TU3A is a novel stress-regulated protein involved in cognition and emotional behavior

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Claudia Liebl

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1. Gutachter: Prof. Dr. Rainer Landgraf

2. Gutachter: Prof. Dr. Gisela Grupe

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Der Zauber steckt immer im Detail.

Theodor Fontane (1819 - 1889)

Für Johannes

Und meine Schwester Petra

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1 Introduction

It is not the strongest of the species that survive,
nor the most intelligent,
but the one most responsive to change.

Charles Darwin (1809 - 1882)

1.1 Stress

Life represents a cumulative exposure to changing and challenging situations. Therefore stress is, in a manner of speaking, an omnipresent influence in the everyday life of all living organisms. One of the first researchers who investigated the biological effects of exposure to adverse external or internal stimuli was Hans Selye (Selye, 1936). Selye introduced the concept of the "general adaptation syndrome", describing a set of physiological responses to various noxious chemical or physical stimuli. He popularized the term "stress" as the state in which the response has been evoked. Selye was influenced by the work of Walter Cannon, who had established the concept of homeostasis. Cannon defined homeostasis as a complex dynamic equilibrium that is constantly challenged by internal or external adverse effects in order to maintain a stable, constant internal environment (Cannon, 1932). Thus, stress can be defined as a state in which homeostasis is actually threatened by stressors or is perceived to be so. In turn, a stressor is specified as a real or perceived demand on the body.

Stressors can be classified into distinct categories. On the one hand, reactive or systemic stressors present a real, physical threat to the homeostasis of the organism, like an immune challenge or pain. Anticipatory stressors, on the other hand, involve appraisal of the threat, include the context of the stimulus, and thereby comprise a certain psychological component, like novel, unpredictable and uncontrollable situations (Dedovic et al., 2009). Different classes of stressors vary in their intensity and are transmitted by different neural circuits (Pacak and Palkovits, 2001; Engelmann et al., 2004; Michaud et al., 2008). In 1988, Sterling and Eyer extended the concept of homeostasis and introduced the term allostasis as the ongoing adaptive efforts of the body to maintain stability (namely homeostasis) in response to stressors (Sterling and Eyer, 1988). While homeostasis describes a state with defined biological set points, allostasis implies achieving stability through change, including

the modulation of those set points in response to a changing environment. The stress response facilitates adaptation and is therefore beneficial for the organism. However, this dynamic adaptation to challenges takes a toll on the organism, which is defined as allostatic load. The latter represents the “wear and tear” the body experiences when allostatic systems are under constant pressure, fail to shut down, or do not respond adequately to the initial challenge (McEwen and Stellar, 1993).

1.2 The physiological response to stress is mediated by two stress systems

The allostatic response to stress involves a complex repertoire of behavioral and physiological adaptive reactions of the organism, which are mainly mediated by two very efficient and highly conserved systems: the autonomic nervous system, providing an immediate but short-lived response, and the hypothalamic-pituitary-adrenocortical (HPA) axis, mediating a relatively decelerated but more persistent reaction. Activation of the autonomic nervous system, in particular the sympatho-adrenomedullary system, mediates the classical “fight-or-flight” response via the release of norepinephrine and adrenaline into the blood (Cannon, 1953; Jansen et al., 1995). The HPA axis mainly contributes to the stress response by releasing glucocorticoids from the adrenal cortex. The autonomic nervous system and the HPA axis act synergistically in response to an acute stressful stimulus, resulting in manifold effects. Those include energy mobilization by glycogenolysis, increased awareness and arousal, improved cognitive function, accelerated motor reflexes as well as enhanced cardiovascular tone and respiratory rate. At the same time they also facilitate suppression of general vegetative functions such as digestion as well as immunity, growth and reproductive function (Ulrich-Lai and Herman, 2009). Thereby, those interlocked systems provide the necessary resources to cope with challenging situations.

1.3 The hypothalamic-pituitary-adrenocortical axis

The HPA axis plays a pivotal role in mediating the stress response of an organism as well as in the terminating the latter by efficient negative feedback. Extensive research over the last decades led to a detailed characterization of the specific HPA axis mechanisms and its main effectors, although many factors and potential relationships are still unknown.

The initial center of the HPA axis in the brain is the hypothalamic paraventricular nucleus (PVN), where a population of hypophysiotrophic neurons synthesizes corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) (Frank and Landgraf, 2008). CRH, a 41-amino acid (aa) neuropeptide, was first isolated in 1981 by Wylie Vale who was influenced by the work of Geoffrey Harris, proposing a controlling influence of the hypothalamus on the stress reaction and suggesting hypothalamic factors as the controlling instances (Harris, 1950; Vale et al., 1981). Via projections from the PVN to the external layer of the median eminence CRH is secreted into the hypophyseal portal circulation within seconds after the stressful stimulus.

In the anterior pituitary this release results in stimulation of adrenocorticotropic hormone (ACTH) from corticotroph cell populations (Engelmann et al., 2004). CRH exerts its action in the anterior pituitary by binding to CRH receptor type 1 (CRHR1) and thereby stimulates the expression of the ACTH precursor molecule proopiomelanocortin (POMC) and triggers the release of ACTH into the circulation. Additionally, AVP exerts a synergistic effect by interacting with V1b receptors, thereby potentiating the ACTH release (Rivier and Vale, 1983; Lamberts et al., 1984).

Circulating ACTH, in turn, stimulates the release of glucocorticoids into the circulation from the the zona fasciculata of the adrenal cortex. In humans, cortisol is the predominant glucocorticoid hormone, while in mice corticosterone is prevailing (Dawson, Jr. et al., 1984; Payne and Hales, 2004). Glucocorticoids are the main effectors of the HPA axis and execute their function by binding to two different receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (de Kloet et al., 1998). HPA axis activity itself is strictly regulated by its principal effector: glucocorticoids exert an efficient negative feedback via rapid MR mediated processes and genomic actions through both, the GR and the MR. At the level of the hypothalamus CRH and AVP expression is inhibited, additionally POMC transcription and ACTH release are suppressed at the level of the anterior pituitary (Keller-Wood and Dallman, 1984; Tasker et al., 2006; Papadimitriou and Priftis, 2009). One of the major regulatory structures in mediating this negative feedback inhibition at the level of the hypothalamic PVN is the hippocampus (Jacobson and Sapolsky, 1991; Furay et al., 2008).

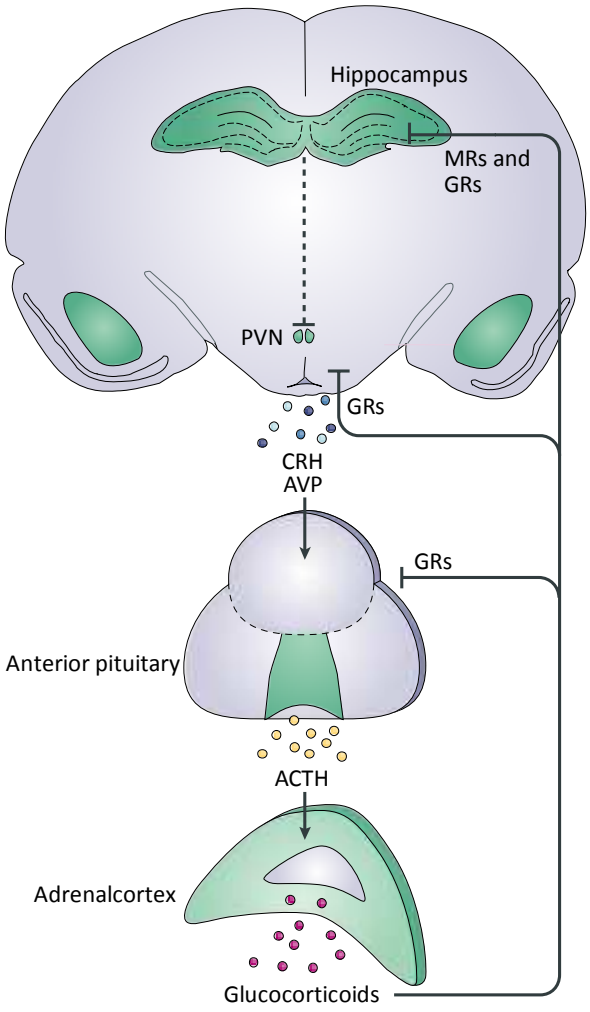

Figure 1.1 The HPA axis

Neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus release corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP). This triggers the subsequent secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, leading to the production of glucocorticoids by the adrenal cortex. The responsiveness of the HPA axis to stress is in part determined by the ability of glucocorticoids to regulate ACTH and CRH release by binding to two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Following activation of the HPA axis feedback loops are triggered at various levels of the system in order to shut the HPA axis down and return to homeostasis. Adapted from Lupien et al., 2009.

Apart from the stress-induced activation of the HPA axis, glucocorticoid levels in the blood display a circadian rhythm with a peak at the onset of the active phase (UNGAR and HALBERG, 1962; Keller-Wood and Dallman, 1984; Van Cauter E., 1990). Diurnal species like humans display the highest plasma glucocorticoid concentrations in the early morning, reaching a nadir in the evening. In nocturnal animals including mice and rats, this pattern is

reversed showing a trough in plasma glucocorticoids in the morning and rising towards a peak in the evening. One major controlling instance for this circadian rhythm is the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus (Moore and Eichler, 1972; Cascio et al., 1987; Kalsbeek et al., 1996). However, circadian rhythmicity of plasma glucocorticoid levels is not promoted by constant secretory activity or inactivity, but is rather induced by pulsatile episodes of glucocorticoid release with an approximate frequency of 1 hour (Lightman et al., 2008; Droste et al., 2008). The varying magnitude of the pulse therefore defines the ascending glucocorticoid concentration during inactivity and the decreasing glucocorticoid level during the activity phase (Veldhuis et al., 1989; Windle et al., 1998).

1.4 Corticosteroid receptors and the molecular determinants of their action

The main effectors of the HPA axis are glucocorticoids that mediate their action via the GR and the MR. The GR is expressed nearly ubiquitously throughout the brain with the highest levels in the PVN; in contrast, the MR has a more restricted distribution with the highest density in the hippocampus and only minimal expression in extra-hippocampal sites such as the hypothalamus, the amygdale and the septum (Reul and de Kloet, 1985; Morimoto et al., 1996; Kretz et al., 2001). The lipophilic glucocorticoids readily enter the cell through the cell membrane and bind to cytosolic MRs and GRs. Both receptors are ligand-dependent transcription factors and belong to the steroid nuclear receptor family (Evans, 1988). The GR is mainly selective for endogenous and synthetic glucocorticoids, whereas the MR binds multiple classes of steroids e.g. mineralocorticoids like aldosterone and glucocorticoids (de Kloet et al., 1998; de Kloet et al., 2008). Glucocorticoids bind to both receptors, however the MR exhibits a 10-fold higher glucocorticoid binding affinity than the GR (Reul and de Kloet, 1985). These characteristics results in a different occupancy, and therefore a different activity, of the two receptors. The MR is already occupied at low glucocorticoid levels such as the nadir of the circadian rhythm, while the GR is only occupied during the circadian peak or after stressful stimuli (Reul et al., 1987; Reul et al., 1990). Hence, a fine tuning of glucocorticoid action is possible with MRs mainly maintaining basal activity of the HPA axis whereas GRs mediate the stress response and the negative feedback of the HPA axis.

1.4.1 Corticosteroid receptor structure and their molecular actions

The receptors share a similar modular structure with three structurally distinct functional domains: the N-terminal domain, followed by a central DNA binding domain and the C-terminal ligand-binding domain (Mangelsdorf et al., 1995; Schoneveld et al., 2004; Pippal and Fuller, 2008) (see Figure 1.2). The N-terminal domain contains the ligand-independent trans-activation function, which is important for interactions of the receptor with transcriptional co-regulators. Cellular expression levels and availability of those co-activators and co-repressors influence the transcriptional activity of the GR by directly affecting formation of the transcription initiation complexes accumulated on the promoter-bound GR (Kino, 2007). Binding of glucocorticoids to the ligand-binding domain induces an allosteric change, leading to translocation of the hormone-receptor complex into the nucleus, thereby allowing receptor mediated manipulation of gene expression. The DNA binding domain of the MR is highly homologous with that of the GR, and facilitates binding of the hormone-bound receptor to glucocorticoid response elements (GREs) as homo- or heterodimers (Arriza et al., 1987; Hard et al., 1990; De Bosscher et al., 2003; Meijer et al., 2006).

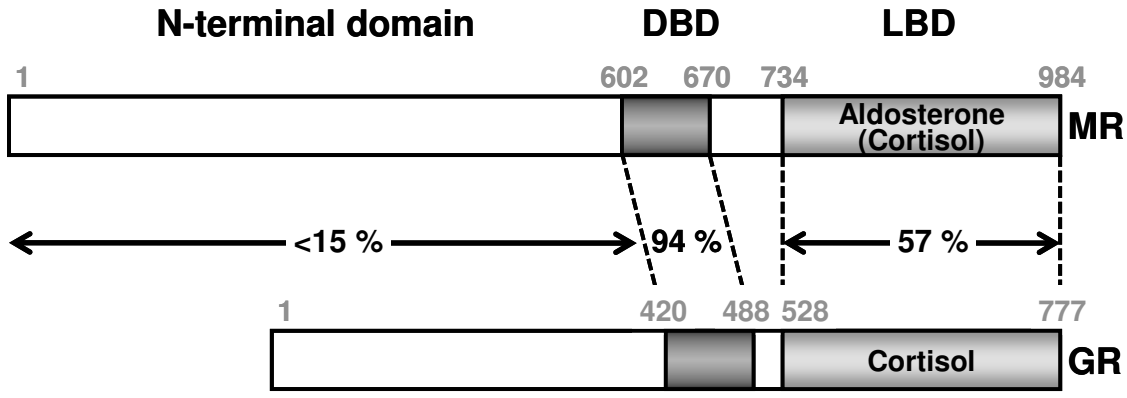

Figure 1.2 Structure of the human GR and MR

Schematic overview of the human GR and MR with the N-terminal domain, the central DNA binding domain (DBD) and the C-terminal ligand-binding domain (LBD) with the percentage of amino acid identity between each domain. Adapted from Pippal and Fuller, 2008.

Classical GREs display a pentadecameric consensus sequence of an imperfect palindrome GGTACAnnnTGTTCT (Nordeen et al., 1990). Binding of the steroid receptor typically leads to transactivation of gene expression. In contrast to this action steroid

receptors can additionally repress target gene expression by direct interaction with negative GREs (Schoneveld et al., 2004). Furthermore, steroid receptors can influence gene expression by interacting with DNA-bound transcription factors in a regulatory complex, both with or without direct binding to the DNA (Morsink et al., 2006) (see Figure 1.3).

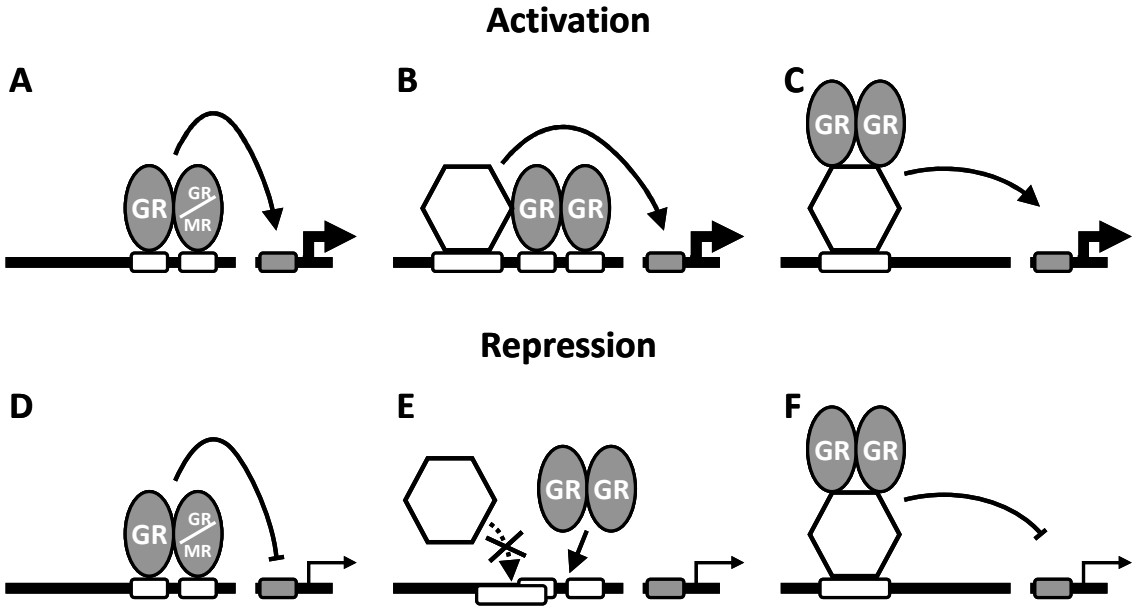

Figure 1.3 Mechanisms of steroid receptor interactions with regulatory elements exemplary for the GR

*Schematic diagrams on the left represent GR induced gene expression activation by (**A**) direct DNA binding of the GR as homo- or heterodimer with the MR, (**B**) direct binding of the GR homodimer and another transcription factor, (**C**) binding to an activating transcription factor. Diagrams below represent repression of gene expression by (**D**) direct binding of the GR as homo- or heterodimer with the MR to a negative GRE, (**E**) direct DNA binding and sterically inhibiting binding of other transcription factors, (**F**) binding to a repressing transcription factor. Adapted from Schoneveld et al., 2004.*

1.4.2 Selective corticosteroid receptor modulators

A great amount of information about glucocorticoid receptor action was gathered using specific receptor activating or blocking approaches. First the purification and later on the synthesis of corticosteroids and the revelation of their structure made it possible to design synthetic glucocorticoid agonists and antagonists leading to a better understanding of the molecular mechanisms of glucocorticoid action (Mason et al., 1936; Clark, 2008). Receptor agonists are defined as ligands binding to a receptor, thereby activating the receptor and producing a pharmacological response. On the contrary, receptor antagonists show an

affinity but no efficacy for the cognate receptors, and binding results in a disrupted interaction leading to inhibition of the agonists (Stephenson, 1997). Due to their anti-inflammatory efficiency numerous synthetic glucocorticoid receptor agonists have been developed over time (Hudson et al., 2008). Among the first synthetic GR agonists were dexamethasone and prednisolone, which are both frequently prescribed as anti-inflammatory agents even today.

In vitro Dexamethasone shows a 14-fold higher affinity for the human GR compared to the endogenous agonist cortisol, but a 8-fold lower affinity for the MR and therefore does not activate the MR with the same potency as the GR (Veldhuis et al., 1982; de Kloet et al., 1984; Rupprecht et al., 1993). Prednisolone has a twofold higher affinity for the human GR, but a similar affinity like cortisol for the MR (Lan et al., 1981; Lan et al., 1982). Therefore dexamethasone represents a very potent and specific GR agonist.

Over time numerous GR and MR antagonist were designed to develop potential treatment strategies for several corticosteroid related disorders. Among the best investigated antagonists are the GR antagonist mifepristone (RU-38486) and the MR antagonist spironolactone (RU-28318). Mifepristone has a high affinity for the GR and the progesterone receptor, but no affinity for the MR (Cadepond et al., 1997). Spironolactone demonstrates a high affinity for the MR and a low affinity for the GR (Rogerson et al., 2003). Both receptor antagonists readily pass the blood brain barrier and binding of mifepristone to the GR or spironolactone to the MR leading to a sterical blockade of transcriptionally necessary motifs that leaves the receptors inactive (Moguilewsky and Philibert, 1984; Pandit et al., 2002; Hu et al., 2005). Both antagonists are frequently prescribed. Mifepristone is admistered as an abortifacient, due to its additional affinity for the progesterone receptor. Spironolactone is commonly used in the treatment of hypertension (Couette et al., 1992). The use of selective receptor agonists and antagonists allows a time and receptor specific manipulation of the HPA axis response in the brain.

1.5 Activating and inhibitory influences on the HPA axis: the underlying neuroanatomical circuits

Stress, especially prolonged or repeated episodes of stress, physically alters the structure and function of brain regions involved in controlling HPA axis responses. To understand the

adaptive and maladaptive consequences of stress it is important to look beyond the PVN at the various brain circuits that integrate the HPA axis response in the brain. Various brain regions and hierarchical pathways act in concert to convey distinct anticipatory (e.g. novelty, noise) and systemic (e.g immune challenge, haemorrhage) stressful stimuli to the PVN. Different stressors evoke varying responses and are directed by different brain circuits. The hypophysotropic neurons of the PVN are generally activated by glutamatergic or noradrenergic afferent stimulation and inhibited by GABAergic inputs (Liposits et al., 1986; van den Pol et al., 1990; Cole and Sawchenko, 2002; Miklos and Kovacs, 2002). Afferents from structures of the limbic system and various hypothalamic regions mediate the initiation and the termination of the stress response. Most of those regions have a complex structure and are composed of several distinct nuclei and subregions. Additionally, most of those regions exert stimulating as well as inhibitory signals to the PVN, depending on the specific subregion and the time point of the stress cascade. In the following chapter only the major regulatory structures will be presented to provide a short excerpt of stress-response mediation in the brain.

1.5.1 Limbic system

Limbic brain structures like the hippocampus, the prefrontal cortex, the amygdala and the septum are strongly involved in emotional, autonomic, motor and cognitive responses linked to memory and motivation (McLachlan, 2009). These structures, with exception of the amygdala, have only little direct anatomical interactions with the PVN. In most cases intervening synapses are required to communicate information to the primary stress effector neurons in the PVN (Herman et al., 2005).

The hippocampus is strongly involved in learning and memory related processes and mediates an important suppressing influence on HPA axis activity. Both MRs and GRs are strongly expressed in the hippocampus, enabling this brain region to react to varying glucocorticoid levels (Reul and de Kloet, 1985; Arriza et al., 1988; Droste et al., 2008). In the mouse brain the MR shows the highest expression intensity throughout the hippocampus. In contrast, the GR is of greatest abundance in the CA1 region of the hippocampus while showing only modest expression in the CA3 region. Stimulation of hippocampal neurons results in decreased neuronal activity in the PVN and prevents glucocorticoid secretion, while hippocampal lesions induce elevated basal levels of glucocorticoids (Knigge, 1961; Rubin et al., 1966; Sapolsky et al., 1991; Herman et al., 1992). The hippocampus is therefore

crucially involved in termination of the HPA axis response by mediating the negative feedback (Feldman and Conforti, 1980; van Haarst et al., 1997). Hippocampal neurons maintain indirect projections to the PVN via the lateral septum, the bed nucleus of the stria terminalis and several hypothalamic regions (Sawchenko and Swanson, 1982; Boudaba et al., 1996; Furay et al., 2008).

The medial prefrontal cortex, a complex structure that is involved in working memory, decision making, planning and behavioral flexibility, as well as in social interactions and emotional processing, contributes an additional inhibitory influence to HPA axis activity (Diorio et al., 1993; Sullivan and Gratton, 1999; Cerqueira et al., 2008). A high expression of GRs is present in the medial prefrontal cortex throughout all subregions, while the MR is not strongly expressed (Ahima et al., 1991; Sanchez et al., 2000; Bizon et al., 2001; Patel et al., 2008). The dorsal subregions of the medial prefrontal cortex, namely the anterior cingulate and the prelimbic cortex, suppress the HPA axis responses to stressors like restraint stress (Diorio et al., 1993; Brake et al., 2000; Radley et al., 2006; Kern et al., 2008). Compared to the inhibitory influence of the hippocampus, lesions in the medial prefrontal cortex have no impact on basal glucocorticoid levels, indicating a stress response specific function of the medial prefrontal cortex (Figueiredo et al., 2003). There is no evidence for direct medial prefrontal cortex projections to the PVN, but indirect innervations proceed via the bed nucleus of the stria terminalis, the nucleus of the solitary tract and several hypothalamic nuclei (Sesack et al., 1989; Spencer et al., 2005).

