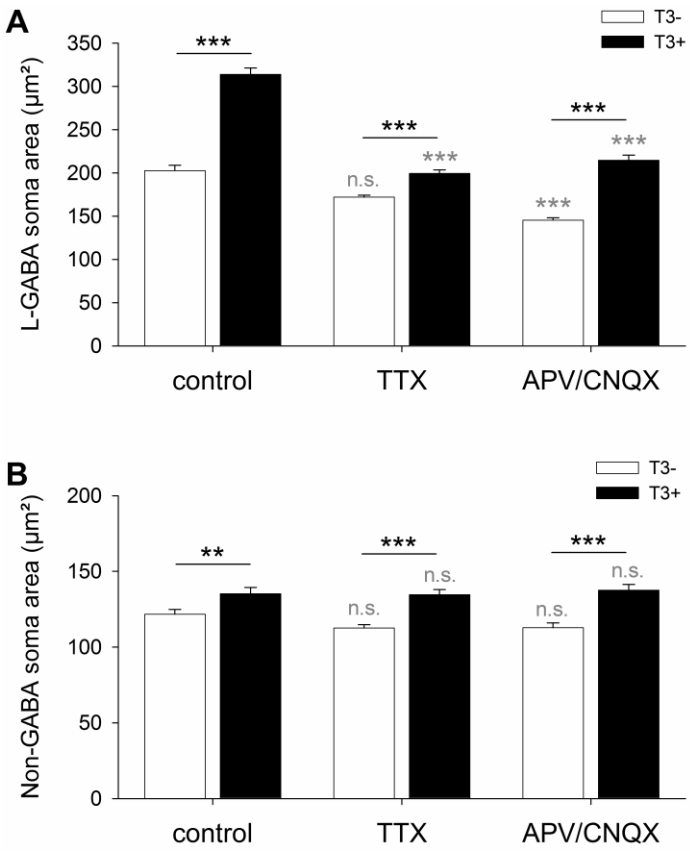

Figure 10: Neuronal activity modulation of T3-mediated growth of L-GABAergic neurons.
A. The blockade of network activity with Na^+ channel blocker TTX or glutamate receptor blockers APV and CNQX during the second week in vitro diminished the soma size of L-GABAergic neurons. However, T3-mediated enlargement of L-GABA soma size occurs also under a chronic activity blockade. L-GABAergic neurons in T3-treated cultures were larger than those in T3-deprived. **B**. Similarly, non-GABAergic neurons were also larger in T3-treated cultures than in T3-deprived cultures when network activity was blocked by TTX or APV/CNQX. In contrast to L-GABAergic neurons, which showed decreased cell size under activity blockade with APV/CNQX in T3-treated and T3-untreated cultures, non-GABAergic neurons' size was not affected by activity blockade. Statistics shown in grey indicate comparison of experimental groups with untreated control cultures of the same hormone group.

The blockade of network activity with TTX or with APV/CNQX significantly diminished the soma size of L-GABAergic neurons in T3-supplemented cultures (Table 3, Fig. 10 A). In T3-deprived cultures, the blockade of network activity with GluR antagonists, but not with TTX, resulted in a significant decrease of L-GABAergic neurons size (Table 3, Fig. 10 A, white bars). These results indicate that neuronal activity modulates the growth of L-GABAergic neurons. However, when network activity was blocked, T3-treated L-GABAergic neurons were also significantly larger than in T3-untreated cultures, suggesting that at least some T3-induced growth occur independently of the activity mediated modulation (Table 3, Fig. 10 A). L-GABAergic neurons developed maximally when both factors, neuronal activity and T3, were present.

Because also non-GABAergic cells showed a small T3-induced increase in soma area (see Fig. 4 F), additionally the soma size of non-GABAergic neurons was measured in cultures treated with TTX or APV/CNQX (Table 3, Fig. 10 B). Similar to L-GABAergic cells, when network activity was blocked by TTX or APV/CNQX, non-GABAergic neurons were larger in T3-treated than in T3-untreated cultures. In contrast to L-GABAergic neurons, the soma size of non-GABAergic neurons was not affected by the activity blockade itself, neither in T3-supplemented nor in T3-deprived cultures (Table 3, Fig. 10 B).

Table 3: Morphometry of L-GABAergic and non-GABAergic neurons in activity blockade experiments

	Hormone deprived (T3-)			Hormone-treated (T3+)			
	Soma area (µm²) Mean ± SEM	N	*P*	Soma area (µm²) Mean ± SEM	N	*P*	*P**
L-GABA neurons							
Drug control	203±6	212		314±7	210		<0.001
TTX	172±2	213	0.218	199±4	211	<0.001	<0.001
APV/CNQX	145±3	190	<0.001	215±6	181	<0.001	<0.001
Non-GABA neurons							
Drug control	122±3	282		135±4	215		0.009
TTX	113±2	303	0.193	135±3	276	0.633	<0.001
APV/CNQX	113±3	187	0.291	138±4	172	0.282	<0.001

N indicates the number of analysed L-GABA neurons (from 7-10 cultures, 4 preparations) and number of Non-GABA neurons (from 3-4 cultures, 2 preparations). *P* refers to the comparison between antagonists treated and untreated neurons; *P** indicates comparison between T3-treated neurons and T3-deprived neurons (MW-RST).

These experiments show first that independent of spontaneous activity, hormones acted as neurotrophic factors for the subpopulations of L-GABAergic and non-GABAergic neurons, and second that the activity blockade dramatically and specifically decreased the T3-mediated L-GABAergic neuronal growth.

4.3 Focus on L-GABAergic interneurons

L-GABAergic interneurons showed enlarged soma size in T3-treated cultures (see Fig. 4 D). The results above suggest that T3 modulation of interneuronal growth is, at least partly, independent from and precedes the increase in spontaneous network activity (see Figs. 9, 10). The morphological properties of L-GABAergic neurons suggest that these cells could synchronize neuronal activity in the network by collecting randomly generated activity and distributing it throughout the network of developing neurons (de Lima and Voigt, 1997; Voigt et al., 2001). Already at a time point when synchronous activity can be first observed in cultured cortical networks (7-8DIV), L-GABAergic neurons show extended axonal arborization that covers a surface of 0.5 mm^2 (Voigt et al., 2001; Opitz et al., 2002).

For further exploration of the hormonal modulation of L-GABAergic growth, low density cultures were generated (Figs. 11 - 14), which contain non-GABAergic and L-GABAergic cells (Fig. 11 A, C), but are virtually free of S-GABAergic interneurons (Fig. 11 B; 1.56 ± 0.73 cells/mm² in $T3^-$ and 1.30 ± 0.73 cells/mm² in $T3^+$). Thus, in these cultures nearly all GABAergic structures visualized by GABA immunocytochemistry originate from L-GABAergic cells (Figs. 12 - 14). Nevertheless, these cultures possess all typical alterations seen after hormone treatment in normal cultures. The density of non-GABAergic neurons was also reduced in T3-treated two-week-old (15DIV) low density cultures (161.71.20 ± 13.43 cells/mm² in $T3^+$ compared with 205. 77 ± 17.69 cells/mm² in $T3^-$, p = 0.008, t-test, mean ± SEM, Fig. 11 C), and the number of L-GABAergic cells remained unchanged (4.94 ± 0.88 cells/mm² in $T3^+$ compared with 7.02 ± 1.41 cells/mm² in $T3^-$, p = 0.12, MW-RST, mean ± SEM, Fig. 11 A). Additionally, as expected, L-GABAergic cells chronically treated with T3 showed increased soma size compared with T3-deprived cultures (Fig. 14 A: 320.33 ± 7.68 µm² in $T3^+$ compared with 277.04 ± 8.77 µm² in $T3^-$; $p < 0.001$, MW-RST, mean ± SEM, n = 80 cells from four cultures, 2 preparations).

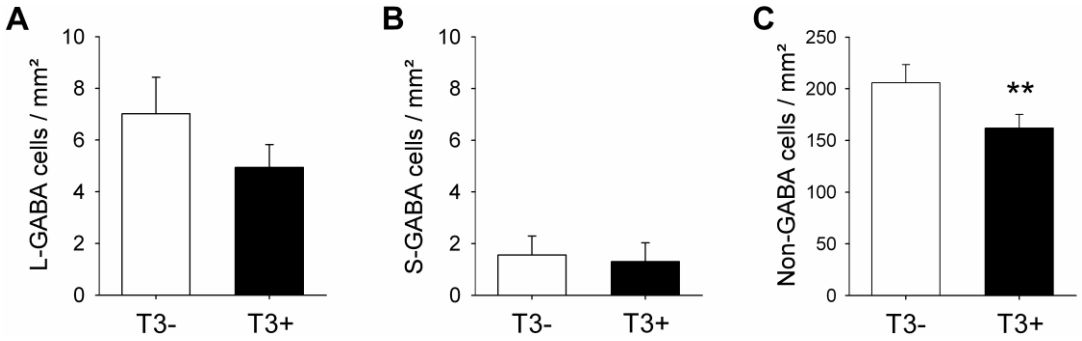

Figure 11: Neuronal population analysis of low-density cultures.
A-C. Low-density cultures contain reduced numbers of non-GABAergic (C) and L-GABAergic cells (A) and are virtually free of S-GABAergic interneurons (B). The density of non-GABAergic neurons was reduced in two-week-old (15DIV) T3-treated low-density cultures (C) compared with T3-untreated cultures, whereas the number of L-GABAergic cells remained unchanged (A).

4.3.1 Dendritic development of L-GABAergic neurons in low density cultures

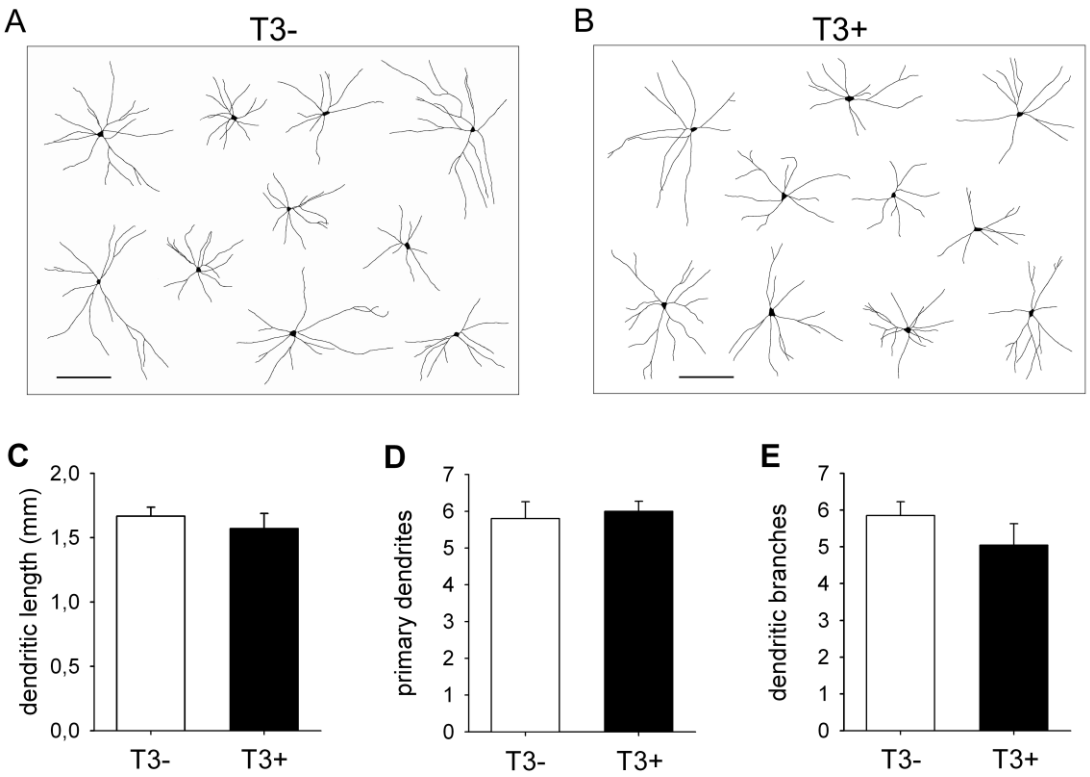

Figure 12: Dendritic growth of L-GABAergic neurons is independent of T3-treatment.
A-B. Representative individual drawings show the dendritic profiles of L-GABAergic neurons from T3-deprived (A) and T3-treated (B) 15-day-old low-density cultures. Scale bar: 200 µm. **C-E.** Neither the total dendritic length of L-GABAergic neurons (C), nor the number of primary dendrites per cell (D) or dendritic branches (E) were changed after T3 treatment.

Besides the quantification of soma area, we measured total dendritic length, the number of primary dendrites, the number of dendritic branches (Fig. 12) and, as an indicator for axonal innervation, the number of GABAergic boutons opposed to unstained non-GABAergic cells (Figs. 13, 14). Neither the total dendritic length (1570.76 ± 117.24 µm in $T3^+$ compared with 1667.01 ± 69.85 µm in $T3^-$, $p = 0.628$, t-test, mean ± SEM) nor the number of primary dendrites per cell (6.0 ± 0.27 in $T3^+$ compared with 5.80 ± 0.46 in $T3^-$, $p = 0.617$, MW-RST, mean ± SEM) nor dendritic branches (5.05 ± 0.58 per cell in $T3^+$ compared with 5.85 ± 0.38 in $T3^-$, $p = 0.318$, t-test, mean ± SEM) of L-GABAergic neurons ($n = 20$ analyzed cells from 4 cultures, 2 preparations) were changed after T3 stimulation (Fig. 14). Changes were observed however in the fine ramifications of L-GABA axons and in the number of GABAergic boutons in the vicinity of non-GABAergic neurons (Fig. 13).

4.3.2 Axonal development of L-GABAergic neurons in low density cultures

L-GABAergic cells typically form long, highly branched axons forming conspicuous baskets and numerous boutons in the vicinity of neuronal cell bodies (de Lima and Voigt, 1997; Voigt et al., 2001). Along their long, richly ramified axons L-GABAergic neurons develop fine axonal arborizations that form putative synaptic boutons at the cell bodies and proximal dendritic regions of non-GABAergic neurons. Electronmicroscopic analysis had previously shown that these varicosities are synaptic contact sites (Voigt et al., 2001). The generation of prominent GABAergic boutons is characteristic for selected subtypes of GABAergic interneurons and proceeds in vivo (Karube et al., 2004) as well as in organotypic culture (Klostermann and Wahle, 1999; Di Cristo et al., 2004; Chattopadhyaya et al., 2004) implying that the occurrence of this characteristic axonal feature is intrinsic to neurons and genetically determined.

While in T3-enriched cultures the axons formed richly arborized nests, including many boutons around small groups of cell somata (Fig. 13 B, D, F), these nests failed to develop in T3-deprived cultures (Fig. 13 A, C, E). This difference was confirmed by the quantification of GABAergic boutons density. The number of GABAergic boutons opposed to non-GABAergic neurons is significantly increased in T3-treated cultures (Fig. 14 B: 14.63 ± 0.52 boutons/cell in $T3^+$ compared with 9.45 ± 0.43 boutons/cell in $T3^-$, $p < 0.001$, MW-RST, mean ± SEM, $n = 80$ fields from 4 cultures, 2 preparations). These

results show that the increased L-GABAergic cell body size mirrors the increased axonal arborization and the increased density in GABAergic boutons in T3-treated cultures.

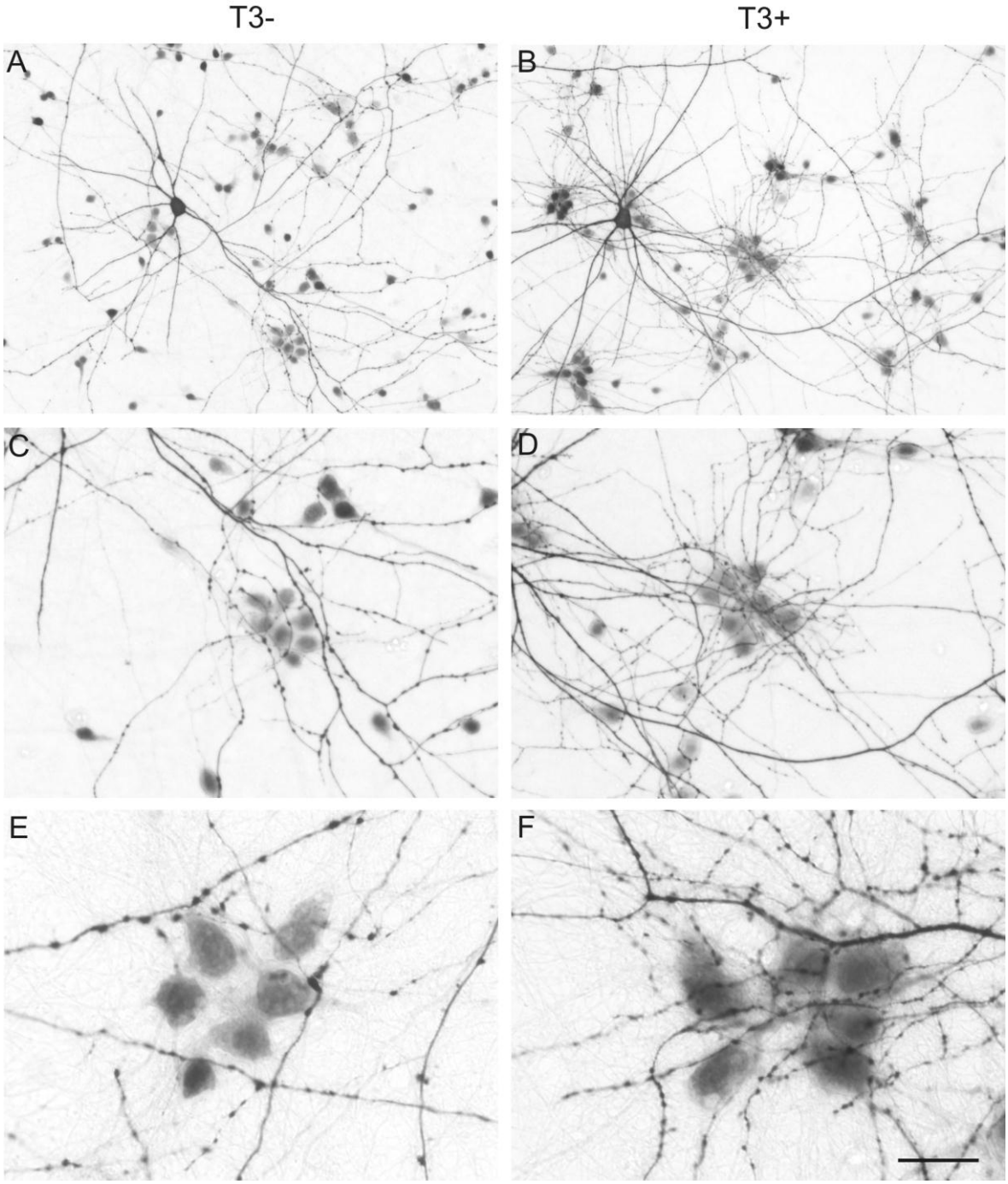

Figure 13: Growth of GABAergic neuropile in T3-treated networks.
Images show GABA immunostained 15-day-old low-density cultures grown in the absence (**A, C, E**) or presence (**B, D, F**) of T3. Because these low-density cultures contain virtually no S-GABAergic interneurons, all GABAergic structures visualized by GABA immunocytochemistry originate from L-GABAergic cells. In T3-treated cultures L-GABAergic neurons (B, D, F) form clear visible nests of axonal arborizations with many boutons around small groups of cells, whereas in T3-deprived cultures these nests are less prominent or absent (A, C, E). Scale bar = 100 µm in A and B, 50 µm in C and D, and 20 µm in E and F.

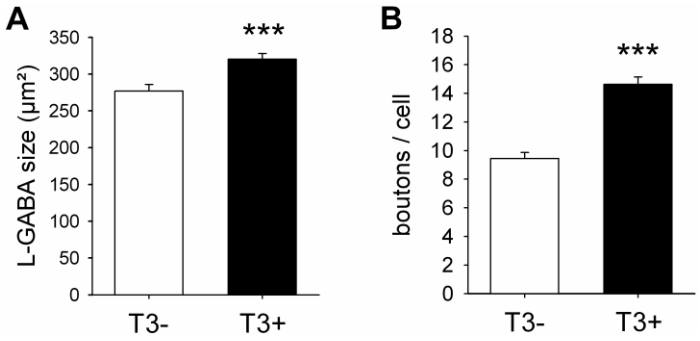

Figure 14: Formation of GABAergic boutons by L-GABAergic neurons.
A. As expected L-GABAergic cells treated with T3 show hypertrophy compared with untreated T3-deprived cultures. **B.** In parallel, the number of GABAergic boutons in the vicinity of non-GABAergic neurons is significantly increased in T3-treated cultures.

4.3.3 T3 modulates the development of L-GABAergic axons and boutons

Next the formation of GABAergic boutons and the distribution of L-GABAergic axonal arbors was analysed in more detail in GABA-immunostained 14-day-old cultures grown in the absence ($T3^-$) or in the presence of triiodothyronine ($T3^+$; Tables 4 - 5; Fig. 15 - 16). Therefore regular cultures (plating density of 300 - 500 neurons/mm²) were used. The total length of GABAergic axons, the number of GABAergic boutons and the number of axonal branches were measured in randomly chosen fields (120 µm x 90µm). Independent of hormonal treatment the axons of L-GABAergic neurons formed a continuous interconnected network. The overall axonal length was reduced by T3 (Fig. 15 C), whereas the number of GABAergic boutons (Fig. 15 D) and the number of axonal branches were increased (Fig. 15 E). In parallel, L-GABAergic neurons showed a prominent enlargement of neuronal soma size in the presence of T3 (Fig. 15 F: 308.45 ± 6.63 µm² in $T3^-$ and 351.07 ± 8.00 µm² in $T3^+$; n = 120 cells; $p < 0.001$; MW-RST). The overall density of L-GABAergic neurons was not changed by hormone treatment (4.42 ± 0.38 cells/mm² in $T3^-$ and 4.38 ± 0.35 cells/mm² in $T3^+$; n = 60 fields; $p = 0.939$; MW-RST), thus the reduction in axonal length did not result from a loss of GABAergic neurons.

