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Localization of RNA-binding Proteins in Mature and Aging Neurons

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1. ABSTRACT

Aging is associated with cognitive decline, depending on structural and functional changes in neuronal networks. Therefore, age-related changes have become an essential factor in the research of learning, memory, and neurodegenerative diseases. The formation and alteration of individual synapses is of essential importance in functional neuronal circuits. Perturbations in the connectivity between cells can have a great effect on processes such as learning and memory formation. Cellular aging has been shown to be both genetically programmed and dependent on damage accumulation, leading to dysfunctions in multiple cellular processes. In neurons, this can result in changes in synapse formation and maintenance. The formation and later the morphogenesis of individual synapses critically depend on the local translation of RNA transcripts. This process depends on the correct function of various RNA-binding proteins (RBPs), involved in RNA- transport, silencing, etc. Here, we propose that age-related changes and damage in various cellular components can lead to perturbations in the function and subcellular localization of such RBPs, and thereby affect synaptic plasticity and consequently can cause neuronal diseases.

In this project, I used primary cultured rat hippocampal neurons held in long-term cell culture to investigate possible age-dependent changes in the cellular distribution of a number of essential RBPs. I applied standard methods of immunocytochemistry and epifluorescence microscopy to label and document the cellular pattern for selected proteins in differentially aged cells.

My experiments revealed an age-dependent change in the distribution of processing bodies (P-bodies). They suggest that large P-bodies in the soma close to the nucleus appear to disassociate or reduce in size during aging. Furthermore, dendritic P-bodies localize to synaptic sites in greater number in aged cells. Their role in RNA degradation and translational control is vital for cellular metabolism, suggesting age-dependent changes in the localization of P-bodies to result in significant alterations in the local translational regulation of synaptic proteins.

Furthermore, a small portion of RBPs were found to undergo changes in nuclear-cytoplasmic shuttling and displayed an increased localization to distal sections of dendrites during aging. Changes in nuclear-cytoplasmic shuttling can affect specific signaling cascades in the nucleus, resulting in altered gene expression. The increased dendritic localization of specific RBPs may be a response to age-dependent synaptic alterations or a side effect of aging. Taken together, this thesis presents age-related changes in the localization of essential RBPs, highlighting the important role of these proteins not only in neurons but also in neuronal aging. Furthermore, my data provide a foundation for further studies addressing the involvement of RBPs in the cellular aging process.

2. INTRODUCTION

2.1 Biological Aging

2.1.1 An Introduction to Aging

Aging is one of the fundamental principles of life, and has become increasingly important in the research of diseases and mortality (Clark, 2012; Tolino et al., 2012). Biological aging is often also referred to as senescence, indicating deteriorating effects opposed to the simple passage of time. Many different theories and definitions for aging have been proposed. Some suggest genetically programmed aging, while others support the theory that aging is a consequence of damage accumulation caused by environmental factors, influencing normal biological processes. A single theory is not sufficient to describe the complex mechanisms underlying aging. Today, a combination of the genetic background and external factors, both biological and cultural are accepted to contribute to the lifespan of an individual. Generally aging can be defined as the period of biological decline that follows the developmental phase in the life of an organism. It encompasses all biological processes of a living organism approaching advanced age, i.e. all of the time-dependent molecular, cellular and tissue specific changes. Advanced aging is thought to go hand in hand with sequential deterioration that occurs in most animals, including weakness, reduced mobility and agility, and a reduction in the ability to respond to activity, stress, or dietary demands, resulting in an increasing homeostatic imbalance (López-Otín et al., 2013; Weinert and Timiras, 2003). A decrease in reproductive capacity and survival probability is usually assumed as well in most but not all species. This imbalance in turn yields an increased susceptibility to environmental conditions and a higher risk of age-associated damage and diseases, resulting in death as the ultimate consequence of aging.

In addition to natural processes, aging due to diseases plays an essential role. A number of diseases such as Alzheimer's or Parkinson's can be triggered in advanced age. Age also carries the risk of greater neuronal deficits after strokes or epileptic seizures. Other diseases, such as Werner's or Bloom's Syndrome accelerate aging (Coppedè and Migliore, 2010; Knoch et al., 2012). Organisms must continuously compensate for the loss of cells to ensure organismal homeostasis. The proliferation of adult stem cells is mainly responsible for tissue renewal, but declines with age. This impairs regeneration and contributes to aging. Therefore, tissues with high cellular turnover are most affected by aging.

However, aging cannot be seen as an irreversible unidirectional process, strictly governed by the accumulation of damage. The lifespan of an organism is mainly encoded in

the genes, but outside stimuli can strongly influence important functions such as the levels of gene expression or cellular metabolism. Thus, aging must be seen as a complex phenomenon characterized not only by decreased fitness and increased susceptibility to diseases, but also by reorganization and optimization, allowing the maintenance of a living organism throughout its life. Therefore, aging depends both on irreversible, but also on reversible processes throughout the lifetime of an organism.

2.1.2 Brain Aging

Past research on aging has brought insight into a number of molecular and physiological changes in multiple organs such as the brain. The aging of the central nervous system (CNS) progresses through alterations by time-dependent degeneration, direct external effects such as stress, or diseases. However, hormonal or physiological changes in other organ systems have been implied as well, e.g. the reduction of estrogen and the synaptic estrogen receptor-α in advanced age (Behl, 2002; Burns et al., 2010). In the CNS, aging leads to an increased impairment and variability in cognitive and motoric capabilities. The gravity of this decline depends on a number of factors, including neurological diseases. There is great variability among individuals, as can be observed in humans who maintain their cognitive or motoric abilities to an advanced age. Others develop cognitive impairments, even in the absence of neurodegenerative diseases. Similarly, studies with aged rodents report differences in the performance of cognitive tasks (Gallagher et al., 1993; Robitsek et al., 2008). In this context, we can distinguish between healthy and neuropathological aging. Healthy aging depends on damaging agents, such as reactive oxygen species (ROS) that accumulate with time and damage DNA or affect processes such as calcium signaling or lipid peroxidation. In turn, age-related diseases such as Alzheimer's, Parkinson's or Huntington's usually depend on genetic factors in addition to environmental influences. Diseases in pathological aging can be triggered by specific factors in advanced age, such as amyloid-β in Alzheimer's (Mattson et al., 2008). The fluctuations in brain aging are thought to be caused to a great extent by the reduced ability of cells to cope with stress, i.e. the cells are less able to respond to damaging changes in the intracellular and local environment. This is an important aspect for brain aging, as neurons are particularly susceptible to stress.

Age-dependent degenerative processes in the brain are associated with structural and functional changes in neuronal networks. In this sense the sites of communication between neuronal cells, termed synapses are of essential importance. Therefore, cellular morphogenesis and the formation of dendrites, which harbor the majority of the up to 10.000 synaptic sites in a single cell, are of particular interest. The reduction of brain volume and

weight, which is found in advanced age, is not due to neuronal loss. However, both neuronal demise and changes in brain volume are associated with pathological memory decline (De Leon et al., 1997; Rusinek et al., 2003). Studies demonstrate that age-related cognitive decline in a healthy brain is most likely governed by changes in synaptic connectivity, rather than by a reduction in the number of neurons. Therefore, the impairments that occur in a normal aging brain are mainly linked to specific synaptic changes in the hippocampus and the prefrontal cortex but not to neuronal decline. The processes mediated by the hippocampus and the prefrontal cortex are those which are most vulnerable to aging. The hippocampus is a part of the limbic system involved in the learning process and memory formation. A decline in memory and hippocampal synaptic plasticity has been linked to aging (Barnes, 2003). The hippocampus is associated with the formation of long-term memory of facts and events. Hippocampal synaptic function can be affected by a number of progressive and possibly irreversible degenerative processes. For example, a reduction in the size of postsynaptic densities has been reported in aged rats with spatial learning disabilities (Nicholson et al., 2004). Other studies claim a decrease in certain receptors in hippocampal neurons. Another example would be age-related changes in place cell firing, associated with spatial memory in the hippocampus (Wilson et al., 2005). These are only a few examples of age-related alterations that have been reported for the hippocampus. Such subtle changes in specific hippocampal synapses can result in grave functional defects. Changes in the number of synapses and synaptic plasticity play an essential role in the formation of a healthy brain during development and processes such as learning and memory, but have also been associated with age-related degeneration. A substantial loss of up to 50 percent of synaptic sites with aging has been shown in brain regions, such as the prefrontal cortex (Peters et al., 2008). Such a reduction in the number of synapses can have multiple effects and has been associated with diseases such as Alzheimer's (Haass and Selkoe, 2007).

Today a number of studies suggest both pharmacological and behavioral strategies, such as a healthy diet and exercise to delay brain aging. For instance, the excessive uptake of calories can result in problems such as obesity, diabetes, or the metabolic syndrome. This accelerates brain aging, by altering hippocampal neurogenesis and long-term potentiation (LTP), resulting in synaptic changes (Stranahan et al., 2008). Disproportionate energy uptake has also been shown to compromise the blood-brain barrier, which may increase the risk of neurodegenerative diseases and strokes (Kanoski et el., 2010). Conditions resulting from an unhealthy diet can alter the levels and rhythmicity of hormones, which in turn affects tissue on a larger scale. For instance, the hormone Ghrelin produced in the stomach has been found to play a role in neuronal plasticity. Ghrelin acts to stimulate feeding, but also enhances hippocampal synaptic density (Diano et al., 2006). The targeted disruption of the ghrelin gene interferes with synaptic function resulting in behavioral defects and deficits in

learning and memory. This finding suggests that altered Ghrelin signaling in obesity plays a critical role in hippocampal function.

Many studies have addressed the effects of specific diets on the brain of rodents. In many cases, restriction in energy uptake was shown to protect against brain aging. Improved learning abilities are associated with long-term dietary moderation (Goodrick, 1984; Fontán-Lozano et. al, 2007). Positive effects on a cellular metabolic level and in signaling activity have been reported as well. The restriction of energy uptake triggers cellular stress response pathways, resulting in the production of various cytoprotective proteins, such as neurotrophic factors, protein chaperones or DNA repair enzymes, which protect neurons against aging (Mattson, 2008; Arumugam, 2010). These are only a few examples how diet can alter brain aging. Specific dietary components can have further defined effects on the brain and influence cognitive skills and aging.

Another important factor found to delay brain aging is both physical and cognitive exercise. Lifestyle factors, including activity can protect neurons against dysfunction or diseases by up-regulating neurotrophic factors, enhancing synaptic plasticity and advocating neurogenesis. Cognitive stimulation and physical exercise have a positive effect on the brain and are associated with a number of signaling cascades maintaining dynamic functions (Park et al., 2011; Stranahan et al., 2010). A healthy lifestyle can not only delay brain aging, but may also enhance the effect of pharmacological treatments against neurodegenerative diseases.

2.2 Cellular Aging

2.2.1 Mechanisms of cellular aging

Cells are the fundamental building blocks of multicellular life forms. Therefore, cell damage, cell death, and regeneration play a crucial role in the aging of an organism. For single cells, aging occurs via the accumulation of changes over time, such as damage to DNA, proteins, or other cell components. Eventually, death ensues when one or more vital cellular processes are altered or decrease to a critical level. In this context, aging is the consequence of the aging processes in all the single cells of an organism and their effects on each other. Therefore, a major part of aging is controlled at a cellular level. The regulation of cellular lifespan is thought to occur at the translational level, via the control of RNA metabolism, essential for homeostasis, differentiation and proliferation.

Cellular aging was first proposed by Leonard Hayflick and Paul Moorhead in 1961 (Hayflick and Moorhead, 1961). They found that human cells cultured *in vitro* arrest

proliferation after an average of 50 divisions. This loss of the ability to divide in normal differentiated cells was termed the 'Hayflick limit' or replicative senescence. Consequentially, Hayflick and Moorhead proposed the concept of an intracellular clock governing this phenomenon in mitotic cells. As this mitotic arrest does not directly depend on the passage of time, but on the number of DNA replications the control mechanism was suggested to be located in the nucleus (Wright and Hayflick, 1975; Muggleton-Harris and Hayflick, 1976).

On the level of DNA, another essential discovery in relation to aging was that DNA-Polymerase is unable to fully replicate the ends of DNA, due to the properties of the enzyme and the replication process (McClintock, 1941). The phenomenon was termed the 'end replication problem'. This would result in the loss of genetic information in each round of DNA replication and eventually lead to fatal errors in basic cell functions. However, as the loss of genetic information during each cell cycle is very unlikely and would quickly result in grave cellular dysfunctions, Olovnikov proposed the existence of telomeres at the end of linear chromosomes, consisting of replicative sequences with no genetic information (Olovnikov, 1973). The length of these telomeric ends would limit the number of possible DNA replications without the loss of genetic information and therefore contribute to cellular aging. The existence of telomeres was first shown in the protozoan *Tetrahymena*. Here, the simple sequence of hexameric repeats TTGGGG was discovered to compose the telomeric ends (Blackburn and Gall, 1978). A very similar sequence was discovered later in human telomeres; TTAGGG (Moyzis et al., 1988). Today, it is known that this sequence is highly conserved between species (Sheng et al., 1995). The main protein responsible for telomere stability and elongation is Telomerase. This ribonucleoprotein (RNP) consists of a catalytic part, the Telomerase Reverse Transcriptase (TERT) and a small RNA functioning as a template for the synthesis of single stranded DNA. The enzyme sequentially adds the telomeric repeats to the 3'-end of DNA in the telomere region. This elongation can offset cellular senescence in mitotic cells. Telomeres can be damaged by a number of mechanisms, including mutations in Telomerase or associated factors and DNA damaging agents, thereby contributing to the aging process.

As is known, the mitotic cell cycle is controlled via cell cycle check points, involving a number of different cyclins acting on cyclin-dependent kinases (CDKs) (Fisher et al., 2012). Most cells in an organism, e.g. hippocampal neurons are in a post mitotic state, set by the inhibition of CDK. As such cells no longer divide, the Hayflick limit does not apply. Therefore, aging mechanisms other than replicative senescence must be involved. This led us to consider two new theories of aging; stochastic aging and programmed aging. The stochastic model suggests an aging process via the accumulation of damage to DNA, proteins, and other cell components caused by various external and internal factors. In contrast, programmed aging supports the model of a genetically programmed aging process,

regulated via an endogenous cellular clock, i.e. activation and inactivation of specific genes at different time points during an organism's life. This model is supported by the fact that different species have a different speed of aging and maximum lifespan. The following pages provide a closer insight into the most important mechanisms found to contribute to the aging process.

2.2.2 Gene expression as a regulating mechanism for aging

On an endogenous cellular level aging has been proposed to involve changes in gene expression patterns throughout the life of an organism. Such changes could affect maintenance, repair, or defense mechanisms of cells. A great deal of genetic work has been done in a number of model organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster;* however, comparatively little is known for mammals.

One important conserved pathway, which has been brought into connection with aging, involves the NAD*-dependent histone deacetylase (HDAC) named Sir2 in budding yeast. Histone acetylation and deacetylation are known epigenetic modifications that involve the acetylation or deacetylation of lysine residues on the N-terminal tails of nucleosomes (Konsoula and Barile, 2012). The acetylation of lysine residues typically decreases the positive charge of histones via the enzyme histone acetyltransferase (HAT), resulting in a decreased association with the negatively charges DNA molecule, yielding a more relaxed chromatin state and making genes more accessible for transcription. In contrast, histone deacetylation HDACs such as Sir2, results in a more condensed chromatin state, rendering genes inaccessible for transcription. In yeast, Sir2 has been found to inactivate certain genomic regions such as the mating type loci, the telomeres or ribosomal DNA (rDNA). Tandem repeats in the yeast's genome can form extrachromosomal rDNA circles by recombination and excision. Such circles accumulate in the cell during aging, but are most likely an effect and not a cause of aging. Overexpression of Sir2 has been found to extend the lifespan of worms and flies. Sir2 homologous in mammals have not yet been shown to affect aging. Epigenetic modifications are an intriguing way to modify the expression of genes during the lifetime of an organism and have become increasingly interesting in the research of aging.