The amygdala is a heterogeneous structure, which is implicated in a wide variety of functions including integration of emotional information, memory processing, attention and anxiety. In contrast to the hippocampus and the prefrontal cortex the amygdala exerts mainly a stimulating influence on HPA axis activity. Both the GR and to a lesser extent the MR are present in the amygdala, providing a target for glucocorticoid action (Arriza et al., 1988; Patel et al., 2000). The medial and basolateral nuclei of the amygdala are primarily stimulated by forced swim or restraint stress paradigms (Dayas et al., 2001). Both nuclei innervate the medial parvocellular PVN directly by a limited number of projections or indirectly via other intervening regions like the bed nucleus of the stria terminalis or the hypothalamic medial preoptic area (Feldman et al., 1990; Canteras et al., 1995; Cullinan et al., 1996).

The lateral septum, composed of dorsal, intermediate and ventral subdivisions, is involved in the regulation of various affective and motivational processes and is an essential nodal point for integrating cognitive information (Sheehan et al., 2004). Along with the hippocampus, its principal afferent source, the lateral septum exerts an inhibitory influence on HPA activity (Risold and Swanson, 1996). The MR is abundantly expressed in the whole lateral septum; in addition the GR is expressed widely in various neurons of the lateral septum, although GR expression levels are especially high in the dorsal and ventral subdivisions (Arriza et al., 1988; Aronsson et al., 1988; Ahima et al., 1991). Damage to the lateral septum results in enhanced HPA responsiveness to acute stress but does not affect basal glucocorticoid levels (Seggie et al., 1974; Dobrakovova et al., 1982). The lateral septum innervates numerous PVN-projecting anterior and lateral hypothalamic nuclei as well as the peri-PVN region (Risold and Swanson, 1997).

The bed nucleus of the stria terminalis is an important joint for numerous brain regions involved in HPA axis activity (Cullinan et al., 1993). This region contains many distinct subdivisions that regulate HPA axis activity in opposing directions. Posterior subdivisions of the bed nucleus of the stria terminalis act mainly inhibitory, whereas anterior subdivisions operate excitatory (Dunn, 1987; Crane et al., 2003). The GR is abundantly expressed in the bed nucleus of the stria terminalis, the MR is also present, but only in a limited number (Arriza et al., 1988; Herman, 1993). The bed nucleus of the stria terminalis exhibits strong direct projections to the medial PVN (Sawchenko and Swanson, 1983).

1.5.2 Hypothalamic regions

The PVN is strongly innervated by numerous hypothalamic regions, which have among other functions an integrative role for stimuli from limbic brain circuits. While the GR is consistently expressed almost everywhere in the hypothalamus, the MR shows only very low expression throughout the hypothalamus (Arriza et al., 1988; Han et al., 2005). An important hypothalamic integrative region is the area directly surrounding the PVN. This peri-PVN area is strongly activated after stress and exerts mostly inhibitory innervations towards the PVN (Boudaba et al., 1996; Herman et al., 2002; Cullinan et al., 2008). Additionally, the medial preoptic area and the dorsomedial hypothalamic nucleus are both exercising an inhibitory input towards the PVN and are activated by restraint stress and forced swimming (Roland and Sawchenko, 1993; Cullinan et al., 1996). The arcuate nucleus is involved in regulating the energy balance and stimulates the HPA axis activity by direct connections with the PVN in

answer to positive as well as negative energy balance (Leibowitz et al., 1988; Dhillo et al., 2002). The mamillary nuclei, in particular the ventral premammillary nucleus, located in the posterior hypothalamus shows the highest hypothalamic MR expression. The mamillary nuclei are stimulated by anticipatory stressors and innervate the PVN heavily (Cullinan et al., 1995).

1.6 Maladaptive consequences of stress

From an evolutionary point of view, the organisms' stress response is highly adaptive, preparing the individual to cope with life threatening situations by mobilizing energy resources and focusing the attention towards survival under adverse conditions. However, the demands of our modern world have changed, presenting mainly prolonged challenges of a more psychosocial nature, like work stress. Whether the stress experience is beneficial or maladaptive for the individual is determined by several intertwined factors. These include the duration and the intensity of the stressor, the ontogenetic stage, the genetic predisposition, and the life history, which eventually culminate into how the stressor is perceived and determine the individuals' extent of the allostatic load.

Short episodes of acute, controlled stress are in principle positive for an individual by enhancing cognitive performance and memory, thereby contributing to adaptation during acute challenges by increased vigilance and promoted learning processes (Diamond et al., 1992; Roozendaal et al., 2002). On the other hand, prolonged or repeated episodes of stress, especially of an unpredictable and uncontrollable nature, present a major risk factor for several serious illnesses, ranging from metabolic and cardiovascular diseases to mood disorders like depression or anxiety disorder (Baum and Posluszny, 1999; Khot et al., 2003; Glei et al., 2007; Schmidt et al., 2008; Calabrese et al., 2009). Chronic stress is known to induce morphological changes in the brain, like alterations in dendritic morphology, hippocampal atrophy and changes in neurotransmitter receptor density (McEwen and Magarinos, 2001; Sapolsky, 2003). While these alterations are mostly reversible after termination of the stress there are also some persisting changes like hormone receptor densities as well as long lasting behavioral alterations, indicating a programming effect of stressful experiences for the future (Magarinos and McEwen, 1995; McEwen, 2001; Sterlemann et al., 2008). Not only the duration, but also the developmental state at the time point of the stressful experience is an important factor for the processing and the

consequences of the stressor. Stressful or traumatic experiences during development are strongly associated with health problems later in life, including somatic symptoms and a predisposition for mood disorders (Neigh et al., 2009). Genetic factors are also known to contribute to the prevalence of human psychopathologies, yet there is little understanding of how genes interact with stressful experiences to shape the individual pathophysiology (Kendler et al., 2003).

1.7 Stress as a risk factor for psychiatric disorders

Stressful life events are generally considered as having precipitating effects on the development of human psychopathologies such as post-traumatic stress disorder (PTSD), schizophrenia, anxiety disorder and major depression (McLaughlin et al., 2009). Major depression is a chronic, often recurring and potentially life threatening illness and has an estimated lifetime prevalence of 10 % to 20 % (Kessler et al., 2003; Alonso et al., 2004; Gonzalez et al., 2010). The World Health Organization now ranks major depression as one of the most burdensome diseases in the world. Moreover, major depression is predicted to be the second leading cause of disability worldwide and even the leading cause of disability in high-income nations in 2030 (Kessler et al., 2003; Mathers and Loncar, 2006). Major depression occurs idiopathically, shows heterogeneous progress and until today, there is only limited understanding of its aetiology. Core symptoms of major depression include depressed mood, anhedonia, irritability, difficulties in concentrating and abnormalities in appetite and sleep (Nestler et al., 2002). Those symptoms significantly reduce the quality of life of people suffering from depression and their social milieu.

There are many safe and effective treatments for major depression available, including antidepressant drugs, electroconvulsive seizures, psychotherapy and deep brain stimulation, but they are far from ideal. Common antidepressants, such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NRIs) or serotonin and norepinephrine reuptake inhibitors (SNRIs) show a multitude of side effects and they need to be administered for two to six weeks before a clinical benefit is perceptible (Krishnan and Nestler, 2008). Additionally, fewer than 50 % of all patients achieve full remission, even after combination of multiple medications (Trivedi et al., 2006; Berton and Nestler, 2006).

Furthermore, there is a high comorbidity between major depression and other mood disorders like anxiety disorders and schizophrenia (Buckley et al., 2009). A large number of

patients suffering from major depression also experience significant symptoms of anxiety disorders and vice versa (Gorman, 1996; Lieb et al., 2005; Francois et al., 2010). Furthermore, most antidepressant drugs also have an anxiolytic effect, which suggests at least a partial similarity of the underlying pathophysiological origins of anxiety disorder and major depression (Kent et al., 1998).

Altered HPA axis activity is a frequent feature of patients with major depression. A large number of depressed patients show HPA axis hyperactivity, which is thought to be related with an impaired feedback inhibition of this system (Sallee et al., 1995; Plotsky et al., 1998; Holsboer, 2000; Lupien et al., 2007; Pariante and Lightman, 2008; Marques et al., 2009). Aside from that, successful treatment with antidepressants is associated with HPA axis normalization and persisting HPA axis dysregulation following successful treatment correlates with increased relapse rates (Holsboer and Barden, 1996; Zobel et al., 1999; McKay and Zakzanis, 2009). The fact that stress clearly is a major risk factor for severe psychopathologies indicates the need for a better understanding of the adaptive and maladaptive mechanisms of the HPA axis response to provide better strategies to treat or even prevent maladaptive consequences of stress.

1.8 Consequences of stress during development

Numerous studies showed that HPA axis function during life is highly dependant on the developmental history (Levine, 1957; Meaney et al., 1996; Pryce and Feldon, 2003; Gillespie et al., 2009). Early life experiences can have a detrimental or beneficial impact on an individual's brain and behavioral development, depending on the manner and the developmental time point of the experience as well as the later environmental context. Early aversive events such as disrupted maternal care is thought to impair normal HPA axis function (Ladd et al., 2004; Neumann et al., 2005), while mild early stimulation like increased maternal care can be beneficial for HPA axis function later in life (Francis et al., 1999; Panagiotaropoulos et al., 2004). There is clearly a programming effect of early life experiences, which is likely to facilitate adaptation to the expected postnatal environments (Gluckman and Hanson, 2004). However, aversive consequences like mood disorders may occur if the early programming is not well matched to the actual environmental demands in adult life. The brain shows high plasticity and is under constant changes, especially during ontogeny. Stressful early life events during critical developmental time points may therefore

predispose the individual for increased stress-susceptibility during the rest of the life. Exposure to childhood trauma is linked with altered reactivity to stress, cognitive deficits and an increased risk for psychopathologies like major depression or schizophrenia in adulthood (Vallee et al., 1996; Heim and Nemeroff, 1999; Agid et al., 1999; Gunnar and Donzella, 2002; Tyrka et al., 2008; Neigh et al., 2009). The developing stress system is therefore of great interest to understand the potentially maladaptive mechanisms of HPA axis programming.

The development and maturation of the HPA axis and its related structures is highly species specific (Kapoor et al., 2006). It was estimated that the human late gestation phase corresponds with the rodent postnatal period (Fox, 1964; Dobbing and Sands, 1979; Hagberg et al., 2002)., which comprises the first two weeks after birth and is characterized by a relative quiescence of the HPA axis (Diez et al., 1976). During this so-called stress hyporesponsive period (SHRP) basal corticosterone levels are very low and mild stressors, like exposure to a novel cage or injections, are unable to induce peripheral HPA axis activity. Maintenance of the SHRP is dependant on maternal social and physical inputs like licking or nursing. Only severe stressors like maternal separation or an immune challenge are able to induce a robust HPA axis response (del Rey et al., 1996; Schmidt et al., 2002).

Modeling early-life stress in non-human primates and rodents is a valuable tool to elucidate the molecular pathways involved in the consequences of disrupting the normal HPA axis activity, thereby enlightening the mechanisms of physiological HPA axis regulation. Early-life manipulations can be principally divided into prenatal and postnatal interventions. Prenatal manipulations include maternal stress experiences, exposing mother and embryo to glucocorticoid excess or deprivation (for overview see (Lupien et al., 2009)). All those manipulations result in persistent changes of the HPA axis response to stress later in life (Weinstock et al., 1992; Henry et al., 1994; Clarke et al., 1994). Postnatal manipulations include postnatal handling, repeated as well as prolonged separation from the mother. Postnatal handling involves brief daily periods of 10 minutes to 15 minutes separation of the pup from the mother and seems to be beneficial for the individual due to increased maternal care in response to the separation (Liu et al., 1997). Effects of handling include a blunted ACTH and corticosterone response to various types of stressors and a faster return to basal corticosterone levels following stressor termination (Meaney et al., 1996). In contrast to the effects of brief handling, repeated prolonged separation for at least 3 hours during the postnatal period present an adverse stimulus for the pups. Those protracted periods of

separation are able to increase HPA activity in the pups and also increase HPA axis reactivity to stressors in adulthood (Plotsky and Meaney, 1993; Ladd et al., 2004). Additionally, a single, prolonged separation of the pups from the dam for 24 hours represents a severe stressor for the offspring (Levine et al., 1991; Schmidt et al., 2002). The pups not only lack the maternal care, but additionally have no access to food during this period (Stanton et al., 1988). Prolonged separation acutely results in robust HPA axis activation in mice; however the magnitude of HPA axis activation is dependent on the duration of the separation. In mice, elevated corticosterone levels are present after 4 hours of maternal separation and increase gradually during the first 12 hours of separation. After 12 hours the corticosterone levels remained elevated and show an increase again after 24 hours of maternal separation (Schmidt et al., 2004). Single prolonged maternal separation for 24 hours has also been shown to induce long lasting behavioral effects in rats (Oitzl et al., 2000). Prolonged maternal separation is therefore an ideal tool to model early adverse experiences and its consequences on adaptive and maladaptive stress system mechanisms.

1.9 The mouse as a model organism

Stress profoundly influences an individual's well-being and represents a strong etiologic factor for various severe illnesses including mood disorders. To gain insights into the physiological and pathological aspects of the HPA axis suitable experimental material is necessary. On the one hand, blood samples to determine the stress hormone concentrations are easily obtained from humans and also cognitive tests can be applied with less effort in humans to assess data of acute effects of stress. On the other hand, information about stress induced structural as well as gene expression changes in the brain, as the central center of the HPA axis, have to rely on postmortem samples or non invasive techniques like magnetic resonance imaging. While those studies contribute valuable information, they are disadvantageous concerning access to information about former case history of the individual, present difficulties in matching the correct controls and it is nearly impossible to link specific events to observed brain physiology. Additionally, obvious ethical reasons prevent the modeling of adverse experiences in humans. Preclinical studies in animal models are therefore crucial and necessary to gain vital information on normal and pathological aspects of the stress response. Animal studies allow the development of experimental protocols in which animals are submitted to acute and/or chronic stress under controlled

conditions and cause–effect relationships between stress and its impact on the brain and the behavior can be demonstrated.

The mouse as a model organism presents a number of convincing advantages and is today the premier animal model to study the genetic basis of multiple human diseases (Peters et al., 2007). The genomes of humans and mice are composed of approximately 2.9 and 2.5 billion nucleotides, respectively and are both encoding about 30,000 genes. Furthermore, for 99 % of the human genes mouse orthologs have been identified (Waterston et al., 2002). Moreover, human and mouse genomes exhibit multiple chromosomal regions with the same organization and regulation of genes (Tecott, 2003). Additionally, mice show an average life span of about 2 years, they are reproductively competent by 6-7 weeks, possess a short gestation period of approximately 3 weeks, have large litter sizes and their small body size enables the economical husbandry of large numbers of animals. Furthermore, over the last 100 years vast knowledge about mouse genetics and behavior has been collected.

In 1909 Clarence Little began the development of the first inbred mouse strain and until today over 450 different inbred strains have been established (Paigen, 2003). Inbred strains are characterized by continued sibling mating for more than 20 generations with all animals being descended from a single breeding pair. Inbred strains are essentially genetical identical and homozygous at every genetic locus. Commonly utilized inbred strains like the BALB/c, DBA and C57Bl6/N strains are very well characterized with regard to their genetic, phenotypical and behavioral features (Festing, 1975). Contrary to inbred strains, outbred mice originate from non-sibling mating, with less than 1% inbreeding per generation, to maintain a maximum of heterozygosity in the offspring. Commonly used outbred stocks include Black Swiss, NIH Swiss and CD1 mice. Due to their relative genetic variability outbred mice offer the possibility to examine effects in a genetically heterogeneous population. Nevertheless outbred stocks are closed colonies derived from a limited gene pool and show genetic variability only within these limits (Chia et al., 2005). Hence, inbred mice with their defined genetic background and outbred mice, which resemble more the heterogenous genetic variety of human populations, offer different advantages.

Taken together, mice provide the resources and tools for understanding the genetic, molecular and cellular basis of human disease and normal biological processes.

1.10 TU3A, a novel stress-inducible gene in the mouse brain

Preliminary studies during the author's diploma thesis, aimed at the identification of novel stress-regulated genes in the mouse brain, identified TU3A (Tohoku University cDNA clone A on chromosome 3) as an interesting stress-regulated candidate gene for further studies (Liebl, 2006). TU3A was identified as one of the genes most strongly up-regulated by maternal separation in the mouse brain, using gene expression profiling in the PVN at postnatal day 9 (Liebl et al., 2009). Its widespread expression in the brain, both during postnatal development and adulthood encouraged us to study its specific role in stress-related central nervous system (CNS) function in more detail.

Initially, the human gene TU3A was identified as a putative tumor suppressor gene in renal cell carcinoma by two different groups (Yamato et al., 1999; Wang et al., 2000). Yamato et al. termed the gene TU3A and Wang et al. named it DRR1 (Down-Regulated in Renal cell carcinoma 1); furthermore this gene was named FAM107A (family with sequence similarity 107, member A) according to the HUGO gene nomenclature committee. In this thesis, the gene will be referred to as TU3A according to the first publication concerning the gene. The human gene is located on the short arm of human chromosome 3, at the chromosomal locus 3p21.1 (Wang et al., 2000). Several tumor suppressor genes have been identified at the short arm of human chromosome 3 (Erlandsson et al., 1990; Latif et al., 1993). Indeed, TU3A shows a loss of expression or a decreased expression in several tumor cell lines or primary tumor samples (Yamato et al., 1999; Wang et al., 2000; van den et al., 2006). This loss of expression is not due to genetic alterations, but is likely mediated by epigenic mechanisms. As a matter of fact, Awakura et al. showed that the inactivation of TU3A in different renal cell carcinoma cell lines was caused by promoter hypermethylation, an epigenetic mechanism often involved in loss of gene function in established tumor suppressor genes (Awakura et al., 2008). Recovery of TU3A expression by transfection resulted in suppressed cell proliferation in different tumor cell lines, confirming the involvement of TU3A in cell growth regulation in tumor cell lines (Wang et al., 2000; Zhao et al., 2007).

Awakura et al. described 3 different transcript variants for the human TU3A that all encode for the same 144 aa sequence with a molecular weight of approximately 17.5 kD (Awakura et al., 2008). Orthologues of the human TU3A have been identified in 36 species, among them african clawed frog, mouse, rat and several non-human primates (Zhao et al.,

2007). The mouse orthologue consists also of 144 aa and shows 90 % sequence identity to the human protein (confirmend by NCBI Blast Altschul et al., 2005). The high identity and similarity of the murine and human TU3A indicates a conserved, analogous function of the protein. The TU3A protein contains a domain of unknown function (DUF1151) of 117 aa, which is highly conserved in different species and has a coiled coil motif (Schülke JP, 2009) (Figure 1.4 B). Coiled coil structural motifs are protein-protein interaction domains suggesting a functional interaction of TU3A with other proteins (Lupas and Gruber, 2005). Furthermore, bioinformatical promoter analysis of the TU3A gene revealed several GREs, which are highly conserved in mouse and human TU3A (Dietrich Trümbach, Helmholtz Zentrum München, Institute for Developmental Genetics, unpublished data), suggesting a regulatory influence of glucocorticoids on gene expression (Figure 1.4).

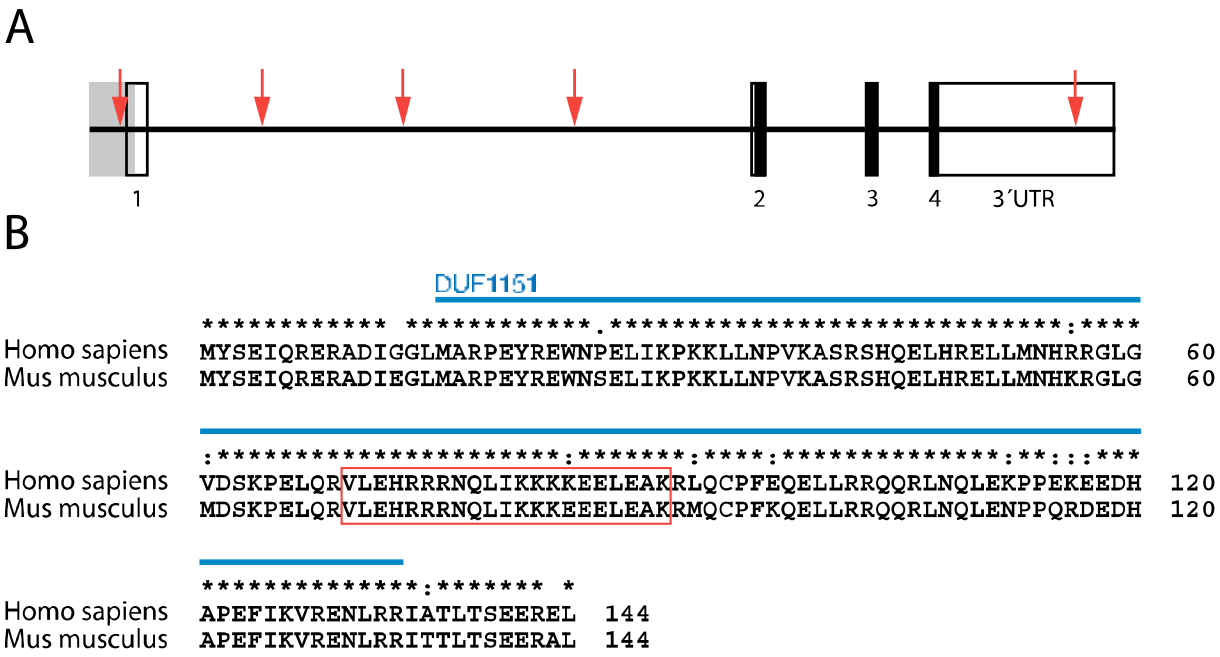

Figure 1.4 Structure and sequence of TU3A

*(**A**) Scheme of the inverted exon-intron structure of the 13.5 kb primary transcript of TU3A is shown with the promoter region marked in grey followed by four exons and the 3'UTR. The red arrows indicate experimentally verified GRE binding sites. (**B**) Comparism of the human and murine TU3A protein sequence. The sequence includes a protein domain of unknown function (blue line) and a coiled coil motif (red box). Asterisks (*) indicate amino acid residues that are conserved across both species, colons (:) indicate strong similarity between human and murine protein and dots (.) indicate weak similarity. This figure has been kindly provided by Dr. Dietrich Trümbach.*

Another research group in our institute (RG Rein, Chaperones) identified actin as a potential interaction partner of TU3A and could further demonstrate a bundling effect of TU3A on actin fialents (Schülke JP, 2009). Actin is the major cytoskeletal protein expressed in pre- and postsynaptic structures and actin dynamics have been implicated in the modulation of synaptic plasticity (Cingolani and Goda, 2008). TU3A is expressed in most human tissue samples with the highest expression in the brain (Yamato et al., 1999; Wang et al., 2000; Su et al., 2004). During embryonic development of *Xenopus laevis* the TU3A expression is present at all stages and gradually increases in the ectoderm and mesoderm. The strongest expression in *Xenopus laevis*, like in human tissue samples, was found in the brain and in the heart (Zhao et al., 2007). Another study using human embryonic stem cells in a neural differentiation culture system found TU3A specifically expressed at distinct time points during neural development. TU3A expression was significantly increased at day 17, where the cells formed neural tube-like rosettes, compared to earlier developmental stages (Pankratz et al., 2007). These findings suggest a role of TU3A during CNS development in different species. Additionally, a postmortem study of mRNA expression in dorsolateral prefrontal cortex tissue of patients suffering from schizophrenia or bipolar disorder showed an increased TU3A expression in both pathophysiologies (Shao and Vawter, 2008).