Under both conditions, non-GABAergic neurons clustered into small groups and form cell rich and cell poor areas, whereas GABAergic neurons usually distributed between these clusters. In T3-treated cultures, GABAergic axonal arborizations and GABAergic boutons tended to concentrate around these groups of non-GABAergic neurons (Fig. 15 B1 - B3), whereas L-GABAergic axons in T3-untreated cultures were distributed more homogenously throughout the neuronal network (Fig. 15 A1 - A3).

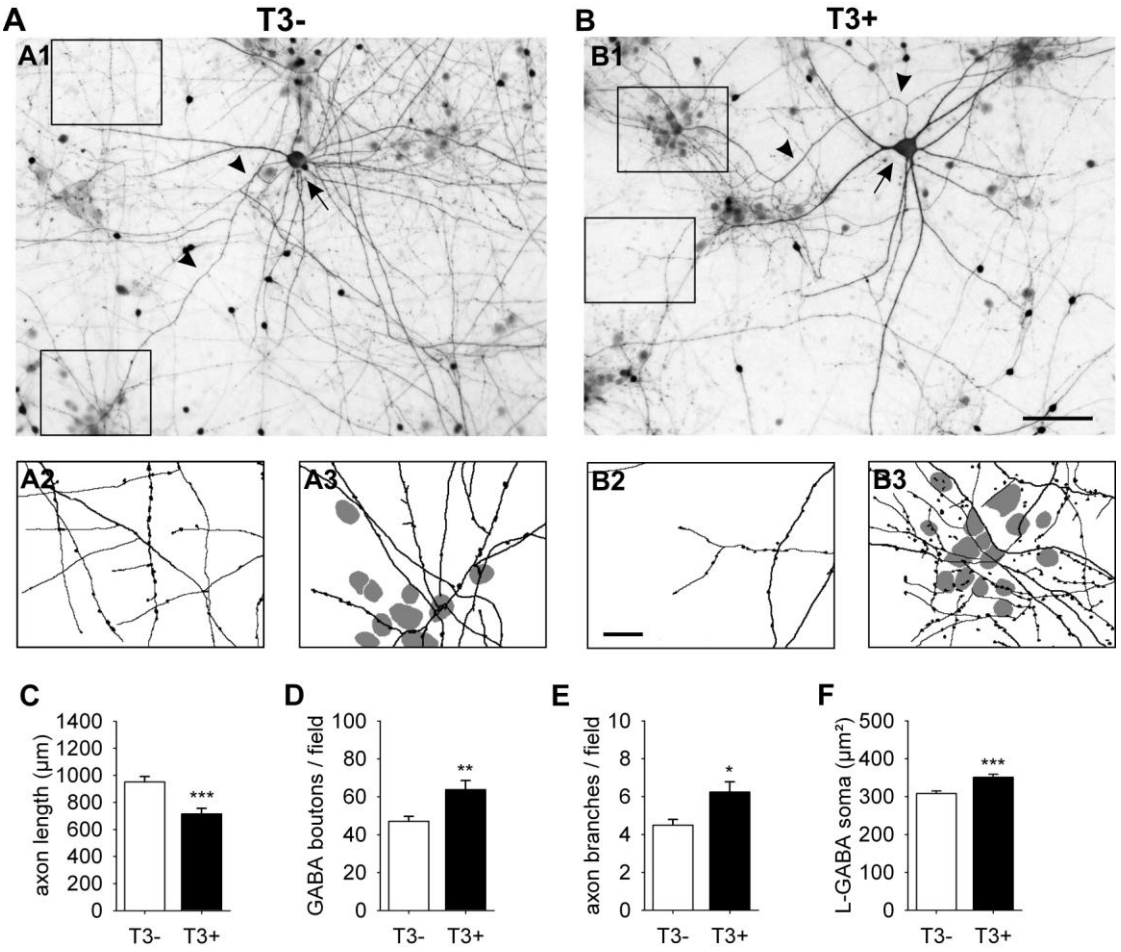

Figure 15: T3 modulation of L-GABAergic neurons´ axonal growth and soma size.
A-B. Images show GABA immunostained 14-day-old cultures grown in the absence (A1) or presence (B1) of T3. Each image shows a L-GABAergic neuron (arrow) with its conspicuous axonal arborizations (arrow heads) surrounded by smaller S-GABAergic neurons and unstained non-GABAergic neurons. In T3-stimulated cultures L-GABAergic neurons selectively target groups of neuronal cell bodies with their axons (B3) but sparsely innervate cell free regions (B2). In contrast, in T3-deprived cultures both cell-free (A2) and cell-rich (A3) regions are equally covered with L-GABAergic axons. Scale bar: (B1) 100 µm in A1 and B1; (B2) 25 µm in A2, A3 and B2, B3. **C-F.** Quantification of L-GABAergic neurons in T3-treated and T3-deprived cultures. Total axonal length per field (C), mean bouton (D) and branch density (E) and L-GABAergic soma size (F) per field. See also Table 4 (controls).

To test the hypothesis that axonal parameters vary according to the distribution of neuronal cell bodies, the total length of GABAergic axons, the number of GABAergic boutons and the number of axonal branches were correlated to the number of non-GABAergic cells. In cultures chronically treated with T3 the axonal length (Fig. 16 A; $p < 0.001$; $r^2 = 0.180$, LR), the number of boutons (Fig. 16 B; $p < 0.001$; $r^2 = 0.396$, LR) and the number of axonal branches (Fig. 16 C; $p < 0.001$; $r^2 = 0.437$, LR) were well correlated with the local cell density. In contrast, in cultures grown in the absence of T3 none of the

parameters were correlated with the local cell density (axonal length: $p = 0.998$, $r^2 < 0.0001$, LR; GABAergic boutons: $p = 0.110$, $r^2 = 0.0434$, LR; axonal branches: $p = 0.079$, $r^2 = 0.0524$, LR).

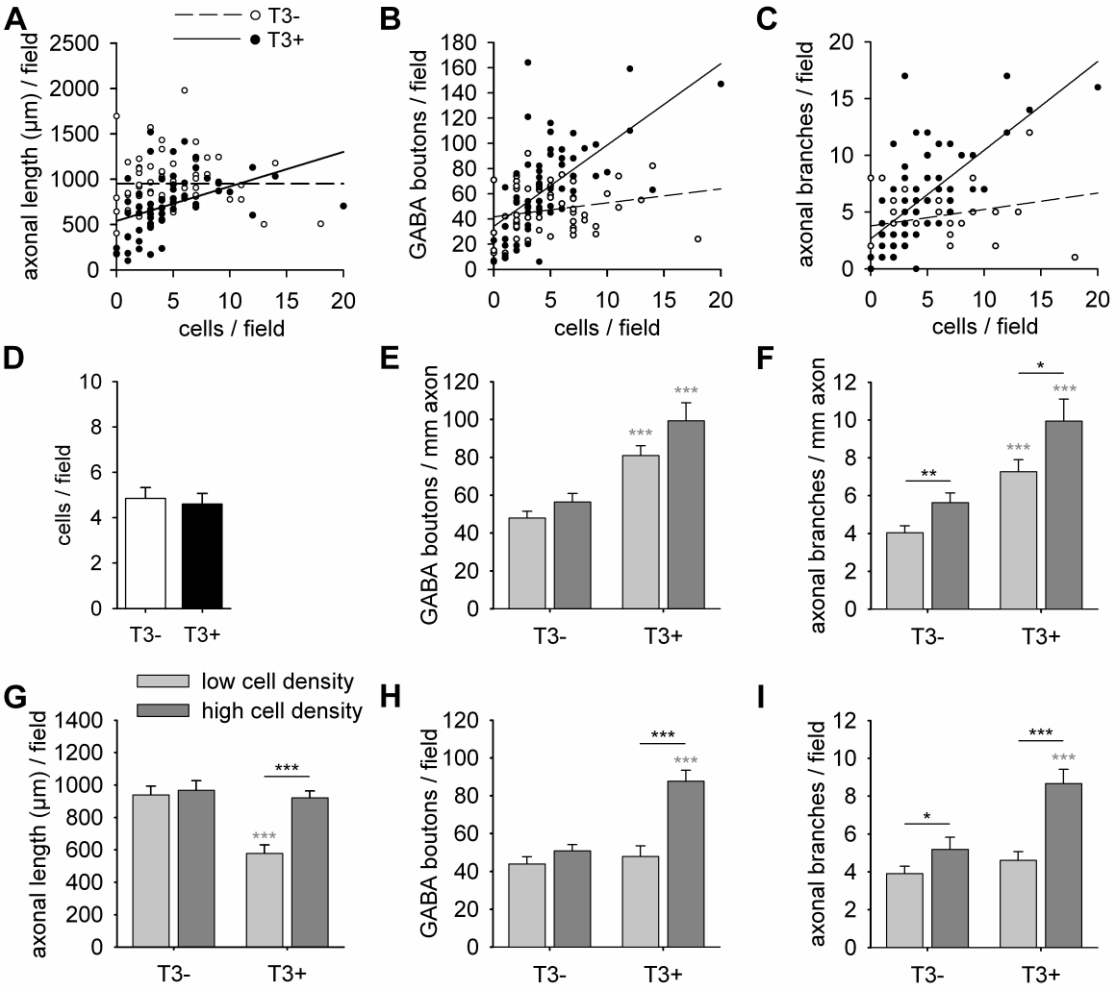

Figure 16: Different distribution of L-GABAergic axons in T3-treated and T3-deprived cultures.
A-C. Scatter plots indicate that the axonal length (A), the number of GABAergic boutons (B) and the number of axonal branches (C) are dependent on the number of non-GABAergic neurons in T3-stimulated ($T3^+$) but not in T3-deprived cultures ($T3^-$). Each dot represents one randomly chosen field (120 µm x 90 µm). Lines indicate best linear fit. **D.** The mean cell density of all measured fields was used as criteria to analyze data in subgroups with low and high cell densities (**E-I**). **G** shows total axonal length per field. **E** and **F** show boutons and branches density per axon segment length, and **H** and **I** show boutons and branches density per field. Asterisks show statistical significance between fields with low and high cell density as indicated by lines. Grey asterisks display significant differences between $T3^-$ and $T3^+$ cultures. See also Tables 4, 5 (controls).

For further quantitative analysis the data were grouped into fields with low cell density (≤ 4 cells/field) and fields with high cell density (> 4 cells/field) according to the mean density of non-GABAergic cells (4.85 ± 0.48 cells/field in $T3^-$ and 4.60 ± 0.47 cells/field in $T3^+$; Fig. 16 D - I; Tables 4 - 5).

Axonal Length. There was no difference in the mean total length of GABAergic axons between fields with low and high cell density in $T3^-$ raised cultures (Fig. 16 G, Table 4). High cell density fields in T3-treated cultures showed a total GABAergic axonal length per field similar to the fields in T3-untreated cultures. In contrast, the GABAergic axonal length in low density fields in T3-treated cultures was significantly reduced. Thus, the decreased mean axonal length in T3-treated cultures (Fig. 15 C) resulted from axonal pruning in low density fields (Fig. 16 G).

GABAergic boutons. T3-treated cultures showed an increased number of GABAergic boutons along axons in both high and low cell density areas, compared with those fields in T3-untreated cultures (Fig. 16 E; Table 5). However, no difference was found in the number of boutons per axonal length between high and low density fields in each of the treatment groups. Considering the number of boutons per field (Fig. 16 H; Table 4), no difference was detected between high and low density fields of T3-untreated cultures. In contrast, the number of GABAergic boutons per field was selectively enriched in fields with high cell density in $T3^+$ cultures.

Axonal branches. The number of axonal branches per axonal length was significantly increased by T3-treatement irrespective of cell density (Fig. 16 F; Table 5), indicating a general increase in axonal branching by T3. In contrast to GABAergic boutons, the local cell density promotes branching of GABAergic axons in $T3^-$ and $T3^+$ cultures. The increase in axonal branching in high density fields was very pronounced in the presence of T3 (Fig. 16 I, Table 4).

These results suggest a twofold effect of T3 treatment on the axon growth of L-GABAergic neurons: on the one hand T3 favored the formation of GABAergic boutons and axonal branches; on the other hand T3 induced a selective reduction in axonal length in fields with low cell density. As a result, in T3-treated cultures GABAergic boutons and axonal branches were significantly increased in the vicinity of non-GABAergic neurons.

Table 4: Interactions of T3 and BDNF on the axonal growth of L-GABAergic neurons.

	T3-			T3+			
	mean±sem (n)	*P#*	*P§*	*mean±sem (n)*	*P#*	*P§*	*P**
Axon length (µm per field)							
total							
control	951 ± 40 (60)			715 ± 42 (60)			<0.001
K252a	1020 ± 50 (60)	0.277		943 ± 38 (60)	<0.001		0.216
BDNF	994 ± 42 (60)	0.406		762 ± 47 (60)	0.453		<0.001
Low density							
Control	938 ± 54 (33)			577 ± 53 (36)			<0.001
K252a	871 ± 120 (15)	0.561		923 ± 126 (7)	0.012		0.796
BDNF	935 ± 52 (38)	0.967		561 ± 46 (37)	0.817		<0.001
High density							
Control	966 ± 59 (27)		0.722	921 ± 43 (24)		<0.001	0.539
K252a	1070 ± 52 (45)	0.208	0.086	945 ± 40 (53)	0.708	0.852	0.056
BDNF	1094 ± 69 (22)	0.165	0.070	1084 ± 45 (23)	0.011	<0.001	0.909
GABA boutons (per field)							
Total							
Control	47.1 ± 2.6 (60)			63.8 ± 4.8 (60)			0.009
K252a	57.8 ± 2.7 (60)	0.005		82.9 ± 3.3 (60)	0.001		<0.001
BDNF	66.8 ± 3.1 (60)	<0.001		74.0 ± 5.9 (60)	0.181		0.836
Low density							
Control	43.9 ± 3.7 (33)			47.9 ± 5.6 (36)			0.569
K252a	46.5 ± 6.4 (15)	0.660		78.1 ± 9.4 (7)	0.029		0.016
BDNF	59.6 ± 3.8 (38)	0.005		43.3 ± 3.7 (37)	0.498		0.003
High density							
Control	50.9 ± 3.3 (27)		0.180	87.7 ± 5.8 (24)		<0.001	<0.001
K252a	61.6 ± 2.8 (45)	0.024	0.032	83.5 ± 3.5 (53)	0.524	0.602	<0.001
BDNF	79.4 ± 4.2 (22)	<0.001	<0.001	123.4 ± 5.1 (23)	<0.001	<0.001	<0.001
Axon branches (per field)							
Total							
Control	4.48±0.30 (60)			6.23±0.55 (60)			0.040
K252a	2.83±0.26 (60)	<0.001		3.63±0.31 (60)	<0.001		0.076
BDNF	4.22±0.30 (60)	0.500		4.82±0.46 (60)	0.073		0.565
Low density							
Control	3.91±0.39 (33)			4.61±0.65 (36)			0.885
K252a	2.13±0.50 (15)	0.006		2.71±0.61 (7)	0.213		0.306
BDNF	3.61±0.34 (38)	0.588		2.92±0.32 (37)	0.092		0.263
High density							
Control	5.19±0.46 (27)		0.042	8.67±0.76 (24)		<0.001	<0.001
K252a	3.07±0.30 (45)	<0.001	0.074	3.75±0.33 (53)	<0.001	0.205	0.241
BDNF	5.27±0.49 (22)	0.833	0.004	7.87±0.71 (23)	0.447	<0.001	0.005

P# indicates p-value of drug treatment groups vs. untreated controls of the same hormone and density category. *P§* refers to p-value high density vs. low density of the same hormone and drug category. *P** shows p-value T3-treated vs. T3-untreated of the same drug and density category. (*n*)

number in brackets indicates the number of fields analysed per condition. All data represent mean ± SEM. Statistics were made with t-test or MW-RST.

Table 5: Normalized density of GABAergic boutons and axonal branches per axonal length.

	T3-			T3+			
	mean±sem (n)	*P#*	*P§*	*mean±sem (n)*	*P#*	*P§*	*P**
GABA boutons (per mm axon)							
Total							
Control	51.8 ± 2.8 (60)			88.4 ± 5.0 (60)			<0.001
K252a	58.8 ± 2.2 (60)	0.053		89.9 ± 2.4 (60)	0.433		<0.001
BDNF	68.3 ± 2.4 (60)	<0.001		92.7 ± 4.1 (60)	0.505		<0.001
Low density							
Control	48.1 ± 3.5 (33)			81.1 ± 5.1 (36)			<0.001
K252a	54.9 ± 2.7 (15)	0.264		87.8 ± 7.8 (7)	0.583		0.001
BDNF	64.5 ± 4.1 (38)	<0.001		78.3 ± 4.6 (37)	0.685		0.014
High density							
Control	56.4 ± 4.5 (27)		0.142	99.5 ± 9.4 (24)		0.224	<0.001
K252a	60.2 ± 2.6 (45)	0.436	0.311	90.1 ± 2.5 (53)	0.961	0.763	<0.001
BDNF	74.9 ± 3.1 (22)	0.002	0.032	116.0 ± 4.9 (23)	0.001	<0.001	<0.001
Axon branches (per mm axon)							
Total							
Control	4.75±0.32 (60)			8.34±0.61 (60)			<0.001
K252a	2.67±0.21 (60)	<0.001		3.87±0.28 (60)	<0.001		<0.001
BDNF	4.35±0.30 (60)	0.451		6.05±0.45 (60)	0.003		0.003
Low density							
Control	4.04±0.36 (33)			7.27±0.63 (36)			<0.001
K252a	2.36±0.23 (15)	0.008		3.20±0.80 (7)	0.009		0.304
BDNF	3.99±0.52 (38)	0.926		5.29±0.61 (37)	0.027		0.075
High density							
Control	5.64±0.50 (27)		0.009	9.94±1.16 (24)		0.032	<0.001
K252a	2.78±0.24 (45)	<0.001	0.392	3.96±0.30 (53)	<0.001	0.394	0.004
BDNF	4.97±0.43 (22)	0.335	0.112	7.26±0.56 (23)	0.046	0.032	0.003

P# indicates p-value of drug treatment groups vs. untreated controls of the same hormone and density category. *P§* refers to p-value high density vs. low density of the same hormone and drug category. *P** shows p-value T3-treated vs. T3-untreated of the same drug and density category. (*n*) number in brackets indicates the number of fields analysed per condition. All data represent mean ± SEM. Statistics were made with t-test or MW-RST.

4.3.4 Contribution of BDNF to T3-mediated L-GABAergic axon growth

Previous results showed complementary effects of T3 and neuronal activity in the regulation of L-GABAergic neurons development (chapter 4.2.6). The neurotrophin BDNF is well known as a mediator of activity-dependent effects and has been shown to be an important regulator of interneuron development (Palizvan et al., 2004; Patz et al., 2004; Woo and Lu, 2006). Additionally, BDNF expression has been suggested to be regulated by T3 (Koibuchi et al., 1999; Sui et al., 2010). Thus, BDNF might act as a mediator of T3 actions on early GABAergic neurons during neocortical network development.

To investigate the interplay between BDNF signalling and T3-mediated growth of L-GABAergic neurons either exogenous BDNF (50 ng/ml) or the tyrosine kinase inhibitor K252a (100nM) were added to the cultures, alone or in combination with T3 (Figs. 17 - 18; Tables 4 - 5). K252a is a high-affinity inhibitor of the BDNF receptor TrkB (tropomyosin-related kinase B; Tapley et al., 1992) and has been shown to inhibit BDNF-mediated plasticity of GABAergic neurons (Rutherford et al., 1997; Jin et al., 2003; Palizvan et al., 2004; Patz et al., 2004; Peng et al., 2010). In the present experiments, TrkB signalling was inhibited during the second week *in vitro*. During this time synapse formation of L-GABAergic axons takes place and early synchronous network activity is present (de Lima and Voigt, 1997; Voigt et al., 2001; Opitz et al., 2002).

First, GABA-immunostainings revealed that K252a inhibited the characteristic axonal distribution pattern induced by T3-stimulation (Fig. 17). While GABAergic cells formed highly branched axon terminals with numerous boutons around non-GABAergic cell bodies sparing cell-free regions in T3-treated control cultures (Fig. 17 B), this typical pattern was completely inhibited by K252a (Fig. 17 D). In T3-untreated cultures the incubation with K252a did not affect axonal distribution (Fig. 17 A, C). To test whether differences between T3-treated and untreated cultures were induced by T3-mediated changes in BDNF levels, sister cultures were stimulation with exogenous BDNF (50ng/ml) during the same time period. Interestingly, BDNF application apparently did not change axonal distribution in T3-deprived cultures (Fig. 17 E), but further promoted bouton formation and axonal branching in the presence of T3 (Fig. 17 F).

To quantify the axonal parameters under these conditions we again measured the total axonal length, the number of GABAergic boutons and the number of axonal branches in randomly chosen fields after the addition of K252a or BDNF (Fig. 17 - 18; Tables 4 – 5; control cultures are the same as in Fig. 16).