Another conserved mechanism involved in aging is the insulin/IGF-1 pathway (Piriz et al., 2011), which is increasingly recognized as a regulator for maximum lifespan. Mutations in insulin-like signaling in worms, flies and the growth hormone IGF-1 in mice have been shown to extend the lifespan of these organisms (Tatar et al., 2003). Defects in IGF-1 and the insulin receptor were found in Alzheimer's disease, indicating a possible resistance to insulin

and IGF-1 signaling (Moloney et al., 2010). The IGF-1 receptors have been show to increase in aging neurons, leading to an increased production of the amyloid β -peptide, a principal event in the pathology of Alzheimer's (Haass and Selkoe, 2007). However, the influence of this pathway on aging is assumed to be a side effect, and not directly related to the aging process.

Random genetic or epigenetic modifications can strongly alter the expression pattern of multiple genes, thereby contributing to aging. Furthermore, such changes can strongly alter the way single cells react to outside stimuli. The following section will describe the influence of such environmental stimuli on cells.

2.2.3 DNA Damage and Reactive Oxygen Species

Carrying genetic information, DNA is of central importance to life itself and indispensible for all associated mechanisms. Therefore, DNA damage and the accumulation of mutations have been proposed to play a central role in the deterioration of organisms and the aging process (Failla, 1957). A correlation between aging and the increase of DNA mutations and chromosomal abnormalities has been show for mice and humans in multiple studies (e.g. Martin et al., 1985; Dollé and Vijg, 2002; Esposito et al., 1989; Lu et al., 2004). The role of DNA damage in aging remains debatable. It could be either a cause or simply an effect of aging. Perturbations in DNA damage repair have been reported to alter the rate of aging (Hart and Setlow, 1974; Grube and Bürkle, 1992). A recent publication indicates DNA double-strand break repair via non-homologous end joining as a central repair mechanism for age-related damage (Freitas et al. 2011).

However, the increase of DNA damages with age is not only due to a decrease in DNA repair, but also depends on the increase of DNA damaging agents such as reactive oxygen species (ROS) (Hamilton et al. 2001). These are reactive molecules containing oxygen. ROS can be free radicals, i.e. atoms with unpaired electrons or oxidizing agents. They include superoxide anion radicals, hydroxyl radicals, nitric oxide, and peroxynitrite (Floyd and Hensley, 2002). These are highly reactive molecules that can damage a number of cellular components. ROS can be formed by normal cellular metabolic processes as well as by a number of exogenous environmental influences, such as ultraviolet light or ionizing radiation. The time-sequential interaction of these damaging agents with cell components has been proposed to play a crucial role in cellular aging (Harman, 1956). This hypothesis was termed the 'Free Radical Theory of Aging'. On a cellular level mitochondrial metabolism is essential in the production of ROS. In mitochondria, ROS have not only been shown to increase with age (Chen et al., 1972) but further to cause damage, resulting in a reduction in

mitochondrial number and sequentially in decreased ATP and NAD⁺ production (Nohl et al., 1978; Tauchi and Sato, 1968; Mattson et al., 2008). The superoxide radical H₂O₂ was shown to originate directly from the mitochondrial respiratory system (Chance et al., 1979). There it significantly damages mitochondrial DNA, lipids, and proteins of the inner membrane involved in the electron transport chain. Additionally, it can generate other ROS, e.g. hydroxyl radicals, leading to a chain reaction resulting in further damage (Pryor, 1973; Tappel, 1973).

The most reactive of such ROS is OH, which strongly interacts with the nucleotides and the deoxyribose of DNA, thereby altering DNA structure (von Sonntag, 1987). Such modifications in the bases occur frequently and are usually corrected by DNA repair mechanisms. But an age-dependent increase in oxidative damage has been shown in the brains of rats and humans (Wolf et al., 2005; Mecocci et al., 1993). This could be argued to be a result of the inability of aged cells to react to the increasing rate of DNA damage.

Protein oxidation or modification by ROS is known to increase with age and has an essential part in neuronal demise in Alzheimer's disease, but also in healthy aging. Oxidative damage affects membrane bound proteins to a great extent in aged animals with cognitive disabilities and in model organisms for neurodegenerative diseases (Nicolle et al., 2001; Guo Q et al., 1999). Lipid peroxidation products can covalently modify membrane bound proteins important for cellular ion homeostasis, resulting in degeneration and dysfunction of the cells (Mattson, 2009). Particularly in neurons, such processes can result in an excitatory imbalance, facilitating the onset of an age-related imbalance (Kapogiannis and Mattson, 2011). In addition to the accumulation of damaged proteins, impairments in protein degradation, often following a stressful phase of homeostatic imbalance, has been connected to aging (Bingol and Sheng, 2011). Due to their high reactivity, ROS are thought to drive the aging process as they accumulate with time. In neurons, oxidative stress and the resulting deficits can result in uncontrolled excitation and facilitate aging.

In conclusion, it has been shown that DNA damage, as described above, is essential to cellular aging. Other contributing factors are increase oxidative stress, perturbations in cellular energy metabolism, impaired cellular calcium signaling, and the excessive accumulation of damaged proteins and organelles (Mattson and Magnus, 2006). All these aspects in conjunction govern the intricate progression of cellular aging. These fundamental processes are independent of the individual genetic background and common for all cell types. Additionally, hormonal factors strongly influence aging, suggesting a broader physiological view of neuronal aging that is not isolated from other organ systems in the body. On a larger scale, the aging and demise of individual cells strongly influences tissue homeostasis and integrity, e.g. atrophy from cell loss, leading to a decline in organ functions, diseases, and eventually to the death of an organism.

2.3 Aging in long-term cell cultures

Cells growing in culture over an extensive period of time, past the point of optimal viability have been widely used as a model for aging. This method is a very direct way to study cellular mechanisms of aging in both mitotic and post-mitotic cell cultures. Stripped from functional tissue and the physiological environment of an organism, these cells exhibit accelerated aging and are directly available for imaging, genetic experiments, biochemical studies, etc. However, there is some debate in the scientific community, whether such long-term cell cultures can be used as a model for natural aging or might more likely display pathological aging processes.

Numerous experiments have been performed on cultured human fibroblasts, T lymphocytes, and a number of other mammalian cell cultures to investigate molecular mechanisms of aging. Such studies show that normal dividing cells have a restricted proliferation capacity in culture (Hayflick, 1961), and have since been used as a model for aging. However, the question remains whether the mechanisms discovered in cells undergoing replicative senescence can be applied to post-mitotic differentiated cells. Replicative senescence, as proposed by the 'Hayflick Limit', does not apply to cultures of primary hippocampal neurons as used in this study. Neurons are post-mitotic and do not divide in culture. Today, the progress in the culturing of highly differentiated cells allows the use of long-term primary cultures as a model for aging (Kuroda et al., 1995). Once stabilized in culture, surviving primary hippocampal neurons undergo a two week period of maturation after which they start to age and remain constant for up to 30-35 days in vitro (DIV), before they start to decline. During maturation, these cells produce an extensive dendritic network and undergo synaptogenesis, resulting in physiological properties similar to those recorded in situ or in tissue slices (Kuroda et al., 1995; Basarsky et al., 1994; Banker and Goslin, 1998). During aging, the cells display an increase in the size of the soma, a broadening of the dendrites, and eventually the accumulation of vacuoles in the soma and the fragmentation of dendrites. The rate of mortality in long-term neuronal cell cultures increases exponentially according to 'Gompertz law' of mortality.

Oxidative damage, as previously described, is essential not only for living organisms but also in cell cultures. In particular, an increase in oxidized proteins has been shown in neuronal cell cultures. The lowest levels of oxidative damage are exhibited between 9-20 DIV. In this period, mitochondrial oxidative metabolism increases, but the damage due to free radicals does not (Aksenova et al., 1999). This suggests that such young neurons are able to cope with elevated oxidative stress by oxidative metabolism or that the formation of free radicals is low at this stage. In aged neurons, the content of protein carbonyls increases dramatically after 20 DIV. Such modified proteins can lose the specificity of their function or

become completely dysfunctional. For instance, a gradual increase of the creatine kinase but a decrease in its activity at advanced age has been reported (Aksenova et al., 1999). This increase in expression and the relative decrease in activity suggest the accumulation of nonfunctional molecules in the aging cell. The same study reports a gradual increase in the expression of β-actin molecules up to 15-20 DIV. After three weeks in culture, the amount of β-actin starts to decline drastically. This may reflect a change in the relation of the synthesis and degradation of this molecule, and in turn possible age-related deteriorations of cytoskeletal components. Other studies make a strong case for a role of elevated voltage-gated calcium channel activity in aged cells, resulting from an increase in channel density due to a change in gene expression (Porter et al., 1997). Elevated Ca²⁺ influx has been shown to have a neurotoxic effect. These age-related changes may drastically influence the viability of hippocampal neurons in long-term cell cultures.

One of the essential factors in cell culturing is the culture medium. Today, the B27supplemented neurobasal medium is widely used for primary cultures of neurons and neuronal cell lines. This neurobasal medium was developed form Dulbecco's Modified Eagle's Medium and optimized for hippcampal neurons (Banker and Goslin, 1998). The medium offers conditions optimal for cell survival and neurite outgrowth, while reducing the number of glia cells to 0.5% compared to other media. The medium contains vitamin E, glutathione, catalase, superoxide dismutase, and transferrin, which leads to an increased resistance to biological oxidants. Therefore, the medium has not been recommended for studies of neuronal survival involving free radical damage and oxidation (Xie et al., 1999). However, other studies using B27-supplemented neurobasal medium, as described in the previous paragraph, report an increase of oxidative damage with advancing age. This suggests that while the neurobasal medium reduces the amount of oxidative stress, ROS still play an essential role in the aging of neuronal cell cultures. Another, more recent paper investigated the ultrastructure and morphology of primary cultured rat neurons in neurobasal medium (Robert et al., 2012). The authors confirm previous reports of synapse formation between cells, via electron microscopy and further conclude that the ultrastructure of cultured neurons resembles that of neurons in vivo. This suggests that cultured neurons are a useful model to study molecular mechanism such as aging.

Considering that aging has been tightly linked to protein oxidation, among other damaging processes, the increasing oxidative stress reported in long-term neuronal cell cultures, provides a model not only for natural aging, but possibly for age-associated neurodegenerative disorders related to oxidative damage. Aging is a major risk factor for many neurodegenerative diseases. Neurons are post-mitotic cells, making the brain particularly vulnerable to age-related changes that affect its function. Therefore, the study of long-term neuronal cell cultures provides the opportunity to gain insight into major molecular

and cellular mechanisms of neuronal demise and age-related neurological disorders in the senescent brain.

2.4 The role of RNA-binding Proteins in Learning and Memory; implications for neuronal aging

The process of learning and memory is of fundamental significance to living organisms and is becoming increasingly important in the research of aging and age-associate neuronal diseases. Yet the molecular mechanisms behind learning and memory are only just starting to be understood (Kandel, 2001).

The formation of long-term memory (LTM) is proposed to occur via the change in signaling activity of individual synapses and the consequent alterations in neuronal networks in an experience-dependent manner (Govindarajan et al, 2006; Redondo and Moris, 2011). The hippocampus, a brain region which is part of the limbic system in mammals, is responsible for the processing of experiences and the formation of memory. The memory in turn is not stored in the hippocampus, but in the temporal cortex. The hippocampus acts as a filter to process new and access old memories. The molecular mechanisms in neurons, essential for the formation of a memory are the activation of gene expression in the nucleus and the local synthesis of proteins at specific synaptic sites (Kandel, 2001). In combination, these two mechanisms are proposed to alter the molecular composition, structure, and in turn the function of individual synapses. Although the translation of proteins occurs mainly in the soma close to the nucleus, local protein synthesis is thought to be induced by synaptic activation at synapses in distal parts of dendrites. The localization of polyribosomes to synapses, along with the presence of specific transcripts supports this model (Steward and Levy, 1982). This allows the local synthesis of specific transcripts, thereby altering single synapses in proximity and in turn synaptic plasticity (Kiebler and DesGroseillers, 2000). This mechanism can modify a synapse morphologically and functionally, thereby contributing to memory consolidation (Martin and Ephrussi, 2009). The specific localization of RNA transcripts has the advantage of targeting the protein to a restricted area, while it may not be expressed in other sections of a cell.

The past decades have brought increasing experimental evidence in various model organisms for the importance of RNA localization to subcellular sites far from the nucleus for a number of biological mechanisms such as axes specification, cell fate determination, or cellular migration (St Johnston, 2005). The regulated translocation of RNA is especially

important in polarized cells, such as differentiated neurons and is involved in axon path finding, dendrite branching, and the formation of dendritic spines.

In order for local protein synthesis to take place far from the nucleus, mRNA transcripts must first be transported from the soma to distal dendritic sites. The presence of mRNA close to synapses makes them directly available for local translation upon demand. This is a very economic way to regulate locally restricted protein synthesis. Here the distribution of specific RNA transcripts is essential in the maintenance of individual synapses. RNA transport and localization has been shown to occur via diffusion and trapping as well as by active transport along cytoskeletal structures. In neurons the transport of RNA into dendrites is thought to occur actively via motor proteins moving along microtubules. RNAs intended for transport have been shown to carry cis-acting localization elements in the 3'untranslated region (3'-UTR) of the transcript. Once a transcript exits the nucleus, RBPs recognize such localization elements. In a subsequent step, the RNA is proposed to undergo a repackaging process with a number of RBPs and associated factors into larger granules, forming transport ribonucleoprotein particles (RNPs), thereby rendering the transcript translationally repressed and functional for transport (Kiebler and Bassell, 2006). These RNPs then translocate along microtubules via motor proteins. Finally they are either proposed to be anchored close to their final destination at synapses or to patrol the dendrite until translated upon demand.

RBPs of different functions, such as spatial and temporal translational control play an important role in these mechanisms, effectively transporting RNA to their intended destinations. The localization elements in the 3'-UTR of the RNA transcripts can be very simple, but in many cases, e.g. CaMKIIa, they may fold into complex secondary structures. Such structures can contain multiple important sites. Therefore, the composition of RBPs binding to these specific elements is essential and is assumed to link RNA to the translocation machinery, including molecular motors. To date many RBPs involved in RNA localization have been identified, usually via genetic screens or biochemical approaches (e.g. Kiebler and DesGroseillers, 2000; Bassell and Kelic, 2004; St Johnston, 2005). Such RBPs include Staufen2, ZBP1 or FMRP, which among others are the topic of this thesis. While the specific role in RNA transport remains elusive for many of these proteins, their involvement is widely accepted today. Functionally, they are greatly diverse. While some recognize singlestranded sequences, others bind to complex secondary RNA structures or both (Stefl et al., 2005; Lude et al., 2007). These proteins contain various RNA-binding domains as well as protein interacting domains, capable of acting independent of RNA binding. The great functional diversity of the RBPs involved in the translocation of RNA transcripts provides an insight into the complexity of the mechanism. The formation of a functional transport RNP not only depends on the binding of RNA, but also on the interaction of the bound RBPs and associated factors.

As described, RBPs play an essential role in the structuring of RNAs, the formation of transport RNPs, and translocation. The loss of certain RBPs has been shown to result in a reduction in synapse number (Kiebler and Bassell, 2006). Therefore, they are essential for the structural and functional remodeling of individual synapses during the formation of long-term memory (LTM). Interestingly, RBPs have also been shown to play a role in cellular aging. Perturbations in the transcription, translation, or localization of these proteins, caused by cellular aging, as previously described, can consequently greatly affect the molecular mechanisms of learning and memory, as an organism reaches advanced age. In this thesis, a number of important RBPs, involved in RNA transport, RNA degradation, RNA mediated silencing, translational control, and possibly synaptic spine morphogenesis were screened in rat primary hippocampal neurons aging in cell culture, in an attempt to identify molecular changes during aging.