The increasing TU3A expression during neural development as well as the pronounced expression of TU3A in the adult brain, together with the altered TU3A expression in human psychopathologies presents TU3A as an interesting candidate to explore its function in the brain in context of stress.

2 Aims of the thesis

The HPA axis mediates adaptive as well as maladaptive consequences of stress and a plethora of genes is involved in these processes. The aim of this thesis is the detailed characterization of TU3A, a novel stress-inducible transcript, in context of its potential function in the central stress system. Specifically, the following questions will be addressed:

- Characterization of the basal TU3A mRNA and protein expression patterns during postnatal development and adulthood in the mouse brain.

- Effects of stress on TU3A expression with special respect to different types of stressors, mouse strains as well as gender influences and investigation of the regulatory mechanism underlying the stress-induced gene expression.

- Influence of TU3A on the modulation of emotional and cognitive performance by means of adeno-associated virus mediated overexpression of TU3A in the adult hippocampus.

3 Animals, material and methods

3.1 Animals

Experiments were carried out with C57Bl6/N or CD1 mice. All animals were obtained from Charles River (Charles River Laboratories, the Netherlands) at the age of 11 weeks and had a habituation period of at least 1 week prior to testing. Animals were single housed in mouse polycarbonate cages (450 mm x 250 mm x 200 mm) containing standard sawdust bedding, under a 12L:12D cycle (lights on at 6 a.m.) with constant temperature (23 ± 2 °C) and humidity (55 ± 5%) conditions. Standard rodent diet (No. 1324 Altromin GmbH, Germany) and tap water were provided *ad libitum.*

The offspring of C57Bl6/N mice was utilized for postnatal development studies. Two 12 weeks old females were mated with one male in a standard cage. Pregnant females were single housed and transferred to clean polycarbonate cages containing sawdust and two sheets of paper towels for nesting material during the last week of gestation. Pregnant females were examined for litters daily between 9 a.m. and 10 a.m. If litters were found, the day of birth was defined as day 0 for that litter.

The experiments were carried out in accordance with European Communities Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

3.2 Experimental design and background

3.2.1 Analysis of TU3A mRNA expression pattern in the neonatal and the adult mouse brain

This study was designed to obtain a detailed gene expression profile in the developing and adult brain. For TU3A gene expression mapping throughout the developing brain, four animals were sacrificed on postnatal day 9 under basal conditions. For gene expression mapping across the adult brain, four animals at the age of 12 weeks were sacrificed under basal conditions between 9 a.m. and 12 a.m. Half of the brains were cut in the coronal plane,

the other half in the sagittal plane with a slice thickness of 18 µm. Every third section of the neonatal brain and every fourth section of the adult brain was used for *in situ* hybridization (see chapter 3.6.1) to obtain a detailed overview across the brain. The different anatomical brain regions were identified by comparison of autoradiographs as well as photomicrographs of cresyl violet counterstained slices with the mouse brain atlas (Paxinos and Franklin, 2001), with the assistance of the textbook comparative vertebrate neuroanatomy (Butler and Hodos, 1996). In addition, the developing mouse brain reference atlas from the Allen institute for brain science was consulted for neonatal brain regions (http://developingmouse.brain-map.org/atlas/index.html). The relative intensities of TU3A mRNA expression were evaluated using ImageJ software (ImageJ 1.42, National Institutes of Health, USA) in at least two sections for each region. Signal intensities were rated into one of the following categories: - not expressed, + weak signal, ++ moderate signal and +++ strong signal.

3.2.2 Analysis of TU3A protein expression in the adult mouse brain

This study was designed to assess TU3A protein expression in addition to the mRNA expression in the adult mouse brain. For TU3A protein expression analysis, four animals at the age of 12 weeks were perfused under basal conditions (see chapter 3.3). Half of the brains were sectioned in the coronal plane and the rest in the sagittal plane. Brains were cut in 30 µm thick sections and every third section was used for free floating immunohistochemistry (see chapter 3.6.2) to obtain a detailed overview of protein expression in specific brain regions. Half of the sections were counterstained with hematoxylin to identify the different anatomical brain regions by comparison of photomicrographs with the mouse brain atlas (Paxinos and Franklin, 2001), with the assistance of the textbook comparative vertebrate neuroanatomy (Butler and Hodos, 1996).

3.2.3 Analysis of TU3A mRNA expression during postnatal ontogeny

The aim of this study was to achieve a comparative TU3A mRNA expression analysis at different stages of postnatal brain development up to adulthood. A total of eight litters and additionally eight male mice at the age of 24 weeks were used in this study. Two litters, respectively, were randomly assigned to one of the following aging groups: postnatal day 1, 6, 12 and 18. Every aging group included eight animals (four male and four female pups) obtained from two litters to exclude litter specific effects. All animals were killed under basal

conditions between 9 a.m. and 12 a.m., in case of young animals immediately after separation from their mother. Coronal brain sections from the region of the hypothalamic PVN and the dorsal hippocampus were used for *in situ* hybridization (see chapter 3.6.1).

3.2.4 Maternal separation in neonatal C57Bl6/N mice

To confirm and expand preliminary results from the authors diploma thesis (Liebl, 2006), TU3A mRNA expression was examined in several brain regions following maternal separation at postnatal day 9 by *in situ* hybridization (see chapter 3.6.1). In this study, a total of 16 C57Bl6/N mice (eight males and eight females) originating from four litters were used. Litters were randomly assigned to either a maternally non-separated (control group) or 24 hour maternally separated condition (see chapter 3.4.1). All pups from one litter were sacrificed on postnatal day 9 by decapitation between 9 a.m. and 12 a.m. and trunk blood was collected immediately after the 24 hours deprivation period or immediately after separation from the mother. The brains were sectioned at the level of the hypothalamic PVN, the dorsal hippocampus and the cerebellum.

3.2.5 Different stress paradigms in the adult C57Bl6/N mice

This study addressed the question if TU3A gene expression is, analogous to the neonatal situation, also stress-inducible in the adult mouse brain in regard to different stressors. A total of 24 male C57Bl6/N mice were used and assigned randomly to a restraint stress, 24 hours of food deprivation (see chapter 3.4) or undisturbed control group (n = 8 per group). Animals were sacrificed between 7 a.m. and 12 a.m.; animals of the restraint stressed group were sacrificed 4 hours after beginning of the restraint stress. Trunk blood samples were collected to examine plasma corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.6 Restraint stress in adult C57Bl6/N mice

To examine the effects of restraint stress on TU3A mRNA expression in more detail, the TU3A gene expression was examined at different time points following 30 minutes of restraint stress (see chapter 3.4.3). In total, 24 male C57Bl6/N mice were used for this study. The onset of the restraint stress was between 8 a.m. and 10 a.m. Animals were either sacrificed under basal conditions from 9 a.m. to 12 a.m. (control group) or 1 hour, 4 hours

and 8 hours after beginning of the restraint stress with six animals per group. Trunk blood samples were taken to examine plasma corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.7 Food deprivation in male CD1 mice

To exclude a strain specific effect of stress on TU3A gene expression in C57Bl6/N mice, this study was done with the CD1 outbred mouse strain, another commonly studied mouse strain. TU3A gene expression was examined after 24 hours of food deprivation (see chapter 3.4.2). In total, 16 male CD1 mice were used in this study. Animals were either exposed to 24 hours of food deprivation (see chapter 3.4.2) or remained undisturbed, with eight animals per group. Food deprivation started between 9 a.m. and 10 a.m. Trunk blood samples were taken to examine plasma corticosterone levels (see chapter 3.6.3) and brains were investigated at the level of the hypothalamic PVN and the dorsal hippocampus using *in situ* hybridization (see chapter 3.6.1).

3.2.8 Chronic social stress in male CD1 mice

In contrast to the influence of acute stress paradigms the effect of chronic stress on TU3A gene expression was examined in this study, employing a chronic social stress paradigm (see chapter 3.4.4). In this study, a total of 24 male CD1 mice were used, from which 12 animals underwent the chronic social stress procedure compared to 12 control animals. Animals were decapitated directly after termination of the seven weeks of chronic social stress. Trunk blood was collected to examine plasma corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.9 Food deprivation in female C57Bl6/N mice

This study was conducted in adult female C57Bl6/N mice to assess a potential gender-specific effect of stress on TU3A gene expression. A total of 16 female C57Bl6/N mice were used. Female mice were either deprived of food for 24 hours (see chapter 3.4.2) or remained undisturbed as control group (n = 8 animals per group). The onset of the food deprivation paradigm was between 9 a.m. and 10 a.m. Trunk blood was collected to examine plasma

corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.10 Dexamethasone treatment in adult C57Bl6/N mice

To study if TU3A mRNA expression is also pharmacologically inducible, male C57Bl6/N mice were treated with the synthetic GR agonist dexamethasone. TU3A mRNA expression was examined at different time points after the injection. A total of 48 animals were used in this study, which were subcutaneously injected between 8 a.m. and 10 a.m. with either 130 µl of 10 mg/kg body weight dexamethasone (Ratiopharm, Ulm, Germany) or 0.9 % saline as vehicle injected control group. The dexamethasone concentration was based on publications that proved this dosage sufficient to induce effects in the brain (Barbany and Persson, 1992; Lee et al., 2006). Animals of both treatment groups were sacrificed 1 hour, 4 hours, 8 hours and 24 hours after injection with six animals per group. Trunk blood was sampled to assess plasma corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

Additionally, potential effects of dexamethasone treatment on TU3A protein levels were examined. Therefore, an independent group of 18 male C57Bl6/N mice were treated with dexamethasone. Animals received a subcutaneous injection of 130 µl dexamethasone (10 mg/kg body weight) or 0.9 % saline as control group between 8 a.m. and 10 a.m. Animals remained undisturbed in their home cage until sacrifice 8 hours after the treatment. Brains were dissected directly after decapitation of the animals and samples of the hippocampus, the hypothalamus, the cortex and the cerebellum were immediately placed in lysis buffer on ice for subsequent protein immunoblotting (see chapter 3.6.4).

3.2.11 Corticosteroid receptor antagonist treatment during maternal separation in neonatal mice

This study addressed the question, if the stress-induced TU3A up-regulation could be blocked by corticosteroid receptor antagonists in neonates. A total of eight litters including 19 male and 21 female C57Bl6/N pups were used in this study. Litters were randomly assigned to either maternal separated (see chapter 3.4.1) or non-separated control condition. At postnatal day 8, all pups received two subcutaneous injections, always 16 and 8 hours before the sacrifice at postnatal day 9, to guarantee a sufficient and stable

antagonist concentration. One third of every litter was treated with either 30 µl of the vehicle polyethylene glycol, the MR antagonist spironolactone (50 mg/kg body weight) or the GR antagonist mifepristone (100 mg/kg body weight), respectively. Antagonist dosages were based on previous studies, that proved successful blockade of the GR and the MR (Schmidt et al., 2005; Garcia-Bueno et al., 2008). At postnatal day 9, between 9 a.m. and 10 a.m., pups were sacrificed by decapitation and their gender was determined. In case of non-separated controls, the mother was removed from home cage directly before decapitation. Trunk blood and brains were collected for determination of corticosterone (see chapter 3.6.3) and gene expression using *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.12 Corticosteroid receptor antagonist treatment during food deprivation in adult mice

In this study the effects of corticosteroid receptor antagonists on TU3A gene expression in the adult brain were examined. A total of 48 male C57Bl6/N mice were randomly assigned to either the undisturbed control condition or 24 hours of food deprivation (see chapter 3.4.2). Animals received two subcutaneous injections of 130 µl of either the vehicle polyethylene glycol, the MR antagonist spironolactone (50 mg/kg body weight) or the GR antagonist mifepristone (100 mg/kg body weight). Antagonist dosage was the same as in neonatal mice (see chapter 3.2.11). Injections were done 16 and 8 hours before sacrifice to ensure sufficient and stable substance concentration. All animals were sacrificed directly at the cessation of the 24 hours of food deprivation between 7 a.m. and 8 a.m. Trunk blood was sampled to assess plasma corticosterone levels (see chapter 3.6.3) and brains were processed for *in situ* hybridization (see chapter 3.6.1) at the level of the hypothalamic PVN and the dorsal hippocampus.

3.2.13 TU3A overexpression in the hippocampus

To gain more insights into a putative function of TU3A in the brain, region-specific overexpression of TU3A in the hippocampal formation was achieved by using a recombinant adeno-associated virus (rAAV). Neurotrophic viral vectors such as rAAV represent an ideal tool for long-term, region specific modulation of gene expression in the CNS (Janson et al., 2001). To this day, the replication defective rAAV has been proven to be non-toxic, elicits

only very low immunogenicy and appears innocuous to normal brain function (Kaplitt et al., 1994; Terzi and Zachariou, 2008).

3.2.13.1 Viral vector construct

Viral overexpression of TU3A in the hippocampus was achieved using a rAAV1/2 vector (GeneDetect, New Zealand) containing the CAG – TU3A – IRES-eGFP-WPRE-BGH-polyA expression cassette (containing the 435 bp coding sequence of TU3A, NCBI CCDS ID CCDS26813.1). The chimeric rAAV1/2 vector combines characteristics of both AAV1 and AAV2 viral serotypes (Hauck et al., 2003) and is especially efficient in transfecting CNS cells (Kiyota et al., 2009). The same vector construct was used for the control group, expressing only eGFP (Figure 3.1). Virus production, amplification and purification were done by Genedetect.

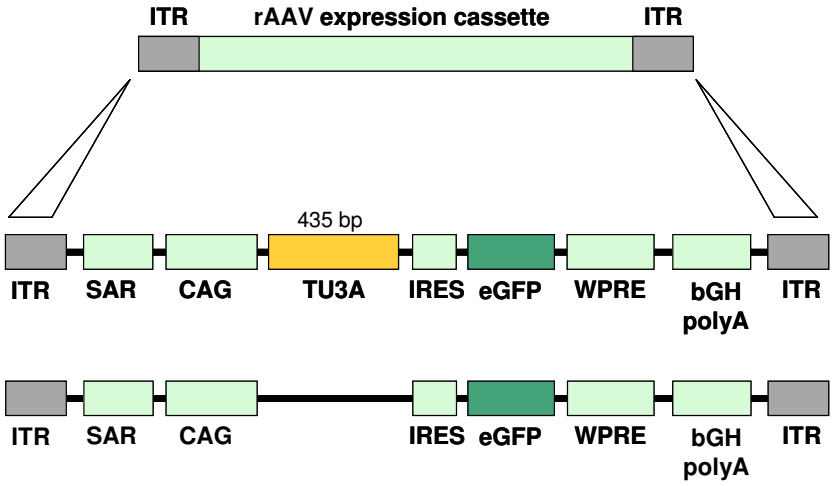

Figure 3.1 rAAV expression vector constructs

The upper part shows an rAAV1/2 vector containing the 435 bp TU3A coding sequence and eGFP, the lower part shows the solely eGFP expressing control vector construct. For optimized gene expression, the vector incorporates the following regulatory elements: rAAV2 inverted terminal repeat (ITR) sequences, a scaffold attachment region (SAR) element, the hybrid chicken B-actin / CMV enhancer (CAG) promoter region, an internal ribosome entry site (IRES), a cis-acting woodchuck post-transcriptional regulatory element (WPRE) and a bovine growth hormone polyadenylation signal sequence (bgh-polyA).

3.2.13.2 Stereotaxic intra-hippocampal injection

Viral injection was performed according to Monroy (Monory et al., 2006). In short, 12 weeks old C57Bl6/N mice were deeply anesthetized with isoflurane (Curamed Pharma GmbH,

Germany), inserted into a stereotaxic frame (TSE systems GmbH, Germany) and the surface of the skull was exposed. Holes were drilled bilaterally through the skull to expose the dura marter, targeting the following coordinates: 1.7 mm posterior to bregma, 1.8 mm lateral from midline, and 1.8 mm below the dura marter (Paxinos and Franklin, 2001). One microliter of either rAAV-TU3A or rAAV-eGFP (titers of 1.3×10^{12} genomic particles/ml) was bilaterally injected at the level of the dorsal hippocampus at 0.06 µl per minute by glass capillaries with tip resistance of 2 - 4 MΩ. Glass capillaries were removed after 5 to 10 minutes following the injection, to avoid reflux. The wound was sewn up and medicated with iodine. Following surgery, mice were treated for 5 days with the analgetic agent Metacam (Boehringer Ingelheim Pharma, Germany) at the dosage of 0.5 mg/kg body weight via the drinking water.

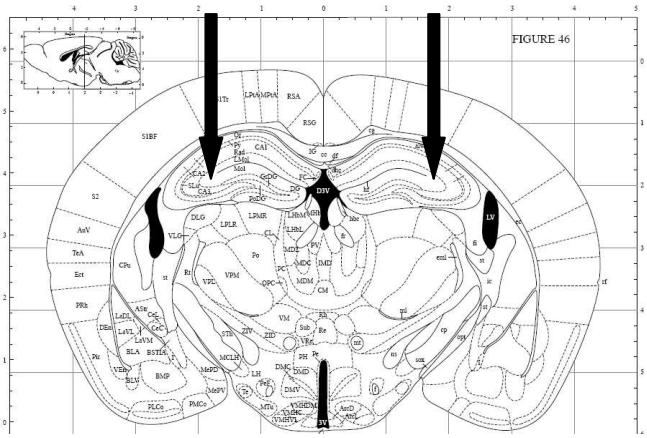

Figure 3.2 Schematic overview of the injection site

Black arrows represent the injection sites into the dorsal hippocampus, adapted from (Paxinos and Franklin, 2001).

3.2.13.3 Testing procedure

All animals were allowed a recovery phase of 3 to 4 weeks before the onset of testing to ensure recovery from the surgery and guarantee sufficient transgene expression. Animals were split into three different sets to conduct different test batteries (n = 15 per group). Behavioral tests within the test batteries were arranged beginning with the least to the most stressful test. Appropriate recovery periods between tests were included to minimize confounding variables (Crawley, 2008).

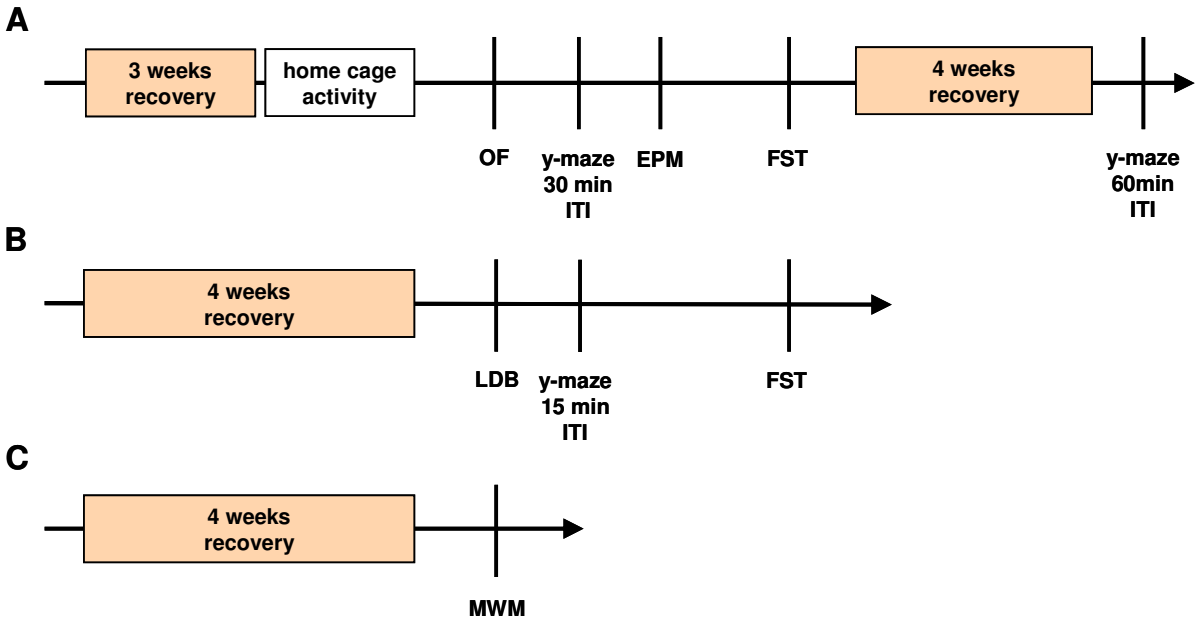

Figure 3.3 Timetable of behavioral testing

*(**A**) Shows the test battery for the first set of TU3A overexpressing animals and control group. (**B**) Represents the test battery for a second set of animals. (**C**) Shows the testing setup for the third set of animals. (OF) open field test, inter-trial interval (ITI), elevated plus-maze (EPM), forced swimming test (FST), light-dark box test (LDB), morris water maze test (MWM).*

In one set of animals, home cage activity was measured over the course of 1 week. Open field testing, y-maze testing (30 minutes inter-trial interval) and plus-maze testing was conducted on consecutive days. After a short recovery period, the forced swimming test was performed and tail blood samples were taken 30 and 60 minutes after testing, basal blood samples were taken after 1 week of undisturbed recovery. Another y-maze test was conducted with a 60 minutes inter-trial interval after 4 weeks of recovery in the same set of animals. A second set of animals undertook light-dark box testing followed by y-maze testing with a 15 minutes inter-trial interval. The Morris water maze test was performed with a third set of animals (see Figure 3.3). A detailed description of the conducted behavioral tests can be found in chapter 3.5. Animals were sacrificed under basal conditions and perfused (see chapter 3.3) for immunochemical confirmation of TU3A overexpression (see chapter 3.6.2).

3.3 Sampling procedure

The time between the first disturbance of the animals and the sampling procedure was in all cases less than 1 minute. Adult animals were anesthetized with isoflurane (Abbott GmbH &

Co. KG, Germany) and decapitated, neonatal animals were quickly decapitated without anesthesia. Trunk blood from all animals was collected individually in labeled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). In case of repeated blood sampling, tail blood was collected in EDTA coated 50 µl capillary tubes without anesthesia by inflicting a small incision at the bottom of the tail using a razor blade (Fluttert et al., 2000). All blood samples were kept on ice and were later centrifuged for 15 minutes at 6,000 rpm at 4 °C. Plasma was transferred to clean, labeled 1.5 ml microcentrifuge tubes. All plasma samples were stored frozen at – 20 ^{o}C until the determination of plasma corticosterone levels. Corticosterone was measured using a standard radio immuno assay kit (see chapter 3.6.3).