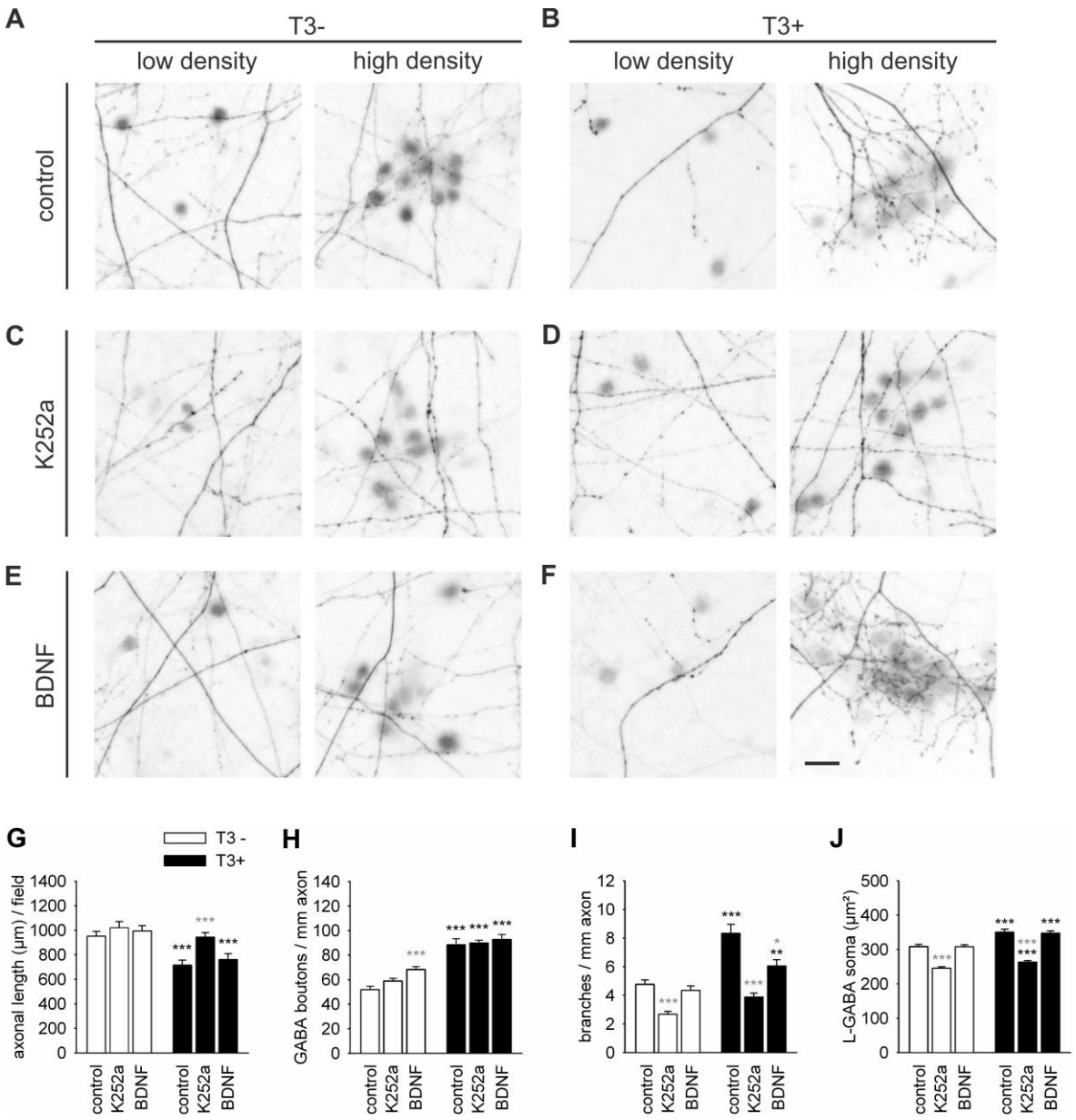

Figure 17: Effects of T3 and BDNF on the distribution of L-GABAergic axons and boutons.
A-F. Images show GABAergic innervation patterns of areas with low or high cell density in T3-untreated and T3-treated 14-day-old cultures, comparing controls (**A-B**) with cultures treated with K252a (**C-D**) or BDNF (**E-F**). **G-J** shows the quantification of the effect of K252a and BDNF treatment considering the mean of all fields: axonal length (**G**), relative number of GABAergic boutons along axons (**H**), relative number of axonal branches (**I**), and L-GABAergic neurons cell body size (**J**) for both $T3^-$ and $T3^+$ cultures. Scale bar = 20 µm.

Axonal Length. In T3-treated cultures K252a prevented the reduction of axonal length in fields with low cell density (Fig. 18 C, right side) and thereby the overall reduction of mean axonal length per field (Fig. 17 G), whereas BDNF application did not change overall axonal length. Interestingly, the addition of exogenous BDNF to T3-deficient cultures also did not change the total axonal length per field (Fig. 17 G; Table 4).

Accordingly, treatment with K252a inhibited the positive correlation of axonal length with the local cell density in T3-supplemented cultures ($p = 0.057$; $r^2 = 0.0612$, LR; Fig. 18 B, C right side; Table 4) but had no effect in T3-deprived cultures ($p = 0.321$; $r^2 = 0.0170$, LR; Fig. 18 A, C left side; Table 4). BDNF application apparently did not change axonal distribution in T3-deprived cultures (Fig. 17 E, G), failing to establish a significant positive correlation of axonal length and cell density in $T3^-$ cultures ($p = 0.062$; $r^2 = 0.0588$, LR; Fig. 18 A, C left side; Table 4). In the presence of T3, the stimulation with exogenous BDNF promoted the correlation of axonal length with the cell density ($p < 0.001$; $r^2 = 0.468$, LR; Fig. 17 F; 18 B, C right side; Table 4).

Boutons. The blockade of TrkB receptors did not change the relative density of boutons along axons (Fig. 17 H; Table 5). The application of exogenous BDNF increased the relative density of GABAergic boutons along axons in T3-deficient cultures, but this increased bouton density was still smaller than bouton density in T3-treated cultures. The T3-induced positive correlation of GABAergic boutons per field with the local cell density was inhibited by K252a ($p = 0.191$; $r^2 = 0.0292$, LR, Fig. 18 E, F right side; Table 4), and, as expected, there was no correlation in T3-untreated cultures after K252a treatment ($p = 0.088$; $r^2 = 0.0493$; Fig. 18 D), although a small difference in boutons density was detected, when fields with high or low cell density were compared (Fig. 18 F, left side; Table 4). Exogenous BDNF application increased the bouton density in fields with high cell density independent of T3 presence ($T3^-$: $p < 0.001$; $r^2 = 0.255$, LR; $T3^+$: $p < 0.001$; $r^2 = 0.706$, LR; Fig. 18 D, E, F; Table 4). However, the increase was much more pronounced in the presence of T3.

Axonal Branches. Finally, the number of axonal branches per axonal length decreased significantly in the presence of TrkB receptor antagonist in both T3-untreated and T3-treated cultures. Moreover, the increased axonal branching due to T3-treatment was prevented by K252a (Fig. 17 I, Table 5). Different from the distribution of axonal length and GABAergic boutons, after incubation with K252a the number of axonal branches per field was still positively correlated with cell density in T3-treated and T3-untreated cultures ($T3^+$: $p = 0.005$; $r^2 = 0.131$, LR; $T3^-$: $p = 0.022$; $r^2 = 0.0866$, LR; Figs. 18 G, H; Table 4), even though the correlation indexes were quite small. After BDNF stimulation the branch numbers were well correlated to the neuron density in both $T3^+$ and $T3^-$ cultures ($T3^+$: $p < 0.001$; $r^2 = 0.483$, LR; $T3^-$: $p < 0.001$; $r^2 = 0.186$, LR; Fig. 18 G, H; Table 4). However, the correlation index was higher in T3-treated cultures. The comparison of fields with low and high cell density (Fig. 18 I) again illustrates that the

number of branches is enhanced in high-density areas by T3 in the presence of BDNF signalling.

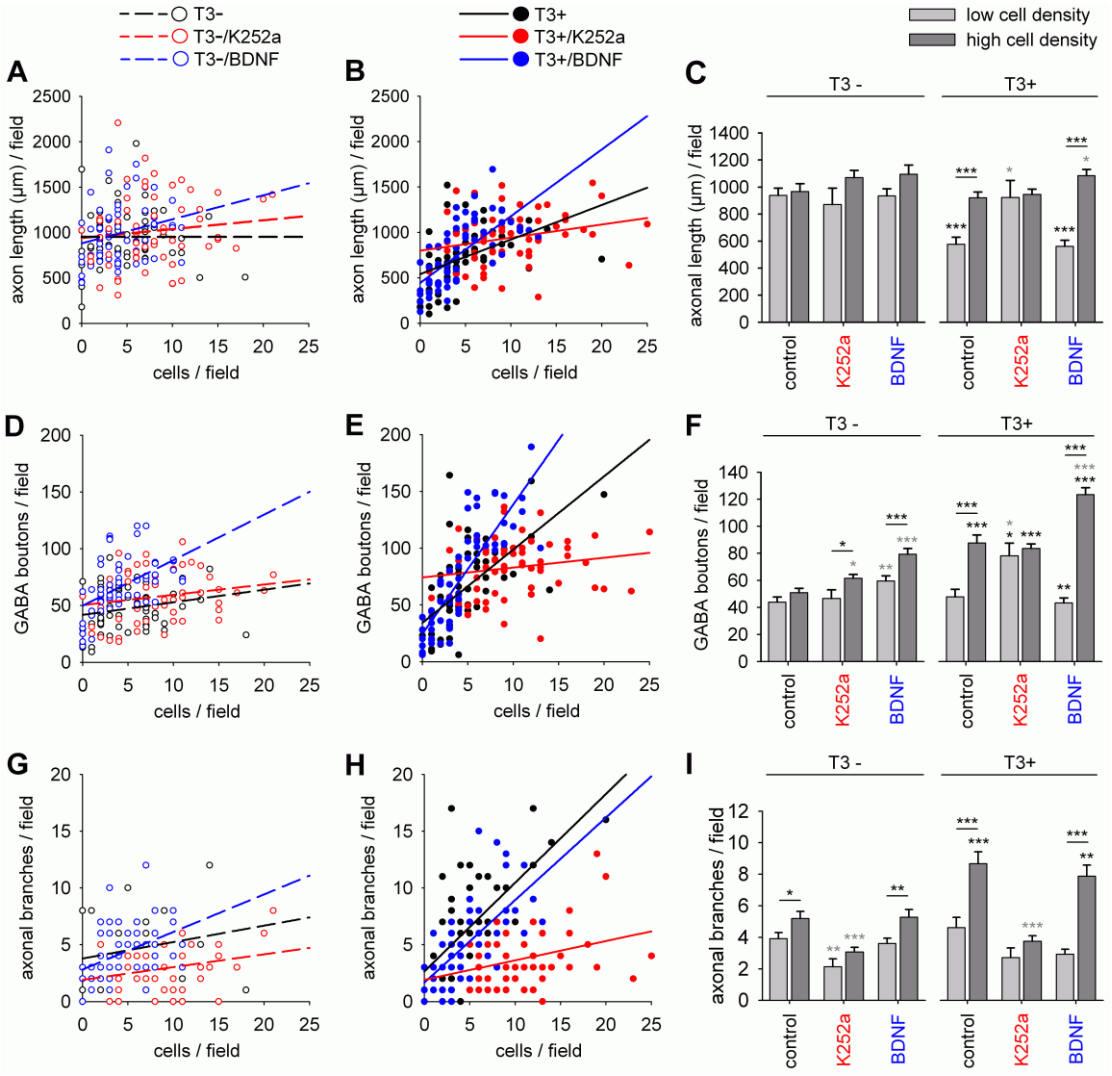

Figure 18: L-GABA axon growth and bouton formation in the presence of T3 and BDNF.
Scatter plots show distribution of axonal length (**A, B**), GABAergic boutons (**D, E**) and axonal branches (**G, H**) of $T3^-$ (open dots) and $T3^+$ cultures (filled dots) when treated with K252a (red) or exogenous BDNF (blue). Lines indicate best linear fit. Black dots and lines display data and best linear fit of untreated control cultures as shown in Fig. 20 A-C. **C, F, I.** As in Fig. 20 E-I, quantitative data were grouped into fields with low (≤ 4 cells/field) and fields with high cell density (> 4 cells/field). **C** shows total axonal length per field. **F** shows boutons density per field, and **I** shows branches density per field. Asterisks show statistical significance between fields with low and high cell density as indicated by lines. Black asterisks directly above bars display significant differences between $T3^-$ and $T3^+$ cultures of the same treatment and density category. Gray asterisks compare K252a or BDNF treated cultures with untreated controls of the same hormone and density category. For more details see also Table 4.

Soma size. Previous results suggested that the increased number of GABAergic boutons correlates with a simultaneous growth of neuronal soma (see Fig. 14). Therefore the soma size of L-GABAergic neurons was additionally measured in this set of experiments after application of K252a or BDNF (Fig. 17 J; n = 120 neurons, 6 cultures). The mean soma size of L-GABAergic neurons in control cultures was 308.4 ± 6.6 µm² in $T3^-$ and 351.1 ± 8.1 µm² in $T3^+$ (cf. Fig. 15 F: $p < 0.001$, MW-RST). The TrkB inhibitor K252a reduced the soma size to 245.6 ± 4.4 µm² in $T3^-$ ($p < 0.001$, MW-RST compared with $T3^-$ control) and 263.6 ± 4.6 µm² in $T3^+$ ($p < 0.001$, MW-RST compared with $T3^+$ control). Thus T3-treated neurons were still larger than T3-deprived neurons even when BDNF signalling was blocked ($p < 0.001$, t-test), revealing a direct neurotrophic effect of T3 on L-GABA soma growth. In contrast the application of exogenous BDNF did not further promote somatic growth in T3-deprived cultures (307.8 ± 6.1 µm², $p = 0.754$, MW-RST compared with $T3^-$ control) or in T3-treated cultures (347.9 ± 6.4 µm², $p = 0.565$, MW-RST compared with $T3^+$ control; $p < 0.001$, t-test compared with BDNF-treated $T3^-$).

In conclusion, the results suggest that T3 promotes the somatic growth and the concomitant increase in relative bouton numbers along axons independently of BDNF signalling. In T3-untreated cultures BDNF stimulation can only partially rescue deficits in bouton density. In contrast, the pruning of GABAergic axons from low cell density areas and the branching of axons, and thereby the characteristic accumulation of boutons around non-GABAergic cells, were prevented by TrkB receptor antagonist K252a. Since the BDNF-dependent pruning of GABAergic axons occurred only in T3-treated cultures and could not be induced by BDNF stimulation in T3-deprived cultures, this process seems to be synergistically regulated by T3 and BDNF.

4.4 T3 modulation of GABA signalling maturation

The proper function of GABAergic signalling is not only controlled by the density and distribution of GABAergic synapses. Its polarity and strength also depends on the intracellular chloride concentration in the postsynaptic cell, which is regulated by the cation chloride cotransporters NKCC1 and KCC2 (Kahle et al., 2008; Blaesse et al., 2009; see also chapter 2.1.4). The aim of the next experiments was to study the influence of T3 on the functional maturation of the GABAergic system by estimating the developmental

expression of KCC2 and NKCC1 and its implication in the shift from depolarizing to hyperpolarizing GABAergic signalling.

4.4.1 Expression of KCC2 and NKCC1

In cultured networks the developmental GABA shift occurs between 9 and 20DIV (Baltz et al., 2010). Therefore the expression of NKCC1 and KCC2 was determined in Western blot experiments (Fig. 19) in 7 to 21-day-old cultures. GAPDH protein level was used as a loading control, and NKCC and KCC2 data were normalized to the mean expression of 7DIV old T3-untreated cultures to calculate the developmental expression profile.

The results showed that NKCC1 became downregulated between 14 and 21 DIV in $T3^-$ as well as in $T3^+$ cultures (Fig. 19 A, B; n = 5 blots; two preparations). The NKCC1 level in $T3^-$ cultures increased slightly from initially 100 ± 23.8 % at 7DIV to 130 ± 28.7 % at 14DIV (p = 0.442, t-test compared with 7DIV) and then dropped to 27 ± 4.8 % at 21DIV (p = 0.017, t-test compared with 7DIV). Similarly, the NKCC1 protein level of $T3^+$ cultures increased slightly from 102 ± 13.1 % at 7DIV to 152 ± 51.8 % at 14DIV (p = 0.374, t-test compared with 7DIV) and decreased to finally 42 ± 10.9 % at 21DIV (p = 0.008, t-test compared with 7DIV). The protein levels were not different between $T3^-$ and $T3^+$ cultures at any age (p = 0.933, t-test at 7DIV; p = 0.714, t-test at 14DIV; p = 0.242, t-test at 21DIV).

In contrast, the expression of KCC2 was regulated in a hormone-dependent manner (Fig. 19 B, C). In $T3^-$ cultures KCC2 decreased from initially 100 ± 14.0 % at 7DIV to 41 ± 9.6 % at 14DIV (p = 0.006, t-test compared with 7DIV) and finally to 65 ± 16.0% at 21DIV (p = 0.127, t-test compared with 7DIV). In $T3^+$ cultures the expression of KCC2 increased from 215 ± 56.2 % at 7DIV to 419 ± 63.5 % at 14DIV (p = 0.027, t-test compared with 7DIV) and then slightly decreased to 315 ± 71.8 % at 21DIV (p = 0.301, t-test compared with 7DIV). The expression of KCC2 was increased in $T3^+$ cultures compared to $T3^-$ cultures at 14 and 21DIV (p = 0.132, MW-RST at 7DIV; p = 0.002, MW-RST at 14DIV; p = 0.007, t-test at 21DIV).

The results indicate that T3 affects the expression of NKCC1 and KCC2 in a transporter specific manner. KCC2 expression is highly dependent on the presence of T3 suggesting a prominent role for thyroid hormone in chloride homeostasis and therefore also in GABAergic signalling.

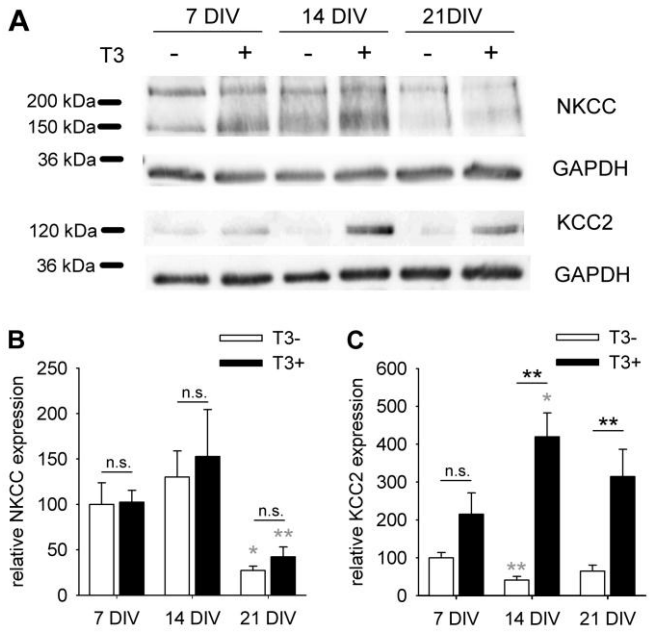

Figure 19: KCC2 and NKCC1 expression in T3-stimulated and T3-deprived cultures.
A. Representative Western Blots of 7 to 21-day-old cultures stained for NKCC1 or KCC2 as indicated. GAPDH levels were used as a loading control, and NKCC and KCC2 data were normalized to the mean expression of 7DIV old T3-untreated cultures to calculate the developmental expression profile. **B.** Developmental downregulation of NKCC1 protein levels was independent of T3 treatment. **C.** Expression of KCC2 was increased in T3-treated cultured compared with T3-untreated cultured at 14 and 21DIV. Asterisks show statistical significance between $T3^-$ and $T3^+$ cultures as indicated by lines. Grey asterisks display significant differences of 14-day-old or 21-day-old cultures compared with 7-day-old cultures of the same hormone group.

4.4.2 GABA signalling shift

The developmental downregulation of NKCC1 and concomitant upregulation of KCC2 lowers the intracellular chloride concentration and shifts GABAergic signalling from depolarizing to hyperpolarizing (Ben-Ari et al., 2007; Blaesse et al., 2009). Following the T3-dependent differences in KCC2 expression the developmental shift in GABA signalling should also be affected. The developmental changes in $GABA_A$ receptor-mediated synaptic transmission were tested in calcium imaging experiments by the local application of the $GABA_A$ receptor agonist muscimol (Fig. 20: 10 to 15 fields, 3 independent experiments).

Following the high intracellular chloride concentration muscimol evoked strong calcium transients in young neurons (Fig. 20 A). At 6 DIV virtually all neurons were depolarized by muscimol application (99.1 ± 0.4 % in $T3^-$; 98.0 ± 0.9 % in $T3^+$, n = 10 fields, p = 0.622, MW-RST). Starting on 9DIV differences in muscimol responsiveness

between the subpopulations of L-GABAergic, S-GABAergic and non-GABAergic neurons became evident (Table 6, Fig. 20). Interestingly, there was no developmental decrease in muscimol responses in the subpopulation of S-GABAergic neurons throughout development. Virtually all S-GABA were activated by muscimol even at 27DIV, the oldest age that has been analysed, and there were no differences between $T3^-$ and $T3^+$ cultures at any time.