2.5 Aims of this Thesis

The objective of this project was to investigate molecular mechanisms underlying neuronal aging. Particularly, changes in learning and memory at an advanced age are of interest to our lab. Considering the important role of RBPs in the maintenance of synapses and synaptic plasticity (Goetze et al., 2006), we proposed that perturbations in the regulation of the gene expression, translational control, and cellular localization of such proteins might significantly contribute to neuronal aging. The project set out to investigate components of the exon junction complex (EJC), RBPs associated with the 80 kDa nuclear cap-binding protein (CBP80), and other important RBPs involved in RNA transport, degradation or translational repression. The primary focus was to assess the cellular localization of these proteins in differentially aged cells *in vitro*, as time-dependent changes in the distribution within neurons may reveal associated mechanisms of aging. To further investigate the distribution, we set out to study their localization to synapses. We applied standard methods of immunocytochemistry and epifluorescence microscopy to visually localize the selected RBPs.

In a parallel project, the double-stranded RBP Staufen2 (Stau2) was used as a marker for ribonucleoprotein particles (RNPs) (Goetze et al., 2006). The preferential expression of this RBP in the nervous system makes it particularly interesting in the context of neuronal aging. Time-dependent changes in the composition of Stau2-associated RNPs may have an influence on aging at a cellular level.

In this context, we investigated three distinct aspects concerning the localization of the proposed RBPs during aging:

- 1. Localization of RBPs within neurons in relation to nucleus, cytoplasm, and proximal or distal parts of dendrites.
- 2. Localization of RBPs to synapses in distal sections of dendrites.
- 3. Localization of RBPs to dendritically localized Stau2-containing RNPs.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Buffers and Solutions

Phosphate buffered saline (PBS)

8,0 g NaCl

0,2 g KCl

1,44 g Na₂HPO₄

ddH₂O to 1 L

pH 7.4

Hank's balanced salt solution (HBSS)

20 mM HEPES

2 mM CaCl₂

5.4 mM KCI

1 mM MgCl₂

135 mM NaCl

1 mM Na₂HPO₄

5.6 mM glucose

in ddH_2O

pH 7.3

<u>NMEM + B27</u>

1x MEM (modified Eagle's medium)

26 mM NaHCO₃

1 mM sodium pyruvate

200 mM L-glutamine

33 mM D-glucose

2% B27 supplement

 ddH_2O

pH 7.4

DMEM+HS

DMEM (Dulbecco's modified Eagle's medium)
10% Horse Serum (HS)
1 mM sodium pyruvate
200 mM L-glutamine

Blocking solution (BS) for immunostainings

2% BSA2% fetal calf serum0.2% fish skin gelatinePBS

Further Reagents

Trypsin/EDTA

Paraformaldehyde (PFA)

Triton X-100 0,1%

DAPI nucleic acid staining solution

Mowiol mounting medium

Barrycidal disinfectant

Ethanol 70 %

3.1.2 Antibodies

The antibodies and dilutions used for the experiments are displayed in **Table 1**. Polyclonal rabbit antibodies had been generated in the lab for Staufen2 (Stau2), Barentsz (Btz), and Pumilo2 (Pum2). These antibodies have been tested and successfully used in various experiments, including immunofluorescence, Western Blotting, and immunoprecipitation. The anti-Staufen2 antibodies were raised against the longest 62 kDa isoform of mouse Stau2 (Zeitelhofer et al., 2008), the anti-Btz antibodies were raised against the C-terminus of Btz (Macchi et al., 2003) and the anti-Pumilio2 antibodies were raised against the N-terminal half of mouse Pum2 (Vessey et al., 2010). The other antibodies used are either commercially available or were kind gifts from scientists to the lab.

Specificity	Species	Company/Laboratory	Dilution used
Primary Antibodies:			
Ago2	Mouse	Abnova	1:500
Btz	Rabbit	M. Kiebler	1:500
CBP20	Rabbit	R. Lührmann	1:500
CBP80	Rabbit	R. Lührmann	1:500
elF4AIII	Rabbit	Abcam	1:500
elF4E	Mouse	BD/Transduction Laboratories	1:500
FMRP	Mouse	Chemicon/Millipore	1:200
GFAP	Mouse	Sigma	1:1000
hnRNPQ	Mouse	Acris	1:500
hnRNPU	Rabbit	Abcam	1.200
Homer1	Guinea pig	Synaptic Systems	1:1000
HuR	Mouse	Santa Cruz	1:500
Magoh	Mouse	Abnova	1:500
Map2	Mouse	Sigma	1:1000
Mov10	Rabbit	Bethyl Laboratories	1:500
PABP1	Rabbit	Cell Signaling	1:500
Pum2	Rabbit	M. Kiebler	1:500
Pur-α	Mouse	M. Kiebler	1:500
RBM14	Rabbit	Aviva Systems Biology	1:500
RCK	Rabbit	MBL	1:500
Stau2	Rabbit	M. Kiebler	1:500
Stau2	Mouse	M. Kiebler	1:500
SynapsinI	Mouse	Synaptic Systems	1:1000
Tau	Rabbit	Abcam	1:1000
Upf1	Rabbit	Wiedemann	1:500
Y14	Mouse	Immunoquest	1:500
YB1	Rabbit	Abcam	1:500
ZBP1	Mouse	S. Hüttelmaier	1:200

Secondary Antibodies:			
Guinea pig	Goat (Cy3)	Dianova	1:1000
Mouse	Donkey (Alexa488)	Invitrogen	1:1000
Mouse	Goat(Cy3)	Dianova	1:1000
Rabbit	Donkey(Alexa488)	Invitrogen	1:1000
Rabbit	Goat(Cy3)	Dianova	1:1000

Tab. 1: List of primary and secondary antibodies used in this study, along with the species and dilutions best applied for experimental conditions for immunofluorescent labeling of fixed cells, according to established lab protocol (Zeitelhofer et al., 2008). Antibodies for Stau2 (Zeitelhofer et al., 2008), Btz (Macchi et al., 2003) and Pum2 (Vessey et al., 2010) were generated in the lab.

3.1.3 Equipment

For dissection of E17 rat hippocampi

Sterile cell culture plastic petri dishes (35 mm and 94 mm)

Sterile falcon tubes (15 ml)

Sterile surgical straight forceps

Sterile fine straight forceps

Sterile circumflexed forceps

Sterile coarse straight scissors

Sterile fine straight scissors

Sterile Vannas spring scissors

Sterile steel spatula

Water bath, prewarmed to 37-°C

Bottom lit stereo microscope

For dissociation and plating of hippocampal neurons and glia cells

Sterile cell culture plastic petri dishes (60 mm)

Sterile cell culture plastic flasks

Sterile glass beakers (20 ml and 150 ml)

Sterile fire polished Pasteur pipettes

Sterile diameter reduced Pasteur pipette

Peleus ball

Sterile glass pipettes (10 ml)

Pipetboy

Automatic pipette (20-200 µI) and tips

Sterile Eppendorf tubes (1.5 ml)

Hemicytometer and coverslip

Water bath, prewarmed to 37-°C

Sterile laminar flow hood

For immunocytochemistry

Automatic pipettes (0.1-2.5 µl, 0.5-10 µl, 20-200 µl, 100-1000 µl) and tips

Sterile falcon tubes (15 ml)

Multi-well culture plates (12 well)

Water bath, prewarmed to 37-°C

Microscopy setup

Microscope Zeiss Axioplan (Carl Zeiss MicroImaging, Inc.)
Objective 63x/1.40NA oil plan-apochromat (Zeiss)
Light Source X-Cite 120 mercury lamp (EXFO)
Camera Olympus F-view II CCD (Soft Imaging Systems)

3.1.4 Animals

To generate hippocampal neurons in culture, timed pregnant rats (*Rattus norvegicus*, Sprague-Dawley CD-SIFA) were used, obtained from Charles River Laboratories (Königshofen, Germany). Rat embryos were dissected at the age of 17 days (E17) (Zeitelhofer et al. 2008).

3.1.5 Software

AnalySIS Five (Soft Imaging System)

MetaMorph 7.5 (Universal Imaging Corp.)

Photoshop CS6 (Adobe)

Excel (Microsoft Office 2007)

3.2 Methods

To assess the localization of the RNA-binding proteins (RBPs) in question within neurons, standard methods of immunocytochemistry and epifluorescence microscopy were applied. In general, the experiments were performed on primary cultured rat E17 hippocampal neurons. Cells were kept in a long-time cell culture, allowed to age, and used at various time points, ranging from mature to aged neurons. These methods provide an effective way to gain a genuine endogenous pattern for the distribution of proteins in mature and aging cells. In addition to the staining of a single epitope, double immunofluorescence stainings were performed, allowing co-localization studies via the detection of two distinct epitopes within the same cell. These double-stainings were performed for each RBP in combination with a synaptic marker to address the question of localization to dendritic spines. Synapsinl and Homer1 were targeted as presynaptic and postsynaptic markers respectively. Furthermore, Staufen2 (Stau2) was used for such co-stainings as a marker for ribonucleoprotein particles (RNPs) (Goetze et al., 2006) to ascertain possible changes in Stau2-associated RNP complexes during aging. DAPI-staining was performed to ensure cells have an intact nucleus and are viable.

3.2.1 Neuronal Cell Culture

Dissection of E17 Rat Hippocampi

The studies were preformed on primary cultured rat hippocampal neurons. The protocol, for maintaining such a neuronal cell culture has been performed in the lab as described by Gary Banker und Kimberly Goslin in *Culturing Nerve Cells* (Banker and Goslin, 1998) and adapted for the lab. A updated version of the original protocol was published in *Nature Protocols* in 2006 (Kaech and Banker, 2006).

Hippocampal neurons were obtained from the hippocampus of E17 rat embryos. The dissection of hippocampi is performed at this developmental stage for numerous reasons. First of all, once the embryonic brain is dissected the meninges can easily be removed, which becomes increasingly difficult in later stages of development. Removing the meninges is essential, as they contain fibroblasts which are not wanted in the final cell culture. In addition, the localization and extraction of the hippocampus is simplified. Furthermore, at this stage the brain tissue has developed an integrity, which allows better handling than in earlier stages.

In E17 rats, the tissue consists largely of neurons. Glia cells, which should be kept at a minimum in the final cell culture, are first generated later during development, then migrate into the hippocampus in greater numbers at later stages. Therefore, the E17 rat brain provides the best developmental stage for dissection and allows the selective enrichment of primary hippocampal neurons over glia cells. However, as development is not consistent in all individuals, the embryos and brains must be inspected at dissection. Especially exposure to extreme stress or sickness during pregnancy can result in developmental delay or influence brain size, brain integrity, etc. of the offspring. Any embryos with conspicuous features in the morphology of body or brain, or inflammation (yellow fluid in the embryonic sack) should be discarded and not use for the cell culture.

All reagents and instruments for cell culturing must be prepared in advance, so the cells can be plated immediately after dissection of the hippocampi. A timed pregnant rat is anesthetized and killed by severing the medulla oblongata. Thereafter, the extraction of the embryos and subsequently the embryonic brains and hippocampi must be performed in an operating room in a sterile environment to avoid contaminations in the cell culture (gloves, face mask, etc.).

Enclosed is the detailed protocol I used to dissect the E17 rat hippocampi, in collaboration with Sabine Thomas:

- 1. HBSS and Trypsin-EDTA is prewarmed in a 37-°C water bath.
- 2. The rat is placed on a sterile surface, and the fur on the abdomen and anal region is disinfected with Barrycidal disinfectant. According to the manufacturer, this should be effective against most bacteria, viruses, or fungi.
- 3. Using sterile large surgical forceps and scissors, the fur is dissected from the abdomen by cutting a V-shape into the epidermis and folding the flap of skin to the top. Disinfect forceps and scissors with 70% ethanol.
- 4. After disinfecting the abdominal wall with 70% ethanol, it can be dissected carefully by cutting in a V-shaped manner. Take care not to harm internal organs, especially the gut, as this will result in immediate loss of sterility.
- 5. Take care while extracting the embryos, and transfer all into a large petri dish (94 mm). Dispose of mother rat in a plastic bag.
- 6. Embryos are dissected from the embryonic sack and placenta with circumflexed forceps and fine scissors and placed into a separate large petri dish filled with prewarmed HBSS. Count the embryos and document. At this point check for any morphological anomalies. Also check the liquid in the embryonic sack for signs of inflammation or infection.
- 7. Cut off the head of an embryo in a dry petri dish. Secure the head with scissors, and pierce it with the forceps at the level of the eyes to hold securely. Dissect the brain by cutting dorsally along the sagittal plane from neck to forehead. Remove the brain using a small steel spatula, and transfer it to a small petri dish (35 mm) filled with prewarmed HBSS. Repeat process with other embryos.
- 8. The dissection of the hippocampi is performed under a stereo microscope with fine forceps and Vannas spring scissors. The hemispheres are isolated, and the meninges are carefully stripped away. Hippocampi are dissected carefully and placed into a falcon tube with prewarmed HBSS and stored in the 37 °C water bath.
- Additionally, two hemispheres can be collected in a HBSS filled falcon tube for glia cell culture.
- 10. The HBSS is decanted from the hippocampi filled falcon tube, Trypsin-EDTA is added to loosen cell contacts, and the tube is placed back into the water bath. Note the time point at which Trypsin-EDTA is added as the incubation period must not exceed 10 minutes.
- 11. Dispose of all petri dishes and their contents in a plastic bag, and dispose appropriately. Disinfect all used instruments and the working surface.

Dissociation and plating of hippocampal neurons and glia cells

Cells are cultured in incubators at 37-°C and 5% CO₂ saturation in NMEM + B27 medium. It has been shown that neurons thrive best when co-cultured with glia cells. As glia cells divide in culture and thus quickly get in the way of any experiment, neurons are first plated separately on coverslips, where they grow and are ready to use when matured. These coverslips are coated with poly-L-lysine, applied with three small paraffin dots and equilibrated with the NMEM + B27 medium in an incubator one day prior to plating. Once the cells are plated and adhere to the coverslips, they are flipped upside down into dishes containing glia cells. The neurons are then located at the underside of the coverslip held up from the surface of the petri dish by the paraffin dots. This way, neurons are cultured in close proximity to glia cells, allowing the exchange of small diffusible substances, but do not physically interact with them.

All necessary preparations to plate primary hippocampal neurons must be done in advance. The medium in culture dishes intended for the primary neurons must be changed from DMEM + HS to NMEM + B27 for equilibration the day prior to cell culture or at the very latest one hour before plating. For glia cells, a culture flask with 15 ml of DMEM + HS is prepared. All necessary reagents are placed in a 37°C water bath, and all instruments disinfected with 70% ethanol and laid out in a laminar flow hood prior to dissection. All steps are performed under the sterile laminar flow hood.