After decapitation, brains of adult animals were dissected from the skull. In case of neonatal animals until postnatal day 12, whole heads (without skin and jaw) were removed. Brains were frozen in isopropane (Carl Roth GmbH, Germany) at - 40 °C and stored at - 80 °C for *in situ* hybridization (see chapter 3.6.1). For immunohistochemistry (see chapter 3.6.2), animals were deeply anesthetized by intraperitoneal injection of ketamin/rompun (Sigma-Aldrich, Germany) and slowly perfused intracardially with 0.9 % saline followed by 4 % paraformaldehyde. Brains were post-fixed overnight in 4 % paraformaldehyde followed by overnight incubation in 20 % sucrose solution at 4 °C and then stored at – 80 °C.

3.4 Stress paradigms

3.4.1 Maternal separation paradigm

Maternal separation took place in a separate room in the animal facility under similar light and temperature conditions as mentioned previously. The initiation day of maternal separation for all experiments was postnatal day 8. If a nest was assigned to maternal separation, mothers were removed from their home cages between 8 a.m. and 10 a.m. The home cage containing the litter and familiar bedding was then placed on a heating pad and maintained at 30 – 33 ^{o}C. Neither food, nor water was available during the separation period. Maternal separation lasted for 24 hours (Schmidt et al., 2002b).

3.4.2 Food deprivation paradigm

Food deprivation presents a very strong stressor for small rodents (Kiss et al., 1994). During this stress paradigm the animals had no access to food for 24 hours. Starting between 9 a.m. and 12 a.m., the standard food was completely removed for 24 hours from the animals and the cages were changed to avoid potential remaining food leftovers in the bedding. The animals had perpetuating access to water during the food deprivation period. The animals of the control group had access to food and water *ad libitum*.

3.4.3 Restraint stress paradigm

Restraint stress presents an inescapable moderately stressful situation for the animals (Jorgensen et al., 1998). A single episode of 30 minutes restraint stress was applied in this stress paradigm. Therefore, mice were placed in a 50 ml Falcon tube, which permitted only space for minimal movements. The Falcon tube was perforated for sufficient ventilation.

3.4.4 Chronic social stress paradigm

The chronic social stress paradigm is based on confronting the animals with a highly unstable and unpredictable social environment during the adolescent period and young adulthood of mice (Schmidt et al., 2007). Male mice CD1 at the age of 26-28 days were used for this study. After a habituation period of 5 days following arrival, the group composition in each cage was changed twice per week for 7 weeks, so that each time four mice from different cages were put together in a new cage. Control mice remained with the same cage mates throughout the whole experiment. Animals were sacrificed directly after cessation of the stress procedure, i.e. 4 days following the last change of the group composition.

3.5 Behavioral test paradigms

All behavioral testing took place in the same room, where the animals were habituated for at least 1 week. All tests were performed during the light phase, between 7 a.m. and 12 a.m. to avoid potential behavioral alterations due to circadian variation of corticosterone levels (Barriga et al., 2001). Recording, tracking and scoring of animal behavior was carried out by the automated video tracking system ANY-maze (ANY-maze 4.5; Stoelting Co., Wood Dale, USA). In case of test settings, where automated scoring was not possible, scoring was performed by an unbiased person, blind to the experimental groups. Between every test,

each applied apparatus was thoroughly cleaned with tap water to exclude olfactory cues as orientation markers.

3.5.1 Home cage activity measurement

General activity including locomotion is a potentially confounding component of murine behavioral performance in numerous test paradigms (Hinojosa et al., 2006). Pre-existing alterations in basal locomotion between two experimental groups are very likely to influence the outcome of anxiety-related, emotional and learning test paradigms. Therefore, basal locomotion was measured under stress-free conditions in a familiar environment, using a home cage activity counter (Ganea et al., 2007). The system consisted of a tilting platform (4 mm x 170 mm x 250 mm) on which the empty home cage was orientated in balance. Each time the animal crossed the centerline of the cage, the platform tipped slightly to the other side. Thereby an electronic switch underneath the platform was activated and tilting events were counted. The margin of the platform movement was reduced to a minimum, so that the animal was not disturbed in its behavior. Locomotion measurement started at 6 a.m. at the onset of the light phase, 12 hours later, at the beginning of the dark phase, the number of counts was recorded and the counter reset. At 6 a.m. the number of counts during the dark phase was recorded.

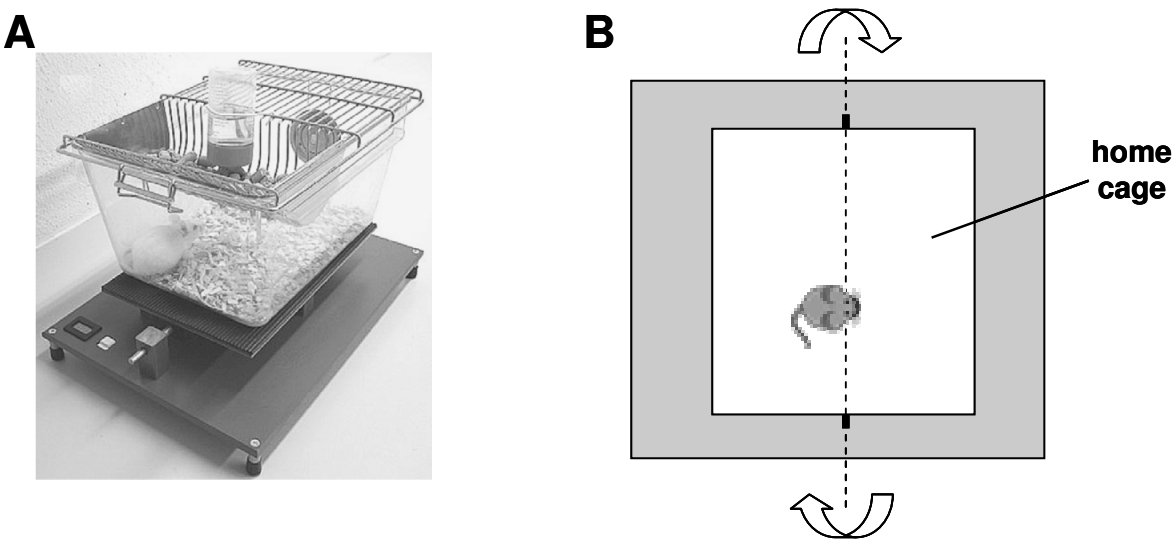

Figure 3.4 Home cage activity measurement

*(**A**) Experimental setup, from Ganea et al., 2007. (**B**) Schematic overview of the apparatus. Crossing of the centerline (dashed line) results in tilting of the platform and recording of this event.*

3.5.2 Open field test

The open field test, originally developed by Hall for rats (Hall, 1934), exploits the conflict between the innate fear that rodents have of the unfamiliar exposed central area of an open field arena versus their natural desire to explore new environments (Choleris et al., 2001). The open field test allowed the characterization of locomotor activity in a novel environment, reflected in the distance traveled and the time of immobility. Additionally, anxiety-related behavior could be examined by the time spend in the exposed center of the apparatus. The testing apparatus consisted of a square, enclosed arena (500 mm x 500 mm x 400 mm) made of gray PVC. For analysis, the area was virtually divided into two distinct sub-fields: a center zone (200 mm x 200 mm) which was illuminated with 20 lux and an outer zone along the walls illuminated with approximately 16 lux. Animals were placed in a corner of the apparatus at the beginning of the testing. Test duration was 15 minutes in total, which was divided into three segments of 5 minutes for a more detailed analysis (Gershenfeld et al., 1997).

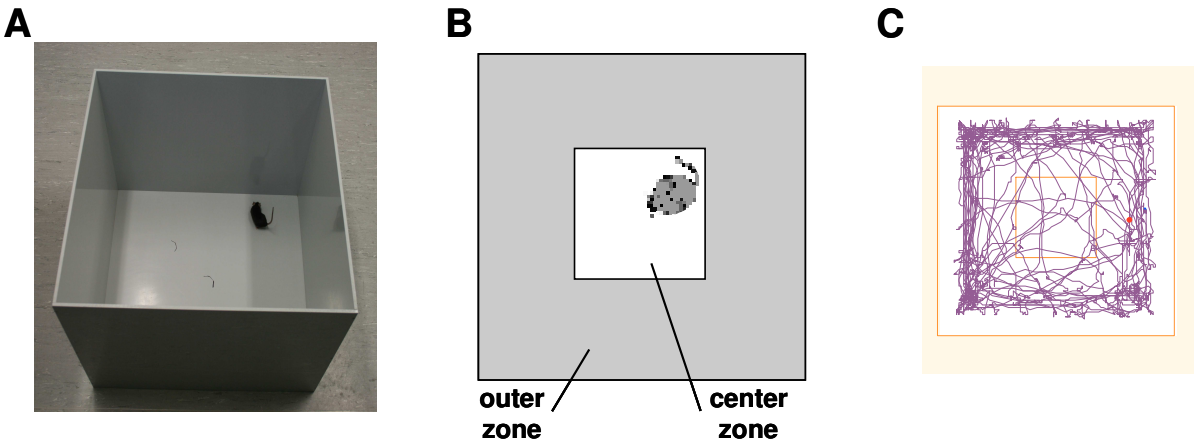

Figure 3.5 Open field test

*(**A**) Experimental setup. (**B**) Schematic overview of the open field apparatus. Gray area represents the outer zone and white area the center zone. (**C**) Representative tracking plot of a 15 minutes open field test. Violet lines display the movement of the animal.*

3.5.3 Light-dark box test

The light-dark behavioral test is based on the natural aversion of mice to brightly illuminated areas in conflict with their spontaneous exploratory behavior in response to a novel

environment (Crawley and Goodwin, 1980; Bourin and Hascoet, 2003). The apparatus was composed of a small, secure and dark compartment (150 mm x 200 mm x 250 mm, dimly lit under 10 lux), connected by a 40 mm long tunnel to a larger, aversive and brightly illuminated compartment (300 mm x 200 mm x 250 mm, lit with 600 lux). At the beginning of a 5 minutes testing phase, the animals were placed in the secure dark compartment facing towards a wall. Entries to a different compartment were defined when both front paws and shoulders were located in the novel compartment. To assess anxiety-related behavior, the time spent in each compartment, the number of entries and the latency of the first entry into the light compartment were measured (Müller et al., 2003).

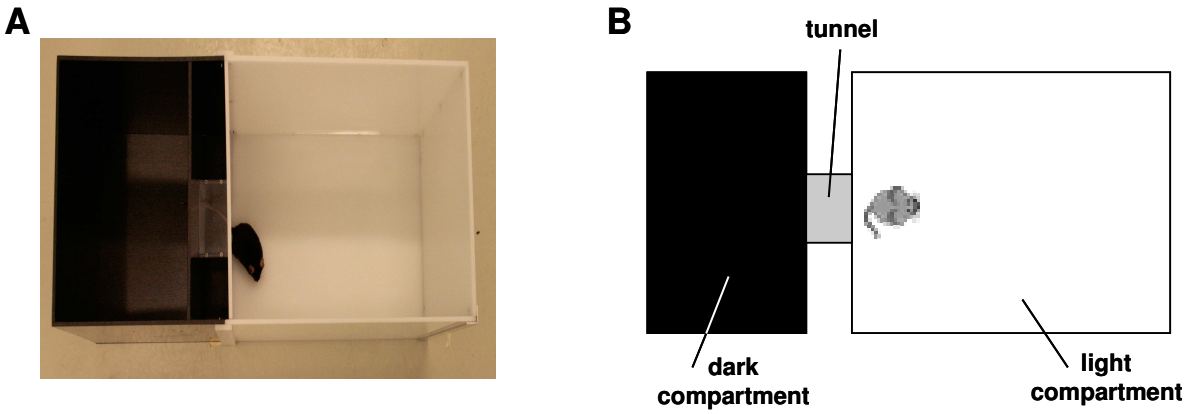

Figure 3.6 Light-dark box test

(A) Experimental setup. (B) Schematic overview of the apparatus. The dark compartment is illustrated in black, the tunnel in gray and the light compartment in white.

3.5.4 Elevated plus-maze test

This well-established test to assess anxiety-related behavior was originally developed by Handley and Mithani in 1984 and is based on rodents propensity towards dark, enclosed areas and their innate fear of elevated, open spaces (Handley and Mithani, 1984). Thereby, the open and closed elevated arms induce an approach–avoidance conflict (Carobrez and Bertoglio, 2005; Roy et al., 2009).

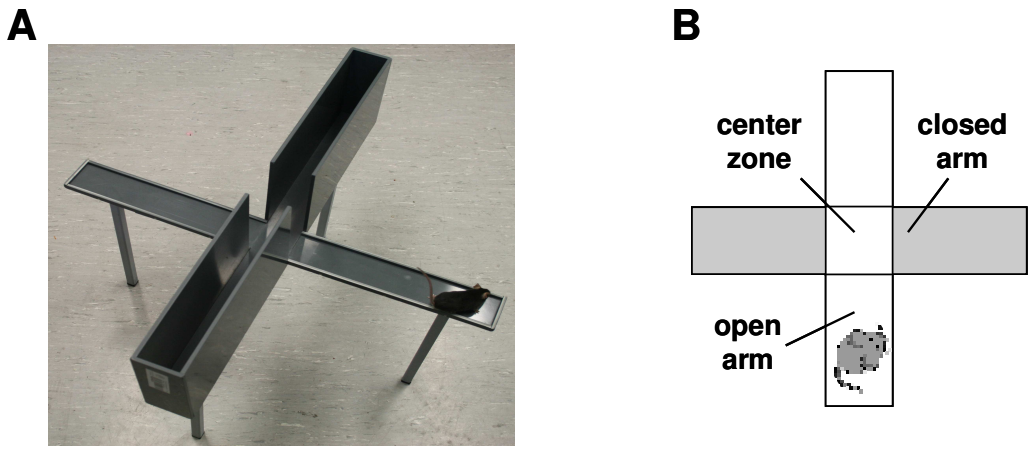

Figure 3.7 Elevated plus-maze

*(**A**) Experimental setup. (**B**) Schematic overview of the apparatus. Gray marked areas represent the closed arms and white area shows the open arms with the center zone in the middle.*

The elevated plus-maze, made of gray PVC, consisted of two opposed open arms (300 mm x 50 mm x 5 mm, each) and two opposed enclosed arms (300 x 50 x 5 mm, each). The arms were connected by a central platform (50 mm x 50 mm), thereby forming a plus sign. The apparatus was elevated approximately 500 mm above the floor. Mice were placed in the center of the apparatus, facing towards one of the closed arms and were allowed to explore for 5 minutes. To examine anxiety-related behavior the percentage of time spent in the open and closed arms were measured.

3.5.5 Y-maze test

The Y-maze test is based on the animals' innate curiosity to explore novel environments. When applied in combination with a two-trial task, hippocampus depended spatial memory can be assessed (Dellu et al., 1992; Conrad et al., 1996). The apparatus consisted of three arms (300 mm x 100 mm x 150 mm), made of gray PVC, which were joined in an angle of 120 ° between two arms, thereby creating three separate zones and a center zone where the arms were joined. The whole apparatus was evenly illuminated with less than 50 lux to avoid preferences based on illumination levels. Each arm was distinctly marked by easily recognizable symbols (triangle, bar and plus-sign). The testing comprised two different trials. During the first trial, referred to as the acquisition phase, one arm was completely blocked by an opaque gray PVC wall. The mouse was placed in the center zone and was allowed to explore the two accessible arms freely for 10 minutes before returning to the home cage.

After intertrial intervals of 15, 30 or 60 minutes mice were re-introduced to the apparatus facing one of the already known arms. During this retrieval phase (5 minutes), all three arms were accessible. Percentage of time spent in the novel arm compared to the familiar arms was used as an indicator for spatial memory performance. Significantly higher percentage than chance level (33.3 %) was rated as successful spatial memory. In case the test was repeated within the same set of animals, the symbols were modified.

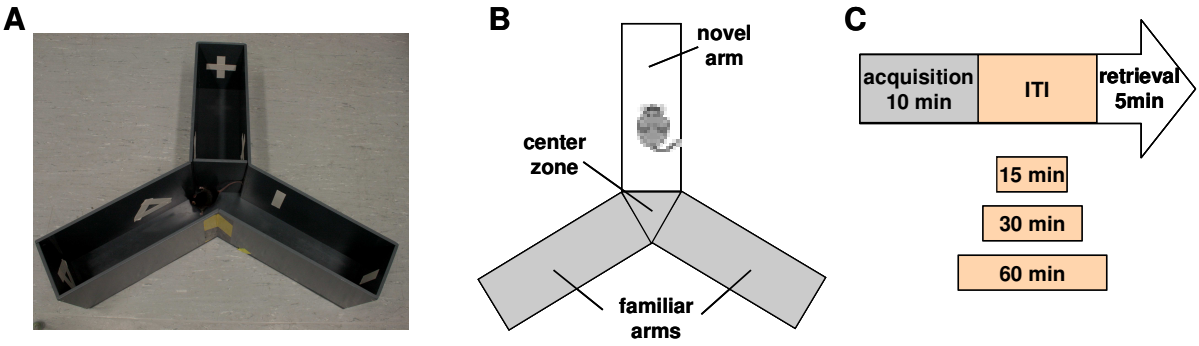

Figure 3.8 Y-maze test

*(**A**) Experimental setup. (**B**) Schematic overview of the Y-maze apparatus, familiar arms (open for exploration during the acquisition phase) are marked in gray, the novel arm (open during retrieval phase) is marked in white. The triangular area represents the center zone. (**C**) Timetable of the Y-maze test procedure, different groups were tested with varied inter-trial intervals (ITI) between acquisition and retrieval phase.*

3.5.6 Morris water maze test

The Morris water maze (MWM), originally developed in 1984 by Morris and colleagues for rats (Morris, 1984), is a frequently used behavioral test paradigm to assess spatial learning and memory (D'Hooge and De Deyn, 2001). To escape the water, which is an aversive environment for mice, the animals have to locate a hidden platform, 9 cm in diameter, using distal spatial cues. The water maze consisted of a circular pool 1 m in diameter filled with 21 ± 1 °C warm water, which was made opaque by addition of chalk. Several clearly visible cues were applied in 50-100 cm distance from the pool to allow spatial orientation. The area of the pool was virtually divided into four quadrants (back left, back right, front left and front right).

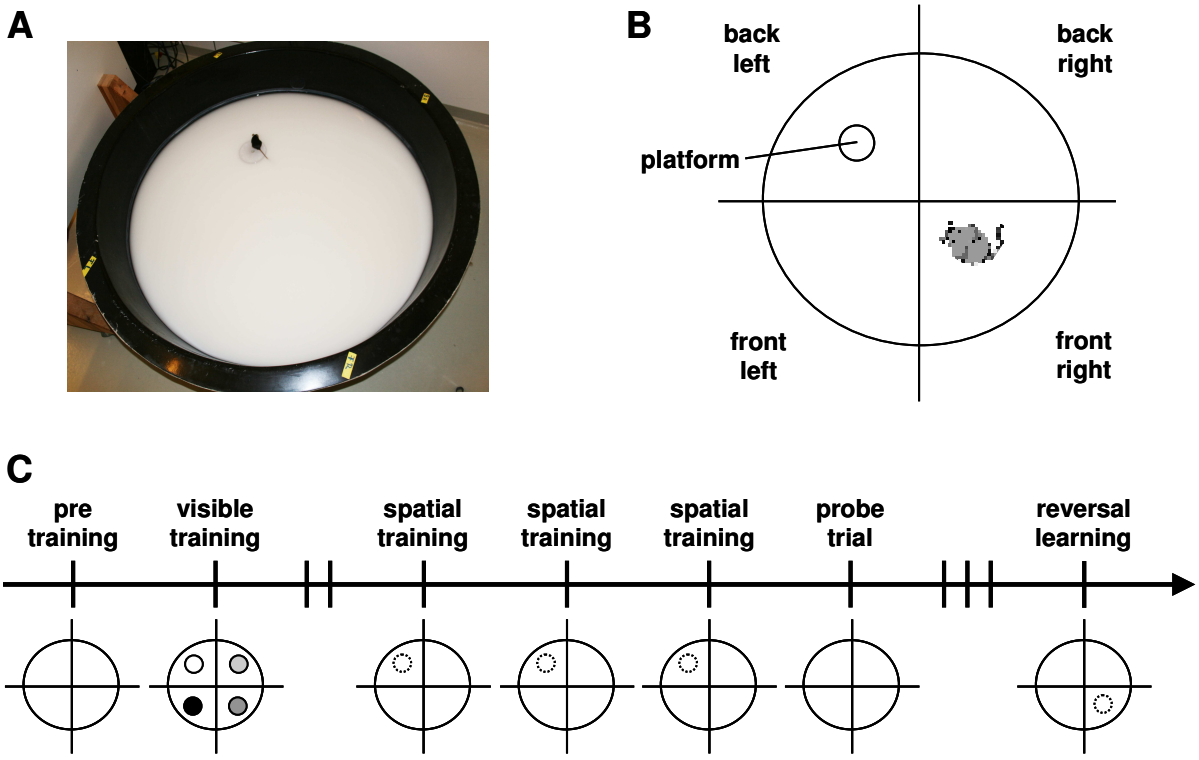

Figure 3.9 Morris water maze

*(**A**) Experimental setup. (**B**) Schematic overview of the apparatus segmented into the four different quadrants. (**C**) Timetable of the MWM test procedure. One tick mark on the time axis represents 1 day. Differently colored circles are indicating the different platform positions, while dashed circles represent the submerged platform.*

Each swimming trial lasted 60 seconds. In case of repeated trials, the inter-trial interval was 10 minutes. After each trial, mice were gently dried and placed into their home cage under red light lamps to avoid hypothermia. The first pre-training day comprised one free swimming trial, where the platform was absent. Mice grew accustomed to the swimming task and potential preferences for specific quadrants could be excluded for later testing. At the second day during the visible training trials, the mice were trained in the concept of finding the apparent platform (platform is visibly elevated 1 cm above the surface of the water). The visible training task consisted of four consecutive trials. The platform was located at a different quadrant for each trial and the starting quadrants of the animals were varying accordingly. The animals were immediately rescued after reaching the platform. If a subject did not reach the platform during the trial, the mouse was placed at the platform for a few seconds before rescued. Following a recovery phase of 2 days, the mice conducted 3 sequential days of spatial training with four trials per day, where the platform was

submerged 1 cm below the surface of the water. During this phase, the platform remained in the same quadrant while the animals started from varying positions. Spatial learning and memory performance was measured as latency to reach the platform. At the subsequent day the platform was removed to conduct a probe trial. Reference memory performance could be determined by the time, the animals spent in the quadrant, where the platform is positioned during spatial training. After 3 days of recovery the platform was positioned in the opposite quadrant, compared to the spatial training, for the reversal learning task. During four trials, the animals' cognitive flexibility to learn a new platform position was tested (Clapcote and Roder, 2004).

3.5.7 Forced swimming test

The forced swimming test (FST) was originally introduced by Porsolt in 1977 as a behavioral method to induce "behavioral despair" in rodents. Antidepressants, as well as other clinical therapies for depression, e.g. electroconvulsive shock, are able to induce a more active coping strategy during testing (Porsolt et al., 1977; Porsolt et al., 1978). Up to day, it is the most widely used test paradigm for screening antidepressant action of compounds as well as depression-like or emotional behavior in different mouse models (Cryan et al., 2004; Petit-Demouliere et al., 2005; Lee et al., 2009b).