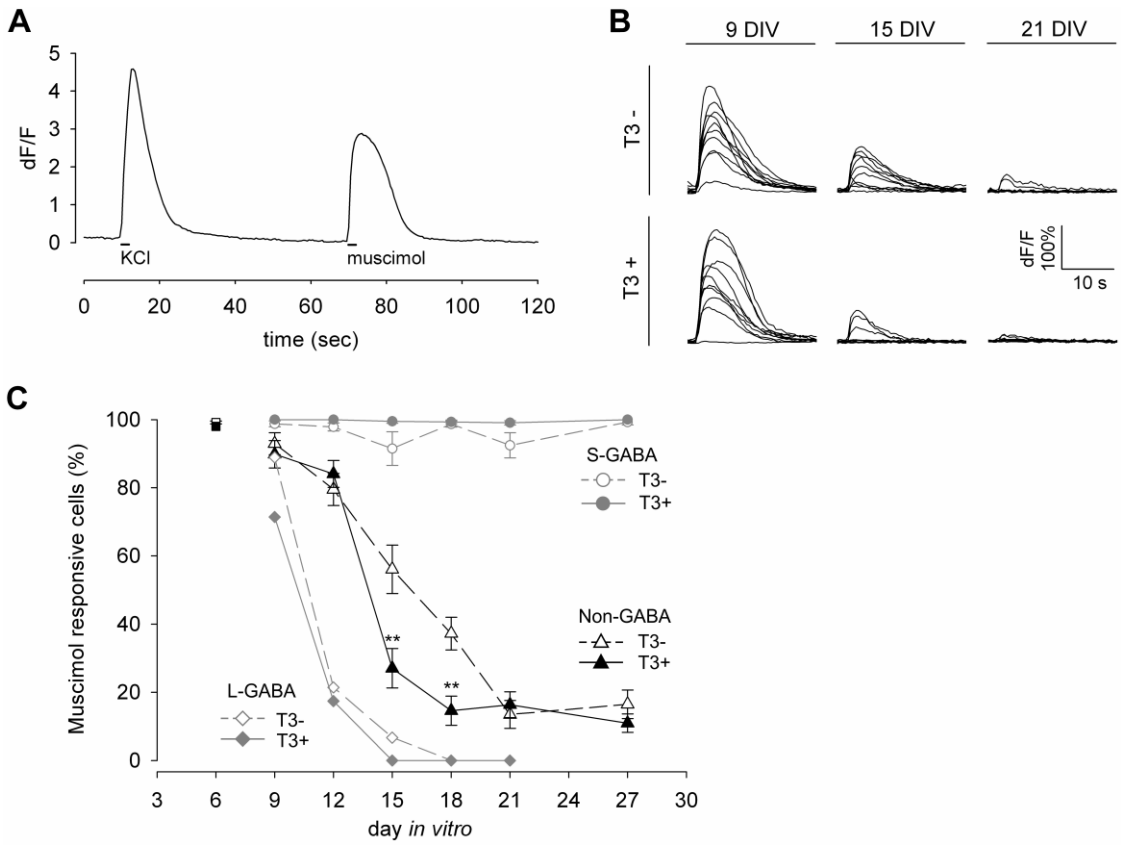

Figure 20: Modulation of GABA signalling shift by T3.
A. Normalized fluorescence of an individual 9-day-old non-GABAergic neuron demonstrates intracellular calcium transients in response to local application of KCl (60mM) and $GABA_A$ receptor agonist muscimol (200µM) during calcium imaging as indicated. **B.** Representative calcium transients of non-GABAergic neurons grown either in $T3^-$ and $T3^+$ in response to muscimol application. **C.** The developmental shift from depolarizing to hyperpolarizing actions of GABA is indicated by the fraction of neurons with a significant increase in fluorescence after muscimol stimulation (*n* = 10-15 fields each). At 6DIV virtually all neurons (black and white rectangles) show calcium transients after muscimol application. Whereas only few L-GABAergic neurons (grey rectangles) can be activated by muscimol application after day 9, virtually all S-GABAergic neurons (gray circles) respond to muscimol during the whole culture period until day 27. However, none of the GABAergic subpopulations was affected by T3 treatment. In contrast, T3 treatment accelerates the developmental downregulation of depolarizing actions of muscimol in non-GABAergic neurons (black triangles). See also Table 6.

In contrast, the subpopulation of L-GABAergic neurons showed a fast and early onset of decrease in responses to muscimol application already after 9DIV. At 15DIV only fewest L-GABAergic neurons showed a calcium influx upon muscimol stimulation. As with S-GABAergic neurons there was no difference between $T3^-$ and $T3^+$ cultured neurons. However it is noteworthy that due to the low overall density in neuronal cultures and the high magnification of recording fields during imaging the number L-GABAergic neurons recorded in the randomly chosen fields was low (in total 125 neurons over all cultures and ages). Since many fields contained only one or two L-GABAergic neurons statistical analysis on this data was not possible.

Table 6: GABA shift experiments

	Non-GABA (% active)			S-GABA (% active)			L-GABA (%)	
	$T3^-$	$T3^+$	*P*	$T3^-$	$T3^+$	*P*	$T3^-$	$T3^+$
DIV								
9	92.9 ± 3.2	89.8 ± 4.0	0.427	98.7 ± 0.9	100 ± 0	0.266	88.9	71.4
12	79.5 ± 4.7	84.1 ± 4.0	0.461	97.8 ± 1.2	100 ± 0	0.356	21.4	17.4
15	56.1 ± 7.1	27.0 ± 5.8	0.004	91.5 ± 4.9	99.4 ± 0.6	0.225	6.7	0
18	37.2 ± 4.8	14.6 ± 4.3	0.002	98.7 ± 0.9	99.3 ± 0.5	0.950	0	0
21	13.5 ± 4.1	16.3 ± 3.9	0.740	92.4 ± 3.7	99.1 ± 0.7	0.439	0	0
27	16.4 ± 4.2	10.9 ± 2.7	0.282	99.2 ± 0.8	100 ± 0	0.728	n.c.	n.c.

Data show the fraction of muscimol responding cells in percentage of the total number of cells. N = 10-15 fields per time point, 3 cultures from 3 preparations; *P* refers to the comparison between T3-treated and T3-untreated neurons with t-test or MW-RST; because of the low cell density no statistics were made for L-GABAergic neurons, n.c., not calculated, because of very low cell density. All data represent mean ± SEM.

Most non-GABAergic neurons were responding to muscimol application at 12DIV without a difference between T3-treated and untreated cultures. After that a remarkable drop in the fraction of responding cells was measured in T3-treated cultures. In contrast, the developmental downregulation was slowed in T3-untreated cultures. Interestingly, the difference between the both treatment groups disappeared at. At 27DIV still a small fraction of non-GABAergic cells was responding to muscimol application.

The results show that the developmental switch of GABAergic signalling proceeded in both treatment groups but was accelerated in the presence of T3. This indicates that the developmental transition in GABAergic signalling in neuronal cultures might be largely driven by the downregulation of NKCC1 protein, since the transition also

takes place in T3-deprived cultures with a low basal level of KCC2 expression. Otherwise, the remarkable decrease in muscimol responding cells around 15DIV in $T3^+$ cultures is timely well correlated to the increase in KCC2 protein expression (cf. Fig. 19 C) confirming a prominent modulatory role for KCC2 in the GABA shift. Interestingly a small population of non-GABAergic neurons and the whole population of S-GABAergic were still responding to muscimol application at the end of the fourth week *in vitro*.

4.4.3 Contribution of GABA signalling to network activity

During early postnatal development synchronous network activity is driven by synergistic actions of GABAergic and glutamatergic neurotransmission, resulting in strong stereotyped synchronous depolarizations (Voigt et al., 2001; Opitz et al., 2002; Ben-Ari et al., 2007). After GABA signalling switched from depolarizing to hyperpolarizing, neuronal activity becomes regulated by a precisely balanced interaction of depolarizing glutamatergic and hyperpolarizing GABAergic signalling (Baltz et al., 2010). Following the differences in KCC2 expression between $T3^-$ and $T3^+$ cultures the contribution of hyperpolarizing GABAergic signalling to network activity might also be affected by thyroid hormones. To analyse the impact of hyperpolarizing GABAergic transmission on network activity, cultures raised in the absence or presence of T3 were treated during the third week *in vitro*, the time when GABA is mainly hyperpolarizing, with $GABA_A$ receptor antagonists BMI and PTX. Spontaneous network activity was measured with calcium imaging technique at 21DIV in the presence of receptor antagonists (Fig. 21 A, Table 7).

T3-treated control cultures showed an increase in burst frequencies compared to T3-untreated control cultures (Fig. 21 B, Table 7) as reported previously for 14-day-old cultures (chapter 4.1.1; Fig. 1). Receptor blockade with BMI and PTX reduced the burst frequency in both T3-untreated as well as T3-treated cultures. However, burst frequency was still increased in $T3^+$ cultures compared to $T3^-$ cultures when $GABA_A$ receptors were blocked. In addition to burst frequencies also the duration of calcium transients was measured since GABA has been shown to play a modulatory role in burst kinetics (Baltz et al., 2010). The mean burst duration in $T3^-$ cultures was not altered by $GABA_A$ receptor blockade with BMI and PTX (Fig. 21 C, Table 7). Interestingly, receptor blockade significantly increased the duration of calcium transients in $T3^+$.

In addition to blockade of GABAergic neurotransmission we used the recently developed specific KCC2 inhibitor VU0240551 (Delpire et al., 2009) to increase the intracellular

chloride concentration and to reduce the hyperpolarizing potential of $GABA_A$ receptor signalling. Corresponding to receptor blockade experiments VU0240551 was added to the culture medium during the third week *in vitro* and was also present during fluorescence recording sessions (n = 15 fields, 3 cultures). The inhibition of KCC2 increased the burst frequency in $T3^-$ cultures (Fig. 21 B, Table 7). The mean frequency of spontaneous network events was also increased in $T3^+$ cultures, but the difference was not significant because of high variability between different cultures. In contrast to burst frequency the inhibition of KCC2 had no effect on the burst duration (Fig. 21 C, Table 7) in both $T3^-$ and in $T3^+$ cultures.

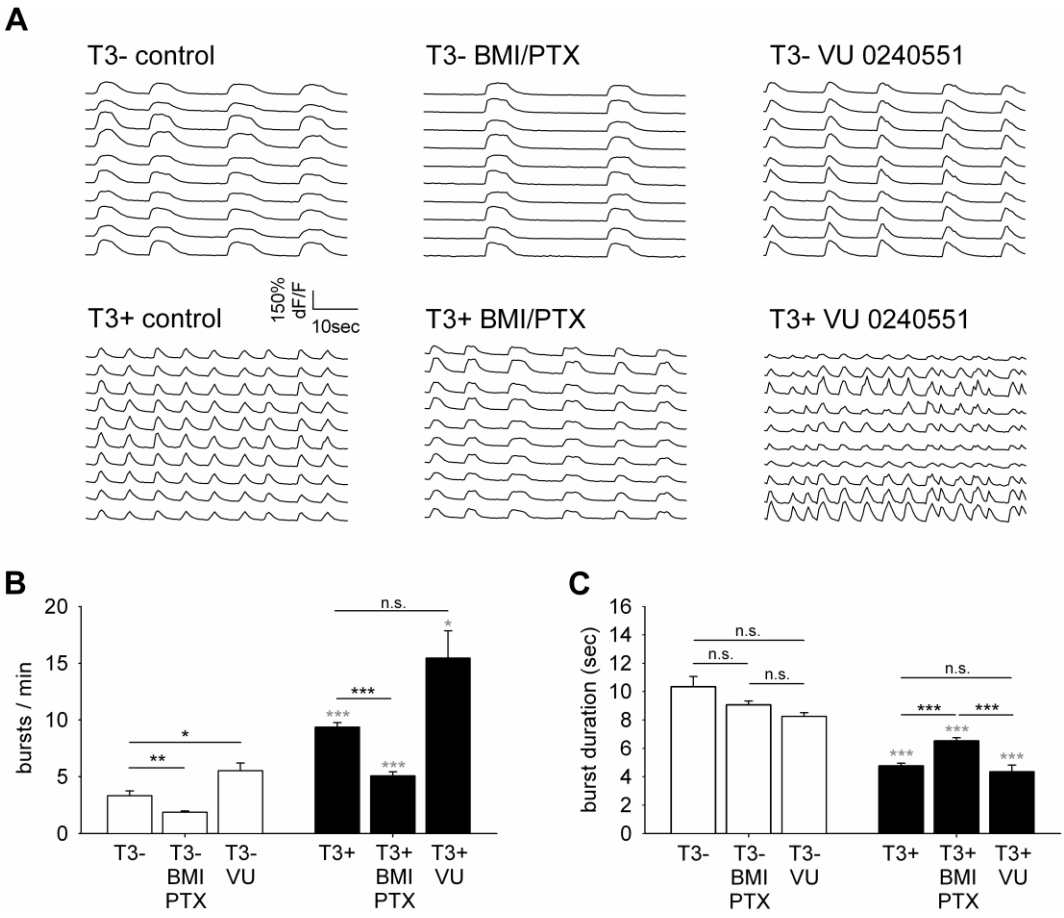

Figure 21: Contribution of inhibitory GABA signalling to network activity.
A. Traces show the change in intracellular calcium signal of individual neurons in 21-day-old cultures. Fluorescence data are expressed as dF/F (background-corrected fluorescence divided by resting fluorescence). Neurons were grown either in the absence or presence of T3 and treated with GABA receptor antagonists BMI + PTX or KCC2 inhibitor VU0240551 from 14-21DIV. Calcium imaging was performed in the presence of the drugs. **B.** Burst frequencies in both T3-treated and T3-untreated cultures were reduced by BMI + PTX but enhanced by VU0240551 **C.** BMI + PTX treatment prolonged calcium transients selectively in T3-treated cultures, whereas VU0240551 did not affect burst duration. Asterisks show significant differences as indicated by lines. Grey asterisks indicate statistical differences between $T3^+$ and $T3^-$ cultures of the same drug category. See also Table 7.

Table 7: Contribution of GABA signalling to network activity

	$T3^-$	$P^{control}$	$T3^+$	$P^{control}$	P^{T3}
frequency (bursts / min)					
control	3.33 ± 0.41		9.37 ± 0.39		< 0.001
BMI/PTX	1.87 ± 0.12	0.004	5.07 ± 0.36	< 0.001	< 0.001
VU	5.53 ± 0.67	0.011	15.47 ± 2.39	0.125	0.014
duration (seconds)					
control	10.34 ± 0.72		4.76 ± 0.18		< 0.001
BMI/PTX	9.07 ± 0.72	0.319	6.53 ± 0.23	< 0.001	< 0.001
VU	8.26 ± 0.26	0.064	4.36 ± 0.46	0.409	< 0.001

N = 15 fields from 3 cultures, 2 preparations; $P^{control}$ refers to the comparison between blocked and control cultures with t-test or MW-RST; P^{T3} refers to the comparison between T3-treated and T3-untreated neurons with t-test or MW-RST; All data represent mean ± SEM.

The experiments confirm that GABAergic signalling has a direct influence on the frequency and duration of network wide spontaneous calcium transients. Similar observations have been made with multi electrode recordings from cultured cortical neurons where acute and chronic blockade of GABAergic signalling reduced burst frequency and prolonged burst duration (Baltz et al., 2010). Furthermore these experiments showed that the burst duration, which is reduced in T3-treated cultures, was only prolonged by $GABA_A$ receptor blockade in T3-treated but not in T3-deprived cultures. This suggests that GABAergic signalling has a larger impact on network activity in T3-treated than in T3-untreated networks, which might result, at least partly, from the enhanced expression of KCC2 in $T3^+$ cultures. In contrast to the complete blockade of $GABA_A$ signalling, reducing the hyperpolarizing drive of GABA signalling by inhibition of KCC2 increased burst frequency, but had no influence on burst duration as recently reported for spontaneous activity in spinal cord networks (Stil et al., 2011). In addition the results suggest that the number or properties of glutamatergic synapses were probably also affected by thyroid hormone application since the burst frequency and duration were different between $T3^-$ and $T3^+$ cultures in the presence of $GABA_A$ receptor antagonists.

4.5 Network changes after early T3 deficiency

A critical period for thyroid hormone signalling has been proposed since lack of T3 during the first two postnatal weeks causes severe and irreversible mental retardation and locomotor deficiencies following cortical, hippocampal and cerebellar malformation (Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Bernal et al., 2003; Venero et al., 2005). However, defects can be rescued by early postnatal administration of high doses of thyroid hormones (Legrand, 1984; Venero et al., 2005; Gilbert et al., 2007).

The previous results show that T3 modulated the development of cortical GABAergic neurons, increased synapse and bouton densities, and enhanced burst frequency during the second week in vitro. As the processes of synaptogenesis (Muramoto et al., 1993; Papa et al., 1995; de Lima et al., 1997), axonal growth (Voigt et al., 2005) and burst frequency development (Baltz et al., 2010) proceed during the third week in vitro, the next question was whether deficits in neuronal development induced by T3 deprivation during the first two weeks may normalize during the third week in vitro or can be rescued by later T3 application.

To address this question four experimental groups were set: a) chronically deprived cultures ($T3^{-}$), b) chronically T3-supplemented cultures ($T3^{+}$), c) cultures supplemented with T3 only during the third week *in vitro* ($T3^{-/+}$), d) and cultures supplemented with T3 only during the first two weeks ($T3^{+/-}$). The neuronal populations and GABAergic morphological development was examined in cultures of the different sets after GABA immunocytochemistry. Additionally, the patterns of spontaneous network activity were examined with calcium imaging.

4.5.1 Development of neuronal populations

The development of GABAergic and non-GABAergic neurons was estimated with a focus on the two subpopulations of GABAergic interneurons, L-GABAergic and S-GABAergic neurons. First, the development of neuronal densities was analysed between 14 and 21DIV (Fig. 22, Table 8; n = 60 fields, 6 cultures, 2 preparations). As previously shown, T3 affects the density of GABAergic and non-GABAergic neurons at 14DIV in a population specific manner (cf. Fig. 4), including a reduction of non-GABAergic neuron number after T3-stimulation.

Table 8: Development of neuronal populations

	cells/mm²	P^{-}	P^{ANOVA}		P^{14DIV}
L-GABA					
14 DIV					
$T3^{-}$	7.32 ± 0.52				
$T3^{+}$	6.15 ± 0.56	0.076			
21DIV					
$T3^{-}$	4.59 ± 0.43		|		< 0.001
$T3^{-/+}$	4.37 ± 0.37		|		< 0.001
$T3^{+/-}$	3.81 ± 0.31		0.196		0.002
$T3^{+}$	3.55 ± 0.38		|		< 0.001
Non-GABA					
14DIV					
$T3^{-}$	666.96 ± 35.25				
$T3^{+}$	464.41 ± 26.52	< 0.001			
21DIV					
$T3^{-}$	410.01 ± 18.22		|		< 0.001
$T3^{-/+}$	370.37 ± 16.12		|		< 0.001
$T3^{+/-}$	369.50 ± 18.61		0.305		0.011
$T3^{+}$	384.84 ± 15.46		|		0.103

		P^{-}	$P^{-/+}$	$P^{+/-}$	P^{14DIV}
S-GABA	cells/mm²				
14DIV					
$T3^{-}$	53.90 ± 2.61				
$T3^{+}$	62.44 ± 4.75	0.294			
21DIV					
$T3^{-}$	52.13 ± 2.70				0.741
$T3^{-/+}$	57.76 ± 3.16	0.178			0.384
$T3^{+/-}$	86.18 ± 4.38	< 0.001	< 0.001		< 0.001
$T3^{+}$	89.00 ± 5.07	< 0.001	< 0.001	0.675	< 0.001
% GABA	% of total				
14DIV					
$T3^{-}$	16.79 ± 0.83				
$T3^{+}$	21.27 ± 1.48	0.028			
21DIV					
$T3^{-}$	18.80 ± 1.03				0.277
$T3^{-/+}$	21.67 ± 1.28	0.107			0.002
$T3^{+/-}$	30.11 ± 1.71	< 0.001	< 0.001		< 0.001
$T3^{+}$	28.29 ± 1.71	< 0.001	0.003	0.435	0.002

P^{-}, p-value of T3 treated groups vs. T3 untreated controls; $P^{-/+}$, p-value of T3 treated groups vs. the $P^{-/+}$ group; $P^{+/-}$, p-value of T3 treated group vs. the $P^{+/-}$ group; P^{14DIV}, p-value of 21DIV cultures vs. 14DIV cultures ($T3^{-}$ and $T3^{-/+}$ vs. $T3^{-}$ at 14DIV; $T3^{+}$ and $T3^{+/-}$ vs. $T3^{+}$ at 14DIV); Statistics were made with t-test or MW-RST. P^{ANOVA}, p-value of 21DIV cultures tested with ANOVA. N = 60 fields each, 6 cultures, 2 preparations. All data represent mean ± SEM.

Indeed, a decrease in non-GABAergic neuron density was observed at 14DIV when cultures were treated with T3 (Fig. 22 B; Table 8). Measurement of neuron density at 21DIV indicated that the neuron number later became similar between $T3^-$ and $T3^+$ due to a delayed decrease in neuronal density in $T3^-$ cultures. The overall reduction in non-GABA density may follow activity-dependent intrinsic cell death programs and is in line with a generally accelerated maturation by T3. Also the L-GABA density was independent from T3 treatment at 21DIV, and already at 14DIV (Fig. 22 A; Table 8).

S-GABAergic interneurons develop later and still proliferate in embryonic cultures usually resulting in a higher density than L-GABAergic neurons. Interestingly there was a remarkably difference in S-GABAergic density at 21DIV (Fig. 22 C; Table 8). The density of S-GABA cultured in presence of T3 during the first two weeks ($T3^{+/-}$) was .not different from chronically T3-treated cultures, but significantly increased to T3-untreated control cultures. In contrast T3-stimulation only during the third week *in vitro* ($T3^{-/+}$) did not increase S-GABA density compared with control cultures. Thus in contrast to L-GABAergic neurons the density of S-GABAergic neurons was dependent on T3. Following the increase in S-GABA density also the total fraction of GABAergic neurons was increased in cultures treated with T3 at least during the first two weeks *in vitro* (Fig. 22 D; Table 8).