Enclosed is the detailed protocol I used to prepare primary hippocampal neurons in culture, in collaboration with Sabine Thomas:

- 1. Decant HBSS from the falcon tube holding the hemispheres.
- 2. Incubate hippocampi no longer than 10 minutes with Trypsin-EDTA. Decant into the falcon tube containing the hemispheres. Incubate the hemispheres for 10-20 minutes in a 37-°C water bath.
- 3. Wash the hippocampi with approximately 10 ml of prewarmed HBSS. When the hippocampi have settled to the bottom of the falcon tube, decant and repeat washing step.
- 4. Decant HBSS so approximately 2 ml remain in the falcon tube. Triturate hippocampi using a peleus ball and a fire polish Pasteur pipette. Pipette up and down about 20 times no longer than 1 min 30 sec until no larger cell aggregates can be seen. Avoid bubbles.
- 5. Use a diameter reduced Pasteur pipette to disassociate cells further by pipetting up and down for one minute. Do not exceed time, as this may induce too much stress on cells. Transfer cells into an Eppendorf tube. If cells are successfully disassociated the liquid should now have a milky appearance.
- 6. Pipette approximately 25 μl of the cell suspension to the sides of a coverslip on a hemocytometer chamber. Count cells in 16 squares. Live cells appear yellow when viewed under the microscope. Multiplication by 1000 yields the cell number in 100 μl. Calculate the appropriate volume for 160,000 cells.
- 7. Plate calculated volume in prepared culture dishes containing 5 ml of NMEM + B27 medium and poly-L-lysine coated coverslips. Move dishes in a cross-like fashion to distribute cells evenly and immediately transfer to incubator.
- 8. Continue processing the glia cell culture. Decant Trypsin-EDTA, and add 5 ml of DMEM + HS with a glass pipette to stop the proteolysis reaction completely. Transfer hemispheres into a 20 ml glass beaker with a 10 ml glass pipette.
- 9. Triturate by pipetting up and down approximately 10 times.
- 10. Transfer the cell solution into the prepared culture flash with 15 ml of DMEM +HS, and immediately place in the incubator.
- 11. Three days after plating, the hippocampal neurons are ready to be flipped onto glia cells cultured in NMEM + B27 medium. Cells are flipped into the dishes holding glia cells in by transferring the coverslips with paraffin dots and neurons facing downward. Paraffin dots ensure spacing between the coverslip and the bottom of the petri dish.

Follow-up Steps for Glia Cell Culture

Three days after plating, the glia cells are washed once with PBS and new medium is applied. One week after plating, the cells are ready to be split and passed into a new culture flask equilibrated with 15 ml of DMEM + HS.

- 1. Wash cells once in prewarmed PBS.
- 2. Add 3 ml Trypsin-EDTA, and pound flask on surface to release cells from bottom until solution becomes milky white (5 10 minutes).
- 3. Add 3 ml DMEM + HS, and pipette up and down 10 times to stop reaction.
- 4. Split cells 1:3 by transferring 2 ml into the new culture flask prepared the previous day.

Three days later, the cells are ready to be split into culture dishes.

- 1. Equilibrate petri dishes with 3 ml DMEM + HS in incubator for one hour.
- 2. Wash cells once in prewarmed PBS.
- 3. Add 3 ml Trypsin-EDTA, and pound flask on surface to release cells from bottom until solution becomes milky white (5-10 minutes).
- 4. Add 3 ml DMEM + HS, and pipette up and down 10 times to stop reaction.
- 5. Pipette approximately 25 µl of the cell suspension to the sides of a coverslip on a hemocytometer chamber. Count live cells, and calculate appropriate volume for 20,000 cells.
- 6. Pipette the calculated volume onto prepared petri dishes with 3 ml DMEM + HS. Move dishes in a cross-like fashion, and immediately transfer to incubator.

3.2.2 Immunocytochemistry

Double immunofluorescence stainings were performed by combining antibodies originating from two different species, targeting the epitopes under investigation. To study the localization of RBPs to synapses, the mouse anti-SynapsinI antibody was used in combination with rabbit antibodies, and the guinea pig anti-Homer1 antibody in combination with mouse antibodies. Similarly, rabbit and mouse anti-Stau2 antibodies were used in combination with mouse and rabbit antibodies for each RBP respectively, to ascertain the localization to Stau2-containing RNPs. Cy3-coupled goat anti-mouse, anti-rabbit and anti-guinea pig antibodies, and Alexa Fluor 488-coupled donkey anti-mouse and anti-rabbit antibodies were used as secondary antibodies. The two secondary antibodies were selected for each experimental combination from two further species, resulting in the application of four antibodies of four different species for each experimental setup. The use of antibodies originating from four different species in such double-staining experiments avoids incorrect binding or aggregation, which in turn would lead to false patterns and imply co-localization where there is none.

The cells used for the experiments were kept in a long-time culture and selected at different time points: 8 days *in vitro* (8 DIV, developing neurons), 14 DIV (dendritic spines established), 22 DIV (many dendritic spines, initial aging), 28-33 DIV (aged neurons). Before each experiment, the cells were assessed under a stereo microscope and selected according to vitality, density, and glia cell percentage.

Following is a description of the established laboratory protocol used for immunofluorescence labelling, as frequently performed and described in previous publications (e.g. Zeitelhofer et al., 2008).

- 1. Warm PBS and a falcon tube containing 3 ml 16% paraformaldehyde (PFA) to 37-°C in a water bath, and thaw the blocking solution (BS).
- Retrieve neurons from the incubator.
- 3. Rinse coverslips shortly into PBS to wash away the medium, and immediately transfer to 4% PFA. Fix cells for 10 15 minutes. In case of a preceding transfection, cover with tinfoil to avoid bleaching.
- 4. Wash cells 3 times for 5 minutes with PBS.
- 5. Permeabilise cells in 0.1% Triton X-100 for 5 minutes.
- 6. Block cells in 100% BS for 30 minutes at room temperature.
- 7. Prepare a dilution of 10 % BS in HBSS at room temperature.
- 8. Prepare the primary antibody in 10% BS at an appropriate concentration.
- 9. Incubate the cells with the primary antibody for 2 hours at room temperature or over night at 4-°C.
- 10. Wash cells 3 times for 5 minutes with PBS.
- 11. Prepare the secondary antibody in 10% BS at an appropriate concentration.
- 12. Incubate the cells with the secondary antibody for 50 minutes at room temperature.
- 13. Wash cells 3 times for 5 minutes with PBS.
- 14. Stain with DAPI for 5 minutes (optional).
- 15. Wash cells 3 times for 5 minutes with PBS.
- 16. Mount coverslips onto microscope slides with 8µl of Mowiol and place at 4-°C.

3.2.3 Microscopy, image processing, and analysis

Images were acquired using a Zeiss Axioplan (Carl Zeiss MicroImaging, Inc.) microscope, the Olympus F-View2 (Soft Imaging System) CCD camera, and the AnalySIS Five (Soft Imaging System) imaging software. For each coverslip, the overall vitality of the cells and the efficiency of the immunofluorescence labeling were assessed. Single cells for imaging were selected according to vitality, dendrite morphology and DAPI-staining. Images were taken at 630-fold magnification with the focal point set upon the dendrites. For each neuron chosen, a picture was acquired in phase contrast and under filters for green, red, and blue fluorescents. To gain further insight into the pattern within the soma and nucleus, the focal plane was adjusted, and a second picture was taken for the green and red fluorescent channels, respectively. For each antibody, images were taken with the same exposure time throughout all experiments to allow the comparison of differently aged neurons.

Once acquired, the images were viewed and processed using the MetaMorph 7.5 (Universal Imaging Corp.) imaging software and assembled with the Adobe Photoshop CS6 software. The images were not modified other than adjustments regarding brightness and contrast. Care was taken only to enhance the image quality and not to alter or lose informational content.

Quantification was performed using the MetaMorph software. Contrast and brightness of the black and white images was optimized to reduce background fluorescence and provide clear patterns for the distribution of each protein. Corresponding images were then overlaid and the two channels artificially colored using the software. For quantification, a 20 µm section of a dendrite, located approximately 20 to 40 µm from the cell body, assuming an average diameter of 20 µm for a cell body was selected. This section was boxed and magnified. The complete number of particles in such a 20 µm segment was manually counted for each channel. In addition, the number of particles found to overlap in the overlaid images were counted and used to calculate the average percentage of overlapping particles in the two channels. Furthermore, images were analyzed for any differences between mature and aged neurons in the distribution of the patterns. The localization of the particles was assessed by addressing a number of categories: presence in the nucleus, presence in the soma, presence in the proximal dendrite, presence in the distal dendrite, presence at dendritic spines, and changes in number or size of particles in a given area.

4. RESULTS

The correct function of RBPs is crucial for a number of important cellular mechanisms, involving processes such as RNA-transport, RNA-degradation, or translational control. In neurons RBPs are essential for the molecular mechanisms underlying learning and memory formation. Here, cellular aging is proposed to affect RBPs leading to perturbations in their associated functions. The aim of this thesis was to investigate the subcellular localization of essential RBPs and to identify age-dependent changes in the distribution of these proteins. The experiments were performed on mature and aging cells of primary cultured rat hippocampal neurons, as routinely cultured in the lab. These neurons were kept in long-term cell cultures up to 33 DIV and selected for the experiments at specified time points at various stages of aging. This approach allowed to investigate these proteins in differentially aged cells and was applied to reveal changes in localization as a direct or indirect effect of aging.

The experiments were designed to investigate the endogenous pattern of the selected RBPs. Standard methods of immunocytochemistry make use of specific antibodies (see **Tab. 1**) to stain cells for the RBPs of interest. These antibodies have been tested and are routinely used in the lab for immunostaining and a number of other antibody based methods (e.g. Kiebler et al., 1999; Macchi et al., 2003; Goetze et al., 2006). The combined use of two different antibodies allows double-staining for two different epitopes in a single cell. This approach was used to assess co-localization and thereby to gain information on the respective relationship of two proteins of interest, to investigate their synaptic localization and their association to neuronal Stau2-containing RNPs. Cells were selected at specified time-points for the experiments, as described in section 3.2.2 of Methods. Images of selected cells and their staining were acquired by epifluorescence microscopy, analyzed, and quantified for age-related changes, as presented in the following pages.

The images presented here provide examples of neurons stained for RBPs which were found to exhibit age-related changes in their cellular localization. In this thesis, I investigated the cellular distribution of RCK, hnRNPQ, HuR, RBM14, CBP80, and ZBP1 during aging. These RBPs were selected for the following reasons.

The RNA helicase RCK is found in P-bodies and stress granules. It functions in translational repression, RNA-degradation, and microRNA induced gene silencing. hnRNPQ (also called Syncrip) is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. hnRNPs in general promote alternative splicing, polyadenylation, and RNA-transport. HuR has been implicated in a number of biological processes and diseases such as cancer. As a member of the ELAVL protein family, HuR binds AU-rich elements in the 3'-UTR, thereby stabilizing the mRNA. RBM14 may function as a general nuclear coactivator

enhancing transcription through other coactivators, but can also work as a repressor. Furthermore, RBPM14 is a splicing modulator. CBP80 is a component of the nuclear capbinding protein complex. This complex functions in splicing, 3'-end processing, nuclear export, and nonsense mediated mRNA decay. ZBP1 has been found to play a role in RNA-localization, RNA-stability, and translational control. For the full list and results of all the RBPs investigated, refer to Tables 2 and 3.

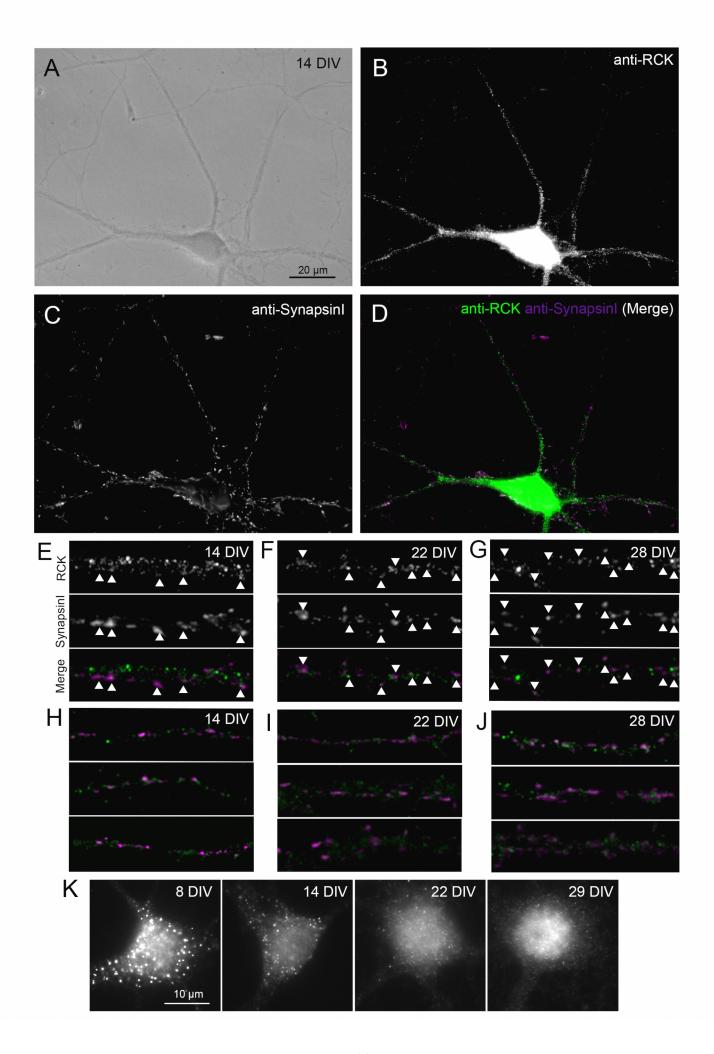


Fig. 1: Changes in RCK-distribution indicate reduction in size or disassembly of P-bodies during aging and increased localization to dendritic spines. (A-D) The images show a 14 DIV old neuron. Phase contrast (A), RCK staining (B), SynapsinI staining (C) and an overlay of B and C (D) are seen. (E-G) The boxes show 20 μm segments of selected dendrites from neurons ranging from 14 DIV, 22 DIV up to 28 DIV. Co-localization events of RCK and SynapsinI increase with age (white arrows). (H-J) Additional images provide further examples, for the point made in images E-G. (K) Selected images are examples for typical RCK-patterns in the cell lumen at different stages of aging. Note the change in pattern and distribution.

First, I analysed the localization of the ATP-dependent RNA helicase RCK, also known as DEAD box protein 6 (DDX6) or p54. It is used as a P-body marker, and has a relation to Stau2 particles as shown recently (Zeitelhofer et al., 2008; Cougot et al., 2008). The experiment was performed to address the localization of RCK in different regions of a neuron, particularly to synapses. Therefore, double-immunostainings were performed on mature and aged neurons. Cells were labeled with rabbit anti-RCK and mouse anti-SynapsinI primary antibodies, targeting the corresponding epitopes.

Images in Fig. 1 provide representative examples for all neurons stained for RCK and recorded under the microscope. Images A-D show a typical vital cell at 14 DIV in phase contrast with the equivalent staining for RCK, SynapsinI, and the corresponding overlay. When comparing representative 20 µm dendrite sections (shown in images E-G and H-J), an increase in co-localization events of RCK and SynapsinI, indicated by white arrows, can be observed with progressing age. Note how the pattern for the RCK and SynapsinI particles remains comparable between ages, but co-localization increases. This finding indicates an increased association of RCK to synapses in aged neurons, which is supported by the corresponding data in Table 2 (see below). Images shown represent representative examples for the average data in Table 2 collected from all neurons analyzed. Panel K provides insight into the RCK pattern as distributed in the lumen. The four time points, ranging from mature (8 DIV) to aged neurons (29 DIV), show an obvious change in the staining pattern. The large particles present at 8 DIV appear to decrease in size and/or dissociate with increasing age, as shown in the sequential pictures. This variation in pattern was highly reproducible throughout the experiments and found in all cells imaged. Taken together, the observed changes in synaptic localization and in the distribution of the pattern in the cells lumen clearly show age-dependent effects.