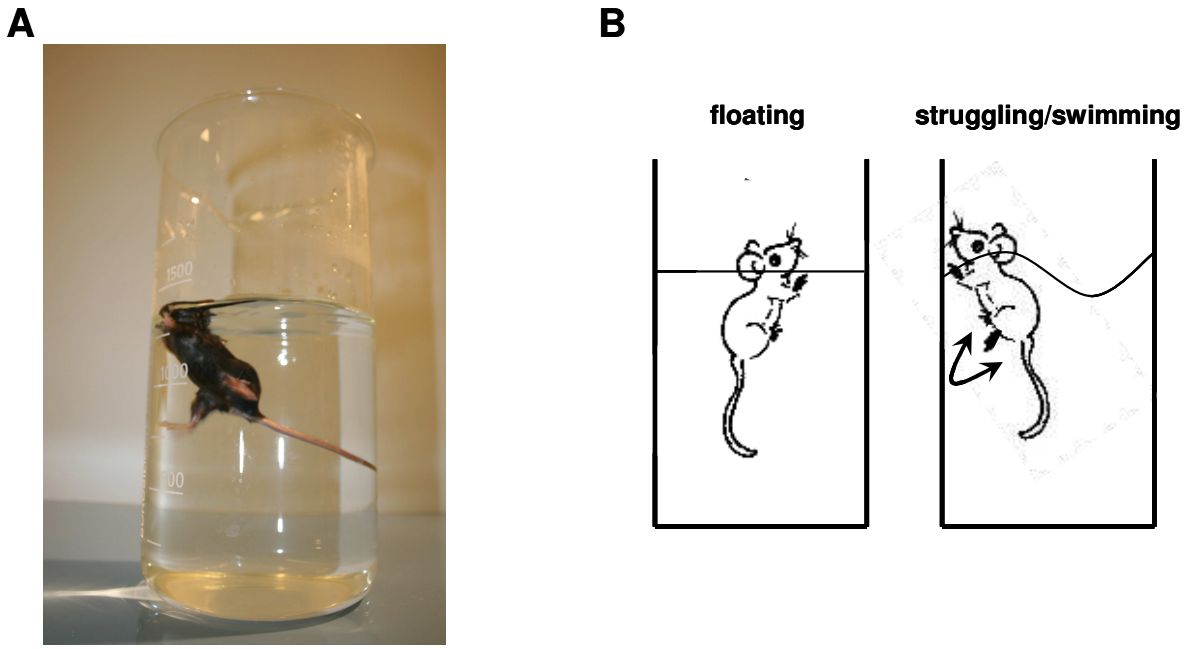

Figure 3.10 Forced swimming test

(A) Experimental setup. (B) Schematic overview of the different behavioral parameters. Floating represents a passive coping strategy with minimal movement while struggling and swimming are indicators of a active stress coping strategy.

The animals were gently placed in a glass beaker (height 240 mm, diameter 130 mm) filled with 21 ± 1°C water up to a height of 15 cm. Thereby the animal was not able to sense the bottom of the glass with its tail or paws and could not escape the aversive situation. After a five minutes testing period, the animals were removed from the water, dried and placed back into their home cage. The FST presents a strong stressor for mice. In the first minutes of testing the animals generally exhibit an active, escape oriented behavior (struggling or swimming). After a while, the mice show increased immobility (floating), reflecting despair (Sillaber et al., 2008). Thereby, the duration of active versus passive behavior served as an indicator for the animals coping strategy in an adversive situation.

3.6 Molecular methods

3.6.1 In situ hybridization

TU3A mRNA expression was assessed using *in situ* hybridization. The *in situ* hybridization technique is commonly used to detect gene expression semi-quantitatively in brain slices utilizing labeled antisense ribonucleotide probes, which are complementary to the investigated mRNA sequence. The applied cRNA probe for TU3A contains 486 base pairs covering exons 1 to 4 of the gene. The antisense ribonucleotide probe was designed using the Primer3 software (Rozen and Skaletsky, 2000) with GTGGAGGGAAGGAGAAGGAC as forward primer and TCCCGGACTTTGATGAACTC as reverse primer. The TU3A cDNA template was generated by polymerase chain reaction (PCR) amplification from mouse hippocampal tissue and subsequent cloned into a pCR® II-TOPO® vector (Invitrogen, USA) according to manufacturers' instructions and afterwards linearized using XhoI and SacI restriction enzymes. The template was sequenced (Sequiserve, Germany) and the correct sequence confirmed by NCBI Blast (Zhang et al., 2000). A corresponding sense probe was designed similarly as a negative control. Ribonucleotide probes were labeled with ^{35}S-UTP (Hartmann Analytic, Germany) by *in vitro* transcription using the sp6 RNA polymerase and tested on mouse brain tissue. Absence of expression after hybridization with radioactive labelled sense probe confirmed the specifity of the antisense probe signal.

Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm. The sections were thaw-mounted on superfrost plus slides (Menzel, Germany), dried and kept at -80 °C. *In situ* hybridization was performed as described previously (Schmidt et al., 2002;

Schmidt et al., 2007). Briefly, for pre-hybridization all solutions were prepared with DEPC H_2O (1% DEPC in H_2Odest.). Sections were post-fixed in 4 % paraformaldehyde in PBS on ice (in case of neonatal tissue 0.5 % glutaraldehyde was added), and acetylated using 0.25 % acetic anhydride in 0.1 M TEA with 0.36 % HCl. For neonatal tissue, a second acetylation step with 0.25 % acetic anhydride in 0.1 M TEA was performed. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. Tissue sections (four brain sections per slide) were saturated with 100 µl of hybridization buffer (50% deionized formamide, 20 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA (pH 8.0), 10% dextran sulfate, 0.5 mg/ml tRNA, 0.2 mg/ml fragmented herring sperm DNA, 1 % Sodiumthiosulfat, 1 % SDS, 0.01% Denhardt's medium) containing approximately 2x 10^6 cpm ^{35}S-UTP labelled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. For post-hybridization, the sections were rinsed in 2x SSC, treated with RNase A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1x SSC for 1 hour at 65 °C and dehydrated through increasing concentrations of alcohol.

For data analysis sections were exposed to Kodak BioMax MR films (Eastman Kodak Co., USA) for 1 to 8 days. Films were fixed and developed, subsequently digitalized and the radiation-induced blackening was measured utilizing ImageJ (ImageJ 1.42, National Institutes of Health, USA). Analysis was carried out semi-quantitatively by the means of relative gray intensities as a measure of relative mRNA expression. Every region was at least measured twice and unspecific background staining measured at the slide was subtracted. In case of a cell specific mRNA expression analysis, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., USA) and exposed at 4 °C for approximately 3 days. Slides were developed and counterstained with cresyl violet to identify different brain regions. Gene expression was represented by precipitated silver particles. Slides were examined with a bright-field microscope and photomicrographs taken with Axiocam and Axiovision software (Carl Zeiss, Germany).

3.6.2 Immunohistochemistry

Immunohistochemistry was used to detect the TU3A protein distribution and its cellular location in tissue sections. This technique is based on introducing antibodies, that are binding highly specifically to the antigens of interest. The antibody-antigen binding results either directly in a coloring reaction or indirectly by employing a second antibody. The indirect approach, used in the following protocol, presents a signal amplification step and

leads thereby to increased signal sensitivity. The second antibody specifically recognizes epitopes of the host species of the first antibody and is either directly linked to a fluorochrome (e.g. immunofluorescence) or conjugated to a small molecule triggering enzyme catalyzed chromogene precipitation (e.g. immunoperoxidase) (Coons and Kaplan, 1950; Hsu et al., 1981).

To asses TU3A protein expression, a polyclonal antibody against TU3A was raised in rabbits, since there was no antibody commercially available. TU3A was equipped with a N-terminal poly-histidine sequence and a C-terminal FLAG sequence and purified by affinity chromatography. Vaccination and purification was done by Biogenes (Biogenes, Germany). Antibody specificity was verified using immunoblot analysis (see chapter 3.6.4), where the antibody recognized in mouse brain lysate a protein the size of 17.5 kDa, equivalent to the predicted size of TU3A. Additionally, in cell culture experiments the antibody detected specifically cells overexpressing TU3A. The creation of recombinant TU3A, purification and specifity verification was done by Jan Schülke (Schülke JP, 2009).

For immunohistochemistry, serial free-floating sections of 30 µm were cut at -20 °C in a cryostat microtome and kept in cryoprotection solution (25 % glycerol, 25 % ethylene glycol, 50% PBS) at -20 °C until immunofluorescence or immunoperoxidase staining was performed. All of the following steps were interposed by repeated washes in PBS and all reagents were diluted in PBS unless otherwise specified. For immunofluorescence, slices were incubated in 0.2 % Triton-X 100, followed by 1 hour blocking in 5 % normal goat serum at room temperature (RT) . Incubation with the polyclonal rabbit-anti-TU3A (1:200) and chicken-anti-GFP (1:1000, Abcam, UK) antibodies was performed overnight at 4 °C, followed by 1 hour incubation at 4 °C in the dark with the secondary antibodies Alexa Fluor 555 anti-rabbit and Alexa Fluor 488 anti-chicken (1:200, Invitrogen, Germany). Sections were rinsed in distilled water, mounted on superfrost plus slides and covered with Vectashield mounting medium (Vector Laboratories, Burlingame, USA) containing DAPI for counterstaining.

For immunoperoxidase staining the VECTASTAIN ABC kit (Vector Laboratories, USA) was used according to manufacturers' instructions. Briefly, pre-incubation in 0.6 % H_2O_2 was followed by 0.2 % Triton X-100 treatment and blocking for 1 hour in 5 % normal goat serum at RT. Incubation with the polyclonal rabbit-anti-TU3A (1:200) was administrated overnight at 4 °C followed by incubation with the biotynilated secondary goat-anti-rabbit (1:300) antibody. After 1 hour incubation of the avidin-biotin complex, diamino benzidine (DAB)

(Sigma-Aldrich, Germany) was applied. Staining duration was designated according to signal intensities. Sections were rinsed in distilled water, mounted on superfrost plus slides and covered with Distrene-80 Plasticizer Xylene (DPX) (BDH Laboratory Supplies, UK).

3.6.3 Radioimmunoassay

Plasma corticosterone levels were measured by radioimmunoassay (RIA) using a commercially available double antibody kit (ImmunoChemTM Double Antibody Corticosterone 125I RIA Kit, MP Biomedicals, USA) with a sensitivity of 6.25 ng/ml. The RIA represents a competitive binding assay utilising the fact, that endogenous corticosterone and radioactive (^{125}I) labelled corticosterone compete for binding to a limited amount of anti-corticosterone antibody. High corticosterone concentration in the sample results in lower binding of a defined amount of labelled corticosterone to the antibody. As a consequence, the magnitude of labelled antibody-corticosterone-complex is inverted proportional to the amount of endogenous corticosterone. Separation of antibody-bound corticosterone and free, labelled corticosterone is done by addition of a second antibody specific for the first antibody followed by centrifugation. The unbound corticosterone fraction is discarded and the radioactivity of the precipitated double antibody-corticosterone complex is quantified by a Gamma-counter (COBRAII AutoGamma, Canberra-Packard GmbH, Germany). The resulting value is compared to a standard curve of known corticosterone concentrations, thereby revealing the absolute concentration of corticosterone in the sample (Shimizu et al., 1983). Analysis was performed according to the manufacturers' instructions. 10 µl of each plasma sample was used either in 1:100 or 1:200 dilutions, depending on the expected value of the sample to obtain results in the linear span of the standard curve.

3.6.4 Western blot

The western blot or immunoblot technique allows detection and quantification of specific proteins from tissue homogenate with antibodies (Burnette, 1981). Brain samples were homogenized in 300 µl lysis buffer (50 mM Tris/HCl (pH 7.5), 250 mM Sucrose, 1 mM EDTA, 1:100 protease inhibitor mixture P2714 from Sigma-Aldrich) per 5 mg tissue using a tissue homogenizer (VWR International, USA). Samples were centrifuged for 10 minutes at 8000 rpm followed by 10 minutes centrifugation at 7,000 rpm to separate non-disintegrated tissue, mitochondrial material and lysosomes. The supernatant contained the total protein

fraction and was stored at –20 °C for further processing. Protein concentration was photometrically determined with the BCA protein assay (Thermo Scientific Pierce, USA) according to manufacturers' instructions.

Proteins were separated by their molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Per lane, 25 µg protein were loaded on to a 14 % SDS-PAGE gel. The negatively charged SDS attaches to the proteins and leads to their movement towards the positively charged electrode through the acrylamide mesh of the gel. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schüll, Germany) and detected by means of chemiluminescence. Briefly, nonspecific binding to the membrane was blocked by 5 % nonfat milk in TBST buffer followed by incubation over night at 4 °C with the polyclonal rabbit-anti-TU3A antibody (1:2,000) and a polyclonal goat-anti-actin antibody (1:5,000). After several washing steps with TBST, incubation with the secondary donkey anti-rabbit antibody (1:10,000) for TU3A and donkey-anti-goat antibody (1:2,000) (Amersham Biosciences, USA) conjugated to horseradish peroxidase was done at RT. Signals were visualized applying the ECL system (Amersham Biosciences, USA) according to manufacturers instructions. For data analysis, the membrane was exposed to Hyperfilm-ECL (Amersham Biosciences, USA), signals were digitalized with a densitometer (Biorad, Germany) and band intensity determined with Kodak 1D Image analysis software. The data was normalized to the actin concentration and expressed as fold regulation compared to the control group. The western blots were performed by Jan Schülke.

3.7 Statistical analysis

All data were statistically analyzed using SPSS 16.0 (SPSS Inc., USA). The data were depicted as means + SEM (standard error of the mean). The level of significance was set at $p < 0.05$. Simple group comparisons were performed using the two-tailed unpaired t-test. All data concerning more than two groups were tested by the appropriate analysis of variance (ANOVA) model followed by Bonferroni post-hoc analysis. In case of three group comparism one-way ANOVA was applied. When more than two groups and additional variables were tested, a two-way ANOVA was conducted. In the event of multiple time points a repeated-measures ANOVA was performed.

4 Results

4.1 Neuroanatomical distribution of TU3A in the mouse brain

To assess the neuroanatomical distribution of TU3A in the brain, mRNA expression analysis in the neonatal and adult brain was carried out; additionally TU3A protein expression was examined in the adult brain. Furthermore, to estimate potential expression differences due to age, TU3A mRNA expression was compared at different postnatal developmental stages as well as into adulthood in brain regions relevant for central regulation of HPA axis function.

4.1.1 TU3A mRNA expression in the neonatal mouse brain on postnatal day 9

In the neonatal brain a moderate basal TU3A mRNA expression was present in almost all areas of the *prosencephalon*, except in the thalamus, where only very low TU3A expression was observed. However, TU3A expression was very prominent in the *telencephalon* and the *diencephalic* hypothalamus. In the *telencephalon,* TU3A was most prominently expressed in the cortex throughout all six horizontal cortical layers with a higher expression in layer IV (the inner granule cell layer) and the caudate putamen (Figure 4.1 A).

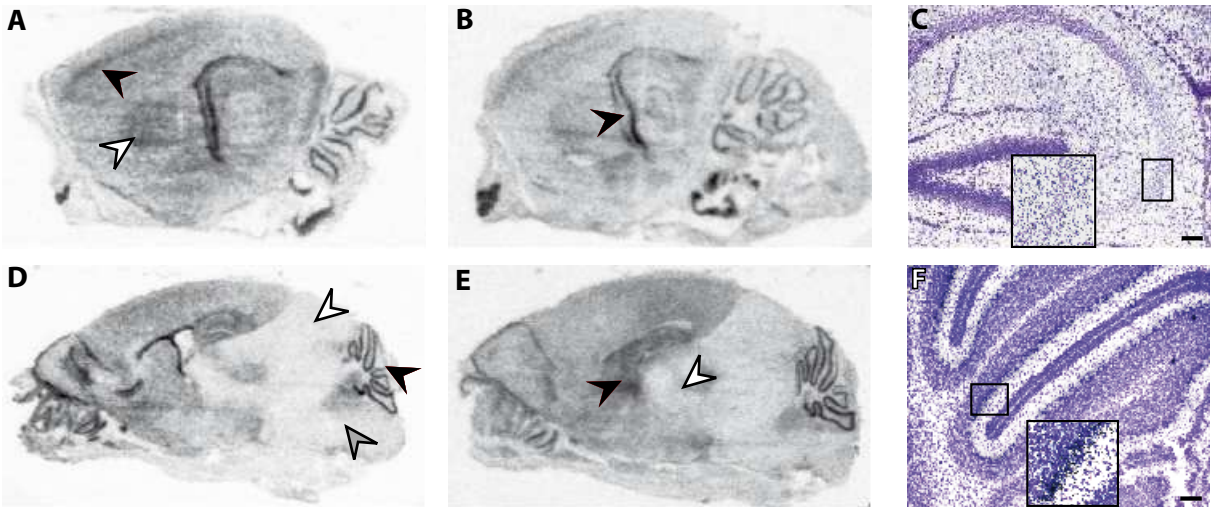

Figure 4.1 Representative TU3A mRNA expression in sagittal sections of the neonatal mouse brain

*(**A**) Autoradiograph of sagittal section cut approximately 3.12 mm lateral of the midline. Pronounced TU3A mRNA expression is visible in layer IV of the cortex (black arrowhead), and in the caudate putamen (white arrowhead). (**B**) Autoradiograph of sagittal section cut approximately 2.87 mm lateral of the midline showing strong TU3A expression in the hippocampal CA3 region (black arrowhead). (**C**) Bright field photomicrograph of sagittal hippocampal section*

*counterstained with cresyl violet. TU3A mRNA expression is represented by black silver grains. The black box indicates the magnified CA3 region with strong TU3A mRNA expression in the pyramidal cell layer. (**D**) Autoradiograph of sagittal section approximately 1.87 mm lateral of the midline. Most parts of mesencephalon (white arrowhead) and the rhombencephalic pons (grey arrowhead) express virtually no TU3A mRNA. A pronounced expression is present in the cerebellum (black arrowhead). (**E**) Autoradiograph of sagittal section cut approximately 1.25 mm lateral of the midline. Strong TU3A mRNA expression is visible in the septum (black arrowhead), while most of the thalamus (white arrowhead) shows little TU3A mRNA expression. (**F**) Bright field photomicrograph of sagittal cerebellar section counterstained with cresyl violet, TU3A mRNA is represented by black silver grains. The black box indicates the magnified part, showing strong TU3A mRNA expression in the molecular layer and the Purkinje cell layer. Scale bars are 100 µm.*

TU3A mRNA expression followed an anterior-posterior gradient with virtually no expression in the *mesencephalon* and the *rhombencephalic* pons and medulla oblongata at postnatal day 9 (Figure 4.1 D and E). The strongest TU3A mRNA expression in the postnatal brain could be observed in the Purkinje cell layer of the cerebellum (Figure 4.1 D and F), followed by the hippocampal CA3 region (Figure 4.1 B and C), the thalamic habenular nuclei and the lateral septum (Figure 4.1 E). White matter structures like the anterior commissure, the corpus callosum and the fornix expressed only very low TU3A mRNA levels.

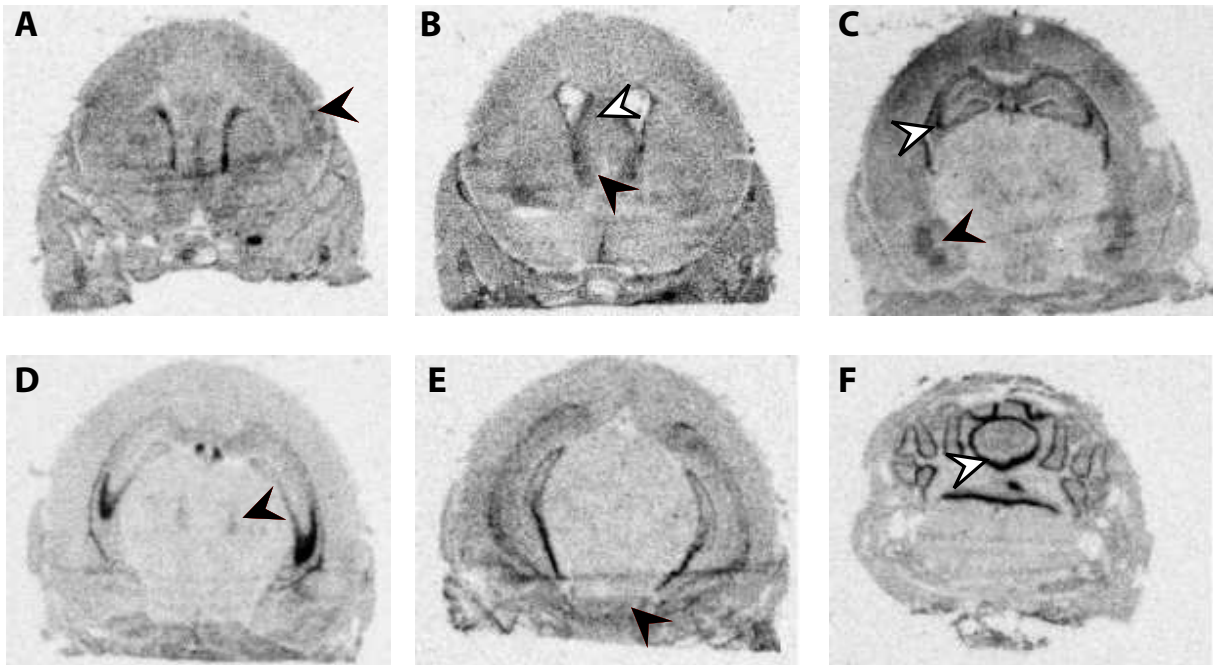

Figure 4.2 Representative TU3A mRNA expression in coronal sections of the neonatal mouse brain

*(**A**) Autoradiograph of coronal section cut approximately 1.20 mm anterior of bregma. Pronounced TU3A mRNA expression is visible in the horizontal layer IV of*

*the cortex (black arrowhead). (**B**) Autoradiograph of coronal section cut approximately 0.18 mm anterior of bregma. The dorsal part (white arrowhead) and the ventral part (black arrowhead) of the lateral septum are showing a pronounced TU3A mRNA expression. (**C**) Autoradiograph of coronal section cut approximately 1.70 mm posterior of bregma. Strong TU3A mRNA expression is visible in the amygdala (black arrowhead) and the hippocampal CA3 region (white arrowhead). (**D**) Autoradiograph of coronal section cut approximately 1.82 mm posterior of bregma. TU3A shows distinct gene expression in the parafascicular thalamic nucleus (black arrowhead). (**E**) Autoradiograph of coronal section cut approximately 2.82 mm posterior of bregma. The mammillary nucleus (black arrowhead) shows a pronounced TU3A mRNA expression. (**F**) Autoradiograph of coronal section cut approximately 4.08 mm posterior of bregma. The Purkinje cell layer of the cerebellum (black arrowhead) shows the most pronounced TU3A mRNA expression in the neonatal brain.*

Coronal brain sections were used for a more detailed mRNA expression analysis throughout the forebrain and cerebellum. Several thalamic and hypothalamic nuclei were identified for a detailed list of all identified regions including relative expression intensities of TU3A mRNA expression in the *telencephalon* and the cerebellum see Table 4.1.