Noteworthy, the density of S-GABAergic neurons was only by trend and not significantly increased at 14DIV, in contrast to the initial experiments where already at 14DIV a mark T3-mediated increase in S-GABA number was measured (cf. Fig. 4). Nevertheless, at 21DIV a large difference between T3-treated and untreated cultures was established suggesting that the development of S-GABAergic neurons might be somehow slowed in this set of experiments. The reason for this is not entirely clear, but one explanation might be the addition of glia cells to cultures that grow older than two weeks, since glia cells have been suggested to inhibit neuronal development (de Lima and Voigt, 1999).

In summary, these results suggest that the final number of S-GABAergic neurons was established during the first two weeks *in vitro*. T3 promoted the integration of S-GABAergic neurons into the network but only during an early critical period. T3 stimulation after the second week *in vitro* was not sufficient to promote S-GABA survival. This is in line with previous reports that the survival of small GABAergic neurons depends on glutamatergic activity and neurotrophins during a restricted period of early network development (de Lima et al., 2004; de Lima et al., 2007).

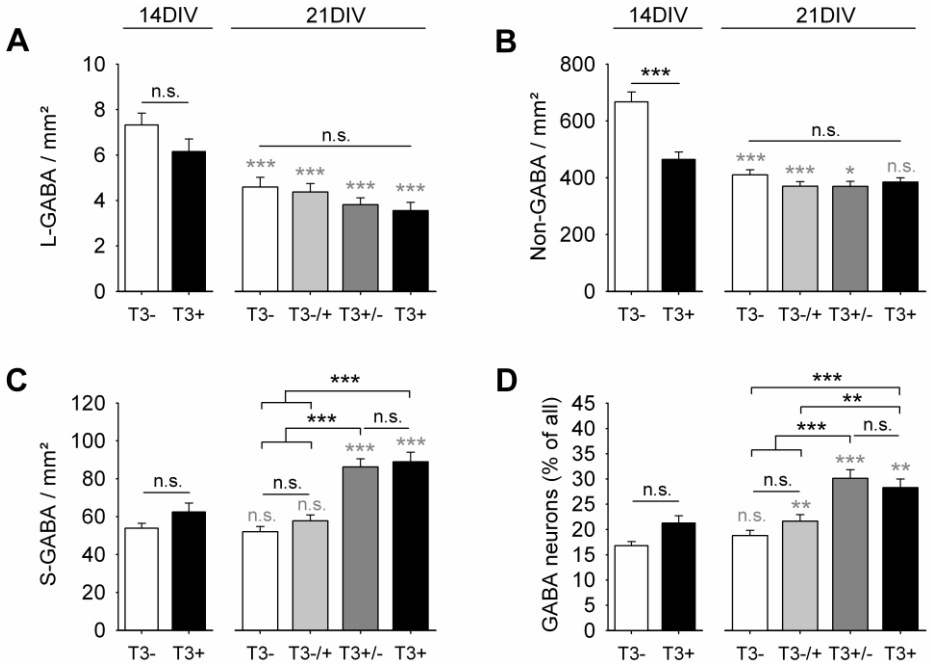

Figure 22: T3-stimulation increases the density of S-GABAergic neurons at 21DIV.
A-C. Neuronal cell densities in 14- and 21-day-old cultures were regulated by T3 in a subpopulation dependent manner. Whereas the densities of L-GABAergic (A) and non-GABAergic neurons (B) were not different at 21DIV, S-GABAergic neurons (C) show a prominent increase in cell number when cultured in T3-containing medium during the first two weeks in vitro. T3 treatment after 14DIV does not rescue S-GABAergic neuron density at 21DIV. **D.** According to the change in S-GABAergic neuron density, also the overall fraction of GABAergic neurons is increased at 21DIV in cultures treated with T3 during the first two weeks in vitro. Asterisks indicate differences between T3-treated and untreated cultures as indicated. Grey asterisks display differences between 14- and 21-day-old cultures. See also Table 8.

Interestingly the T3-sensitive period for S-GABA establishment seems to be timely correlated with the depolarizing period of GABAergic signalling (cf. Fig. 20). Depolarizing GABA signalling has neurotrophic effects potentially by inducing the expression and release of BDNF (Owens and Kriegstein, 2002; Ben-Ari et al., 2007). To demonstrate that the survival of S-GABA density might depend on the release of neurotrophins during spontaneous network activity S-GABA densities were calculated in 14-day-old cultures treated with the TrkB inhibitor K252a during the second week (Fig. 23 A: n = 60 fields, 6 cultures, 2 preparations). The S-GABA density was dramatically reduced by K252a from 100.87 ± 6.01 cells/mm² in $T3^-$ controls and 112.38 ± 6.39 cells/mm² in $T3^+$ controls to 2.38 ± 0.39 cells/mm² in $T3^-$ ($p < 0.001$, MW-RST) and 3.97 ± 0.67 cells/mm² in $T3^+$ ($p < 0.001$, MW-RST). The data confirm that S-GABAergic neurons are strongly dependent on neurotrophin support during the second week *in vitro*.

For complementation also the densities of L-GABAergic and non-GABAergic neurons were assessed. BDNF signalling blockade by K252a during the second week did neither affect the density of L-GABAergic neurons (Fig. 23 B: 4.42 ± 0.38 cells/mm² in $T3^-$ controls; 4.38 ± 0.35 cells/mm² in $T3^+$ controls, $p = 0.939$, MW-RST compared with $T3^-$ controls; 5.11 ± 0.49 cells/mm² in $T3^-$ + K252a, $p = 0.616$, MW-RST compared with $T3^-$ controls; 5.11 ± 0.45 cells/mm² in $T3^+$ + K252a, $p = 0.353$, MW-RST compared with $T3^+$ controls; $p = 0.747$, MW-RST compared with $T3^-$ + K252a), nor the density of non-GABAergic neurons (Fig. 23 C: 707.75 ± 46.07 cells/mm² in $T3^-$ controls; 521.41 ± 28.54 cells/mm² in $T3^+$ controls, $p < 0.001$, t-test compared with $T3^-$ controls; 763.31 ± 52.65 cells/mm² $T3^-$ + K252a, $p = 0.605$, MW-RST compared with $T3^-$ controls; 552.95 ± 30.95 cells/mm² in $T3^+$ + K252a, $p = 0.455$, t-test compared with $T3^+$ controls; $p = 0.004$, MW-RST compared with $T3^-$ + K252a). Thus, the results demonstrate that exclusively the survival of S-GABAergic neurons was dependent on BDNF signalling during the second week in culture, whereas the numbers of L-GABAergic and non-GABAergic neurons were not sensitive to BDNF deprivation.

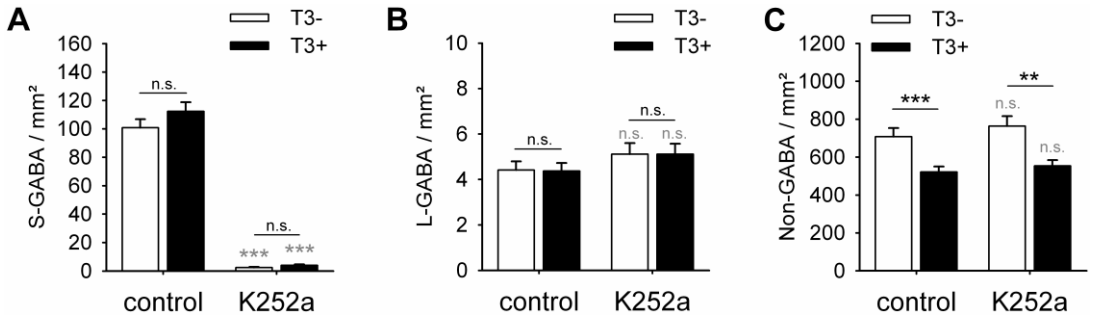

Figure 23: Survival of S-GABAergic neurons is dependent on BDNF signalling.
A-C. Treatment with K252a during the second week *in vitro* dramatically reduced the density of S-GABAergic neurons at 14DIV (A), but not of L-GABAergic (B) and non-GABAergic (C) neurons. Asterisks show significant differences as indicated. Asterisks indicate differences between T3-treated and untreated cultures as indicated. Grey asterisks display differences between K252a treated and control cultures.

4.5.2 Development of neuronal soma sizes

In addition to neuronal densities we measured the development of neuronal soma sizes between 14 and 21DIV as a general indicator of neuronal growth (Fig. 24, Table 9; $n = 120$ neurons, 6 cultures from 2 preparations). As already shown (see Figs. 4, 14, 15) L-GABAergic neurons showed a prominent T3-mediated enlargement of soma sizes at 14DIV (Fig. 24 B). However, in 21-day-old cultures the difference in soma sizes of L-

GABA vanished (Fig. 24 A, B) due to a progressive prominent growth between 14 and 21DIV. Also S-GABAergic (Fig. 24 C) and non-GABAergic (Fig. 24 D) neurons showed a prominent growth between 14 and 21 days *in vitro*, but no difference in soma sizes between the experimental groups was observed at 21DIV (Table 9).

These experiments indicate that T3 accelerates the somatic growth of L-GABAergic neurons and to a lesser extent that of S-GABAergic neurons, but cell sizes in all experimental groups reaches the same final size at 21DIV.

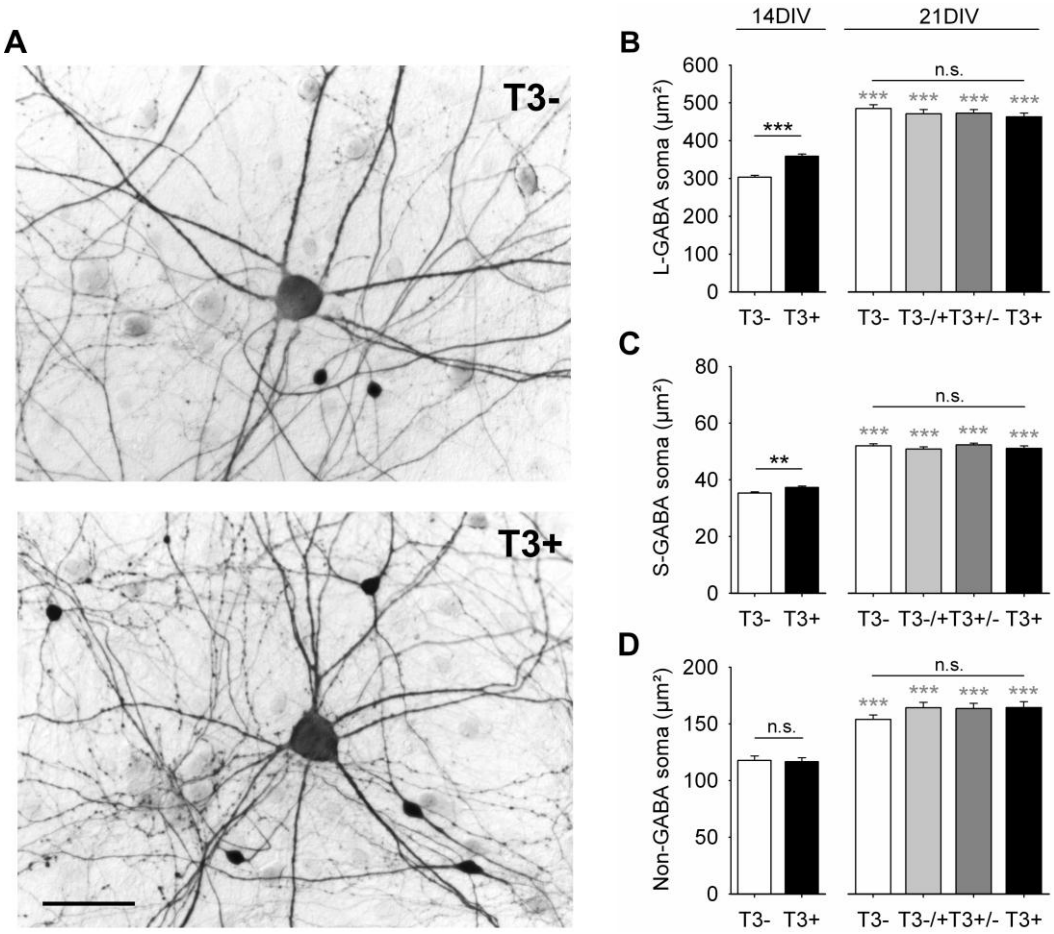

Figure 24: T3-treatment did not affect neuronal soma size at 21DIV.
A. Images show GABA immunostained 21-day-old cultures grown in the absence (top) or presence (bottom) of T3. Both images show a central L-GABAergic neuron surrounded by smaller S-GABAergic neurons and unstained non-GABAergic neurons. Scale bar = 50 µm. **B-D.** Soma sizes of neuronal subpopulations increase with culture time. All subpopulations show a significant soma enlargement between 14 and 21DIV. However, there were no differences between T3-treated and untreated cultures at 21DIV. Asterisks show significant differences as indicated. Grey asterisks display differences between 14- and 21-day-old cultures of the same hormone category. See also Table 9.

Table 9: Development of neuronal soma sizes

	soma size (µm²)	*P**	*P* ^{14DIV}
L-GABA			
14 DIV			
$T3^-$	303.64 ± 5.10		
$T3^+$	358.87 ± 6.27	< 0.001	
21DIV			
$T3^-$	485.11 ± 9.99	|	< 0.001
$T3^{-/+}$	471.36 ± 11.28	|	< 0.001
$T3^{+/-}$	472.62 ± 9.94	0.401	< 0.001
$T3^+$	462.98 ± 10.14	|	< 0.001
S-GABA			
14DIV			
$T3^-$	35.31 ± 0.46		
$T3^+$	37.28 ± 0.54	0.006	
21DIV			
$T3^-$	51.99 ± 0.73	|	< 0.001
$T3^{-/+}$	50.84 ± 0.79	|	< 0.001
$T3^{+/-}$	52.34 ± 0.63	0.201	< 0.001
$T3^+$	51.14 ± 0.88	|	< 0.001
Non-GABA			
14DIV			
$T3^-$	117.80 ± 3.83		
$T3^+$	116.56 ± 3.65	0.797	
21DIV			
$T3^-$	153.84 ± 4.15	|	< 0.001
$T3^{-/+}$	164.17 ± 4.90	|	< 0.001
$T3^{+/-}$	163.51 ± 5.26	0.230	< 0.001
$T3^+$	164.28 ± 4.78	|	< 0.001

*P**, p-value of T3 treated groups vs. T3 untreated controls tested with t-test or MW-RST at 14DIV and ANOVA at 21DIV; *P* ^{14DIV}, p-value of 21DIV cultures vs. 14DIV cultures ($T3^-$ and $T3^{-/+}$ vs. $T3^-$ at 14DIV; $T3^+$ and $T3^{+/-}$ vs. $T3^+$ at 14DIV). N = 120 neurons each, 6 cultures, 2 preparations. All data represent mean ± SEM.

4.5.3 Development of L-GABAergic axons and boutons

Next we asked whether the initially described T3-mediated changes in axonal development might be also transient effects. We first calculated the mean density of GABAergic boutons in 21-day-old cultures. Because of the highly increased density of the GABAergic neuropil at 21DIV it was not possible to reliably estimate the length and branch numbers of GABAergic axons. At 14DIV (Fig. 25 A) the mean number of boutons was increased in T3-treated cultures (47.05 ± 2.55 boutons/field in $T3^-$; 63.80 ± 4.79 boutons/field in $T3^+$; n = 60 fields, p = 0.009, MW-RST). Like the soma size also the overall number of GABAergic boutons formed by L-GABAergic neurons did not differ

between T3-treated and T3-untreated cultures at 21DIV (Fig. 25 B: 90.86 ± 6.37 boutons/field in $T3^{-}$; 83.63 ± 7.42 boutons/field in $T3^{-/+}$; 75.55 ± 8.22 boutons/field in $T3^{+/-}$; 76.48 ± 10.06 boutons/field in $T3^{+}$; n = 40 fields; p = 0.512, ANOVA).

In 14-day-old cultures GABAergic axons showed a characteristic concentration in cell rich areas in the presence of T3 (cf. Figs. 15, 16). Correlating the number of GABAergic boutons to the local cell density at 21DIV (Fig. 25 C) suggested that this characteristic distribution was gradually also established in older T3-deprived cultures ($T3^{-}$; $p < 0.001$; $r^2 = 0.308$; $T3^{-/+}$; $p < 0.001$; $r^2 = 0.374$; $T3^{+/-}$; $p < 0.001$; $r^2 = 0.548$; $T3^{+}$; $p < 0.001$; $r^2 = 0.574$). However, there were still differences in the correlation indexes and in the slopes of the regression lines between the different experimental groups.

According to the mean cell number (Fig. 25 D; 3.40 ± 0.40 cells/field in $T3^{-}$; 3.55 ± 0.38 cells/field in $T3^{-/+}$; 3.93 ± 0.46 cells/field in $T3^{+/-}$; 3.65 ± 0.43 cells/field in $T3^{+}$; n = 40 fields each) the data were again grouped into fields with low (≤ 3 cells/field) and high cell density (> 3 cells/field). The results confirm that independent of hormonal treatment axons were concentrated in regions with more than 3 cells/field (Fig. 25 E; in boutons/field, brackets indicate number of fields: 69.88 ± 8.99 (17) in $T3^{-}$ low-cell-density and 106.39 ± 7.49 (23) in $T3^{-}$ high-cell-density, p = 0.003, t-test; 57.63 ± 7.31 (19) in $T3^{-/+}$ low-cell-density and 107.14 ± 10.13 (21) in $T3^{-/+}$ high-cell-density, $p < 0.001$, t-test; 48.64 ± 8.79 (22) in $T3^{+/-}$ low-cell-density and 108.44 ± 10.60 (18) in $T3^{+/-}$ high-cell-density, $p < 0.001$, t-test; 37.73 ± 10.12 (22) in $T3^{+}$ low-cell-density and 123.83 ± 11.04 (18) in $T3^{+}$ high-cell-density, $p < 0.001$, t-test).

However, the increase in bouton number in fields with high cell density compared with that in fields with low cell density revealed that T3 stimulation during at least the first two weeks *in vitro* had long-lasting effects on axonal distribution (Fig. 25 F). The increase in chronically deprived cultures was 152.24 ± 10.72% (n = 23 fields) and was not further promoted if T3-stimulation was applied only during the third week of culturing (185.91 ± 17.57%, n = 21 fields, p = 0.103, t-test compared with $T3^{-}$). T3 presence during the first two weeks (222.97 ± 21.80%, n = 18 fields, p = 0.003, t-test compared with $T3^{-}$) as well as chronic T3 treatment (328.23 ± 29.26%, n = 18 fields, $p < 0.001$, MW-RST compared with $T3^{-}$) significantly increased the difference between fields with high and low cell density. The increase in chronically treated cultures was significantly larger than in cultures from reversal experiments ($p = 0.007$, t-test compared with $T3^{+/-}$; $p < 0.001$, t-test compared with $T3^{-/+}$) indicating that continuous T3 presence was essential for the optimal axonal adjustment.

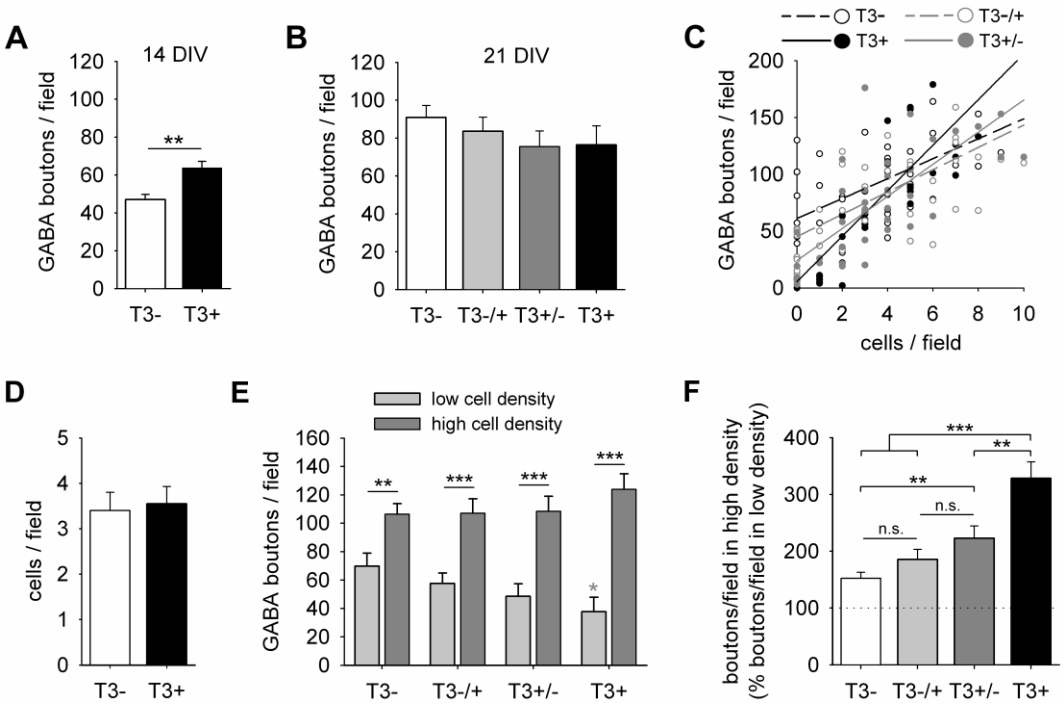

Figure 25: Development of L-GABA axons and boutons at 21DIV.
A. T3 enhances the overall density of GABAergic boutons in 14-day-old cultures. B. In 21-day-old cultures the overall number of GABAergic boutons is not different between culture conditions. C. In all experimental groups the number of GABAergic boutons was dependent on the local density of non-GABAergic neurons, but the slope of the regression line was highest in chronically T3-treated cultures. D. According to the mean neuron number at 21DIV data were grouped into fields with low (≤ 3 cells/field) and high (> 3 cells/field) cell densities. E. In all culture conditions the number of GABAergic boutons was significant higher in fields with high cell density compared with fields with low cell density. F. The normalized difference in bouton numbers (boutons in fields with high cell density in percent of boutons in fields with low cell density of the same experimental group) was significantly increased compared with T3-untreated cultures when neurons were treated with T3 during the first two weeks *in vitro*. Asterisks show significant differences as indicated. Grey asterisks display statistical difference compared to $T3^-$ (E).