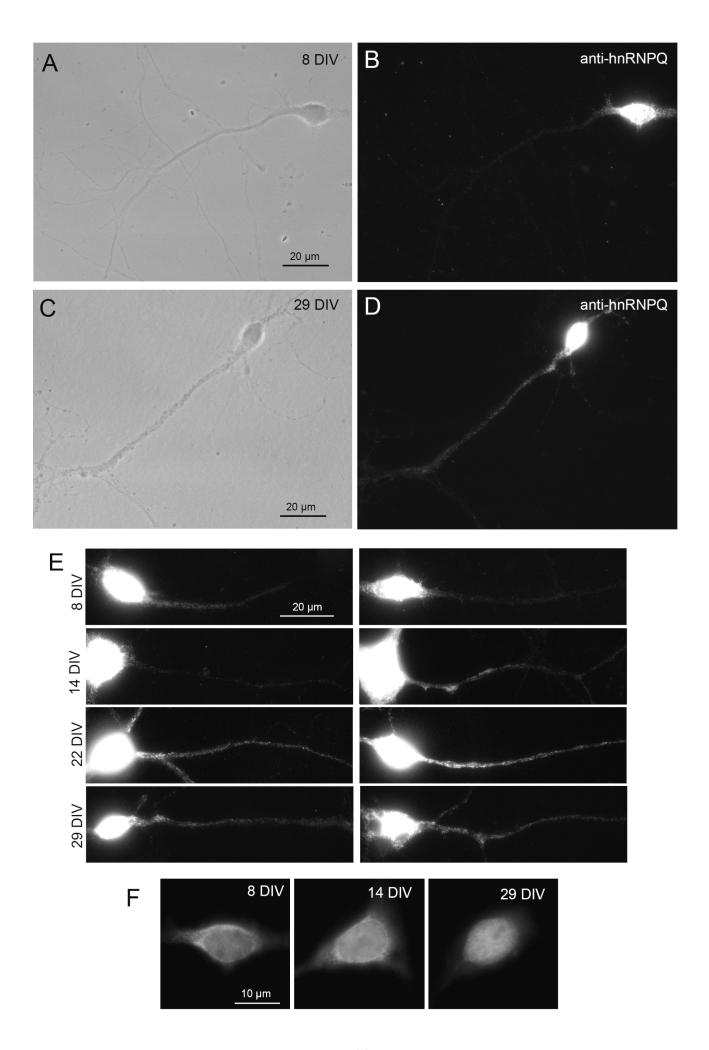


Fig. 2: Localization of hnRNPQ to distal parts of dendrites and relocalization to the nucleus are shown to be age-dependent. (A-D) The images show a 8 DIV and a 29 DIV cell in comparison, including phase contrast (A, C) and hnRNPQ staining (B, D). (E) Further examples for hnRNPQ staining are given in this pannel, showing increased localization to dendrites in aged cells. (F) Additionally, relocalization to the nucleus can be observed during aging.

The images shown in **Fig. 2** present the data collected for the heterogeneous nuclear ribonucleoprotein Q (hnRNPQ), also frequently referred to as Syncrip. This protein has been previously implicated with dendritic mRNA localization in neurons (Kanai et al., 2004).

The experiments presented here address the localization events of this RBP in relation to the nucleus, cell body and dendrite. Cells were treated with mouse anti-hnRNPQ primary antibody and the Alexa Fluor 488-coupled donkey anti-mouse secondary antibody. Double immunostainings were performed as well to investigate positional correlation with synapses and RNPs. However, no significant age-dependent effects could be found (**Tab. 2** and **Tab. 3**, fluorescence data not shown).

The images provided in Fig. 2 were selected as examples to represent all neurons stained for hnRNPQ and provide insight into the distribution of the protein at different time points. Images in A-D show a mature cell at 8 DIV and a highly aged cell at 29 DIV in phase contrast and corresponding staining for hnRNPQ. When comparing the phase contrast images (A and C), both cells appear to be vital and intact. However, age-related effects on cell morphology are nicely demonstrated here, especially when comparing the dendritic structure. Note the reduced halo around the nucleus, the broadening of the dendrite, and the rough appearance of the cell membrane in the aged neuron. The distribution of hnRNPQ is presented in images B and D. Notice the increased localization to more distal parts of the dendrite in the aged cell. This effect is shown in detail in panel E, showing two examples each for the time points selected during the aging process. As shown little change occurs between time points 8 DIV and 14 DIV. Similarly, the effect between 22 DIV and 29 DIV is minimal. However, a stronger effect can be found when comparing the middle time points 14 DIV and 22 DIV. Overall an increased localization to dendrites can be observed with advanced age. This effect somewhat varies between cells. Whereas some neurons show a low localization to dendritic sites, others show a much stronger and distinct increase. However, the effect was consistent in all cells imaged. Images F provide an insight into the hnRNPQ pattern within the soma and nucleus. Here a relocalization of the protein can be observed from the cytoplasm and nuclear membrane to the interior of the nucleus with increasing age. This effect was stable in all cells imaged. These changes in dendritic and nuclear localization are shown to be age-dependent.

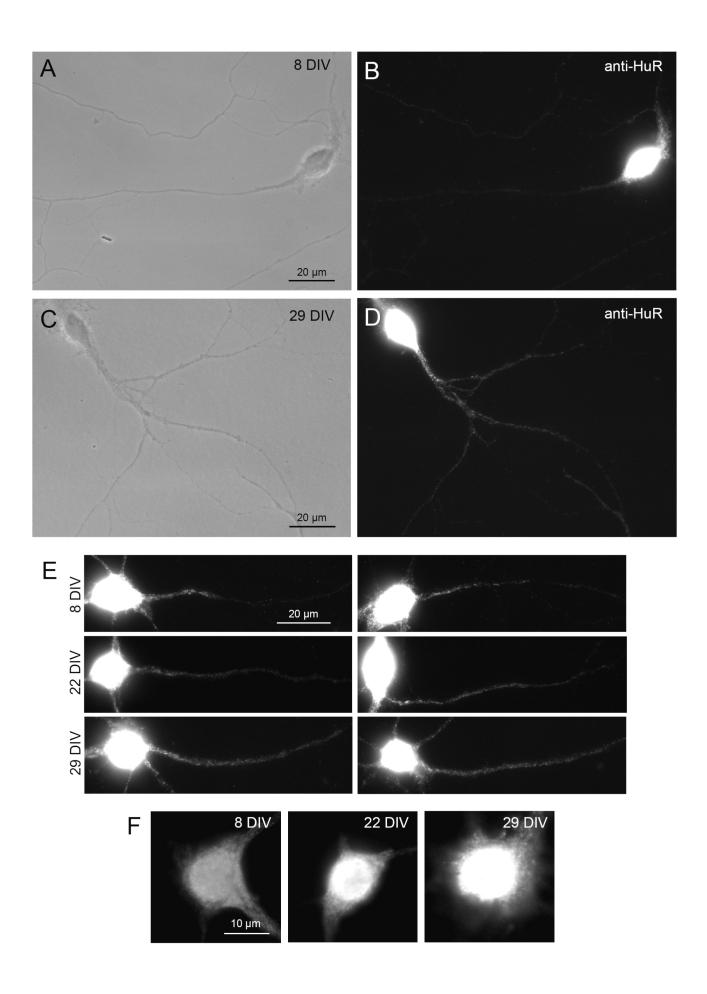


Fig. 3: Localization of HuR to distal parts of dendrites and relocalization to the interior of the nucleus are shown to be age-dependent effects. (A-D) The images show a 8 DIV and a 29 DIV cell in comparison, as well as phase contrast (A, C) and HuR staining (B, D). (E) Further examples are given in the panel, showing increased localization to dendrites in aged cells. (F) Additionally a nuclear relocalization effect can be observed for HuR during aging.

Fig. 3 shows representative examples of the localization for the Hu-antigen R (HuR), also known as ELAV-like protein 1 or Elav-like generic protein. HuR is a member of the Hu RNA-binding proteins. There is evidence that HuR (and HuD) potentially interact with neuronal mRNAs implicated with synaptic plasticity (Tiruchinapalli et al., 2008).

The immunostainings presented were performed to investigate the localization of HuR to different parts of neurons in differentially aged cells. These experiments involved the mouse anti-HuR primary antibody and the Alexa Fluor 488-coupled donkey anti-mouse secondary antibody. Double immunostainings with Stau2 and the synaptic marker Homer 1 revealed no dependent effects in aging (**Tab. 2** and **Tab. 3**, fluorescence data not shown).

Fig. 3 shows images with representative staining for all cells labeled for the HuR protein. Images A-D give phase contrast and the HuR-staining of a mature cell at 8 DIV and a highly aged cell at 29 DIV. The phase contrast images (A and C) prove both cells to be viable, though age-related morphological changes can be observed. The immunostaining of HuR is presented in images B and D. Notice the obvious increase in localization to more distal parts of the dendrite in the aged cell, as previously observed also for hnRNPQ in Fig. 2. More detailed evidence for this effect is presented in panel E. Here, two examples for each time point show an increase in dendritic localization, which becomes obvious at 22 DIV and is most evident in highly aged cells at 29 DIV. This change in dendritic distribution is agedependent and was observed to a variable degree but consistently in all imaged cells. An insight into the HuR localization pattern in the neuronal cell body and nucleus is given in panel F. The focal point was set to show both the soma and the nucleus. The exposure time for these cells was shorter than in previous images. The different time points nicely outline an increase of HuR in the cytoplasm and possibly the nucleus during aging. This effect was stable in all cells imaged. The changes in dendritic and nuclear localization presented in **Fig. 3** clearly shown age-dependent effects.

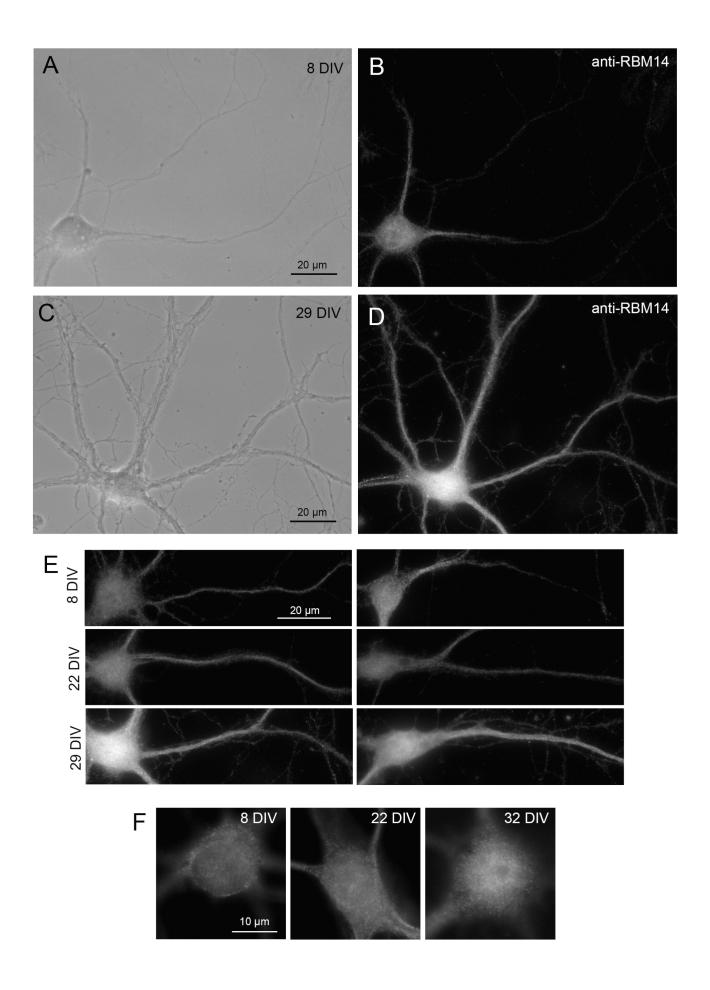


Fig. 4: Increased localization of RBM14 to dendrites and changes in nuclear localization are shown to be age-dependent. (A-D) The images show a 8 DIV and a 29 DIV cell in comparison, as well as phase contrast (A, C) and RBM14 staining (B, D). (E) Further examples are given in the panel, showing increased localization to dendrites in aged cells. (F) Additionally altered nuclear localization can be observed during the aging process.

The data collected for the RNA-binding motif protein (RBM14) is presented in the images in **Fig. 4.** RBM14 is also known as PSP2, SYT-interacting protein or RRM-containing coactivator activator/modulator. There is recent evidence in the lab that RBM14 is found in Stau2-containing RNPs from rat brain (Härtel, Karra et al., submitted). This experiment set out to investigate the localization of RBM14 in mature and aged neurons. Cells were treated with the rabbit anti-RBM14 primary antibody and the Alexa Fluor 488-coupled donkey antirabbit secondary antibody. The quantification of double immunostainings performed can be seen in **Table 2** and **Table 3**, but did not yield any significant effects during aging (fluorescence data not shown).

Fig. 4 shows selected examples that provide insight into the distribution of RBM14 in neurons of various ages. Images A-D show a mature cell at 8 DIV and an aged cell at 29 DIV in phase contrast and corresponding staining for RBM14. Both cells are vital and intact as shown by phase contrast (A and C). The cell in image C, while still viable displays strong morphological hallmarks of aging, such as the broadening of the dendrites, the rough appearance of the cell membrane and the flattening of the soma on the cover slip. Images B and D display the staining for RBM14. An increase in intensity and possibly a stronger localization to the dendrite in the aged cell can be observed. This effect is further outlined in panel E, giving two examples each for the time points selected during aging. Overall a low increased localization to dendrites can be observed with advanced age. This effect was observed weakly in some neurons, with merely dendritic localization of RBM14. Other neurons showed a much stronger and distinct increase in dendritic localization. The effect was consistent in all cells imaged. However, as the effect is very subtle further experiments are necessary to confirm these results. Images F give an insight into the RBM14 pattern within the cell body. Here an increasing localization of the protein can be observed in the nucleus with advanced age. The diffuse pattern in the images makes it difficult to analyze this relocalization, but an effect can clearly be observed and was identified in the majority of cells imaged. These changes in dendritic and nuclear localization show an age-dependent effect.

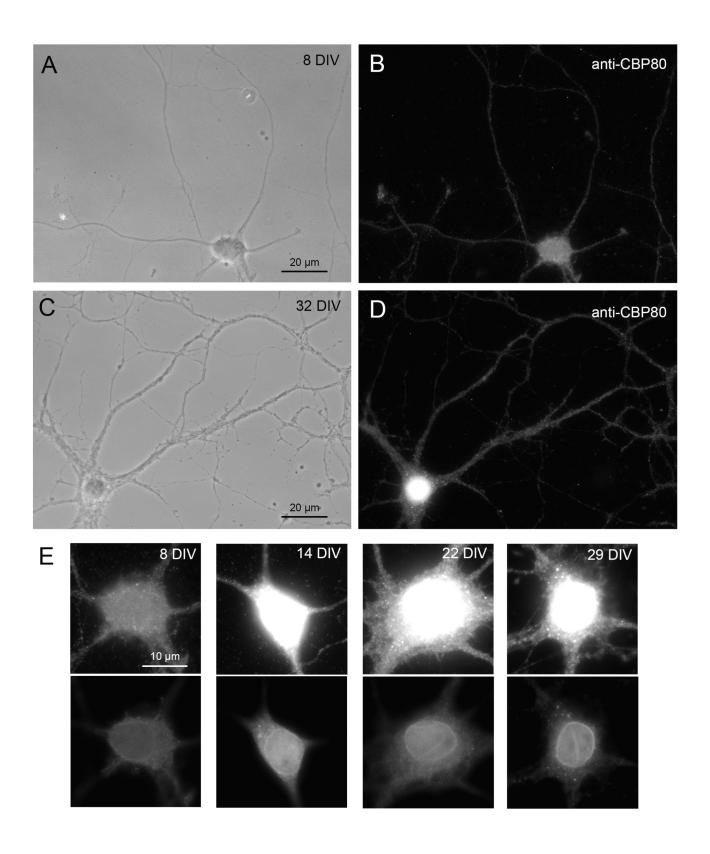


Fig. 5: Increased localization of CBP80 to the nuclear membrane and nucleus occurs at the time point of synapse establishment and is possibly intensified in advanced stages of aging. (A-D) The images show a 8 DIV and a 32 DIV neuron in comparison, as well as phase contrast (A, C) and CBP80 staining (B, D). (E) Increasing localization to the nuclear membrane can be observed at different time points in the cytoplasm (upper images) and the interior of the nucleus (lower images).