4.1.2 TU3A mRNA expression in the adult mouse brain

Corresponding to the neonatal findings, there was a basal expression of TU3A mRNA present throughout the *prosencephalon* in the adult brain with distinct differences in TU3A mRNA expression intensity in individual brain regions. Reflecting the findings in the neonatal brain, only very weak TU3A mRNA expression was visible in most parts of the *rhombencephalic* medulla oblongata and pons, the *mesencephalon* and most of the *diencephalic* thalamus. Compared to those regions, TU3A mRNA expression was more pronounced in the *diencephalic* hypothalamus as well as in most of the *telencephalon* (Figure 4.3 D and E).

Sagittal brain sections of adult mice revealed a strong TU3A mRNA expression throughout the cortex, especially in the subregion of the primary somatosensory cortex barrel field of the parietal cortex (Figure 4.3 A). In contrast to the TU3A mRNA expression in the neonatal brain there were no differences in TU3A mRNA expression apparent between the horizontal layers I, II, III, IV and VI of the cortex, however, the cortical layer V (the inner pyramidal layer) displayed reduced TU3A mRNA expression. The strongest TU3A mRNA expression in the adult brain was detected in the Purkinje cell layer of the cerebellum (Figure 4.3 C and D), the molecular layer of the cerebellum, the CA3 region of the hippocampus (Figure 4.3 B), the habenular nuclei (Figure 4.4 F) as well as in the lateral septum (Figure

4.3 E). In white matter structures like the anterior commissure (Figure 4.4 B), the corpus callosum and the fornix TU3A mRNA expression was very weak or completely absent. Compared to the neonatal brain, TU3A mRNA was less expressed in the adult brain in those structures.

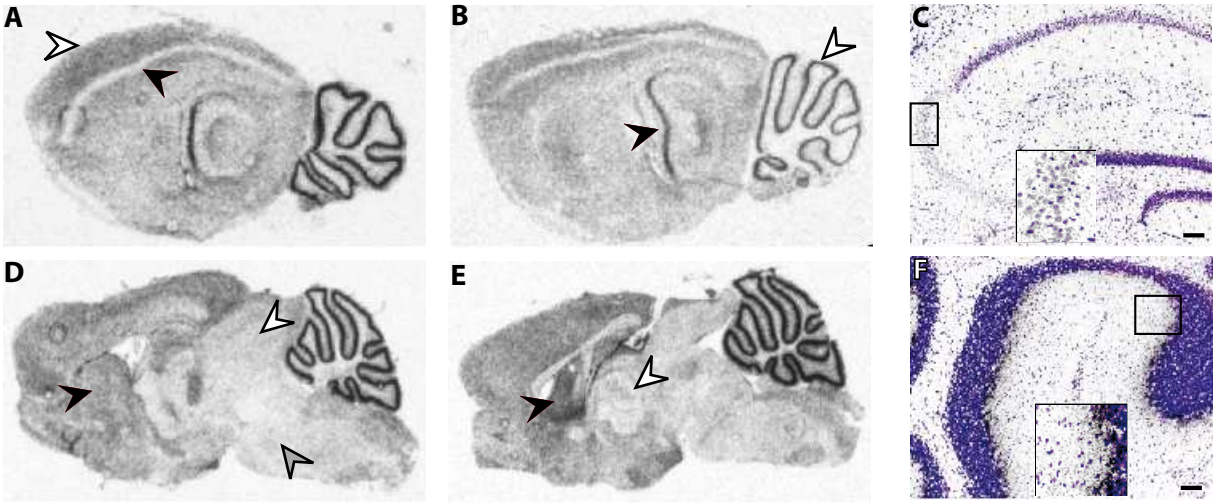

Figure 4.3 Representative TU3A mRNA expression in sagittal sections of the adult mouse brain

*(**A**) Autoradiograph of sagittal section cut approximately 3.25 mm lateral of the midline. Pronounced TU3A mRNA expression is visible in the barrel field region of the primary somatosensory cortex (white arrowhead), weak TU3A mRNA expression becomes apparent in the horizontal layer V of the cortex (black arrowhead). (**B**) Autoradiograph of sagittal section cut approximately 3.12 mm lateral of the midline, showing strong TU3A mRNA expression in the molecular layers and Purkinje cell layers of the cerebellum (white arrowhead) and pronounced TU3A mRNA expression in the hippocampal CA3 region (black arrowhead). (**C**) Bright field photomicrograph of sagittal hippocampal section counterstained with cresyl violet. TU3A mRNA expression is represented by black silver grains. The black box indicates the magnified CA3 region with strong TU3A mRNA expression. (**D**) Autoradiograph of sagittal section approximately 0.96 mm lateral of the midline. While most parts of the mesencephalon (white arrowhead) and the rhombencephalic pons (grey arrowhead) show only little TU3A mRNA expression, a much stronger expression is present in the telencephalon, e.g. in the caudate putamen (black arrowhead). (**E**) Autoradiograph of sagittal section cut approximately 0.24 mm lateral of the midline. Strong TU3A mRNA expression is visible in the septum (black arrowhead), while most of the thalamus (white arrowhead) shows little TU3A mRNA expression. (**F**) Bright field photomicrograph of sagittal cerebellar section counterstained with cresyl violet, TU3A mRNA is represented by silver grains. The black box indicates the magnified part, showing strong TU3A mRNA expression in the molecular layer and the Purkinje cell layer. Scale bars are 100 µm.*

The overall TU3A mRNA expression pattern of the adult brain was very similar to the pattern in the neonatal brain. Although, in some regions the relative TU3A expression intensity varied between the neonatal and the adult brain (for details see Table 4.1). This is most prominently apparent in the lateral septum (Figure 4.4 B) or the mammillary nuclei (Figure 4.4 H).

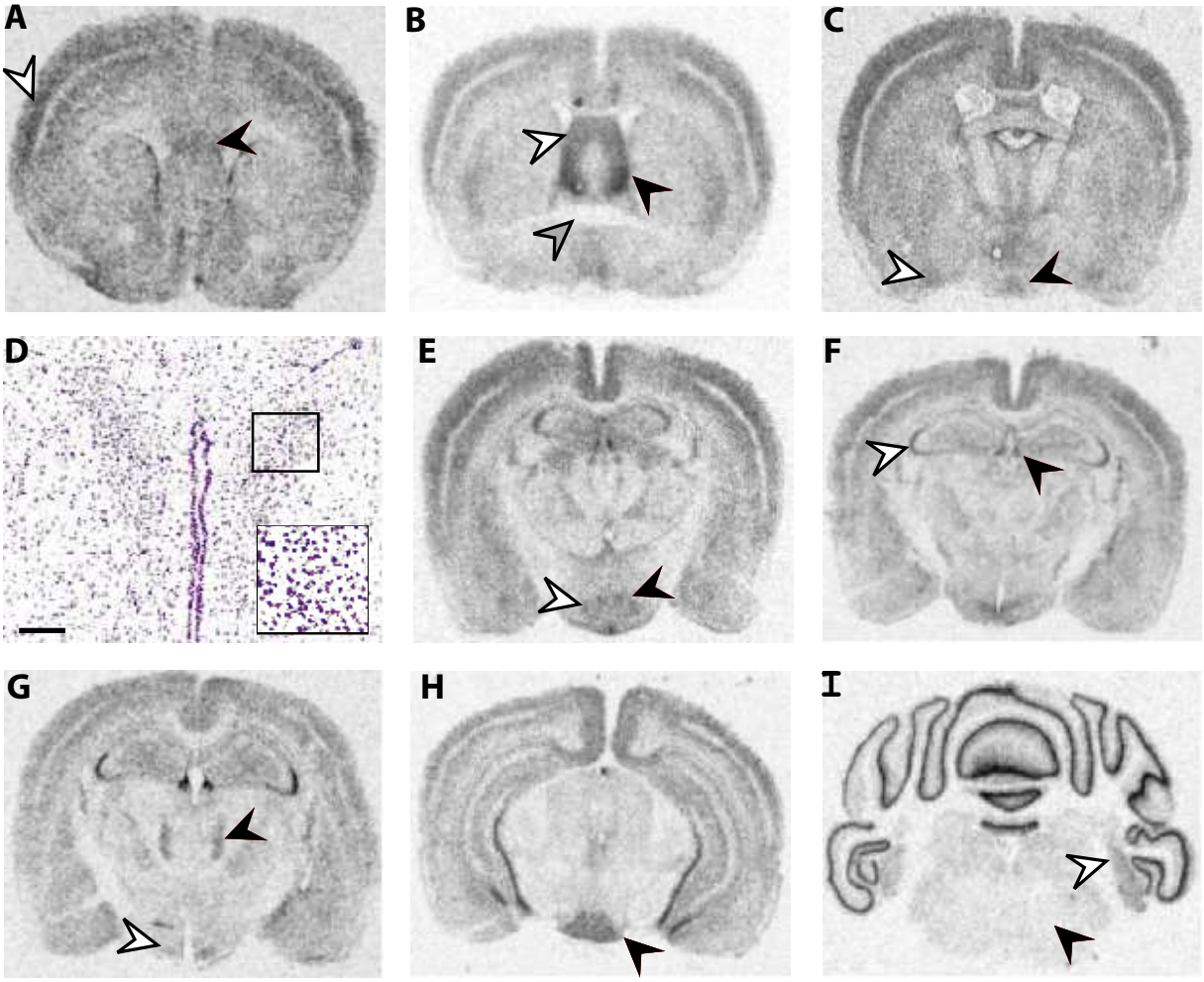

Figure 4.4 Representative TU3A mRNA expression in coronal sections of the adult mouse brain

*(**A**) Autoradiograph of coronal section cut approximately 1.54 mm anterior of bregma. Pronounced TU3A gene expression is visible in the primary somatosensory cortex (white arrowhead) and the prelimbic cortex (black arrowhead). (**B**) Autoradiograph of coronal section cut approximately 0.20 mm anterior of bregma. The dorsal part (white arrowhead) and the ventral part (black arrowhead) of the lateral septum are showing very strong TU3A mRNA expression, while the anterior commissure (grey arrowhead) is devoid of TU3A expression. (**C**) Autoradiograph of coronal section cut approximately 0.58 mm posterior of bregma. TU3A mRNA is moderately expressed in the ventrolateral part of the suprachiasmatic nucleus (black arrowhead) and the nucleus of the lateral olfactory tract (white arrowhead). (**D**) Bright field photomicrograph of cresyl violet counterstained coronal section cut*

*at the level of the hypothalamic PVN. TU3A mRNA expression, represented by black silver grains, is not distinguishable of the the surrounding hypothalamic tissue. The black box indicates the magnified part. Scale bar is 200 µm. (**E**) Autoradiograh of coronal section cut approximately 1.22 mm posterior of bregma. The dorsomedial part (black arrowhead) and the ventrolateral part (white arrowhead) of the ventromedial hypothalamic nucleus show distinct TU3A mRNA expression. (**F**) Autoradiograph of coronal section cut approximately 1.70 mm posterior of bregma. Strong TU3A mRNA expression is visible in the medial habenular nucleus (black arrowhead) and the hippocampal CA3 region (white arrowhead). (**G**) Autoradiograph of coronal section cut approximately 1.82 mm posterior of bregma. TU3A shows distinct gene expression in the parafascicular thalamic nucleus (black arrowhead) and the ventromedial hypothalamic nucleus (white arrowhead). (**H**) Autoradiograph of coronal section cut approximately 3.08 mm posterior of bregma. The mammillary nucleus (black arrowhead) shows a pronounced TU3A expression in the lateral and medial part. (**I**) Autoradiograph of coronal section cut approximately 6.24 mm posterior of bregma. TU3A is very weakly expressed in major parts of the medulla (black arrowhead), but shows distinct gene expression in the dorsal and ventral cochlear nucleus.*

Table 4.1 TU3A mRNA expression in the *telencephalon* and cerebellum

Relative expression levels of TU3A mRNA in adult and neonatal brain regions are expressed in the following categories: - not expressed, + weak signal, ++ moderate signal and +++ strong signal. Only regions that could be clearly assigned in regard of TU3A mRNA expression were included.

Structure	expression level	
	neonatal brain	mature brain
Thalamus		
Habenular nuclei	+++	+++
Reuniens thalamic nucleus	+	+
Parafascicular thalamic nucleus	+	++
Zona incerta	++	++
Hypothalamus		
Paraventricular nucleus	++	++
Peri-PVN region	+	+
Ventromedial hypothalamic nucleus	++	++
Lateral mammillary nucleus	+	+++
Medial mammillary nucleus	+	+++
Septal region		
Lateral septal nucleus dorsal part	++	+++
Lateral septal nucleus ventral part	++	+++
Lateral septal nucleus intermediate part	++	+++
Medial septal nucleus	+	++
Septohippocampal nucleus	++	++

Table 4.1 continued

Structure	expression level neonatal brain	mature brain
Amygdaloid complex		
Basolateral amygdaloid nucleus	++	++
Basomedial amygdaloid nucleus	++	++
Central amygdaloid nucleus	+	+
Neocortex		
Prefrontal cortex		
Infralinbic cortex	+	+
Prelimbic cortex	+	++
Cingulate cortex area 1	+	+
Frontal cortex	++	++
Parietal cortex		
Layers I to III	++	++
Layer IV	+++	++
Layer V	++	-
Layer VI	++	++
Occipital cortex	++	++
Temporal cortex	++	++
Retrosplenial granular cortex	++	++
Retrosplenial agranular cortex	++	++
Hippocampal region		
CA3	+++	+++
CA2	++	+
CA1	++	+
Granular layer of the dentate gyrus	++	+
Polymorhic layer of the dentate gyrus	+	+
Molecular layer of the dentate gyrus	-	+
stratum lacunosum-moleculare	+	+
Stratum radiatum	-	-
White matter structures		
Corpus callosum	+	+
Anterior commissure	-	-
Fornix	+	-
Basal ganglia and related areas		
Caudate putamen	++	++
Globus pallidus	+	+
Bed nucleus of the stria terminalis	+	+
Cerebellum		
Purkinje cell layer	+++	+++
Molecular layer	++	+++
Granular layer	-	-

4.1.3 TU3A protein expression in the adult mouse brain

Several brain regions were examined using immunohistochemistry to assess if TU3A is also expressed at the protein level under basal conditions in adult animals.

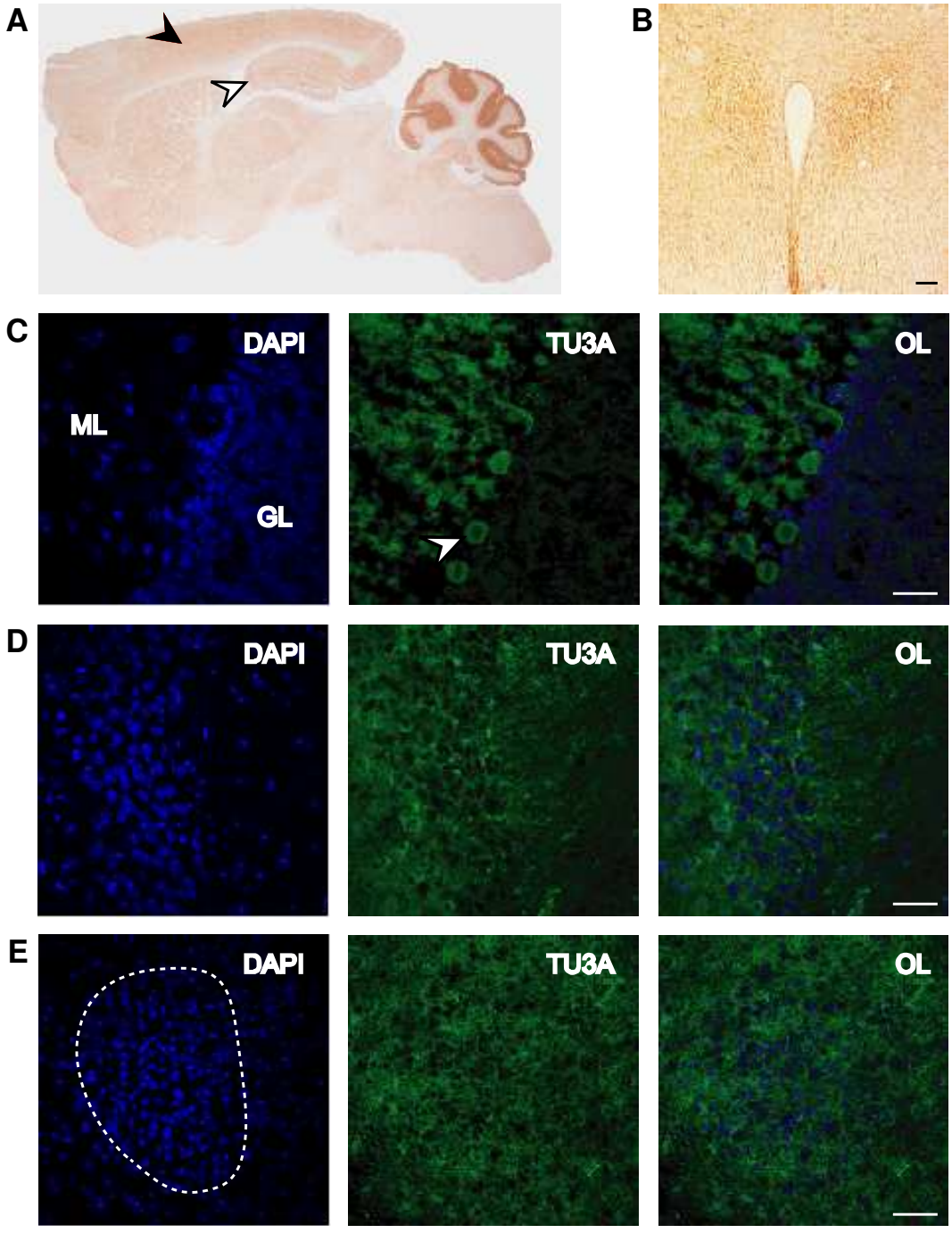

Figure 4.5 Representative photomicrographs illustrating TU3A protein expression in specific regions of the adult mouse brain

(A) Bright field photomicrograph of sagittal section with TU3A staining in brown. TU3A protein is strongly expressed in the cortex (black arrowhead) and the hippocampus (white arrowhead). (B) Bright field photomicrograph of the hypothalamic PVN with TU3A staining in brown. (C) Fluorescence photomicrograph

of the cerebellum. Purkinje cell (white arrowhead) shows strong TU3A protein expression as well as the molecular layer (ML) of the cerebellum. In the granular layer (GL) only very little TU3A protein is evident. (D) Representative photomicrograph showing fluorescence signal for TU3A in the hippocampal CA3 region. (E) Representative photomicrograph showing fluorescence signal for TU3A protein in the hypothalamic PVN. White, dashed line indicates the PVN (left hemisphere). Scale bars are 50 µm, blue color shows DAPI staining and green color represents TU3A protein staining.

In general, the regional TU3A protein expression was consistent with the TU3A mRNA expression pattern of the adult brain. Additionally, the protein expression level matched the observed mRNA intensity: brain regions that showed strong TU3A mRNA expression also exhibited pronounced TU3A protein levels (see Figure 4.5 A). For a more detailed expression we focused on the cerebellum, where TU3A mRNA was abundantly expressed and on HPA axis relevant regions like the hypothalamic PVN and the hippocampus. In the cerebellum we could confirm a prominent TU3A protein expression in Purkinje cells and in the molecular layer (see Figure 4.5 B). In the hippocampal CA3 region the pyramidal neurons were strongly immunoreactive for TU3A, and the protein was mainly present in the cytoplasm and absent in the nucleus (see Figure 4.5). In the cerebellum and the CA3 region of the hippocampus TU3A protein was specifically expressed in distinct neuronal cell populations. The hypothalamic PVN showed moderate TU3A protein staining, reflecting the moderate TU3A mRNA expression under basal conditions (see Figure 4.5 B and E).

4.1.4 TU3A mRNA expression during postnatal ontogeny

To examine the postnatal ontogeny of TU3A mRNA expression in more detail, the mRNA expression was investigated under basal conditions in several HPA axis relevant regions and in the cortex.

TU3A mRNA expression could be detected in the PVN at all examined ages. ANOVA revealed a significant effect of age ($F_{4,36}$ = 3.753, $p<0.05$); at postnatal day 12 TU3A mRNA was significantly decreased compared to postnatal day 1. All other ages showed no significant differences of TU3A mRNA levels in the PVN. A strong TU3A mRNA expression was present in the hippocampal CA3 region at all examined ages. ANOVA revealed a significant effect of age ($F_{4,37}$ = 4.720, $p<0.01$). TU3A mRNA expression was present at postnatal day 1 and increased with time; at postnatal day 12 and 18 TU3A mRNA was significantly higher compared to postnatal day 1. In the cortex ANOVA showed a significant effect of age ($F_{4,36}$ =

13.004, p< 0.001). TU3A mRNA expression was low at postnatal day 1 and 6 and increased significantly at postnatal day 12, 18 and in the adult animals compared to both postnatal day 1 and 6. In the basolateral amygdala no significant differences in TU3A mRNA expression were found between the different examined age groups.

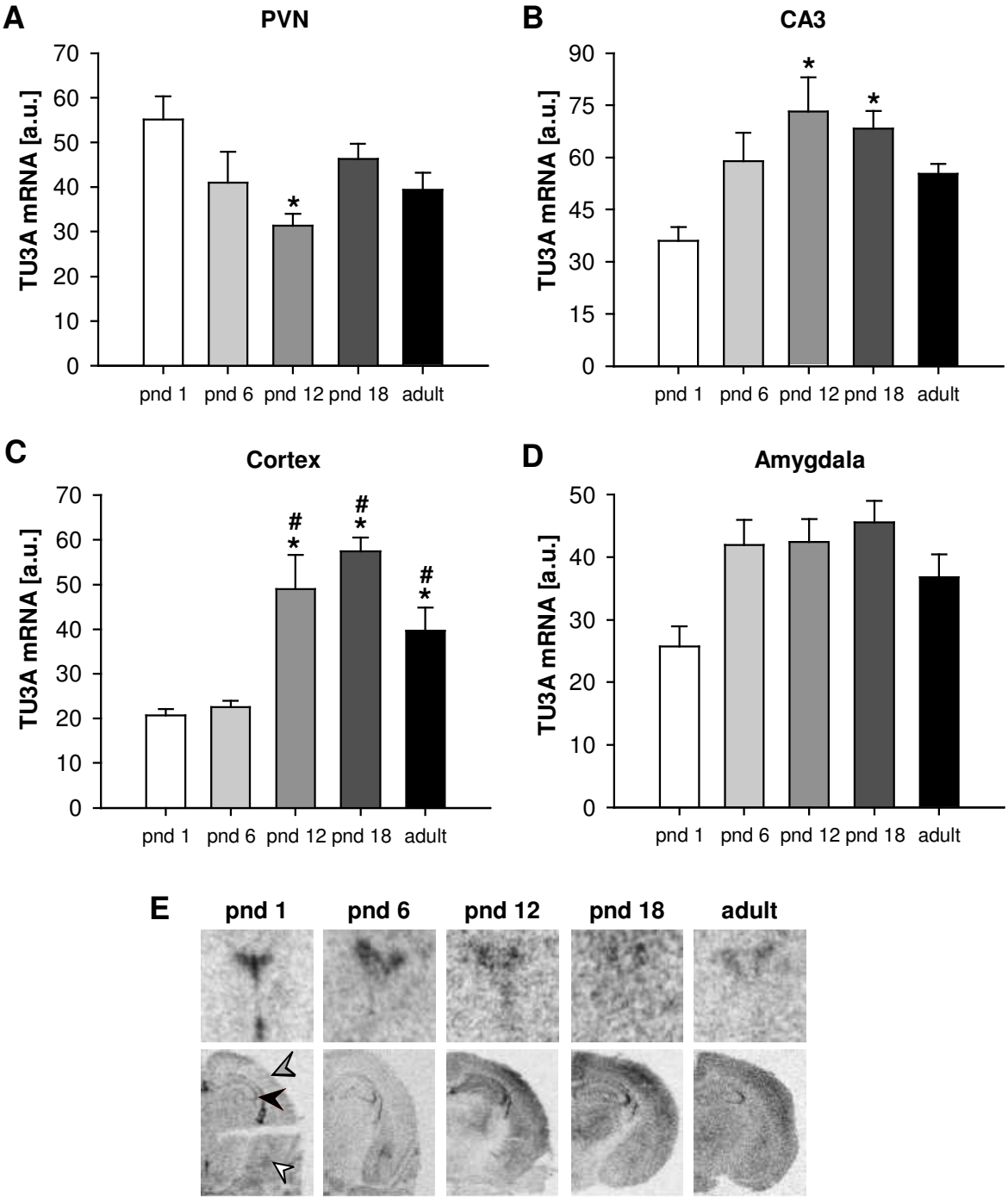

Figure 4.6 TU3A mRNA expression during postnatal ontogeny in different brain regions

*(**A**) TU3A mRNA expression in the PVN under basal conditions. (**B**) TU3A mRNA expression in the hippocampal CA3 region under basal conditions. (**C**) TU3A mRNA expression measured in the cortex. (**D**) TU3A mRNA expression in the basolateral*

*amygdala under basal conditions. (**E**) Representative autoradiographs of the PVN (upper part) and the level of the dorsal hippocampus (lower part). Black arrowhead indicates the hippocampal CA3 region, white arrowhead indicates the basolateral amygdala and grey arrowhead the cortex. * significanlyt different compared to postnatal day (pnd) 1, p<0.05; # significantly different compared to pnd 6, p<0.05.*

4.2 Effect of stress on TU3A mRNA expression in the mouse brain

The effect of stress on TU3A mRNA expression was examined in different brain regions of neonatal mice following maternal separation and in the adult mouse brain using different stress paradigms, mouse strains as well as both genders.