These results demonstrate that the general growth of L-GABAergic neurons, reflected by the soma size and the overall number of GABAergic boutons, is transiently promoted by T3. A concentration of GABAergic boutons around cell bodies of non-GABAergic neurons could be observed in all cultures, but this concentration was more pronounced in cultures that received T3 during the first two weeks of development. Thus, the maturation of L-GABAergic axonal arborizations is most sensitive to T3 during initial phases of network formation. However, optimal axonal growth is dependent on continuous T3 presence.

4.5.4 Spontaneous network activity

Finally we estimated the functional development of neuronal networks supplemented with T3 during different time windows using the calcium imaging technique (Fig. 26: *n* = 20 fields, 4 cultures from 2 preparations). In general all 14 to 21-day-old cultures showed prominent synchronous network events (Fig. 26 A - C).

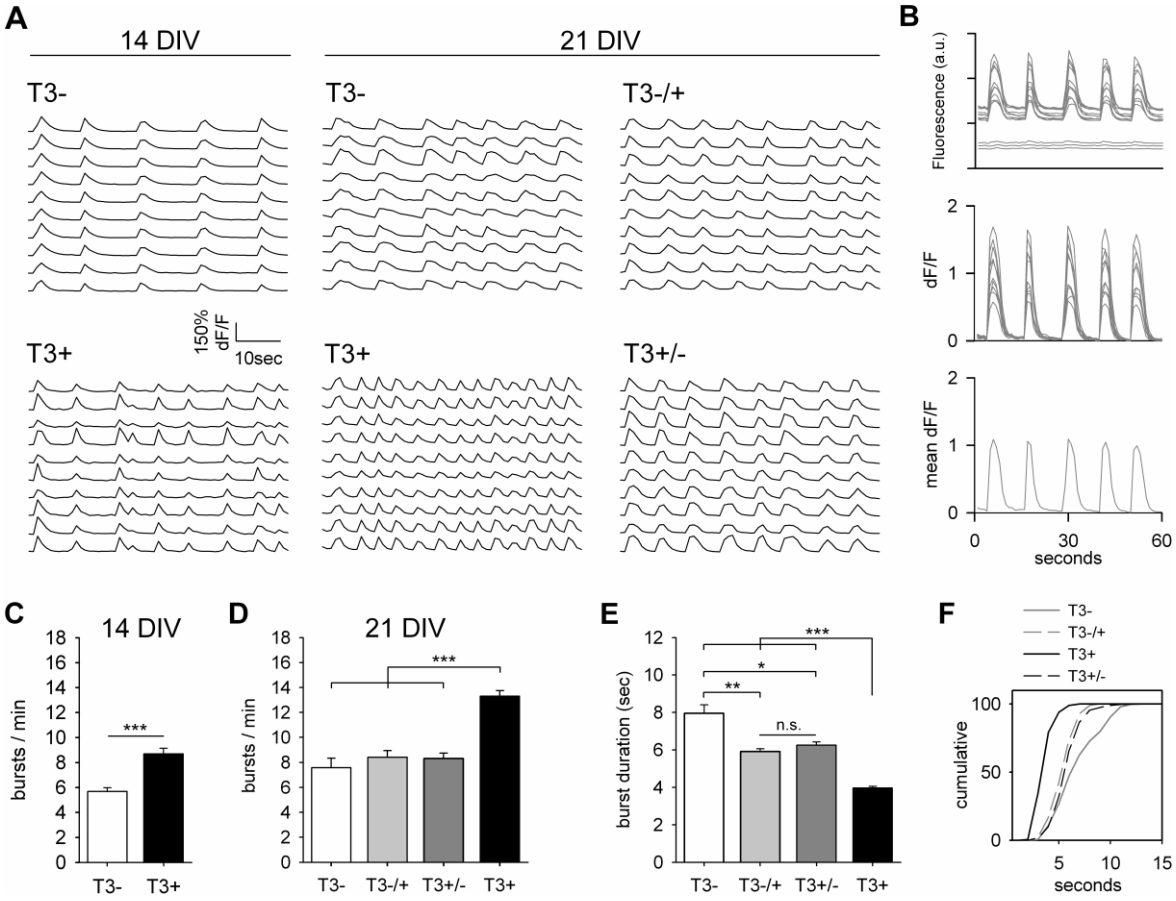

Figure 26: Network activity at 21DIV and reversibility.
A. Traces show the change in intracellular calcium signal of individual neurons from 14- or 21-day-old cultures grown either in the absence or presence of T3 as indicated. Fluorescence data are expressed as dF/F (background-corrected fluorescence divided by resting fluorescence). **B.** Analysis of calcium transients. Top panel: raw fluorescence data from ten individual cells and three background regions. Middle panel: fluorescence data were background corrected and normalized (dF/F) according to the minimal fluorescence level during the sequence. Bottom panel: mean dF/F averaged from the ten individual cells presented above. The mean dF/F trace of each recorded field was used to calculate burst duration. **C.** T3 increases the burst frequency at 14DIV. **D.** The maximal burst frequency at 21DIV was measured in cultures chronically treated with T3. **E.** Burst duration at 21DIV was reduced in reversal experiments compared with T3-untreated cultures and minimal burst duration was observed in T3-treated cultures (*n* = 20 fields). **F.** Cumulative representation of burst durations including all single transients analyzed in mean dF/F data (*n* = 451 bursts in $T3^{-}$: 622 bursts in $T3^{-/+}$; 588 bursts in $T3^{+/-}$; 925 bursts in $T3^{+}$).

However, chronic supplementation of neuronal cultures with T3 clearly enhanced the burst frequencies at 14DIV (Fig. 26 A, B; $T3^{-}$: 5.67 ± 0.31 bursts/min, $T3^{+}$: 8.70 ± 0.45 burst/min, $p < 0.001$, t-test) as well as at 21DIV (Fig. 26 A, C; $T3^{-}$: 7.56 ± 0.77 bursts/min, $T3^{+}$: 13.30 ± 0.45 bursts/min, $p < 0.001$, MW-RST) compared to T3-deprived cultures. Furthermore, T3 treatment only during the third week in vitro did not enhance burst frequencies compared with untreated control cultures ($T3^{-/+}$: 8.41 ± 0.53 bursts/min; $p = 0.273$, MW-RST compared with $T3^{-}$ at 21DIV). Additionally, depletion of the hormone from the culture medium at 14DIV normalized burst frequencies compared with untreated cultures ($T3^{+/-}$: 8.31 ± 0.43 bursts/min, $p = 0.589$, MW-RST compared with $T3^{-}$ at 21DIV). Thus the high burst frequency of chronically T3-treated cultures ($T3^{+}$) could not be achieved by T3 supplementation neither during only the first two weeks *in vitro* ($T3^{+/-}$: $p < 0.001$, t-test compared to $T3^{+}$ with 21DIV) nor during only the third week of culturing ($T3^{-/+}$: $p < 0.001$, t-test compared with $T3^{+}$ at 21DIV).

The duration of calcium transients in 21-day-old cultures was estimated as a general indicator of burst kinetics (Fig. 26 D, E; $n = 20$ fields, 4 cultures from 2 preparations). The mean length of calcium transients in T3-deprived cultures was 7.95 ± 0.45 sec (Fig. 26 D). The duration was significantly decreased already by transient T3 presence during the first two weeks ($T3^{+/-}$: 5.91 ± 0.16 sec; $p = 0.003$, MW-RST compared with $T3^{-}$) or during the third week of development ($T3^{-/+}$: 6.26 ± 0.17; $p = 0.013$, MW-RST compared with $T3^{-}$). Chronic T3-treatment during the entire cultivation period reduced the burst duration to 3.97 ± 0.09 sec ($p < 0.001$, MW-RST compared with $T3^{-}$; $p < 0.001$, t-test compared with $T3^{+/-}$; $p < 0.001$, t-test compared to $T3^{-/+}$). These results indicate that a constant supply of T3 is correlated with an increased burst frequency and a decrease of bursting duration. However, the addition of the hormone after the initial period of development (i.e., only during the third week in vitro [$T3^{-/+}$]) is not sufficient to rescue the network from deficits associated with the absence of T3.

5. Discussion

5.1 Hormonal modulation of network activity and synaptic maturation

5.1.1 Network activity

Converging evidence suggests that during early neocortical development synchronous network activity emerge when neurons become interconnected. Synchronous events with very low frequency develop to reach maximal cellular participation, which includes nearly all neurons (Garaschuk et al., 2000; Opitz et al., 2002; Corlew et al., 2004). As development proceeds, synchronous events with lower cellular participation and higher burst frequency become interposed to events with higher participation, and eventually these slow highly synchronous events disappear (Corlew et al., 2004; Allene et al., 2008; Baltz et al., 2010).

Two-weeks-old cultures treated chronically with triiodothyronine (T3) showed increased frequency of spontaneous bursts of activity and decreased neuronal participation per event, when compared with T3-deprived cultures (Fig. 1). These results strongly suggest that networks treated with T3 show more mature properties than untreated networks, and that T3 actions contribute to the generation of synchronous events with higher frequency and lower neuronal participation. Additionally, at the end of the third week *in vitro* (Fig. 26) chronically T3-treated cultures showed higher burst frequencies but shorter burst durations than untreated cultures.

Synchronous network activity is found transiently in a wide range of brain structures and plays an important role in the establishment of neuronal connections (Garaschuk et al., 2000; Voigt et al., 2005; Allene et al., 2008). It is suggested that early spontaneous activity patterns guide the initial wiring of cortical structures and disappears upon maturation of sensory systems, when experience-dependent mechanisms further refine network connectivity (Katz and Shatz, 1996; Hensch, 2004). Considering the importance of activity-dependent processes in neuronal maturation, early network activity may be an attractive candidate to mediate several indirect effects of T3. Enhanced maturation of network activity may induce additional development of the GABAergic neurons and other activity-dependent processes regarding survival and maturation of neuronal connectivity. For example, by regulating network activity through local increases in synaptogenesis and neuronal growth, T3 may indirectly modulate the formation of long range axonal connections (Voigt et al., 2005). Indeed, T3 has been shown to influence the

establishment of cortical and hippocampal projections potentially by means of activity-dependent mechanisms (Schwegler et al., 1991; Li et al., 1995; Lucio et al., 1997). On the other side, it could not be excluded that a premature increase in spontaneous activity due to an early excess of T3 might convey negative effects like an abnormal stabilization of developing synapses, prevention of normal refinement and specific synaptic elimination, leading to altered circuitry.

5.1.2 Synaptic maturation

The present results show additionally that T3-mediated increase in synchronous activity is paralleled by increases in co-expression of synaptic proteins in pre- and postsynaptic compartments (Fig. 2). The increase in density of glutamatergic and GABAergic synapses following T3 treatment concurs with the idea of network activity maturation.

Thyroid hormones have already been shown to enhances the expression of several presynaptic proteins involved in neurotransmitter release, e.g. Synapsin (Hosoda et al., 2003), SNAP-25 (Zhang et al., 2008), Synaptophysin (Gong et al., 2010) and Synaptotagmin-1 (Wang et al., 2011). Accordingly, Hosoda et al. (2003) showed that exposure to triiodothyronine or thyroxine caused an increase in the frequency of spontaneous synchronous oscillatory changes in intracellular calcium concentration, which correlated with the number of synapses formed. Consequently, deficits in thyroid hormone signalling have been related to impaired long term potentiation (Sui and Gilbert, 2003; Gerges and Alkadhi, 2004; Dong et al., 2005), which potentially might arise from deficits in synaptic proteins.

5.1.3 Axonal arrangement and pruning

The present experiments further show that the number of GABAergic boutons formed by large GABAergic neurons is increased in 14-day-old cultures, suggesting an increase in GABAergic synapses and GABA release (Figs. 13, 14). But not only the number of GABAergic boutons is enhanced in T3-stimulated cultures, GABAergic axons also form conspicuous nests of axonal protrusions around groups of non-GABAergic cells (Figs. 13, 15, 16). These characteristic nests arise from the enhanced formation of GABAergic boutons in the vicinity of non-GABAergic neurons but apparently also from

the pruning of GABAergic axons from cell free regions (Fig. 15, 16). The results suggest that T3 is involved in both the formation of new contacts and the pruning of other ones.

The reorganization of synaptic contacts and pruning of axonal projections is an important process in the establishment and refinement of neuronal networks. Following the initial formation of synaptic connections, many developing circuits undergo a period of regression and refinement, through which some connections are eliminated while others are strengthened (Purves and Lichtman, 1980; Lohof et al., 1996; Zhang and Poo, 2001). It is typical for the initial wiring of neuronal networks that an exuberant number of connections are formed, which are then refined by a selective, often activity-dependent pruning of overwhelming connections (Innocenti and Price, 2005). Interestingly, thyroid hormones have been suggested to play a role in the refinement of exuberant axons (Innocenti and Price, 2005). T3 has been shown to regulate the disappearance of transient callosal projections (Li et al., 1995; Lucio et al., 1997). In animals made hypothyroid during early development, callosal projection which are normally transient, could also be found in adult animals. These T3-mediated processes might be activity-dependent, since the refinement of callosal projection is also regulated by neuronal activity (Wang et al., 2007).

5.2 Hormonal modulation of GABAergic neurons development

The analysis of neuron development was done on three neuronal subpopulations, the L-GABAergic neurons, S-GABAergic neurons and non-GABAergic neurons, which can be easily identified in neuronal cell cultures. Special attention was given to the subpopulations of GABAergic neurons, which develop in close interrelationship with the network activity. The results suggest population specific changes in soma sizes and cell density at 14DIV following thyroid hormone treatment (Fig. 4).

5.2.1 Large GABAergic interneurons

Irrespective of growing conditions, L-GABA neurons have a large cell body, stellate dendritic tree and thick axons forming long range connections. In mature cultures, L-GABAergic neurons show morphological and physiological properties similar to cortical parvalbumin neurons in situ (Voigt et al., 2001; de Lima et al., 2004; Klueva et al., 2008; see chapter 2.2.5).

The present results demonstrate that the morphological maturation of large GABAergic interneurons is highly sensitive to T3 during early postnatal development. T3 stimulation accelerates the formation of GABAergic boutons and has profound long-lasting effects on the distribution of axonal arborisations among non-GABAergic neurons. In the presence of T3 L-GABA axons sprout around cell bodies and are pruned in cell free areas (Figs. 15, 16) resulting in conspicuous nests of axonal protrusions around groups of non-GABAergic cells (Figs. 13, 15, 16). Also the soma size of L-GABAergic neurons was transiently increased in hormone-treated cultures (Figs. 4, 15, 24), suggesting that T3 accelerates the maturation of this interneuron type. But the acceleration of soma size development and bouton formation observed in hormone-treated cultures is presumably not caused by an enhanced overall cell growth, because number and length of dendrites are not changed (Fig. 12).

Accumulating evidence already suggests that parvalbumin expressing interneurons *in vivo* are modulated by thyroid hormone signalling (Berbel et al., 1996; Gilbert et al., 2007; Wallis et al., 2008). Disrupting thyroid hormone signalling by rendering rats hypothyroid or using mice expressing a dominant-negative point mutation in the TRalpha1 gene reduces the number of parvalbumin-immunoreactive neurons as well as the density of parvalbumin-positive terminals and processes in hippocampus and neocortex (Berbel et al., 1996; Gilbert et al., 2007; Wallis et al., 2008). Since parvalbumin neurons have been identified to express thyroid hormone receptors (Guadaño-Ferraz et al., 2003), it is likely that these neurons are a direct target of T3. The present results extend available evidence by showing that already during early cortical network development, when parvalbumin is not present yet, a deficit of thyroid hormone slows the development of this subpopulation of GABAergic neurons.

5.2.2 Small GABAergic interneurons

S-GABAergic neurons are a second and numerically larger subpopulation of GABAergic neurons. These neurons are born later, have smaller fusiform to bipolar or multipolar cell bodies and very thin axons (de Lima and Voigt, 1997; de Lima et al., 2004). They typically express the calcium binding protein calretinin and might therefore be correlates of calretinin/VIP interneurons of the upper cortical layers in situ (Gonchar and Burkhalter, 1997).

In contrast to large GABAergic neurons, the small GABAergic cells showed only minor changes in soma size following T3 treatment (Fig. 4), but a large increase in S-GABA density could be measured already in 14-days-old cultures (Fig. 4). This difference did not normalize in older cultures and could not be rescued by later T3 application (Fig. 22), suggesting that S-GABAergic neurons are irreversibly established until the end of the second week in vitro. As these neurons are a numerically large group, also the relative proportion of all GABAergic neurons was increased in T3-treated cultures (Fig. 4, 22).

In contrast to parvalbumin neurons, which have already been identified to be sensitive to thyroid hormones, little is known about the effects of thyroid hormones on other GABAergic neuron populations. This might be due to the fact, that these smaller, late born interneurons compose a quite heterogeneous group. In culture these neurons are characterized by their small, fusiform cell bodies and their late appearance. Due to their late birth, S-GABAergic neurons are generated in vitro from precursors and increase numerically until the end of the second week in vitro (de Lima and Voigt, 1997; de Lima et al., 2004). Interestingly, it has been shown that these neurons are specifically vulnerable to irreversible elimination from the network during a critical time window (DIV12-14) (de Lima et al., 2004). During this time they strongly depend on glutamatergic activity mediated by NMDA receptors (de Lima et al., 2004) and the presence of neurotrophins (Fig. 23). The present results suggest that thyroid hormones promote the establishment of S-GABAergic neurons during this special time window, potentially indirect by the increase in neuronal activity.

5.2.3 Non-GABAergic neurons

The present study was focussed in the developmental aspects of GABAergic neurons, because GABAergic neurons were recently identified as an important target of thyroid hormone actions and because of the intricate interrelationship of GABAergic neurons and network activity development during early network formation. Nevertheless, the results suggest that also the number of glutamatergic synapses (Fig. 2) and the size of non-GABAergic neurons (Fig. 4) is increased by T3 at 14DIV. Additionally, T3 treatment enhanced the burst frequency even in the presence of blocked GABAergic neurotransmission (Fig. 21), suggesting the modulation of glutamatergic mechanisms. Thus, although the present study did not investigated the long-term changes in

glutamatergic neurons in detail, also non-GABAergic neuron are likely a target of thyroid hormone actions.

This is in line with reports showing that thyroid hormones regulate the development of axonal projections (Li et al., 1995; Lucio et al., 1997), the number of dendritic spines (Ruiz-Marcos et al., 1980, 1982; Nimchinsky et al., 2002), the differentiation of dendrites (Ipiña et al., 1987; Sala-Roca et al., 2008), NMDA receptor expression (Kobayashi et al., 2006; Lee et al., 2003) and the excitability of glutamatergic neurons (Potthoff and Dietzel, 1997; Hoffman and Dietzel, 2004; Niederkinkhaus et al., 2009). Since the differentiation and synaptogenesis of glutamatergic cells is also modulated by activity- and neurotrophin-dependent mechanism (Lessmann et al., 2003; Gottmann et al., 2009), it would be interesting, whether T3-mediated modulation of glutamatergic and GABAergic neurons follow the same mechanisms.

5.2.4 Relevance of GABAergic neurons modulation

GABAergic signalling plays important roles both during development and for adult brain function. During development immature GABAergic signalling regulates neurogenesis (LoTurco et al., 1995; Haydar et al., 2000), cell migration (Behar et al., 2000; Lujan et al., 2005) and neuritogenesis (Groc et al., 2002; Ben-Ari et al., 2007). Many neurotrophic actions of GABA have been related to the depolarizing actions of immature GABAergic signalling (Owens and Kriegstein, 2002; Cancedda et al., 2007; Pfeffer et al., 2009). Depolarizing GABAergic neurotransmission is required for the initiation and propagation of early synchronous network activity. In this process the function of L-GABAergic neurons is of special interest. The presence and the size of L-GABAergic neurons impact the initial development of neocortical networks (Voigt et al., 2001; Baltz et al., 2010) especially promoting the early synchronization of spontaneous activity and the maturation of glutamatergic synapses (Voigt et al., 2001, 2005). Because L-GABAergic cells are key elements in the emergence of early oscillatory network activity, the action of thyroid hormone on their growth contributes to the timely appearance of synchronized network activity. The increased growth and synaptogenesis of early GABAergic neurons lead to the enhanced maturation of network activity, which in turn may induce additional development of the GABAergic neurons and other activity-dependent processes like survival and trimming of neuronal connectivity (de Lima et al., 2004; Ben-Ari et al., 2007).

Moreover, the correct development of the GABAergic system is essential for the balance between excitation and inhibition in adult cortical networks (Buzsáki, 2006). Consequently, dysfunctions of GABAergic signalling have been associated with several pathological conditions that are potentially generated when the balance between excitation and inhibition is impaired, such as autism, mental retardation, epilepsies and schizophrenia (DeFelipe, 1999; Levitt et al., 2004; Lewis et al., 2005; Wonders and Anderson, 2006, Ben-Ari et al., 2007; Woo and Lu, 2006). By modulating the growth and synaptic maturation of GABAergic neurons, T3 might modulate critical aspects of neuronal network formation and maturation, and in turn T3 deprivation might cause multiple and irreversible developmental deviations. Indeed, deficits in the maturation of the GABAergic system reported in mice harbouring a mutant thyroid hormone receptor have been directly correlated to a reduction on gamma oscillation power and locomotor dysfunctions and anxiety (Venero et al., 2005; Wallis et al., 2008).