Fig. 5 shows neurons stained for the 80 kDa nuclear Cap-binding protein (CBP80). CBP80 is the nuclear Cap-binding protein, consisting of two distinct subunits of 80 and 20 kDa, respectively. Furthermore, it has been found in dendritic RNPs in primary neurons (di Penta et al. 2009).

This experiment was performed to investigate the distribution of CBP80 in neurons and its co-localization with synapses and Stau2-containing RNP particles. For the experiment, cells were labeled with the rabbit anti-CBP80 primary antibody followed by the Alexa Fluor 488-coupled donkey anti- rabbit secondary antibody. Double immunostainings revealed no age-related changes in co-localization events (**Tab. 2** and **Tab. 3**, fluorescence data not shown).

Images A-D in Fig. 5 show a mature (8 DIV) and an aged (32 DIV) cell, in phase contrast and the respective staining for CBP80. Similar to previous figures, the phase contrast images (A and C) nicely visualize the morphological differences of vital mature and vital aged cells. The immunostainings for CBP80 (B and D) give examples for the distribution of this RBP in mature and aged neurons. Notice the difference in signal intensity in the nucleus. This effect was found consistently in the cells imaged. Taking age-related morphological changes into account, the distribution in dendrites was found to be stable in most experiments recorded. Images in panel E provide detailed insight into the localization in the nucleus. The four time points displayed each show an immunostaining of the cell body (upper) and an image with the focal point set to the interior of the nucleus (lower). The upper images clearly show a strong increase in cytoplasmic and nuclear localization from 8 DIV to 14 DIV. The strong and diffuse signal makes a more detailed analysis difficult. The lower images were exposed more shortly during imaging, hence the lower signal intensity. Here a strong increase to the nuclear membrane becomes apparent. Intriguingly this effect is distinct from the previous examples given for hnRNPQ, HuR and RBM14, as it primarily affects the nuclear membrane. The images further suggest a relocalization to the interior of the nucleus as well. However this conclusion cannot clearly be drawn from the existing data, as the stronger signal may be a remnant of the localization to other parts of the nuclear membrane. In this case confocal microscopy could provide better insights. It appears that increased localization to the membrane of the nucleus occurs primarily in the early aging process, at the time of synaptic establishment. A further slight increase could be argued from the data in advanced stages of aging.

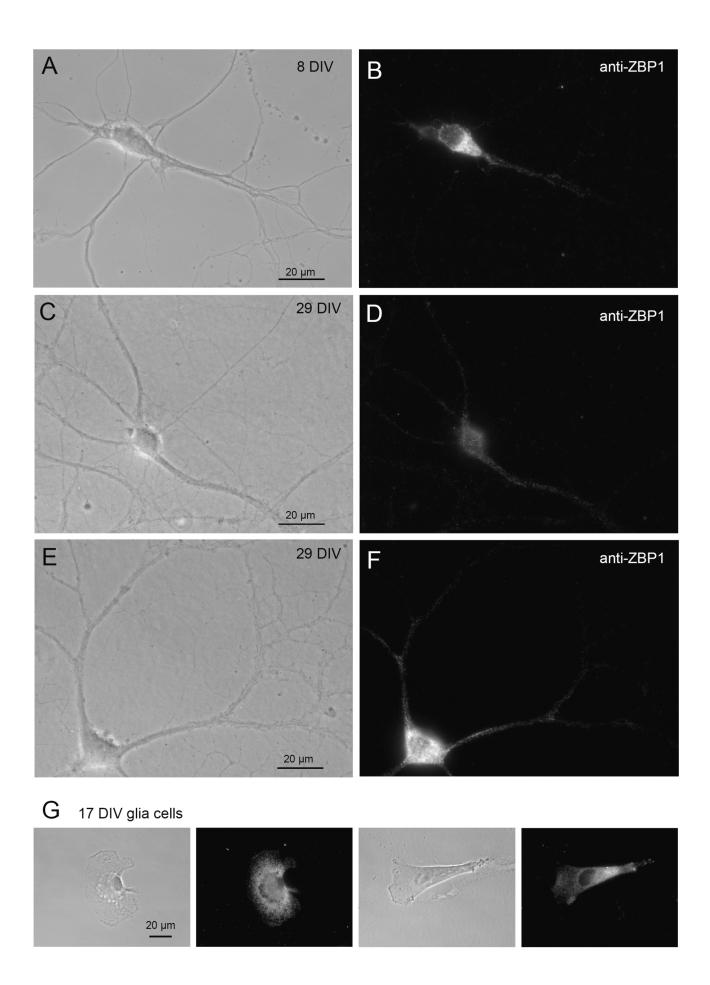


Fig. 6: Localization of ZBP1 in neurons of different age. (A-F) The images show a 8 DIV and two 29 DIV neurons in comparison, as well as phase contrast (A, C, E) and ZBP1 staining, displaying both high and low levels of expression (B, D, F). (G) Further images give insight into the pattern in 17 DIV glia cells.

The images provided in **Fig. 6** present preliminary data collected for the Zipcode-binding protein 1 (ZBP1), also referred to as IGF2 mRNA-binding protein 1. ZBP1 is one of the major RBPs implicated in RNA localization in neurons with a special emphasis on coupling transport to translational control (Hüttelmaier et al., 2005)

The experiment set out to identify the localization of ZBP1 within neurons. Cells were labeled with the mouse anti-ZBP1 primary antibody and the Alexa Fluor 488-coupled donkey anti-mouse secondary antibody. Additionally, double immunostainings were performed as previously described but showed no age associate changes (**Tab. 2** and **Tab. 3**, fluorescence data not shown).

Fig. 6 provides three very different examples of the ZBP1 distribution in neurons. Images A and B show a mature 8 DIV neuron, while images C-F show two 29 DIV neurons with increased age. Phase contrast and immunostaining is provided, respectively, for each cell. The images were selected to visualize the great diversity of the ZBP1 pattern that was found not only in different cells, but also at different ages. The mature cell in image B shows a pattern largely restricted to the soma and proximal parts of the dendrite. Comparing the two 29 DIV cells the heterogeneity of the pattern even at identical age becomes apparent. Image D shows a very weak pattern while image F displays a stronger and broader distribution of the protein. The stainings for ZBP1 imaged at different ages show great variability. In any case, the pattern was found to be weaker with fewer particles than the pattern of other markers used in this study. Often the distribution was restricted to the cell body and in some cases proximal parts of dendrites. Other cases showed a broader distribution to proximal sites. Only on rare occasions were particles identified at terminal sections of dendrites. The patterns remain inconclusive throughout aging and all experiments performed. Differences were also identified between cells of same experiments, treated alike during cell culturing and immunostaining. This variability not only affects neurons, but could be identified in glia cells as well, as seen in panel G. No correlation to aging or the number of synapses and RNPs could yet be established. Therefore, the heterogeneity in the distribution found in ZBP1 remains unresolved and cannot be implicated in the aging process given the present data.

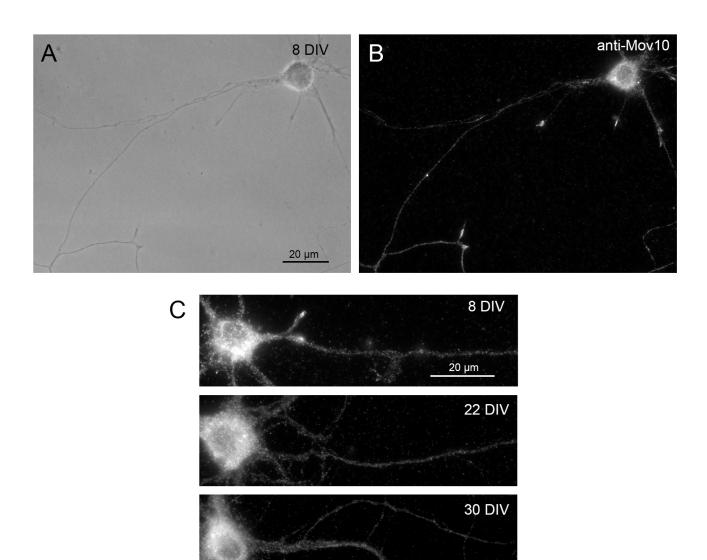


Fig. 7: The localization of Mov10 in neurons displays a very stable and comparable pattern at different stages of aging. (A-B) The images show a 8 DIV cell in phase contrast (A) and Mov10 staining (B). (C) Further examples are given in the panel, showing a comparable localization in the nucleus, soma and dendrites in different stages of aging. The images are presented as a control for a stable protein distribution in comparison to **Fig. 1-6**.

The images in **Fig. 7** present the data collected for the Moloney leukemia virus 10 (Mov10) protein. It is an RNA-dependent helicase that has been previously implicated in local translational control at the synapse (Banerjee et al., 2009) and has been found as part of Stau2-containing RNPs in rat brain (Härtel, Karra et al., submitted). The experiments were conducted to investigate the localization of Mov10 in differentially aged hippocampal neurons. Cells were labeled with the rabbit anti-Mov10 primary antibody and the Alexa Fluor 488-coupled donkey anti-rabbit secondary antibody. Additionally, double-immunostainings were performed with Stau2 and the synaptic marker Synapsinl.

Fig. 7 is presented here as a control-figure, providing an example of a stable localization pattern in comparison to the previously discussed RBPs. Panels A and B in Fig. 7 show a mature 8 DIV cell of good viability, in phase contrast and the respective staining for Mov10. Image B demonstrates the broad and stable distribution of this RBP in both proximal and distal sections of dendrites. A closer look into the localization of Mov10 is provided in the images of panel C. These images are representative examples of cells at three different time points of aging. The distribution of Mov10 is comparable at all three stages, displaying a similar pattern in nucleus, soma, and dendrites. This is especially apparent when comparing the images to the respective time points of other markers in Fig. 1-6. The quantifications in Table 2 and Table 3 additionally account for the stability of Mov10 during the aging process. Furthermore, it suggests there is no age-dependent change in the localization to synaptic sites or neuronal RNP granules. This makes Mov10 a perfect example of an RBP which remains stable during aging, in respect to its cellular distribution and localization. However, the data does not indicate that Mov10 remains untouched by cellular mechanisms of aging, but provides an ideal control for the localization of the previously discussed RBPs.

The experiments conducted in the course of this project yielded a large amount of fluorescence data for each RBP analyzed. The localization of each RBP was investigated at the different time points addressed; in 8 DIV, 14 DIV, 22 DIV and 28-32 DIV neurons. To draw quantitative conclusions from this data set, images were processed, and the particles labeled via immunofluorescence were manually counted as described in section 3.2.3 of Materials and Methods. Quantification is particularly vital for the co-localization studies performed for synaptic markers and Stau2. The data accumulated during quantification is summarized in **Table 2** in respect to synaptic markers and in **Table 3** in respect to Stau2. The numbers given represent the average data for a small 20 µm segment of a dendrite. Therefore, the data does not represent absolute values, but provides significant insight into the relative amount of the particles and co-localization events.

One of the core questions in this project was to assess the localization of RBPs to synapses. The mouse anti-SynapsinI and the guinea pig anti-Homer1 antibodies were used to mark synaptic sites in the neuronal cells. Double-immunostainings with each of the selected RBPs were used to investigate possible co-localization events. **Table 2** shows the data for the quantification of each RBP at respective time points and localization to synaptic markers. The number of co-localizing particles as well as the percentage of RBPs at synapses and the percentage of synapses with RBPs are provided. Section A lists data showing significant age-dependent changes. For the deadbox helicase RCK, please note how the number of particles remains stable with a small window, but the co-localization events increase with increasing age of the neuronal cultures. Section B shows data for RBPs where no significant age-dependent changes could be determined from the quantification. The final section C gives an overview of inconclusive data. Here, further experiments are required to allow any conclusions.

The preferential expression of Stau2 in the nervous system makes this RBP particularly interesting as a marker for neuronal RNPs. Mouse and rabbit anti-Stau2 antibodies were applied in double-immunostainings to assess the localization of each respective RBP to neuronal Stau2-containing RNPs. **Table 3** shows preliminary data for the quantification of co-localization experiments with Stau2. Similarly to **Table 2**, the number of co-localizing particles as well as the percentage of RBPs co-localizing with Stau2 and the percentage of Stau2-RNPs co-localizing with RBPs are given. In accordance with **Table 2**, **Table 3** is divided into the same sections: section A listing data with no significant changes and section B listing inconclusive data. The results shown are considered to be preliminary. Further experiments are required to allow significant statements.

Table 2.1 Significant

	Av. # of	Co-staining with synaptic marker			
	particles/ 20	Av. # of	Av. # of co-	% RBPs at	% synapses
	μm dendrite	synapses	localizations	synapses	with RBPs
RCK 8 DIV	31.1 ± 5	-	-	-	-
RCK 14 DIV	28.7 ± 5.5	$\textbf{22.7} \pm \textbf{6.4}$	5.6 ± 2.0	19.9 ± 7.7	$\textbf{26.4} \pm \textbf{12.1}$
RCK 22 DIV	27.7 ± 5.9	17.5 ± 5.2	7.2 ± 1.8	26.8 ± 10.3	42.2 ± 8.0
RCK 28/30 DIV	29.7 ± 5.2	$\textbf{22.4} \pm \textbf{6.1}$	11. 4 ± 2.7	38.2 ± 9.4	53.4 ± 13.8