4.2.1 Influences of stress on TU3A mRNA expression in specific regions of the neonatal mouse brain

First, the TU3A mRNA expression following 24 hours of maternal separation was examined in in the PVN, the hippocampal CA3 region, the cortex, the amygdala and the cerebellum at postnatal day 9.

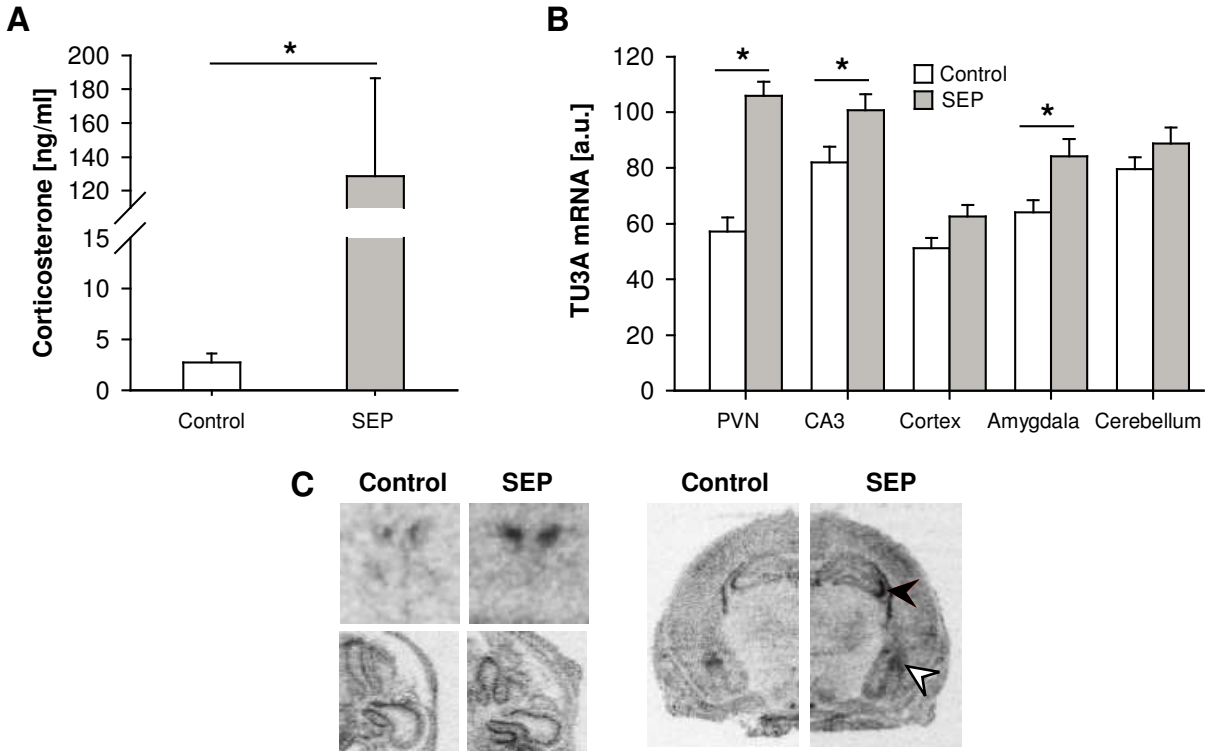

Figure 4.7 TU3A mRNA expression is regulated by stress at postnatal day 9 in various brain regions

*(**A**) Circulating plasma corticosterone levels after 24 hours of maternal separation (SEP) compared to undisturbed control group. (**B**) TU3A mRNA expression*

*measured in the PVN, the hippocampal CA3 region, across all layers of the cortex, the basolateral amygdala and the Purkinje cell layer of the cerebellum. (**C**) Representative autoradiographs of the examined brain regions. The Upper part of the images on the left side shows the TU3A mRNA expression in the PVN, the lower part shows the mRNA expression in the cerebellum. Images on the right side show the CA3 region of the hippocampus (black arrowhead) and the basolateral amygdala (white arrowhead). * significantly different from control group with $p < 0.05$.*

Maternal separation leads to a significant increase in plasma corticosterone levels compared to undisturbed controls (T_{14} = 2.175, $p < 0.05$) (Figure 4.7 A). TU3A mRNA expression was significantly increased following 24 hours of maternal separation in the hypothalamic PVN, the hippocampal CA3 region and the basolateral amygdala (T_{14} = 6.746, $p<0.001$; T_{14} = 2.339, $p<0.05$; T_{14} = 2.608, $p<0.05$, respectively). TU3A mRNA expression was not increased after the stress experience at the level of the cortex (T_{14} = 2.104, $p=0.054$) or the cerebellum (T_{14} = 1.278, $p=0.219$). Additional statistical analysis revealed no differences in plasma corticosterone levels and gene expression between both genders (data not shown).

4.2.2 Influence of different stress paradigms on TU3A mRNA expression in the adult mouse brain

To determine whether TU3A is, additionally to the neonatal situation, stress-regulated in the adult mouse brain the mRNA expression in several brain regions was examined after different stress paradigms. TU3A gene expression was measured in the PVN, the hippocampal CA3 region, the amygdala and the cortex of male C57Bl6/N mice under basal conditions, 4 hours following the onset of 30 minutes restraint stress, or after 24 hours of food deprivation. Circulating plasma corticosterone levels were examined to determine HPA axis activation.

Circulating plasma corticosterone was significantly increased after 24 hours of food deprivation compared to both, control and restraint stressed group ($F_{2,21}$ = 26.794, $p < 0.001$). The corticosterone level in the restraint stressed group did not differ from control levels at the time of sacrifice 4 hours after the onset of stress.

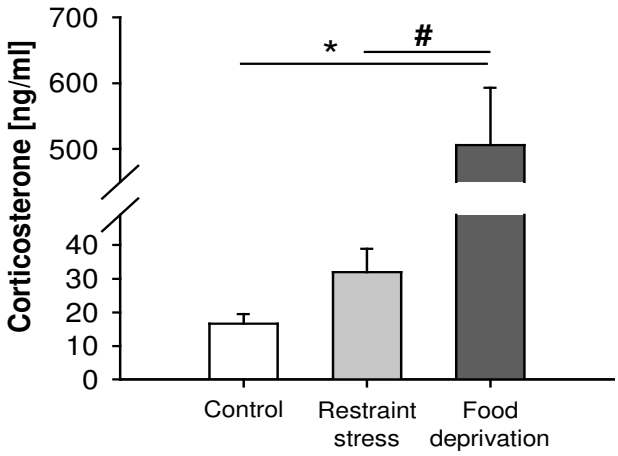

Figure 4.8 Plasma corticosterone levels following different stress paradigms in male C57Bl6/N mice

*Circulating plasma corticosterone levels under basal conditions (control), 4 hours after the onset of a 30 minutes restraint stress paradigm and after 24 hours of food deprivation. * significantly different from control group, $p < 0.001$; # significantly different from restraint stressed group $p < 0.001$.*

In the 24 hours food deprivation group, TU3A mRNA levels in the region of the hypothalamic PVN were significantly elevated compared to both, the control and the restraint stress group ($F_{2,22} = 34.847$, $p < 0.001$) (Figure 4.7 A and B). TU3A mRNA expression was significantly increased following 24 hours of food deprivation in the CA3 region of the hippocampus compared to the control group ($F_{2,23} = 4.525$, $p < 0.05$) (Figure 4.9 C and D). Neither restraint stress, nor 24 hours of food deprivation had a significant effect on TU3A gene expression at the level of the basolateral amygdala (Figure 4.9 E). Additionally, TU3A mRNA expression was significantly elevated after 24 hours food deprivation in a section across layer I, II, III and IV oft the cortex as well as in layer VI compared to both, control and restraint stressed group ($F_{2,23} = 11.398$, $p < 0.05$ and $F_{2,23} = 20.701$, $p < 0.05$ respectively) (Figure 4.9 F). Layer V of the cortex was excluded from measurement, since there was no TU3A mRNA expression detectable. In the following experiments all cortical measurements were performed across all six cortical layers, since no differences between specific layers were detectable.

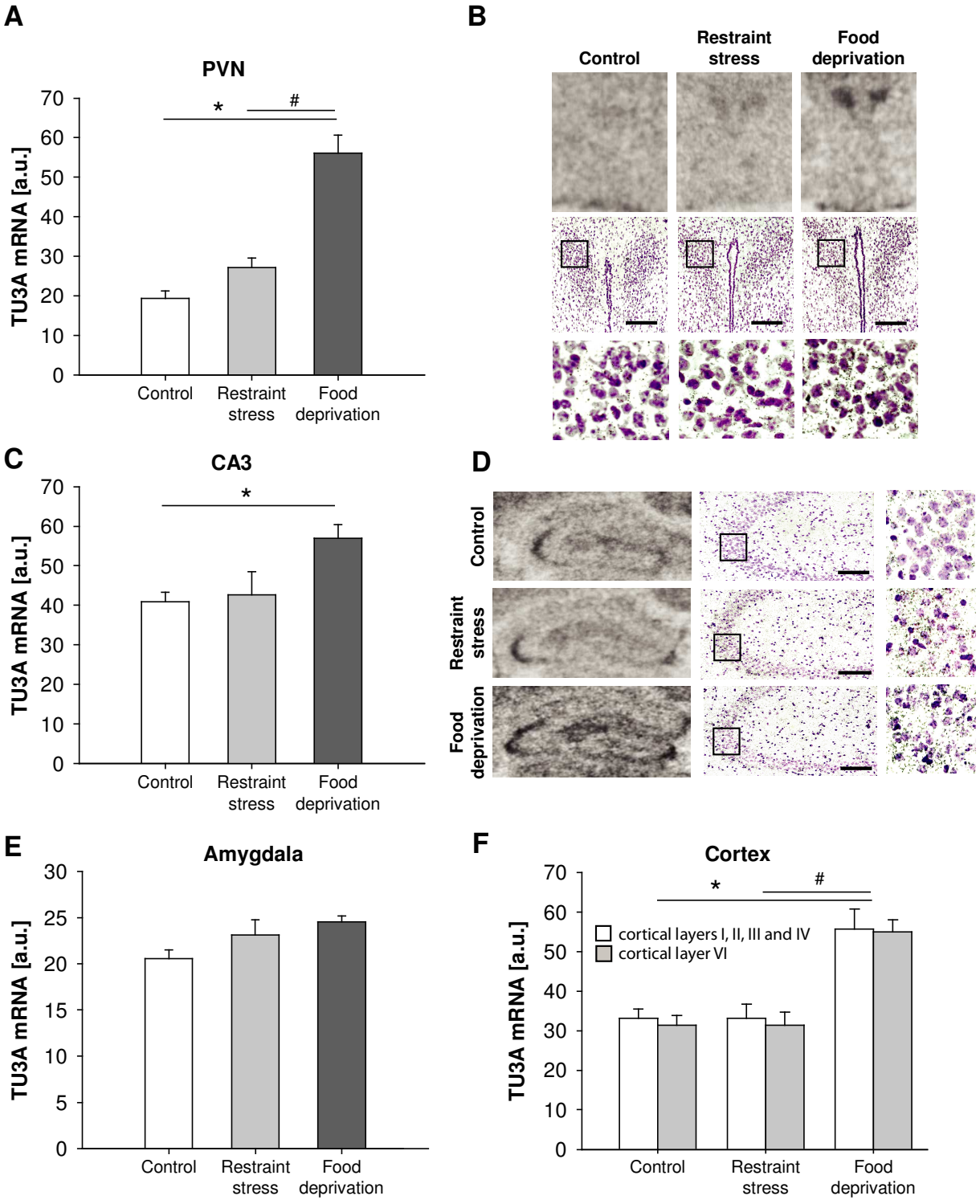

Figure 4.9 Effects of different stress paradigms on TU3A mRNA expression in different brain regions

*TU3A mRNA expression levels measured under basal conditions, 4 hours after the onset of restraint stress or 24 hours of food deprivation in the PVN (**A**), the hippocampal CA3 region (**C**), the basolateral amygdala (**E**) and layer I-IV (white bar) as well as layer VI (grey bar) of the primary somatosensory cortex barrel field (**F**). Representative autoradiographs (upper part) and counterstained photomicrographs of TU3A mRNA expression (middle part) in the PVN. The area of the black squares is enlarged (lower part) (**B**). Representative autoradiographs (left*

*part) and counterstained photomicrographs of TU3A mRNA expression (middle part) in the hippocampal CA3 region. The area of the black squares is enlarged (lower part) (**D**). Scale bars are 250 µm. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from control group, $p < 0.05$; # significantly different from restraint stressed group, $p < 0.05$.*

4.2.3 Time course of TU3A mRNA expression following restraint stress in male C57Bl6/N mice

To examine the temporal profile of TU3A induction after stress, gene expression was measured at various time points following the onset of restraint stress.

The plasma corticosterone levels were significantly elevated one hour after the onset of the restraining paradigm compared to the control group ($F_{3,22} = 4.712$, $p < 0.05$). Plasma corticosterone levels decreased again at 4 and 8 hours post stress and did not differ statistically from the plasma corticosterone level of the control group (Figure 4.10 A).

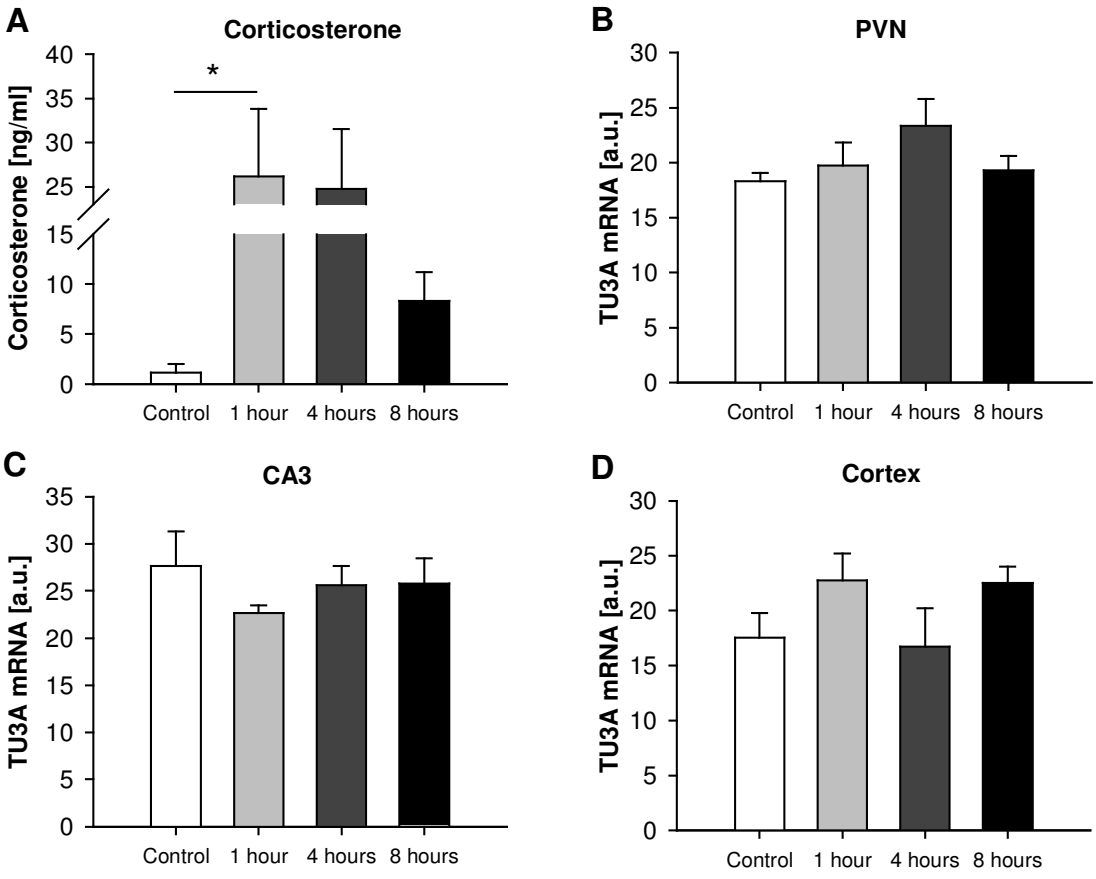

Figure 4.10 Time course of TU3A mRNA expression following restraint stress

*(**A**) Plasma corticosterone levels under basal conditions (control), 1 hour, 4 hours and 8 hours after the onset of 30 minutes restraint stress. (**B**) TU3A mRNA*

*expression measured in the PVN. (**C**) TU3A mRNA expression in the hippocampal CA3 region. (**D**) TU3A mRNA expression in the cortex. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from control group, p < 0.05.*

TU3A mRNA expression was measured in the PVN, the CA3 region of the hippocampus and the cortex. The mRNA expression did not differ significantly from the control group in all three areas at any time point (Figure 4.10 B, C and D). Restraint stress was therefore at no time point able to induce increased TU3A mRNA expression in the examined brain regions.

4.2.4 Influence of 24 hours food deprivation on TU3A mRNA expression in male CD1 mice

To exclude a strain specific effect on stress-induced TU3A mRNA expression particularly for the C57Bl6/N mouse strain, the 24 hours food deprivation paradigm was additionally performed in animals of the CD1 outbred strain.

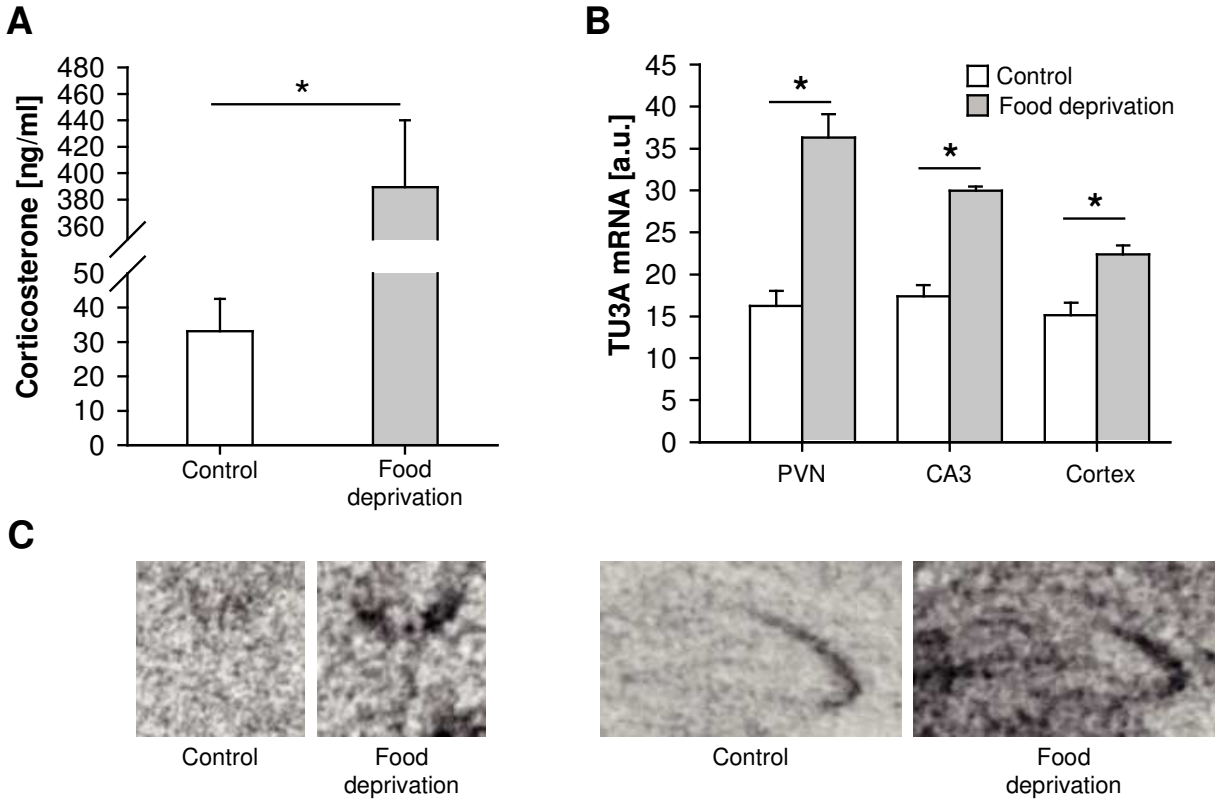

Figure 4.11 Influence of 24 hours of food deprivation on TU3A mRNA expression in male CD1 mice

*(**A**) Plasma corticosterone levels after 24 hours of food deprivation and under control conditions. (**B**) TU3A mRNA expression in the PVN, hippocampal CA3 and*

*cortex. (**C**) Representative autoradiographs of TU3A mRNA expression in the PVN (left) and hippocampus (right). TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from control group, p < 0.05.*

The 24 hours of food deprivation increased the circulating plasma corticosterone levels significantly compared to the control group (T_{13} = 6.484, p < 0.05) in CD1 mice (Figure 4.11 A). As seen in the C57Bl6/N mouse strain, TU3A mRNA expression was significantly elevated in the hypothalamic PVN, the hippocampal CA3 region and the cortex of CD1 mice after 24 hours of food deprivation (T_{10} = 7.463, p < 0.05, T_9 = 8.006, p < 0.05 and T_9 = 3.854, p < 0.05) (Figure 4.11 B and C).