5.3 T3-mediated expression of KCC2 and GABA switch

The depolarizing actions of GABA during early postnatal development originate from a high intracellular concentration of chloride regulated by the two cation chloride cotransporters NKCC1 and KCC2 (Ben-Ari, 2007; Blaesse et al., 2009). The developmental downregulation of NKCC1 and concomitant upregulation of KCC2 represent the onset of mature hyperpolarizing GABAergic signalling by lowering the intracellular chloride concentration. Recently, thyroid hormone signalling has been linked to the expression and function of NKCC1 and KCC2 in hippocampus and auditory system respectively (Friauf et al., 2008; Hadjab-Lallemend et al., 2010).

To examine the influence of T3 on the GABA shift, the developmental expression patterns of NKCC1 and KCC2 protein in T3 treated and untreated cultures were determined. Our results reveal that KCC2 is critically dependent on thyroid hormone signalling since no developmental upregulation of KCC2 protein could be observed in the absence of T3 (Fig. 19). Moreover, the failure of KCC2 upregulation in T3-deprived neurons was correlated to a delayed switch from depolarizing to hyperpolarizing GABAergic signalling in non-GABAergic neurons (Fig. 21), corroborating the idea of a correlation between KCC2 expression and the developmental GABA shift (Rivera et al., 1999; Zhu et al., 2005; Lee et al., 2005). In contrast to KCC2, the developmental downregulation of NKCC1 after the second week *in vitro* was independent of thyroid

hormone presence (Fig. 19). Interestingly, in T3-deprived cultures the developmental GABA shift was correlated with the downregulation of NKCC1, suggesting an important role of NKCC1 downregulation in the regulation of the GABA shift. The results extend recent studies which suggest that thyroid hormone signalling regulates GABAergic function in hippocampus and auditory system, respectively (Friauf et al., 2008; Hadjab-Lallemend et al., 2010).

Deficit in KCC2 has been correlated to epilepsy and spasticity (Munoz et al., 2007; Kahle et al., 2008; Boulenguez et al., 2010) and thus might potentially be related to locomotor dysfunctions reported in mice harbouring a mutant thyroid hormone receptor (Venero et al., 2005; Wallis et al., 2008). Moreover, plasticity of GABAergic signalling can be rapidly induced by the regulation of intracellular chloride through the immediate modulation of KCC2 expression and function by neuronal activity, referred to as 'ionic plasticity' (Wardle and Poo, 2003; Rivera et al., 2004; Blaesse et al., 2009). Following the failure of developmental KCC2 upregulation in T3-deprived neurons this form of plasticity should be limited in the absence of thyroid hormone. Additionally, KCC2 might also be involved in maturation of excitatory synapses since KCC2 located at excitatory synapses (Gulyas et al., 2001) has been suggested to regulate dendritic spine formation by a transport independent mechanism (Li et al., 2007).

Interestingly, thyroid hormones have also been suggested to modulate the Na^+-K^+-ATPase (Bajpai et al., 2001; Pacheco-Rosado et al., 2005; Cheng et al., 2010). This is of relevance, because NKCC1 and KCC2 do not use adenosine triphosphate (ATP) but operate using the electrochemical gradients for Na^+ and K^+ produced by the Na^+-K^+-ATPase. Since also the Ca^{2+}-ATPase is affected by thyroid hormones (Cheng et al., 2010), thyroid hormones are an important regulator of the neurochemical composition and thus the intrinsic properties of neurons.

5.4 Mechanisms of thyroid hormone mediated actions

To analyse the underlying mechanisms of T3 actions in more detail the work was focused on the development of L-GABAergic neurons because of two reasons: first, these neurons show large changes in soma size in initial experiments (Figs. 4), and thus they might be particularly sensitive to thyroid hormone treatment. Secondly, the development of L-GABAergic cells is of special interest, because they are key players in the generation and propagation of network activity (Voigt et al., 2001).

5.4.1 Direct or indirect T3 modulation of GABAergic neurons?

Although a large number of thyroid hormone regulated proteins has been identified (Yen et al., 2006; Takahashi et al., 2008), the question whether changes in protein expression are the direct or indirect result of thyroid hormone action remains unanswered. Only few genes have already been shown to be under direct control of T3 (Bernal et al., 2003). The present data suggest that synchronous network activity enhancement parallels the increased growth of L-GABAergic neurons by thyroid hormone. On the one hand, it is an attractive hypothesis that T3 promotes the maturation and synaptogenesis of L-GABAergic cells, which then, due to the excitatory action of GABA during early development, enhances the network activity. On the other hand, the maturation of GABAergic neurons depends on neuronal activity (Patz et al., 2004; Jin et al., 2003; Chattopadhyaya et al., 2004; de Lima et al., 2004). So it is also feasible that GABAergic maturation is not the cause but rather the consequence of activity enhancement by T3 stimulation.

In favor of the first hypothesis the results show that T3 influences L-GABAergic neurons growth also in the absence of activity (Fig. 10). Moreover, a significant modulation of L-GABAergic interneurons development by thyroid hormones occurs before any measurable alteration of spontaneous activity (Fig. 9). Also the GABAergic boutons density along axons increases in presence of T3 irrespective of local cell density and independently of BDNF activity, although stimulation with exogenous BDNF partially rescued GABAergic bouton formation in absence of T3 (Figs. 16, 17, 18). Thus, growth and bouton formation of L-GABAergic neurons is, at least partly, directly induced by T3 actions. In line with this, thyroid hormone receptors have been found in parvalbumin-positive GABAergic neurons of the hippocampus and neocortex (Guadano-Ferraz et al., 2003).

5.4.2 Genomic or non-genomic actions?

In the classical view the actions of T3 are mediated by nuclear thyroid hormone receptors (TRs). These ligand-modulated transcription factors can directly bind to the DNA and influence gene expression (Yen, 2001; Bernal, 2007), and TRs are expressed in virtually all neurons (Wallis at al., 2010) including parvalbumin interneurons (Guadano-Ferraz et al., 2003). This is in line with the present results showing that thyroid hormone receptor antagonist 1-850 (Schapira et al., 2003) reduces T3 mediated increase in burst

frequency as well as in L-GABA soma size (Fig. 8). Additionally, T3 effects can be induced by very low concentrations (Fig. 6) and burst frequency is not dependent on the acute presence of T3 (Fig. 7), which is also more likely for regulation by genomic actions.

This suggestion is supported by recent studies using mutant mice. Thyroid hormones had already been shown to modulate the development and function of parvalbumin-positive interneurons (Berbel et al., 1996; Gilbert et al., 2007). Similar results were obtained from experiments using mutant mice devoid of all TRs or carrying a mutated form of the TRalpha receptor (Guadano-Ferraz et al., 2003; Venero et al., 2005; Wallis et al., 2008) suggesting a prominent role of nuclear receptors in GABAergic neurons development.

In conclusion, the detailed mechanisms of T3-mediated growth of GABAergic neurons are still unclear, but the results suggest, in line with the literature, that T3 acts primary via nuclear thyroid hormone receptors. However, the contribution of non-genomic hormone actions or the involvement of other growth factors cannot be completely excluded.

5.4.3 Dependence on neuronal activity

The present experiments suggest that independent of spontaneous activity, thyroid hormones act as neurotrophic factors for the subpopulations of L-GABAergic and non-GABAergic neurons. T3-treated neurons were significantly larger in T3-treated then in T3-untreated cultures even when network activity was blocked (Fig. 10, cf. chapter 5.4.1). However, antagonists to ionotropic glutamate receptors and TTX blocked most of T3 effect on L-GABAergic growth and in turn GABAergic neurons developed maximally when both neuronal activity and T3 were present (Fig. 10). Additionally, also in T3-deprived cultures the blockade of network activity decreased the size of L-GABAergic neurons (Fig. 10), suggesting that neuronal activity modulates the growth of L-GABAergic neurons. Indeed, it is well established that the morphological and functional maturation of GABAergic interneurons is dependent on neuronal activity (Huang et al., 2007; Huang, 2009; Cossart, 2010). The results suggest that an intricate hormone-activity interrelationship influences GABAergic neurons development. Thus, either both T3 modulation and network activity function synergistically promoting GABAergic neuron growth or T3-mediated effects are partly activity-dependent.

5.4.4 Contribution of BDNF signalling

A mediator for the hormonal- and activity-dependent modulation of L-GABAergic neurons might be brain derived neurotrophic factor (BDNF), whose expression is said to be regulated by both thyroid hormones (Koibuchi et al., 1999, 2001; Sui et al., 2010) or by neuronal activity (Zafra et al., 1990; Thoenen et al., 1991; Gorba et al., 1999). The neurotrophin BDNF is well known as a mediator of activity-dependent effects and BDNF strongly promotes interneuron development (Marty et al., 1996, 1997; Rutherford et al 1997; Huang et al., 1999; Yamada et al., 2002; de Lima et al., 2004; Palizvan et al., 2004; Patz et al., 2003, 2004; Woo and Lu, 2006).

Indeed, some aspects of GABAergic neurons development are dependent on BDNF signalling. Both the L-GABA soma size and the axonal branching were sensitive to the TrkB receptor inhibitor K252a irrespective of T3 presence (Fig. 18). Furthermore, K252a abolished the positive correlation between axonal parameters and the local cell density in T3-treated cultures and also inhibited the pruning of GABAergic axons from fields with low cell density (Figs. 17, 18).

Thus the neurotrophin BDNF might act as a mediator of thyroid hormone actions since T3 has been suggested to regulate BDNF expression (Koibuchi et al. 1999; Koibuchi and Chin, 2000; Sui et al., 2010). In addition, T3 promotes the maturation of spontaneous network activity (Figs. 1, 4, 26) and BDNF secretion by projection neurons in the cerebral cortex is regulated by neuronal activity (Zafra et al., 1990; Lessmann et al., 2003). Thus, BDNF mediation might explain the intricate interrelationship between T3, L-GABAergic interneurons and network activity development.

Moreover, the present results suggest a synergistic action of T3 and BDNF. The reduction of GABAergic axonal length in low neuronal density fields and thereby the correlation of axonal length and GABAergic boutons with the local cell density occurs only when both T3 and BDNF are present (Figs. 17, 18). In the absence of T3, even stimulation with exogenous BDNF had no influence on the axonal length and only partially enhanced the bouton formation and the correlation of GABAergic boutons with the local cell density (Fig. 18).

Interestingly, it has been demonstrated that an overexpressed unliganded TR prevent nerve growth factor (NGF) induced neurite outgrowth in PC12 cells and raised the hypothesis that unliganded TRs might block neurotrophin-dependent neuronal maturation (Munoz et al., 1993; Bernal, 2007). Similarly, a combination of both NGF and T3 was

necessary to induce the expression of cytoplasmic dynein, a protein that is involved in retrograde axonal transport, in dorsal root ganglion explant cultures (Barakat-Walter and Riederer, 1996). In favour of this hypothesis the present data suggest that unliganded TRs might repress aspects of BNDF-induced maturation of large GABAergic interneurons.

The contribution of BDNF signalling to thyroid hormone actions has also been discussed in context of cerebellar Purkinje cell development (Heuer and Mason, 2003). But in contrast to the present results no interactions of T3 and BDNF could be observed. T3 induced growth of Purkinje cell dendrites also in the absence of BDNF signalling, but TrkB antagonists influenced the patterning of dendrites.

5.4.5 Open questions: Other factors?

As discussed previously, the results suggest that T3 acts primary via nuclear thyroid hormone receptors on the development of GABAergic neurons and network activity. However, the contribution of non-genomic hormone actions or the involvement of other growth factors cannot be completely excluded.

Thyroid hormones have been suggested to induce non-genomic effects by binding to a membrane receptor formed by the integrin alphaVbeta3 and activating the mitogen-activated protein kinase signalling cascade (Bergh et al., 2005; Davis et al., 2005). Additionally, thyroid hormones rapidly modulate protein phosphorylation (Sarkar et al., 2006) and cytoskeletal proteins (Zamoner et al., 2006). Recently, fast and direct non-genomic modulation of glutamatergic and GABAergic signalling by thyroid hormones has been suggested (Losi et al., 2008; Piua and Losi, 2011). In contrast, T3 application in the present experiments had no direct influence on the frequency of network events (Fig. 7). This contrast might be explained by the different methods of investigation and very high doses of thyroid hormones used by Piua and coworkers (Losi et al., 2008; Piua and Losi, 2011). Additionally, non-genomic actions mediated by the integrin alphaVbeta3 are more likely for T4 than for T3 (Cheng et al., 2010).

Furthermore, the contribution of non-neuronal cells to T3-mediated effects is an unsolved issue. On the one hand, thyroid hormones are known to regulate astrocyte maturation (Paul et al., 1996; Anderson, 2001; Mendes-de-Aguiar et al., 2010), which might further modulate neuronal development. On the other side, thyroid hormone actions might depend on the release of trophic factors from glial cells. It has been suggested that the release of basic fibroblast growth factor from glial cells is essential for the T3-mediated

increase in excitability of hippocampal neurons (Niederkinkhaus et al., 2009). Additionally, T3 loses its ability to enhance neurite outgrowth in dorsal root ganglion explant cultures when non-neuronal cells are eliminated from the cultures (Barakat-Walter, 1996). Thus selected stimulating effects of T3 are probably mediated through non-neuronal cells.

5.5 Temporal aspects

5.5.1 Transient effects

Morphological analyses revealed that T3-dependent acceleration of the somatic growth of L-GABAergic neurons (Fig. 24) and the increased formation of GABAergic boutons (Fig. 25) disappeared around 21DIV. This is in line with reports showing that lack of T3 during the first two to three weeks of postnatal development results in a delayed upregulation of specific proteins (Oppenheimer and Schwartz, 1997; Royland et al., 2008; Morte et al., 2010), the later appearance of parvalbumin interneurons in the cortex (Wallis et al., 2008) and a general delay in network development (Venero at al., 2005; Wallis et al., 2008). However, all these parameters normalize during later postnatal development. Thus, several aspects of neuronal development might become independent from T3 signalling after an initial T3-dependent period (Oppenheimer and Schwartz, 1997). Additionally, some homeostatic mechanisms may compensate specific developmental parameters in order to approximate a behaviourally plausible network performance (Turrigiano and Nelson, 2004; Marder and Goaillard, 2006; Grashow et al., 2010).

5.5.2 Long-lasting effects

The characteristic GABAergic innervation of non-GABAergic cell bodies with a sparing of cell free regions (Fig. 25), and the density of late-born S-GABAergic neurons (Fig. 22) were still reduced in 21-day-old T3-deprived cultures. Additionally, the burst frequency was significantly reduced in three week old T3-untreated cultures compared to T3-treated ones (Fig. 26). None of these features could be rescued by T3 application starting after the second week *in vitro*. Thus T3 is essential for selected aspects of cortical interneuron and network activity development during a restricted period of early network formation and T3 deprivation during this time might cause a permanent or at least long-lasting deficit in the network architecture development.

The most severe consequences of thyroid hormone deficits *in vivo* are induced during a developmental period up to the end of second postnatal weeks in rodents. Irreversible mental retardation and locomotor deficiencies follow cortical, hippocampal and cerebellar malformations (Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Anderson, 2001; Bernal et al., 2003). Alterations in the formation of the network structure, especially those alterations concerning excitatory-inhibitory balance and the structure of the synaptic connections may explain the consequences of malfunction in mature networks. However, data about long-lasting consequences of hypothyroidism on GABAergic neurons are controversial. Gilbert et al., (2007) reported chronic decrease in the number of parvalbumin-positive interneurons after thyroid hormone deprivation during early development. In contrast, reduction in parvalbumin expression in mice bearing a mutated TRalpha was transient (Wallis et al., 2008) or could be reversed by high doses of T3 in adulthood (Venero et al., 2005).

5.5.3 Critical period of GABAergic neurons development

Thyroid hormones control several developmental processes, many of them restricted to innate critical periods (Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Bernal et al., 2003). In line with this, the results of the present work show that deficits in GABAergic neuron density, axonal patterning, and burst frequency following early T3 deprivation could not be rescued by T3 application after the second week *in vitro*. However, the mechanisms generating such sensitive periods are still unclear. In general, two principal possibilities are feasible: limitations in the T3 signalling pathways itself or changes in the plasticity of GABAergic neurons.

First, changes in the T3 signalling pathways might create periods with enhanced sensitivity. Indeed, the spatio-temporal pattern of T3 availability appears dynamic and heterogeneous during development (Quignodon et al., 2004). But postnatal T3 levels in serum and brain are homeostatically controlled and quite stable, and are therefore unlikely to close a critical period (Quignodon et al., 2004). The differential expression of thyroid hormone receptors TRalpha and TRbeta is another possible explanation for the time sensitive actions of T3. Especially the TRbeta is expressed more postantally with more restricted spatio-temoral expression pattern, and therefore might act more specifically. However, the thyroid hormone receptors are expressed early in embryonic development and increase with further development without prominent changes during early postnatal

development (Bradley et al., 1992; Oppenheimer and Schwartz, 1997). Finally, TRs bind several coregulators, which represent an important regulation mechanism for thyroid hormone actions. Although the exact expression patterns of these coregulators have not been clarified, the developmental expression patterns of coregulators might be the most interesting candidates to explain developmental changes in thyroid hormone sensitivity (Yen, 2001; Bernal, 2009). Thus in summary, the specific developmental events in T3 signalling remain poorly understood.

Secondly, the change in T3 sensitivity might arise from general alterations in neuronal plasticity. In general, neurons undergo a developmental transition from immature to mature stages (Moody and Bosma, 2005) accompanied by prominent changes in gene expression (Mody et al., 2001; Stead et al., 2006; Semeralul et al., 2006) and neuronal properties (Klueva et al., 2008; Doischer et al., 2008; Okaty et al., 2009). Also the developmental switch in GABAergic signalling results from changes in intrinsic neuronal features (Ben-Ari et al., 2007; Blankenship and Feller, 2010). Thus it is feasible, that neurons might lose some kind of plasticity during this transition. Indeed, age-dependent changes in GABAergic synapse plasticity have been reported (Gubellini et al., 2001; Chattopadhaya et al., 2004). Additionally, the activity-dependent survival of a subpopulation of GABAergic neurons is restricted to a critical period of early development (de Lima et al., 2004). More generally, it is known that the plasticity of adult neurons, in contrast to immature neurons, is limited by several factors on the cellular and molecular level e.g. myelination, perineuronal nets and the excitatory-inhibitory balance (Bavelier et al., 2010).

5.5.4 Role of depolarizing GABA?

The present results suggest that specific aspects of GABAergic neurons development, the establishment of axonal distribution (Fig. 25) as well as the survival of S-GABAergic neurons (Fig. 22), are correlated to a critical phase of early network development (until the end of the second week in vitro). Imaging experiments revealed that this time is well correlated to depolarizing actions of GABAergic signalling (Fig. 20), suggesting that these processes might be limited by the transition from depolarizing to hyperpolarizing GABAergic signalling.

The developmental switch in GABAergic signalling represents a hallmark in network maturation (Ben-Ari et al., 2007; Blankenship and Feller, 2010). Interestingly, the

excitatory actions of GABAergic signalling have been implicated in the early morphological and functional maturation of neurons (Owens and Kriegstein, 2002). Thus, the T3-mediated acceleration of GABAergic neurons growth and synaptogenesis during early development, and thereby a presumably increased GABAergic neurotransmission, might strengthen the neurotrophic actions of depolarizing GABAergic signalling.

Moreover, both long-lasting effects the establishment of axonal distribution (Fig. 25) as well as the survival of S-GABAergic neurons (Fig. 22) were dependent on BDNF signalling (Figs. 17, 18, 23). Thus it is likely that some activity-dependent mechanisms may be involved. Interestingly, depolarizing GABA increases BDNF expression in postsynaptic neurons (Berninger et al., 1995; Obrietan et al., 2002), which might provide an optimal environment for initial interneuron development. GABA itself can stimulate the expression of BDNF, which then in turn facilitates the maturation of GABAergic neurons creating a positive feedback loop. But GABA switches to repressing BDNF expression during the GABA shift (Berninger et al., 1995), what might close the window for the initial GABA-triggered and BDNF-mediated interneuron maturation. Thus there might be a critical phase of BDNF dependence in the development of GABAergic neurons, which ends with the switch in GABAergic signalling. So, if developmental processes like the axonal pruning are synergistically regulated by BDNF and T3, lack of thyroid hormone during the time of depolarizing GABA, the time when depolarizing GABA can stimulate BDNF expression, should hamper the positive effects of this feedback loop and might explain the presence of a critical period.

5.5.5 Relevance of T3 modulation for the in vivo cortical network development

The effects of T3 not only depend on the site of action but also on the developmental timing. T3 deficiency during fetal brain development results in different behavioral and neuroanatomical malformations than during perinatal development. In rodents T3 affects proliferation and migration during prenatal development and differentiation of neurons and synapse formation during perinatal and postnatal development. Multiple genes mediate T3 actions (Alvarez-Dolado et al., 1999; Alvarez-Dolado et al., 2000; Royland et al., 2008; Morte et al., 2010) and it is possible that different mechanisms are involved in successive phases of development (Zoeller and Rovet, 2004; Morreale de Escobar et al., 2004). The present results show that the initial steps of the functional network formation are regulated by thyroid hormone. This specially

concerns the perinatal and the early postnatal period in rodents that correspond to the first half of the third trimester in humans (Zoeller and Rovet, 2004; Khazipov and Luhmann, 2006).