Table 2.2 No change/ not significant

Ago2 8 DIV	30.9 ± 5.1	-	-	-	-
Ago2 22 DIV	29.6 ± 6.4	27.7 ± 7.9	5.7 ± 2.4	19.9 ± 7.4	21.2 ± 7.2
Ago2 29/30 DIV	27.5 ± 7.4	27.2 ± 6.4	8 ± 2.8	31.2 ± 12.7	29.9 ± 11.1
Btz 14 DIV	29.8 ± 5.8	20.9 ± 6.8	7.0 ± 2.6	24.8 ± 9.8	36.2 ± 16.5
Btz 22 DIV	30.3 ± 5.3	24.2 ± 9.3	9.1 ± 2.9	32.4 ± 10.4	39.8 ± 11.3
Btz 32/33 DIV	30.8 ± 5.5	$\textbf{21.2} \pm \textbf{5.6}$	6.1 ± 1.9	20.4 ± 7.2	29.7 ± 9.0
CBP20 8 DIV	27.6 ± 3.7	-	-	-	-
CBP20 22 DIV	25.1 ± 4.5	19.6 ± 4.5	8.9 ± 2.7	36.3 ± 10.3	$\textbf{46.1} \pm \textbf{10.2}$
CBP20 30/32 DIV	25.6 ± 4.2	16.9 ± 3.5	8.3 ± 2.8	$\textbf{36.3} \pm \textbf{11.4}$	48.8 ± 13.5
CBP80 14 DIV	$\textbf{31.5} \pm \textbf{8.1}$	$\textbf{18.1} \pm \textbf{4.9}$	5.5 ± 2.0	$\textbf{17.8} \pm \textbf{6.2}$	$\textbf{31.4} \pm \textbf{12.4}$
CBP80 22 DIV	27.4 ± 5.7	$\textbf{18.0} \pm \textbf{6.1}$	6.1 ± 2.3	24.0 ± 6.9	35.5 ± 11.1
CBP80 30/32 DIV	30.0 ± 4.8	$\textbf{21.6} \pm \textbf{5.9}$	5.4 ± 0.5	18.7 ± 4.3	$\textbf{26.9} \pm \textbf{7.6}$
elF4AIII 14 DIV	$\textbf{28.7} \pm \textbf{3.8}$	$\textbf{18.5} \pm \textbf{3.1}$	6.6 ± 2.9	$\textbf{23.5} \pm \textbf{10.7}$	$\textbf{35.1} \pm \textbf{12.8}$
eIF4AIII 22 DIV	25.9 ± 5.8	$\textbf{20.1} \pm \textbf{4.1}$	4.6 ± 2.3	19.2 ± 11.4	$\textbf{22.9} \pm \textbf{9.1}$
eiF4AIII 32/33 DIV	30.0 ± 6.2	19.5 ± 4.9	7.0 ± 3.7	24.6 ± 9.7	$\textbf{35.4} \pm \textbf{12.6}$
eIF4E 14 DIV	31.0 ± 5.3	$\textbf{26.4} \pm \textbf{7.4}$	$\textbf{7.2} \pm \textbf{2.4}$	$\textbf{23.0} \pm \textbf{7.1}$	$\textbf{29.0} \pm \textbf{11.0}$
eIF4E 22 DIV	$\textbf{27.8} \pm \textbf{3.7}$	$\textbf{23.8} \pm \textbf{7.7}$	6.1 ± 2.2	22.0 ± 6.8	26.1 ± 5.5
eIF4E 29 DIV	28.6 ± 6.9	$\textbf{18.9} \pm \textbf{7.1}$	5.8 ± 1.6	$\textbf{20.8} \pm \textbf{6.2}$	$\textbf{34.1} \pm \textbf{14.1}$
hnRNPQ 14 DIV	26.5 ± 3.1	$\textbf{28.1} \pm \textbf{6.3}$	$\textbf{7.2} \pm \textbf{1.7}$	27.5 ± 6.2	$\textbf{26.6} \pm \textbf{7.1}$
hnRNPQ 22 DIV	27.4 ± 5.3	24.1 ± 8.4	$\textbf{4.7} \pm \textbf{2.1}$	19.8 ± 7.9	20.6 ± 8.7
hnRNPQ 29/31	26.3 ± 4.9	$\textbf{21.9} \pm \textbf{4.5}$	5.1 ± 1.9	$\textbf{20.5} \pm \textbf{8.2}$	$\textbf{24.7} \pm \textbf{11.2}$
HuR 8 DIV	$\textbf{28.6} \pm \textbf{5.6}$	$\textbf{34.8} \pm \textbf{5.1}$	5.8 ± 2.6	19.8 ± 8.3	$\textbf{16.7} \pm \textbf{7.4}$
HuR 14 DIV	29.8 ± 8.4	$\textbf{32.6} \pm \textbf{8.3}$	5.9 ± 1.7	20.0 ± 3.8	19.5 ± 8.2
HuR 22 DIV	29.2 ± 7.3	$\textbf{27.9} \pm \textbf{4.1}$	6.2 ± 2.3	19.8 ± 7.9	22.0 ± 6.5
HuR 29 DIV	26.8 ± 4.8	19.5 ± 6.6	4.8 ± 1.6	19.4 ± 6.1	$\textbf{28.0} \pm \textbf{12.8}$
Mov10 8 DIV	28.3 ± 3.4	-	-	-	-
Mov10 22 DIV	27.9 ± 4.3	18.8 ± 5.6	5.6 ± 2.6	20.5 ± 8.7	30.4 ± 14.5
Mov10 28/29/30	28.4 ± 5.3	$\textbf{22.6} \pm \textbf{4.9}$	$\textbf{7.1} \pm \textbf{2.5}$	25.5 ± 6.0	$\textbf{31.8} \pm \textbf{11.4}$
-	•		•	•	•

	I				
PABP1 8 DIV	31.0 ± 4.6	-	-	-	-
PABP1 22 DIV	28.3 ± 5.5	20.4 ± 6.5	3.3 ± 1.9	11.8 ± 7.4	$\textbf{18.2} \pm \textbf{14.1}$
PABP1 28/30 DIV	24.4 ± 3.7	20.0 ± 5.6	$\textbf{5.2} \pm \textbf{2.6}$	$\textbf{23.5} \pm \textbf{16.5}$	$\textbf{27.6} \pm \textbf{15.1}$
Pum2 14 DIV	24.9 ± 3.9	18.6 ± 3.9	6.7 ± 2.3	$\textbf{27.8} \pm \textbf{10.8}$	37.3 ± 14.0
Pum2 22 DIV	28.8 ± 4.3	-	-	-	-
Pum2 28/29/30	$\textbf{28.1} \pm \textbf{5.7}$	19.6 ± 6.2	$\textbf{7.5} \pm \textbf{3.1}$	$\textbf{27.3} \pm \textbf{13.6}$	$\textbf{38.6} \pm \textbf{12.4}$
Pur-α 8 DIV	31.3 ± 6.0	-	-	-	-
Pur-α 22 DIV	31.3 ± 3.7	24.4 ± 3.8	8.3 ± 2.8	$\textbf{27.1} \pm \textbf{9.0}$	$\textbf{34.3} \pm \textbf{12.0}$
Pur-α 29/ 31 DIV	31.3 ± 4.3	$\textbf{23.5} \pm \textbf{7.4}$	$\textbf{9.8} \pm \textbf{2.6}$	30.7 ± 6.8	$\textbf{42.7} \pm \textbf{6.1}$
RBM14 8 DIV	$\textbf{31.2} \pm \textbf{3.4}$	-	-	-	-
RBM14 22 DIV	29.4 ± 5.8	19.9 ± 6.0	4.0 ± 1.8	$\textbf{14.7} \pm \textbf{6.1}$	20.9 ± 8.4
RBM14 29/32 DIV	30.3 ± 5.4	$\textbf{20.6} \pm \textbf{6.0}$	6.7 ± 2.6	$\textbf{23.9} \pm \textbf{9.6}$	$\textbf{35.4} \pm \textbf{15.5}$
Stau2 14 DIV	$\textbf{30.1} \pm \textbf{4.11}$	15.8 ± 4.3	6.2 ± 2.9	20.7 ± 9.5	$\textbf{39.4} \pm \textbf{17.4}$
Stau2 22 DIV	$\textbf{27.4} \pm \textbf{5.0}$	20.5 ± 9.4	8.5 ± 3.0	30.9 ± 8.8	44.1 ± 9.8
Stau2 32/33 DIV	$\textbf{27.3} \pm \textbf{4.2}$	19.7 ± 4.5	7.5 ± 3.3	$\textbf{28.0} \pm \textbf{13.3}$	$\textbf{37.2} \pm \textbf{12.4}$
Upf1 8 DIV	30.7 ± 5.9	-	-	-	-
Upf1 22 DIV	29.0 ± 5.9	15.5 ± 3.9	$\textbf{8.4} \pm \textbf{2.4}$	30.4 ± 9.9	$\textbf{54.8} \pm \textbf{13.2}$
Upf1 28/29 DIV	$\textbf{28.7} \pm \textbf{6.4}$	16.3 ± 5.8	8.7 ± 3.2	$\textbf{32.0} \pm \textbf{15.8}$	54.3 ± 14.2
Y14 8 DIV	34.2 ± 5.0	-	-	-	-
Y14 22 DIV	30.1 ± 3.7	23.9 ± 5.0	9.7 ± 2.7	$\textbf{31.8} \pm \textbf{9.5}$	40.5 ± 4.4
Y14 29/33 DIV	31.5 ± 4.9	22 ± 6.7	5.7 ± 3.0	18.3 ± 8.2	25.4 ± 8.0

Table 2.3 Inconclusive

FMRP 22 DIV	31.2 ± 6.5	-	-	-	-
FMRP 31 DIV	$\textbf{30.1} \pm \textbf{5.3}$	-	-	-	-
Magoh 8 DIV	$\textbf{29.3} \pm \textbf{2.7}$	-	-	-	-
Magoh 22 DIV	$\textbf{28.4} \pm \textbf{2.8}$	26.6 ± 6.9	$\textbf{7.6} \pm \textbf{2.7}$	$\textbf{26.8} \pm \textbf{10.1}$	28.5 ±6.6
Magoh 29/33 DIV	$\textbf{28.4} \pm \textbf{5.0}$	$\textbf{26.2} \pm \textbf{2.7}$	9.5 ± 2.4	$\textbf{38.2} \pm \textbf{11.2}$	36.4 ± 9.9
YB1 8 DIV	27.5 ± 5.8	-	-	-	-
YB1 14 DIV	$\textbf{28.9} \pm \textbf{5.8}$	25 ± 7.5	$\textbf{7.7} \pm \textbf{2.1}$	$\textbf{27.4} \pm \textbf{7.7}$	$\textbf{33.4} \pm \textbf{14.6}$
YB1 22 DIV	$\textbf{27.5} \pm \textbf{4.7}$	$\textbf{17.5} \pm \textbf{0.7}$	6 ± 1.4	$\textbf{24.8} \pm \textbf{9.9}$	34.1 ± 6.7
YB1 28/30 DIV	26.6 ± 4.6	$\textbf{16.2} \pm \textbf{3.5}$	8.1 ± 2.3	$\textbf{34.0} \pm \textbf{10.0}$	$\textbf{52.0} \pm \textbf{16.1}$
ZBP1 8 DIV	$\textbf{17.0} \pm \textbf{6.0}$	$\textbf{19.9} \pm \textbf{5.4}$	4.6 ± 2.1	30.43 ± 12.9	$\textbf{23.9} \pm \textbf{10.9}$
ZBP1 14 DIV	$\textbf{25.6} \pm \textbf{3.2}$	$\textbf{30.4} \pm \textbf{5.8}$	$\textbf{10.2} \pm \textbf{2.3}$	$\textbf{40.4} \pm \textbf{10.1}$	34.2 ± 8.2
ZBP1 22 DIV	$\textbf{23.3} \pm \textbf{6.4}$	20.3 ± 5.8	4.1 ± 2.1	$\textbf{27.0} \pm \textbf{13.6}$	20.1 ± 8.2
ZBP1 29/30 DIV	$\textbf{19.4} \pm \textbf{4.1}$	$\textbf{15.7} \pm \textbf{3.6}$	$\textbf{6.2} \pm \textbf{1.8}$	33.5 ± 15.9	39.4 ± 13.0

Tab. 2: Quantification of co-localization experiments between the indicated RBP and the respective synaptic markers. The data represents the average number of particles present in a 20 μm segment of the dendrites analyzed. Section (A) lists data showing significant differences at the given stages of aging. Section (B) lists data showing no significant changes, and section (C) lists inconclusive or insufficient data.

Table 3.1 No change/ not significant

	Av. # of	. # of Co-staining with Stau2				
	particles/ 20	Av. # of	Av. # of co-	% RBPs with	% Stau2 with	
	μm dendrite	Stau2	localizations	Stau2	RBPs particles	
Ago2 8 DIV	30.9 ± 5.1	28 ± 5.4	4.8 ± 1.5	16 ± 3.3	17.5 ± 5.1	
Ago2 22 DIV	29.6 ± 6.4	$\textbf{29.1} \pm \textbf{5.4}$	9.5 ± 2.1	32.5 ± 6.2	$\textbf{32.9} \pm \textbf{6.3}$	
Ago2 29/30 DIV	27.5 ± 7.4	27.3 ± 8.3	6.3 ± 2.4	23.1 ± 8.1	23.6 ± 7.5	
CBP20 8 DIV	27.6 ± 3.7	24.8 ± 4.2	7.0 ± 2.1	25.0 ± 8.1	28.4 ± 7.4	
CBP20 22 DIV	$\textbf{25.1} \pm \textbf{4.5}$	25.5 ± 7.5	5.3 ± 2.0	$\textbf{22.0} \pm \textbf{11.2}$	21.4 ± 8.5	
CBP20 30/32 DIV	25.6 ± 4.2	29.3 ± 3.8	8.6 ± 2.3	31.7 ± 8.7	29.5 ± 7.8	
CBP80 8 DIV	28.3 ± 5.5	27.0 ± 5.6	$\textbf{7.4} \pm \textbf{1.7}$	26.5 ± 5.8	28.0 ± 6.4	
CBP80 22 DIV	27.4 ± 5.7	$\textbf{25.4} \pm \textbf{6.4}$	6.5 ± 3.0	$\textbf{22.3} \pm \textbf{10.5}$	$\textbf{26.2} \pm \textbf{12.1}$	
CBP80 30/32 DIV	30.0 ± 4.8	29.4 ± 4.6	9.2 ± 2.5	31.4 ± 9.2	31.7 ± 8.3	
hnRNPQ 8 DIV	22.8 ± 5.3	25.0 ± 3.3	9.5 ±2.6	42.2 ± 8.9	38.3 ± 10.0	
hnRNPQ 22 DIV	27.4 ± 5.3	$\textbf{23.3} \pm \textbf{4.6}$	7.6 ± 1.6	$\textbf{30.8} \pm \textbf{7.1}$	$\textbf{33.5} \pm \textbf{8.4}$	
hnRNPQ 29/30/31	26.3 ± 4.9	25.8 ± 3.8	6.4 ± 1.6	25.5 ± 5.0	25.0 ± 6.7	
HuR 8 DIV	28.6 ± 5.6	$\textbf{25.9} \pm \textbf{5.1}$	7.5 ± 1.4	30.9 ± 8.8	30.6 ± 10.2	
HuR 22 DIV	29.2 ± 7.3	$\textbf{27.8} \pm \textbf{4.2}$	$\textbf{7.2} \pm \textbf{1.8}$	29.4 ± 5.5	26.4 ± 6.3	
HuR 29 DIV	26.8 ± 4.8	$\textbf{28.2} \pm \textbf{6.1}$	8.7 ± 2.1	31.9 ± 8.9	32.5 ± 12.1	
Mov10 8 DIV	28.3 ± 3.4	25.3 ± 8.0	8.6 ± 4.1	30.7 ± 16.0	33.1 ± 10.2	
Mov10 22 DIV	27.9 ± 4.3	26.3 ± 4.5	10.2 ± 3.0	35.0 ± 10.0	39.1 ± 10.3	
Mov10 28/29/30	28.4 ± 5.3	$\textbf{25.1} \pm \textbf{4.4}$	11.2 ± 3.2	39.1 ± 10.3	44.4 ± 8.7	
Pum2 8 DIV	29.7 ± 4.6	20.8 ± 4.3	9.2 ± 3.2	32.5 ± 11.6	44.7 ± 14.4	
Pum2 22 DIV	28.8 ± 4.3	19.3 ± 4.3	8.2 ± 2.0	28.9 ± 6.0	43.7 ± 11.3	
Pum2 28/29/30	28.1 ± 5.7	21.6 ± 4.4	9.3 ± 3.0	33.6 ± 9.7	43.0 ± 9.7	
Pur-α 8 DIV	31.3 ± 6.0	30.2 ± 3.6	10.5 ± 3.5	32.6 ± 10.2	34.9 ± 10.9	
Pur- $lpha$ 22 DIV	31.3 ± 3.7	24.1 ± 2.6	10.2 ± 2.8	33.1 ± 10.4	41.7 ± 9.0	
Pur-α 29/31 DIV	31.3 ± 4.3	26.9 ± 7.4	10.2 ± 3.8	33.6 ± 11.2	37.6 ± 9.5	
RCK 8 DIV	31.1 ± 5	23.9 ± 4.4	5.7 ± 2.1	18.7 ± 6.5	23.3 ± 5.7	
RCK 22 DIV	27.7 ± 5.9	$\textbf{20.3} \pm \textbf{5.9}$	4.1 ± 1.1	15.3 ± 3.2	$\textbf{21.2} \pm \textbf{6.5}$	
RCK 28/30 DIV	29.7 ± 5.2	$\textbf{21.6} \pm \textbf{6.1}$	4.4 ± 1.7	15.8 ± 7.0	$\textbf{21.0} \pm \textbf{7.5}$	

RBM14 8 DIV	31.2 ± 3.4	23.4 ± 5.0	7.8 ± 2.6	26.8 ± 8.2	33.0 ± 8.5
RBM14 22 DIV	29.4 ± 5.8	$\textbf{27.2} \pm \textbf{6.3}$	10.1 ± 3.5	30.6 ± 11.4	$\textbf{37.4} \pm \textbf{10.1}$
RBM14 32/33 DIV	27.3 ± 4.2	19.7 ± 4.5	7.5 ± 3.3	28.0 ± 13.3	$\textbf{37.2} \pm \textbf{12.4}$
Upf1 8 DIV	30.7 ± 5.9	16.8 ± 4.1	5.2 ± 1.6	18.9 ± 5.8	31.3 ± 9.4
Upf1 22 DIV	29.0 ± 5.9	20.4 ± 5.8	5.5 ± 2.7	18.4 ± 8.3	26.4 ± 9.3
Upf1 30 DIV	33.8 ± 5.6	$\textbf{21.4} \pm \textbf{4.8}$	6.5 ± 2.7	19.1 ± 7.3	$\textbf{30.1} \pm \textbf{10.1}$
YB1 8 DIV	27.5 ± 5.8	$\textbf{28.4} \pm \textbf{8.2}$	12.2 ± 3.9	46.0 ± 11.8	43.3 ± 8.8
YB1 22 DIV	27.5 ± 4.7	19.5 ± 5.1	6.5 ± 2.3	23.2 ± 8.3	$\textbf{32.9} \pm \textbf{7.1}$
YB1 28/30 DIV	26.6 ± 4.6	$\textbf{21.6} \pm \textbf{6.4}$	7.6 ± 1.9	$\textbf{25.8} \pm \textbf{7.6}$	36.1 ± 8.0

Table 3.2 Inconclusive

	1				
PABP1 8 DIV	$\textbf{31.0} \pm \textbf{4.6}$	$\textbf{27.7} \pm \textbf{8.0}$	10.1 ± 3.8	33.6 ± 10.3	36.1 ± 8.8
PABP1 22 DIV	28.3 ± 5.5	24.0 ± 4.8	$\textbf{5.2} \pm \textbf{2.9}$	18.3 ± 10.6	$\textbf{21.2} \pm \textbf{11.7}$
PABP1 28/30 DIV	24.4 ± 3.7	25.3 ± 4.7	9.7 ± 2.6	39.7 ± 9.9	38.4 ± 9.3
Y14 8 DIV	34.2 ± 5.0	29.3 ± 4.9	$\textbf{8.4} \pm \textbf{2.9}$	24.8 ± 7.1	28.7 ± 8.9
Y14 22 DIV	30.1 ± 3.7	20.3 ± 8.0	7.3 ± 4.9	25.9 ± 14.2	34.5 ± 11.3
Y14 29, 33 DIV	31.5 ± 4.9	27.1 ± 4.9	9.1 ± 3.0	27.2 ± 5.9	34.5 ± 12.5
ZBP1 8 DIV	17.0 ± 6.0	22.0 ± 4.8	6.4 ± 4.4	33.3 ± 12.8	27.8 ± 14.9
ZBP1 22 DIV	12.7 ± 4.4	20.5 ± 9.7	4.0 ± 1.7	33.3 ± 12.2	23.9 ± 160
ZBP1 29/30 DIV	19.4 ± 4.1	23.0 ± 3.2	6.6 ± 2.0	34.4 ± 9.8	29.3 ± 10.9

Tab. 3: Quantification of co-localization experiments between the indicated RBP and the Stau2 marker for neuronal RNPs. The data represents the average number of particles present in a 20 μ m segment of the dendrites analyzed. Section (A) lists data showing no significant changes, and section (B) lists inconclusive or insufficient data.

5. DISCUSSION

5.1 Changes in RCK-distribution indicate reduction in size or disassembly of P-bodies and increased localization to dendritic spines during aging.

The RNA helicase RCK is frequently used as a marker for processing bodies (P-bodies) in immunofluorescence labeling. P-bodies have been found to function as sites of RNA degradation (Cougot et al., 2004), storage, and translational control (Liu et al., 2005). In mammalian neurons, P-bodies have been detected both in the cell body and in dendrites (Vessey et al., 2006). Here, they have been implied in the translational control of dendritically localized RNAs. The current hypothesis states that dendritic RNAs are stored in P-bodies and can be released and translated upon synaptic stimulation (Zeitelhofer et al., 2008). Considering what is known for the function of P-bodies, the collected data allows speculations for the age-dependent effects found and reported in this thesis. Particularly, the role of translational repression in combination with synaptic localization may have a critical effect in neuronal aging.

A previous study investigated the question whether transport RNPs and P-bodies are of similar composition or distinct from one another (Zeitelhofer et al., 2008; Cougot et al., 2008). The results in this study showed that both transport RNPs and P-bodies are separate, however docking events were frequently observed. The preliminary data for RCK shown in **Table 3** results from experiments using RCK as a marker for P-bodies and Stau2 as a marker for transport RNPs. Note that while some co-localization events were observed, their occurrences are too few to assume a significant association from the data. The results support the previous findings and furthermore indicate no age-dependent changes in P-body and RNP association. However, further experiments, along with live cell imaging will be necessary to address this question in depth.

In this study, age-dependent changes in P-body localization were found both in the cell body and dendrites of neurons (**Fig.1**, **Tab. 2**). The disassembly or the reduction in size of P-body particles identified in the soma (**Fig.1 K**) may affect both local and dendritic localization and translational control. Previous work in the lab revealed an increase in P-body size, dependent on synaptic activity (Zeitelhofer et al., 2008; Georg Ammer, 2011, Master's thesis). This may be congruent with my findings, assuming an age-related change in signaling activity.

The finding of an increased association to synapses in aged cells (**Fig. 1 E**) raises the question how this may affect individual synapses or dendritic spines. This change may result in increased RNA-silencing or RNA-degradation at the synapses of aged cells. The

consequence of increased P-body presence in proximity to synapses cannot be predicted from the existing data and may vary dependent on the associated RNAs or other particle components. To further study these effects, additional experiments will be necessary to investigate other P-body markers such as DCP1, DCP2 or Ago2. The data collected for the Argonaute 2 protein (Ago2) in this study (**Tab. 2**) shows a slight but insignificant age-associated increase to synapses. Therefore, no claims concerning Ago2 can be made here. Cougot et al. showed in 2004 that synaptic activation relocalizes P-bodies to distal parts of dendrites. A recent publication reports increased localization of P-bodies to the postsynaptic density protein 95 (PSD-95) induced by neuronal activation and suggests a role in translational control and synaptic structural plasticity (Oh et al., 2013). Further experiments could address this effect in relation to synapse localization and aging.

Independent of the outcome of future studies, the data collected here suggests that an age-dependent change in this important RNP, as shown in this thesis, can be of vital significance to an aging organism. Alterations in translational repression could strongly influence individual synapses and therefore synaptic plasticity, thereby affecting tissue structure. Such perturbations in translation and the resulting effect on respective tissue could significantly contribute to the natural aging process and furthermore advocate age-associated neuronal diseases.

5.2 Changes in Nuclear-cytoplasmic shuttling effects are linked to aging

Nuclear-cytoplasmic shuttling has been implied in numerous important cellular functions. Cellular stress and mutations in the involved proteins have proven to alter or inhibit shuttling, thereby contributing to a number of known diseases (Kodiha et al., 2005; Federici and Fallini, 2013; Tong et al., 2012). Furthermore, changes in shuttling have been implied in aging (Kovalenko et al., 2010; Eijkelenboom and Burgering, 2013).

To cope with the damage generated by ROS during aging, cell have developed multiple responses. All of these responses involve the activation of specific signaling pathways in the nucleus. Therefore, stress response mechanisms need to be coordinated between the nucleus and the cytoplasm, via the shuttling of macromolecules across the nuclear membrane (Otis et al., 2006; Kodiha and Stochaj, 2012). In neurons, various signaling pathways in the nucleus regulate gene expression associated with long-term structural changes of synapses (Jordan and Kreutz, 2009). The transcriptional regulation of such genes is essential and strongly depends on the correct communication between synapses and the nucleus. Signaling via Ca²⁺ is known to activate transcription in neurons (Greer and Greenberg, 2008), however, the relocalization of cytoplasmic proteins to the

nucleus may also be required to activate specific expression patterns. This communication may occur via the retrograde transport of proteins from a synapse to the nucleus, as suggested by multiple studies (e.g. Deisseroth et al., 2003 and West et al., 2002). Some proteins enriched at synaptic sites are also found in the nucleus and have been shown to relocalize to the nucleus upon synaptic stimulation, suggesting a role in shuttling (e.g. Ch'ng and Martin, 2011; Jordan et al., 2007; Lai et al., 2008). The localization of proteins with nuclear localization signals (NLS) or nuclear export signals signal (NES) to synapses, as found in this study supports this model. Such sequences are carried by the RBPs hnRNPQ, HuR, and CBP80, which are found in my work to potentially undergo shuttling (Fig. 2, 3, and 5). No shuttling sequence is known for RBM14; however, the fluorescent data suggests a similar effect (Fig. 4). The data presented here clearly shows that the nuclear localization of these proteins is not stable, but subcellular changes occur in an age-dependent manner. The cause of these changes, i.e. relocalization from other cellular compartments or de novo synthesis prior to translocation remains unclear and is yet to be determined. Synaptic activity or remodeling could regulate the translation or degradation of proteins carrying a NLS or a NES. Alternatively, different splice variants could result in diverse versions of a protein with or without an NLS (Jordan and Kreutz, 2009). Perturbations in the stability of cytoplasmic anchoring proteins may be another reason why shuttling sequence-bearing factors accumulate in the nucleus. In any case, my data suggest a nuclear-cytoplasmic shuttling mechanism resulting in changed distribution patterns during aging.

In the context of aging, the shuttling of proteins to the nucleus, as presented in this study, could be due to a response to an age-related damage of proteins in other sections of the cell, possibly distal dendrites. Therefore, the reactivation of signaling pathways in the nucleus could contribute to replacing proteins and thus compensate for increasing damage. Also, age-related changes or malfunctions in phosphorylation, splicing or protein synthesis and degradation can have an influence on localization. The underlying mechanisms and the effects resulting from the altered shuttling and changes in localization of the proteins implied here are yet to be explored in sequential studies.

5.3 Increased localization of RBPs to distal parts of dendrites is associated with aging

The RBPs hnRNPQ, HuR and RBM14 are shown here to increase in distal parts of neurons during aging (**Fig. 2**, **3** and **4**). However, there seems to be significant variation in the intensity of the observed effect between different sets of neurons. The changes in dendritic localization of these proteins are accompanied by alterations in nuclear-cytoplasmic shuttling of the same proteins as described above.

The great fluctuations shown in the dendritic distribution of ZBP1 (**Fig. 6**) could not be linked to aging in this study. No stable effects were identified. The localization of this RBP may rely on synaptic activity or on other associated factors. Previous data suggest that ZBP1 is primarily expressed in young, but not old neurons in culture (Donnelly et al., 2011). Therefore, a role in aging can neither be implied nor excluded.

The increase in dendritic localization as shown for hnRNPQ, HuR and RBM14 is in many cases a small but stable effect, which may both be a cause of or a response to aging. This effect could result from *de novo* synthesis, the relocalization of proteins or a combination of both. For example, HuR has been shown to undergo a nuclear to cytoplasmic transition in response to stress. Thereby, HuR is proposed to enrich in the cytoplasm where it increases the stability of RNA transcripts and modulates their translation (Kim et al., 2008; Fan and Steitz, 1998). An increased dendritic localization of HuR, as seen in the aged cells of **Fig. 3** may reflect a response to stress induced aging.

My findings are striking considering dendrites harbor the majority of synaptic sites in neuronal cells. Such changes may have strong local influences on synapses either directly or via translational control and in turn affect synaptic plasticity. The outcome is hard to predict and require further investigation. However, an increase in dendritic protein concentration or the localization of proteins to distal sections can be assumed to strongly influence synaptic composition.

5.4 Conclusions

This thesis has investigated a number of important RBPs in neurons, in relation to their localization in mature and differentially aged cells. The majority of RBPs investigated exhibit stable distribution during the aging process in cell culture. However, age-related effects were detected as shown by the examples presented here, clearly highlighting the important role for RBPs not only in neurons but also in neuronal aging. Considering the diverse and essential functions of RBPs, relocalizations or malfunctions in neurons must elicit grave effects. The collected data allows speculations for effects in aging and advocates successional studies in this context. The functions of RBPs in neurons are essential, and merit further consideration in the aging process and age-related neuronal diseases.

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Zusammenfassung

Die Abnahme kognitiver Fähigkeiten geht mit dem Altern eines Organismus einher und ist abhängig von strukturellen und funktionellen Änderungen in neuronalen Netzwerken. Daher sind altersabhängige Veränderungen in der Erforschung von Lernen, Erinnerung und neurodegenerativen Erkrankungen von großer Bedeutung. Die Bildung und Änderung individueller Synapsen ist essenziell für ein funktionelles neuronales Netzwerk. Veränderungen in der Konnektivität von Zellen können einen großen Einfluss auf Prozesse wie Lernen und der Bildung einer Erinnerung haben. Das Altern auf zellulärer Ebene ist genetisch programmiert, aber auch abhängig von der Anhäufung zellschädigender Stoffe, die zur Fehlfunktion multipler zellulärer Prozesse führen. In Neuronen kann dies zu einer Änderung in der Bildung von Synapsen und deren Erhaltung führen. Die lokale Translation von RNA-Transkripten ist wesentlich für die Bildung und dann anschließend für die Morphogenese individueller Synapsen. Dieser Prozess ist von der korrekten Funktion verschiedener RNA-binde Proteine (RBPs) abhängig. Altersabhängige Veränderungen und die Schädigung verschiedener Zellkomponenten können zu einer fehlerhaften Funktion und subzellulären Lokalisation solcher RBPs führen. Dies kann sich auf die synaptische Plastizität auswirken und infolgedessen neurodegenerative Erkrankungen bewirken.

In diesem Projekt verwendete ich primär kultivierte hippocampale Neuronen der Ratte in Langzeitzellkulturen, um mögliche altersabhängige Veränderungen in der zellulären Verteilung einiger wichtiger RBPs zu untersuchen. Dazu machte ich Gebrauch von standardisierten Methoden der Immunozytochemie und Epifluoreszenzmikroskopie, um die zelluläre Verteilung ausgewählter Proteine in Zellen unterschiedlicher Altersstufen zu markieren und zu dokumentieren.

Meine Experimente zeigten eine altersabhängige Veränderung in der Verteilung von processing bodies (P-bodies). Große P-bodies im Soma, in der Nähe des Nukleus, scheinen während des Alterns der Zelle zu zerfallen oder an Größe zu verlieren. Weiters lokalisieren dendritische P-bodies im Alter verstärkt zu synaptischen Strukturen. Die Beteiligung dieser P-bodies an RNA-Abbau und Translationskontrolle ist essenziell für den zellulären Metabolismus. Daher können altersabhängige Veränderungen in deren Lokalisation signifikant in die lokale Translation synaptischer Proteine eingreifen.

Eine kleine Gruppe an RBPs zeigten Veränderungen in Transportmechanismen zwischen Nukleus und Zytoplasma (nuclear-cytoplasmic shuttling) und außerdem eine verstärkte Lokalisierung zu distalen Sektionen der Dendriten in alternden Neuronen. Veränderungen im nuclear-cytoplasmic shuttling können spezifische Signaltransduktionskaskaden im Nukleus beeinflussen und so in die Genexpression eingreifen. Die Zunahme in der dendritischen Lokalisation spezifischer RBPs kann eine

Antwort auf altersabhängige synaptische Veränderungen, oder aber eine Nebenerscheinung des Alterns sein. Zusammenfassend präsentiert diese Diplomarbeit altersabhängige Veränderungen in der Lokalisation essenzieller RBPs und demonstriert so die wichtige Rolle dieser Proteine, nicht nur in Neuronen, sondern auch in neuronalem Altern. Weiters bieten meine Daten eine Grundlage für weitere Forschung, um die Rolle von RBPs in zellulären Alterungsmechanismen anzusprechen.

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