Since most effects of T3 actions have been observed during early development thyroid hormones have been suggested to accelerate and synchronize neuronal maturation (Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Bernal et al., 2003). If developmental processes occur out of phase, windows of opportunity may be missed and irreversible maladaptive changes may occur. The present data show that T3 promotes the expression of KCC2 and the development of GABAergic synaptic network, partially mediated by BDNF signalling. Developmental impact of T3 on GABAergic network formation is most relevant in the limited period of early network activity characterized by the depolarizing actions of GABA. As the BDNF-mediated upregulation of KCC2 is apparently limited to early development (Aguado et al., 2003; Rivera et al., 2004; Ludwig et al., 2011), it might be coordinated with T3-mediated interneuron growth. Taken together, the results of the present study corroborate the idea that T3 accelerates neuronal maturation, and by doing that, contribute to the timely synchronization of critical aspects of network activity and GABAergic system development during the early neocortical development. This highlights the importance of thyroid hormone signalling during early network development.

5.6 Conclusions

Taken together, the results show that T3 contributes to the morphological and functional maturation of early cortical networks by regulating the development of the cortical GABAergic system.

T3-deprivation during the first two weeks induced long-lasting deficits in the axonal patterning of L-GABAergic neurons and the density of small late born GABAergic neurons. Additionally, the expression of the potassium chloride cotransporter KCC2 was retarded in T3-untreated networks. These alterations in the formation of the network structure, especially those alterations concerning excitatory-inhibitory balance and the structure of the synaptic connections, may explain the consequences of malfunction in mature networks following deficits in T3 signalling.

The results also demonstrate that cultures chronically treated with T3 showed increased frequency of spontaneous bursts of activity and more mature properties than

untreated networks. Network activity in turn plays an important role in the maturation of developing neuronal structures (see chapter 2.1.3). Thus it is feasible that disturbances of early network activity provoke abnormalities that might explain at least some developmental effects of T3. This hypothesis could provide a framework to understand and further investigate the physiological and structural changes both due to deficits or excess of thyroid hormones.

Recovery experiments highlighted the importance of thyroid hormone signalling during early network development. They suggest that the development of GABAergic neurons and network activity is especially sensitive to thyroid hormone modulation during the first two weeks in cultures, and that developmental deficits following early T3 deprivation might not be rescued after this initial T3 sensitive period. These results corroborate the idea that T3 accelerates neuronal maturation and thereby contributes to the timely synchronization of critical developmental processes.

5.7 Perspectives

"*There is excitement ahead for thyroid research in neuroendocrinology*!"
Graham R. Williams (2008)

5.7.1 Role of T3 in synaptic plasticity

The present results highlighted the function of thyroid hormones in synaptic plasticity, especially in the formation of GABAergic boutons and axons. Although thyroid hormones have already been shown to modulate the expression of several synaptic proteins and the structure of neuronal axons, dendrites and dendritic spines (see chapter 2.3.4), their distinct role in synaptic plasticity is still unknown. An unresolved issue is whether thyroid hormones act in an instructive or a permissive fashion. Do they directly enhance synaptic maturation or are they simply permissive factors, setting the framework for activity-dependent maturation, e.g. by modulating the threshold for synaptic plasticity? Interestingly, T3 and also T3 binding sites have been shown to be located at synaptic sites (Dratman et al., 1976, 1982, 1987; Mashio et al., 1982) and thyroid hormones rapidly modulate protein phosphorylation in cerebrocortical synaptosomes (Sarkar et al., 2006), rising the still unanswered question whether T3 might even function as a neurotransmitter (Dratman and Gordon, 1996). A contribution of T3 to synaptic plasticity further raises the question, where and when T3 is released in the brain. A large amount of T3 is generated locally in the brain by deiodination of T4 in astrocytes (Guadano-Ferraz et al., 1997;

Williams, 2008). It will be of interest whether the release of this T3 occurs in a consecutive, paracrine fashion (Gereben et al., 2008; Freitas et al., 2010) or is triggered by specific events, e.g. neuronal activity. Thus there are several unresolved questions which have to be addressed to establish the exact role of thyroid hormones in activity-dependent synaptic arrangement, especially during early development.

5.7.2 Signalling pathway

The present work highlights the interactions of thyroid hormone signalling and actions mediated by neuronal activity, especially those induced by BDNF. The present data suggest that T3 interacts with BDNF signalling pathways and thus suggest an important role for T3 in activity-dependent plasticity. However the exact interaction sites of these pathways are still unclear, but their identification might considerably improve our understanding of thyroid hormone function on synaptic plasticity and neuronal maturation.

Control of BDNF expression. Thyroid hormones have been suggested to regulate the expression of BDNF (Koibuchi et al., 1999, 2001; Shulga et al., 2009; Sui et al., 2010). Because the upregulation of BDNF expression occurs in a fast (Sui et al., 2010) and promoter specific fashion (Koibuchi et al., 1999), a direct mechanism of T3-mediated BDNF expression is feasible. However, the identification of a thyroid hormone response element in the BDNF promoters is still missing. But only the identification of this specific binding site can prove the direct regulation of BDNF expression by T3.

Interaction of T3 and BDNF. In addition to the direct regulation of BDNF, the signalling pathways of thyroid hormones and BDNF offer several interaction sites, which have to be taken into account when investigating the mechanism of thyroid hormone actions.

(1) The actions of thyroid hormones are thought to be mainly mediated by nuclear receptors, which are ligand-dependent transcription factors. Unliganded receptors can function as potent repressors of gene expression by binding corepressors with histone deacetylase activity (Yen, 2001; Bernal, 2007; Williams, 2008). Thus, the deacetylation and thereby inaccessibility of DNA might hinder BDNF-mediated transcription of genes. Indeed, such a mechanism has already been suggested to regulate neurotrophin-dependent maturation of PC12 cells (Munoz et al., 1993; Bernal, 2007).

(2) It has recently been shown that TRs can also be localized in the cytosol (Storey et al., 2006) and bind to a subunit of the phosphoinositide-3-kinase (PI3K; Moeller et al.,

2006). The PI3-kinase is an important downstream factor of BDNF signalling (Gottmann et al., 2009; Minichiello, 2009) and is essential for axonal development and synaptic plasticity (Cosker and Eickholt, 2007; Hoeffer and Klann, 2009). T3 binding modulates the PI3K and mammalien-target-of-Rapamycin (mTOR) pathway in non-neuronal (Moeller et al., 2006) and neuronal cells (Cao et al., 2009; Sui et al., 2008) and might thereby regulate BDNF-mediated induction of the same pathway.

(3) T3 directly regulates the expression of a set of proteins, e.g. the Ca^{2+}/calmodulin-dependent protein kinase IV (CamKIV), the calmodulin binding protein RC-3/neurogranin, and the growth associated protein GAP-43 (reviewed in Bernal et al., 2003; Bernal, 2009), which take important places in synaptic plasticity and therefore might modulate BDNF-dependent plasticity (Benowitz and Routtenberg, 1997; Minichiello, 2009). At least, thyroid hormones have been suggested to control the expression of neurotrophin receptors (Roskoden et al., 1999).

These pathways have been identified very recently and have not been analysed in detail. Thus the detailed mechanisms and their contribution to thyroid hormone actions are still unclear and have to be investigated.

5.7.3 Clinical relevance

The objects of the present work have been related to different psychiatric disorders. Thus, deeper understanding of the mechanisms of the intricate interrelationship of GABAergic neurons, thyroid hormones and network activity, especially during early development, might be helpful to better understand the causes of psychiatric disorders and might give rise to improved prevention and therapeutic strategies.

Thyroid hormones. Lack of thyroid hormones has been correlated to a set of developmental disorders with cretinism as its most severe form (World Health Organization, 2004). Furthermore T3 deficiency has also been linked to depression, anxiety and ADHD (Kirkegaard and Faber, 1998; Morreale de Escobar et al., 2004; Vermiglio et al., 2004; Carvalho et al., 2009; Pilhatsch et al., 2010). Mild hypothyroidism caused by iodine deficiency led to reduced intellectual ability (Delange, 2000; de Benoist et al., 2008) and is the world's greatest single cause of preventable brain damage. Consequently, the maintenance of adequate iodine levels is a great public health challenge (World Health Organization, 2004). In addition, thyroid hormone application might be a treatment strategy in neurological disorders. Indeed, T3 is commonly used to accelerate or

augment standard antidepressant trials (Carvalho et al., 2009). Thyroid hormones might also be a potential therapeutic approach for human peripheral nerve injuries (Barakat-Walter, 1999). T3 treatment accelerated and improved the regeneration of sciatic nerves and the recovery of sensory and motor functions after peripheral nerve transection in rats (Barakat-Walter, 1999).

GABAergic neurons. Dysfunctions of GABAergic signalling can result in several pathological conditions, such as autism, mental retardation, epilepsies and schizophrenia (DeFelipe, 1999; Levitt et al., 2004; Lewis et al., 2005; Wonders and Anderson, 2006; Ben-Ari et al., 2007; Woo and Lu, 2006). In addition, deregulated excitatory GABAergic signalling has been implicated in the genesis of neuropathic pain, neonatal seizures, temporal lobe epilepsy and seizures that occur after ischemic–hypoxic insult (Kahle et al., 2008). Thus, the understanding of GABAergic neurons plasticity will be helpful in the prevention and treatment of psychiatric disorders related to GABAergic signalling.

Furthermore, maturation of the GABAergic system has been correlated to the closure of the 'critical period' of cortical plasticity and inhibitory GABAergic signalling is suggested to function as a 'brake on plasticity' (Huang et al., 1999; Bavelier et al., 2010). Resetting the inhibitory-excitatory balance might provide a strategy to reactivate neuronal plasticity (Hensch, 2005; Bavelier et al., 2010).

Early spontaneous network activity. Early spontaneous network activity is thought to have an important role in the development of circuits. Disruption of normal firing patterns during development, for example by fetal alcohol exposure, prevents the normal development of primary sensory systems (Stromland et al., 2004; Medina et al., 2005). An understanding of the mechanisms that mediate spontaneous activity could have profound implications for the clinical treatments of pregnant women and will help to prevent neuropathologies associated with fetal exposure to neuroactive pharmacological agents (Blankenship and Feller, 2009).

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7. Appendix

7.1 Solutions and media

7.1.1 General buffer

Phosphate buffered saline (PBS)

137 mM NaCl
2.6 mM KCl
8.1 mM Na_2HPO_4
1.4 mM KH_2PO_4
Aqua dest. (pH 7.4)

artificial cerebrospinal fluid (aCSF)

140 mM NaCl
5 mM KCl
1.5 mM CaCl2
0.75 mM MgCl2
1.25 mM NaH2PO4
20 mM d-glucose
15 mM HEPES/NaOH
Aqua dest. (pH 7.4)

7.1.2 Solutions and media for cell culture

Hank's medium	Hank´s balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free; Gibco by Invitrogen, Carlsbad, CA, USA) 20 mM HEPES (Carl Roth, Karlsruhe, Germany) pH 7.4
Dissociation buffer	0.5% trypsin/EDTA (Gibco) in Hank's medium
Astrocyte culture medium	Dulbecco's modified Eagle medium (DMEM, Gibco) 10% fetal calf serum (FCS; Gibco)
N2 neuron culture medium	75% DMEM (Gibco) 25% Ham's F12 (Gibco) N2 supplements (Gibco)

7.1.3 Solutions for immunocytochemistry

Tris/metabisulfite solution	Aqua dest. 0.05 M Tris (pH 7.5) 0.85% sodium metabisulfite
Preincubation	Tris/metabisulfite solution 3% bovine serum albumin 10% normal goat serum 0.6% Triton X-100
Primary antibody	Tris/metabisulfite solution 3% bovine serum albumin 10% normal goat serum 0.6% Triton X-100
PBS washing buffer	0.1 M PBS (pH 7.4)
Secondary antibody	0.1 M PBS (pH 7.4) 10% normal goat serum 2% bovine serum albumine 5% sucrose 0.3% Triton X-100
Peroxidase reaction	50 mM Tris-HCl in Aqua dest. 0.01% 3, 3' diaminobenzidine tetrahydrochloride 0.004% H_2O_2 1% Nickel ammonium sulfate 50 mM Imidazole

Modifications for synapse stainings:

PBS washing buffer	0.01 M PBS (pH 7.4)
Preincubation	0.01 M PBS (pH 7.4) 10% normal goat serum 5% sucrose 2% bovine serum albumine 0.25% Triton X-100
Antibody solution	0.01 M PBS (pH 7.4) 10% normal goat serum 5% sucrose 2% bovine serum albumine 0.25% Triton X-100

7.1.4 Solutions for proteinbiochemistry

RIPA (Radio Immuno Precipitation Assay) lysis buffer

stock solution	150 mM NaCl 1% Igepal 0.5% Sodium deoxycholate (Doc) 0.1% sodium dodecyl sulfate (SDS) 10% Glycerol in 50 mM Tris-HCl (pH 8.0)
working solution	RIPA stock solution 4% Protease inhibitor (Complete) 0.5% phenylmethanesulfonyl fluoride (PMSF)

8% SDS-Page (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

6.6 ml Aqua dest.
3 ml 1.5 M Tris-HCl, pH 8.8
60µl 20% (w/v) SDS
2.4 ml 40% Acrylamide/bisacrylamide
60 µl 10% (w/v) Ammonium persulfate (APS)
12 µl Tetramethylethylenediamine (TEMED)

SDS-Page sample buffer	6.7 ml Aqua dest.
stock solution	300 µl 2M Tris-HCl, pH6.8 1,0 ml Glycerol 1,0 ml 20% SDS 500 µl of 0,1% Bromphenol blue solution
working solution	stock solution 5% ß-Mercaptoethanol

Running-puffer (10x stock)	Aqua dest. 192 mM Glycine 25 mM Tris-base 0.1% SDS pH 8.6
working solution	Aqua dest. 10% stock solution

Transfer buffer (stock)	Aqua dest. 48 mM Trizma-base 39 mM Glycine 0.0375% SDS pH 8.6
working solution	Transfer buffer 20% Methanol

Immunoblot

Blocking solution
0.1 M PBS
5% milk
1% GNS
0.1% Tween20

Washing buffer (PBST)
0.1 M PBS (pH 7.4)
0.1% Tween20

Antibody solution
0.1 M PBS
1% milk
1% GNS
0.1% Tween20

7.2 Drugs / Pharmacological reagents

Drug	Abbrev.	Concentr.	Manufacturer
triiodothyronine	T3	15 nM	Sigma-Aldrich, St. Louis, MO, USA
Corticosterone	Cort	0.1 µg/ml	Sigma-Aldrich, St. Louis, MO, USA
tetrodotoxin	TTX	1 µM	Alomone Labs, Jerusalem, Israel
D-(-)-2-amino-5-phosphonopentanoic acid	APV, D-AP5	12.5-50 µM	Tocris Cookson, Ellisville, MO, USA
6-cyano-7-nitroquinoxaline-2,3-dione disodium	CNQX	2.5-10 µM	Tocris Cookson, Ellisville, MO, USA
Potassium chloride	KCl	60 mM	Merck KGaA, Darmstadt, Germany
Muscimol		200 µM	Sigma-Aldrich, St. Louis, MO
Brain derived neurotrophic factor	BDNF	50 ng/mL	Pepro Tech, Rocky Hill, NJ, USA
K252a	-	100 nM	Calbiochem by Merck KGaA, Darmstadt, Germany
1-850	-	1 µM	Merck KGaA Darmstadt, Germany
(-)-bicuculline methiodide	BMI	20 µM	Sigma-Aldrich, St. Louis, MO, USA
Picrotoxin	PTX	10 µM	Tocris Cookson, Ellisville, MO, USA
VU0240551	VU	4 µM	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

7.3 Primary antibodies

Antibody	Host	Dilution ICC	Dilution WB	Cat. No./ clone	Manufacturer
GABA	mouse	1:200	-	clone 5A9	Chemicon, Temecula, CA
Pan-Shank	mouse	1:100	-	clone N23B/49	UC Davis/NIH NeuroMab Facility, Davis, CA
GluR2	mouse	1:100	-	clone L21/32	UC Davis/NIH NeuroMab Facility, Davis, CA
Gephyrin	mouse	1:1000	-	No. 147 011	Synaptic Systems, Göttingen, Germany
Synapsin 1,2	rabbit	1:500	-	No. 106 002	Synaptic Systems, Göttingen, Germany
KCC2	rabbit	-	4 µg/ml	Cat. KCC21-A	Alpha Diagnostic International Inc, San Antonio, TX
KCC2	mouse	-	1-10 µg/ml	clone N1/12	UC Davis/NIH NeuroMab Facility, Davis, CA
NKCC	mouse	-	330ng/ml	clone T4	Hybridoma Bank, Iowa City, IA
GAPDH	mouse	-	1:1000	clone 1D4	Covance, Princeton, NJ

7.4 Secondary antibodies and peroxidases

Antibody	Host	Dilution ICC	Dilution WB	Cat. No.	Manufacturer
anti-mouse	goat	1:200	-	SMI-5010C	Convance Inc, Princenton, NJ
Peroxidase-anti peroxidase	mouse	1:200	-	405	Sternberger, Baltimore, MD; now Convance Inc, Princenton, NJ
anti-mouse Cy2	goat	1:400	-	115-225-003	Dianova, Hamburg, Germany
anti-rabbit Cy3	goat	1:400	-	115-165-003	Dianova, Hamburg, Germany
anti-mouse HRP	goat	-	1:10000	P0447	Dako, Glostrup, Denmark
anti-rabbit HRP	goat	-	1:2000	P0448	Dako, Glostrup, Denmark
StrepTactin HRP	-	-	1:10000	161-0380	Bio-Rad Laboratories Inc., Hercules, CA

7.5 Lab instruments and equipment

Instrument	Application	Manufacturer
Axiophot 2	Microscope	Zeiss, Oberkochen, Germany
Spot slider	CCD camera	Diagnostic instruments, Sterling Heights, MI, USA
Axiovert S100 TV	Microscope	Zeiss, Oberkochen, Germany
CoolSNAP ES	CCD camera	Roper Scientific, Ottobrunn, Germany
UniBlitz	Shutter	Visitron Systems, Puchheim, Germany
STG 2008	Stimulus generator	Multi Channel Systems, Reutlingen, Germany
Axioskop Standard WL	Microscope	Zeiss, Oberkochen, Germany
Camera Lucida		Zeiss, Oberkochen, Germany
Hp Officejet pro L7580	Scanner	Hewlett-Packard Company, Palo Alto, CA, USA
EBU-4000	Semi-dry-blotter	C.B.S. Scientific, Del Mar, CA, USA
Mini-Protean Tetra Cell	Electrophoresis Cell	Bio-Rad Laboratories Inc., Hercules, CA
GeneGnome5	ECL detection	Syngene by Synoptics Ldt, Cambridge, UK
BB 6220 CU	Incubator	Heraeus, Hanau, Germany
Hera cell 240	Incubator	Thermo Electron Corp., now Thermo Fischer Scientific Inc., Waltham, MA

7.6 Software

Software	Version	Supplier
Metamorph	7.0	Molecular Devices, Sunnyvale, CA, USA
Matlab	7.5	MathWorks, Natick, MA, USA
MiniAnalysis	6.0.3	Synaptosoft, Decatur, GA, USA
HP SolutionCenter	Officejet L7500 series	Hewlett-Packard Company, Palo Alto, CA, USA
SigmaStat	2.03	SPSS Inc., Chicago, IL, USA
SigmaPlot	8.0	SPSS Inc., Chicago, IL, USA
QuantityOne	4.5.0	Bio-Rad Laboratories Inc., Hercules, CA, USA
Excel	2003, SP3	Microsoft Corp., Richmond, WA, USA
CorelDraw	15	Corel Corp., Ottawa, ON, Canada

8. Figure and table index

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10. Curriculum Vitae

Personal data

Name:	Dipl.-Biol. Sören Westerholz
Date of birth:	04.01.1980
Place of birth:	Herford
Nationality:	German

School education

1986 – 1990	Ev. Grundschule Halstern
1990 – 1999	Städtisches Gymnasium Löhne
	Final degree: ‚Abitur‘

Basic military service

07/1999 – 04/2000	Panzergrenadierbataillon 192 in Ahlen

University education

10/2000 – 09/2001	Otto-von-Guericke University Magdeburg
	Study course: ‚Sport & Technik‘
10/2001 – 05/2006	University of Bielefeld
	Study course: Biology
	Final degree: Diploma
11/2005 – 05/2006	Otto-von-Guericke University Magdeburg
	External diploma thesis at the Institute of Biology

Doctoral thesis

07/2006 – 04/2007	Otto-von-Guericke University Magdeburg
	Institute of Biology
05/2007 – 08/2011	Otto-von-Guericke University Magdeburg
	Institute of Physiology

11. Publications

Scientific publications:

Riedel A, **Westerholz S**, Braun K, Edwards RH, Arendt T, Härtig W (2010) Vesicular glutamate transporter 3-immunoreactive pericellular baskets ensheath a distinct population of neurons in the lateral septum. J Chem Neuroanat. 36(3-4):177-90.

Westerholz S, de Lima AD, Voigt T (2010) Regulation of early spontaneous network activity and GABAergic neurons development by thyroid hormone. Neuroscience. 168(2):573-89.

Submitted manuscripts:

Westerholz S, de Lima AD, Voigt T. Thyroid hormone regulation of GABAergic axonal growth during early cortical network development. Under review.

Westerholz S, de Lima AD, Voigt T. Thyroid hormones regulates GABAergic interneurons growth and signaling shift during early cortical network development. Under review.

12. Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation mit dem Thema

‘Thyroid hormone modulation of early neocortical network development’

selbständig verfasst, nicht schon als Dissertation verwendet und die benutzten Hilfsmittel und Quellen vollständig angegeben habe.
Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.