4.2.5 Influence of chronic social stress on TU3A mRNA expression in male CD1 mice

So far, only acute stress paradigms and their influence on TU3A mRNA expression were examined. To investigate the effects of chronic stress on TU3A mRNA expression, a chronic social stress paradigm was applied in male CD1 mice (see chapter 3.4.4).

Seven weeks of chronic social stress lead to significantly increased circulating plasma corticosterone levels compared to the unstressed control group (T_{19} = 2.125, p < 0.05) (Figure 4.12 A). In contrast to the acute food deprivation stress paradigm, TU3A mRNA expression was not elevated in the PVN, the hippocampal CA3 region or the cortex after seven weeks of chronic social stress (Figure 4.12 B).

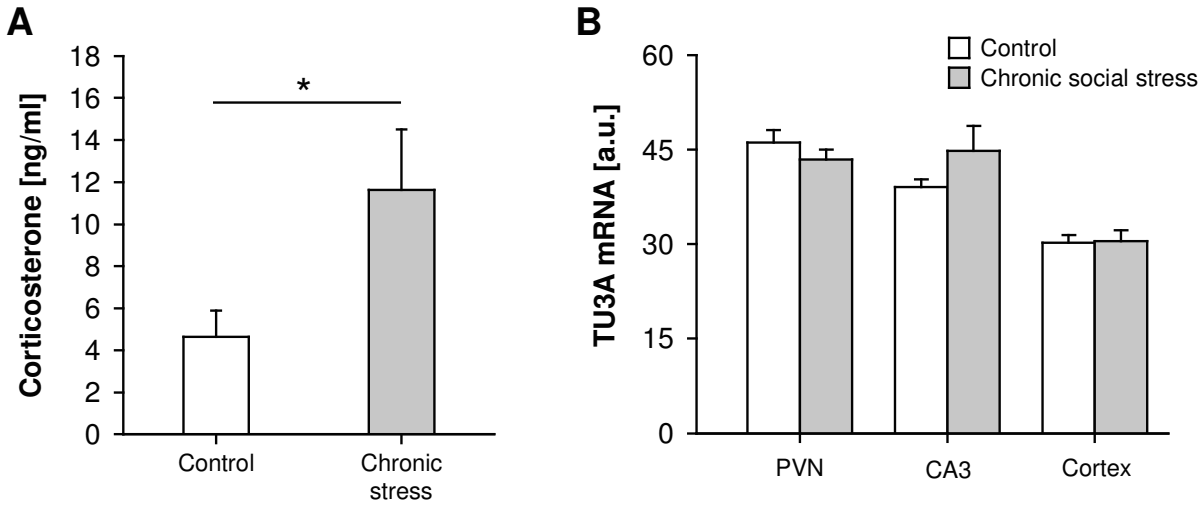

Figure 4.12 Effects of chronic social stress on TU3A mRNA expression in male CD1 mice

*(**A**) Plasma corticosterone levels after seven weeks of chronic social stress and under control conditions. (**B**) TU3A mRNA expression in the PVN, the hippocampal*

*CA3 region and the cortex. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from control group, p < 0.05.*

4.2.6 Influence of 24 hours food deprivation on TU3A mRNA expression in female C57Bl6/N mice

To examine gender specific effects on stress-induced TU3A mRNA expression, the 24 hours food deprivation paradigm was also performed in female C57Bl6/N mice.

24 hours of food deprivation resulted in significantly elevated plasma corticosterone levels in female C57Bl6/N mice compared to the undisturbed control group (T_{13} = 6.725, $p < 0.05$) (Figure 4.13 A). Corresponding to the situation in male mice, TU3A mRNA expression following 24 hours of food deprivation was significantly increased in the PVN, the CA3 region of the hippocampus and in the cortex compared to the control group (T_{14} = 20.198, $p < 0.05$, T_{14} = 6.003, $p < 0.05$ and T_{14} = 6.067, $p < 0.05$) (Figure 4.13 B).

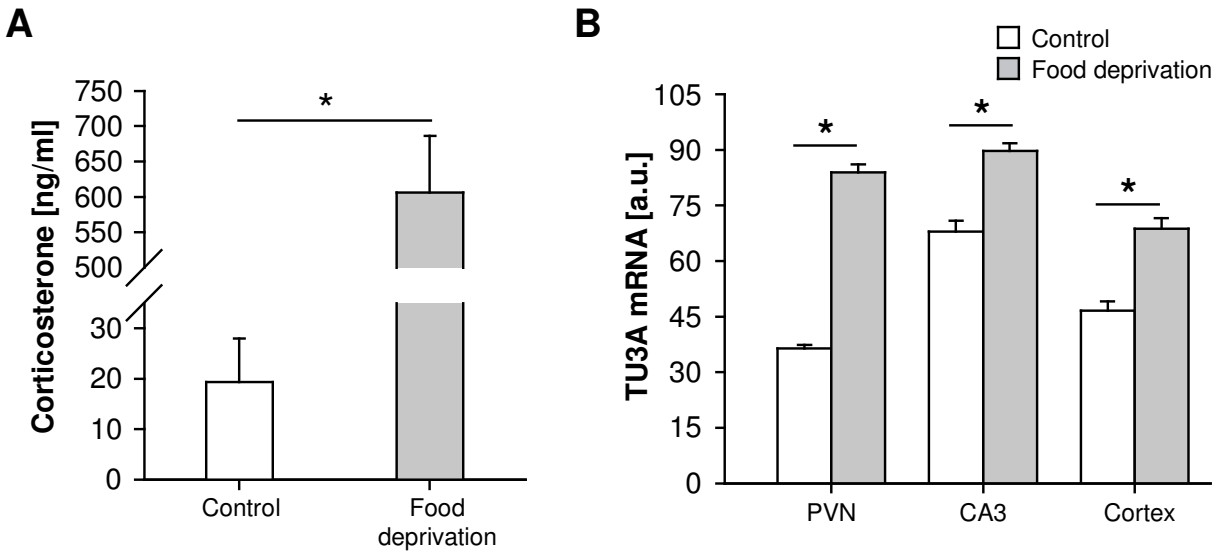

Figure 4.13 Influence of 24 hours food deprivation on TU3A mRNA expression in female C57Bl6/N mice

*(**A**) Plasma corticosterone levels after 24 hours of food deprivation and under undisturbed control conditions. (**B**) TU3A mRNA expression in the PVN, the hippocampal CA3 region and the cortex. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from control group, p < 0.05.*

4.3 Effect of glucocorticoids on TU3A mRNA expression

To investigate the involvement of corticosteroid receptors in the regulation of TU3A transcription adult mice were treated with a GR agonist. Additionally, adult as well as neonatal mice were treated with corticosteroid receptor antagonists during stress.

4.3.1 Influence of the GR on TU3A mRNA and protein expression

To study whether the stress-induced increase of TU3A mRNA expression can be induced pharmacologically via activation of the GR, adult male C57BL6/N mice were treated with dexamethasone, a potent synthetic glucocorticoid receptor agonist.

For plasma corticosterone, ANOVA revealed a significant effect of time ($F_{3,47}$ = 10.579, $p < 0.05$), treatment ($F_{1,47}$ = 62.645, $p < 0.05$) and time*treatment interaction ($F_{3,47}$ = 12.489, $p < 0.05$). Dexamethasone injection decreased plasma corticosterone levels significantly 4 and 8 hours following the treatment compared to the control group (T_{10} = 4.209, $p < 0.05$ and T_{10} = 7.579, $p < 0.05$). Plasma corticosterone levels in the dexamethasone treated group did not differ from the control group 24 hours following dexamethasone injection (Figure 4.14 A).

For TU3A mRNA expression in the PVN, ANOVA showed a significant effect of time ($F_{3,47}$ = 32.089 $p < 0.001$), treatment ($F_{1,47}$ = 78.17, $p < 0.001$) and time*treatment interaction ($F_{3,47}$ = 14.551, $p < 0.001$). TU3A mRNA expression was significantly increased in the hypothalamic PVN 4 and 8 hours following dexamethasone treatment compared to vehicle treated animals (T_{10} = 6.006, $p < 0.05$ and T_{10} = 7.931, $p < 0.05$, respectively). After 24 hours, the TU3A mRNA expression decreased again to the level of the control animals (Figure 4.14 B). For TU3A mRNA expression in the CA3 region of the hippocampus, ANOVA showed an effect of treatment ($F_{1,47}$ = 15.933, $p < 0.001$) and a time*treatment interaction ($F_{3,47}$ = 4.275, $p < 0.01$). TU3A mRNA expression was significantly increased 4 and 8 hours after dexamethasone treatment compared to control group (T_{10} = 2.885, $p < 0.05$ and T_{10} = 4.348, $p < 0.05$, respectively) (Figure 4.14 C). For the mRNA expression in the cortex, ANOVA revealed a treatment effect ($F_{1,47}$ = 45.377, $p < 0.001$). TU3A mRNA expression was significantly elevated 4, 8 and 24 hours following dexamethasone treatment (T_{10} = 2.492, $p < 0.05$, T_{10} = 5.855, $p < 0.001$ and T_{10} = 3.734, $p < 0.01$, respectively) (Figure 4.14 D).

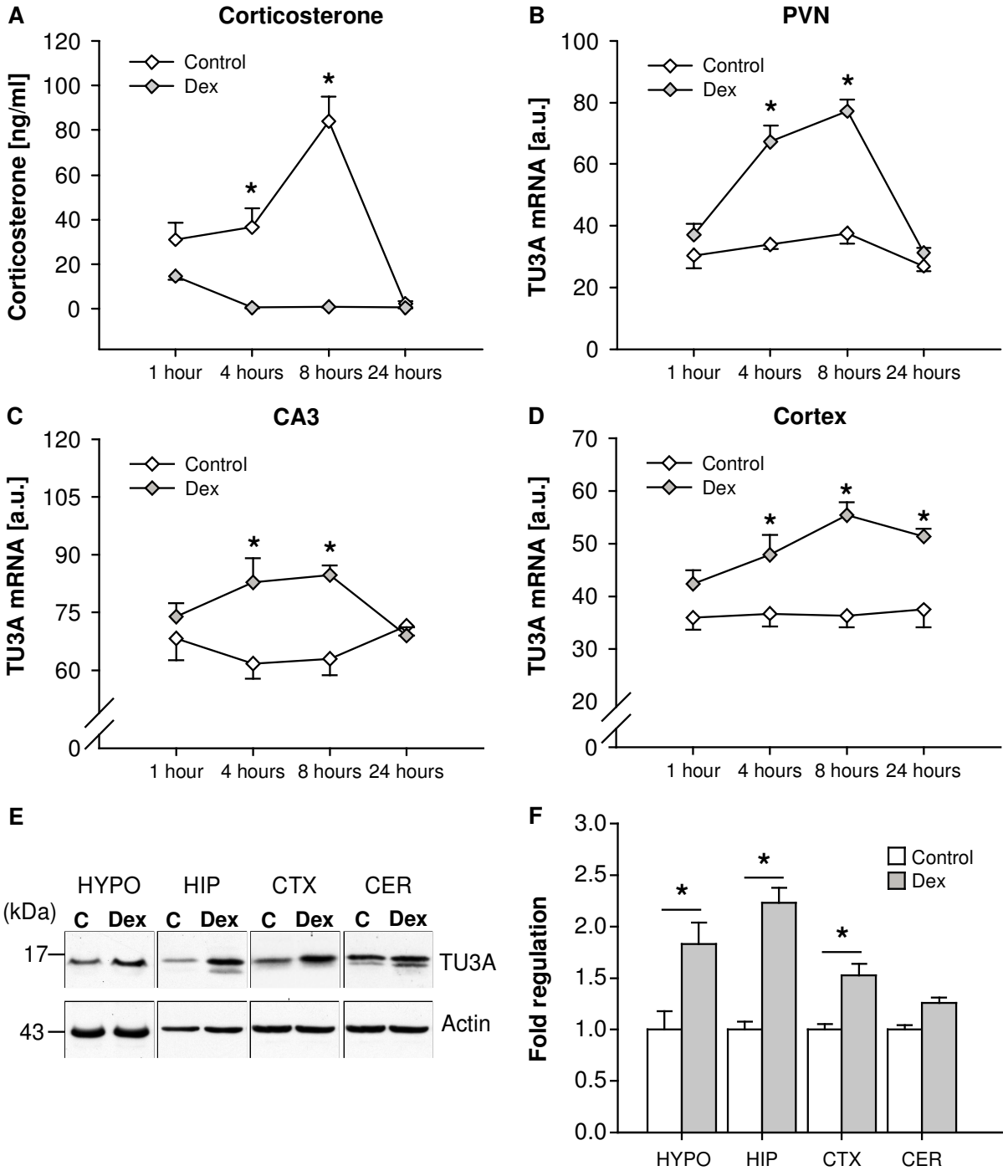

Figure 4.14 Influence of dexamethasone treatment on TU3A mRNA and protein expression

*(**A**) Plasma corticosterone levels at different time points after subcutaneous dexamethasone or vehicle treatment. (**B**) TU3A mRNA expression levels in the PVN. (**C**) TU3A mRNA expression levels in the hippocampal CA3 region. (**D**) TU3A mRNA expression levels in the cortex. (**E**) Representative western blots of brain-lysates showing TU3A and actin protein levels 8 hours after dexamethasone or vehicle injection. (**F**) TU3A protein level, normalized to the actin signal, expressed as fold regulation of vehicle-treated control. TU3A mRNA expression is displayed as*

*arbitrary units [a. u.]; * indicates significant difference from control group, $p < 0.05$; hypothalamus (HYPO), hippocampus (HIP), cortex (CTX) and cerebellum (CER).*

To examine if the observed dexamethasone-induced up-regulation of mRNA results in a elevated TU3A protein level, western blot analysis of brain samples was performed 8 hours after dexamethasone treatment. Reflecting the findings at mRNA level, TU3A protein content was significantly elevated 8 hours after dexamethasone treatment in the hippocampus, the hypothalamus and the cortex ($T_{16} = 2.513$, $p < 0.05$, $T_{15} = 3.055$, $p < 0.01$ and $T_{16} = 4289$, $p < 0.01$) (Figure 4.14 E and F). Dexamethasone treatment had no effect on the amount of TU3A protein in the cerebellum ($T_{16} = 0.834$, $p = 0.416$) (Figure 4.14 E and F).

4.3.2 Effect of corticosteroid receptor antagonist treatment on TU3A mRNA expression during maternal separation in neonatal mice

To test whether the stress-induced TU3A mRNA increase is mediated by corticosteroid receptors in neonates, C57BL6/N mice were treated with a GR antagonist or a MR antagonist during maternal separation.

For plasma corticosterone levels, ANOVA revealed effects of treatment ($F_{2,46} = 20.677$, $p < 0.05$) and condition ($F_{1,46} = 51.413$, $p < 0.05$). The 24 hours of maternal separation resulted in elevated plasma corticosterone levels among all three treatment groups compared to the non-separated control groups (vehicle: $T_{12} = 4.828$, $p < 0.05$, GR antagonist: $T_{15} = 2.72$, $p < 0.05$ and MR antagonist: $T_{14} = 5.563$, $p < 0.05$). In the non-separated condition, GR antagonist treatment lead to significantly elevated plasma corticosterone levels compared to vehicle treatment and MR antagonist treatment ($F_{2,26} = 23.992$, $p < 0.05$) (Figure 4.15 A).

With regard to mRNA expression in the hypothalamic PVN, ANOVA showed an effect of treatment ($F_{2,43} = 16.944$, $p < 0.05$), condition ($F_{1,43} = 8.424$, $p < 0.05$) and treatment*condition interaction ($F_{2,43} = 4.831$, $p < 0.05$). The 24 hours of maternal separation resulted in elevated TU3A mRNA expression in the vehicle treated group ($T_9 = 2.346$, $p < 0.05$) as well as the MR antagonist treated group ($T_{15} = 2.82$, $p < 0.05$), compared to the undisturbed control groups of both conditions. Under separation conditions the GR antagonist treatment resulted in significant lower TU3A mRNA levels compared to both, the vehicle and the MR antagonist treated group ($F_{2,19} = 18.164$, $p < 0.05$). There was no significant difference between separated and control group under GR antagonist treatment

(T14 = 1.040, p = 0.316). The GR antagonist treatment therefore prevented the stress-induced elevation of TU3A mRNA expression in the hypothalamic PVN (Figure 4.15 B).

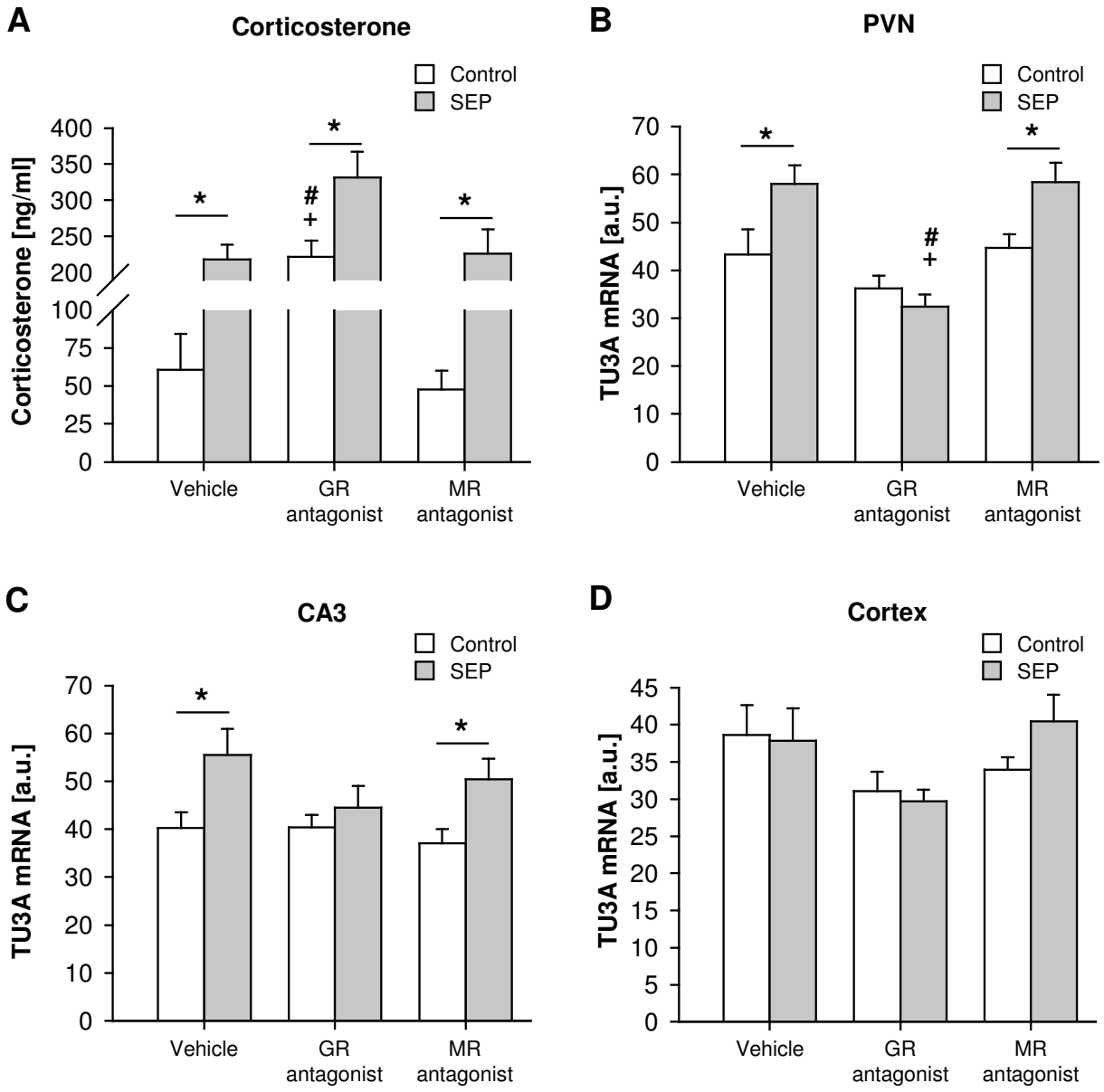

Figure 4.15 Effect of corticosteroid receptor antagonist treatment on TU3A mRNA expression during maternal separation in neonatal mice

*Circulating plasma corticosterone levels (**A**) and TU3A mRNA expression in the PVN (**B**), the hippocampal CA3 region (**B**) and the cortex (**C**) following 24 hours of maternal separation (SEP), compared to non-separated control group, after vehicle, GR antagonist (mifeprostone) or MR antagonist (spironolactone) treatment. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from the non-separated control group, p < 0.05; # significantly different from the vehicle treated group, p < 0.05; + significantly different from the MR antagonist treated group, p<0.05.*

In the hippocampal CA3 region, ANOVA revealed a significant effect of condition ($F_{1,46}$ = 9.609, $p < 0.05$) and a treatment*condition interaction ($F_{2,46}$ = 3.593, $p < 0.05$). Maternal separation increased TU3A mRNA expression significantly compared to non-separated controls in the CA3 region of the hippocampus in the vehicle and the MR antagonist treated group (T_{11} = 2.467, $p < 0.05$ and T_{15} = 2.67, $p < 0.05$), but not in the GR antagonist treated group (T_{15} = 0.492, p = 0.630). Thus, the GR antagonist treatment prevented the maternal separation induced TU3A mRNA expression up-regulation at post natal day 9 in the hippocampal CA3 region (Figure 4.15 C).

There was no significant effect of treatment or condition on TU3A mRNA expression in the cortex of neonatal mice (Figure 4.15 D).

4.3.3 Effects of corticosteroid receptor antagonist treatment on TU3A mRNA expression during 24 hours of food deprivation in the adult mouse brain

To examine if the stress-induced TU3A mRNA expression increase is comparable to the neonatal situation, also mediated by corticosteroid receptors in adult animals, adult male C57Bl6/N mice were either treated with a GR antagonist or an MR antagonist during 24 hours of food deprivation.

With respect to plasma corticosterone levels, ANOVA revealed an significant effect of treatment ($F_{2,43}$ = 5.409, $p < 0.05$). Under control conditions the GR antagonist treatment lead to significantly elevated plasma corticosterone levels compared to vehicle treatment ($F_{2,22}$ = 5.804, $p < 0.05$).

For TU3A mRNA expression in the PVN, ANOVA showed an effect of condition ($F_{1,39}$ = 25.648, $p < 0.05$) and condition*treatment interaction ($F_{2,39}$ = 8.075, $p < 0.05$). Under food deprived conditions the GR antagonist treatment resulted in significantly lower TU3A mRNA levels compared to the vehicle treated group ($F_{2,19}$ = 3.615, $p < 0.05$). TU3A mRNA expression was significantly elevated following 24 hours of food deprivation in the vehicle treated group (T_{10} = 5.311, $p < 0.05$) and the MR antagonist treated group (T_{12} = 2.343, $p < 0.05$), but not in the GR antagonist treated group (T_{12} = 0.101, $p < 0.921$). GR antagonist treatment prevented the stress-induced TU3A mRNA up-regulation in the PVN, while treatment with a MR antagonist had no effect on stress-induced TU3A mRNA expression. For the hippocampal CA3 region and the cortex ANOVA revealed no significant effects of treatment or condition.

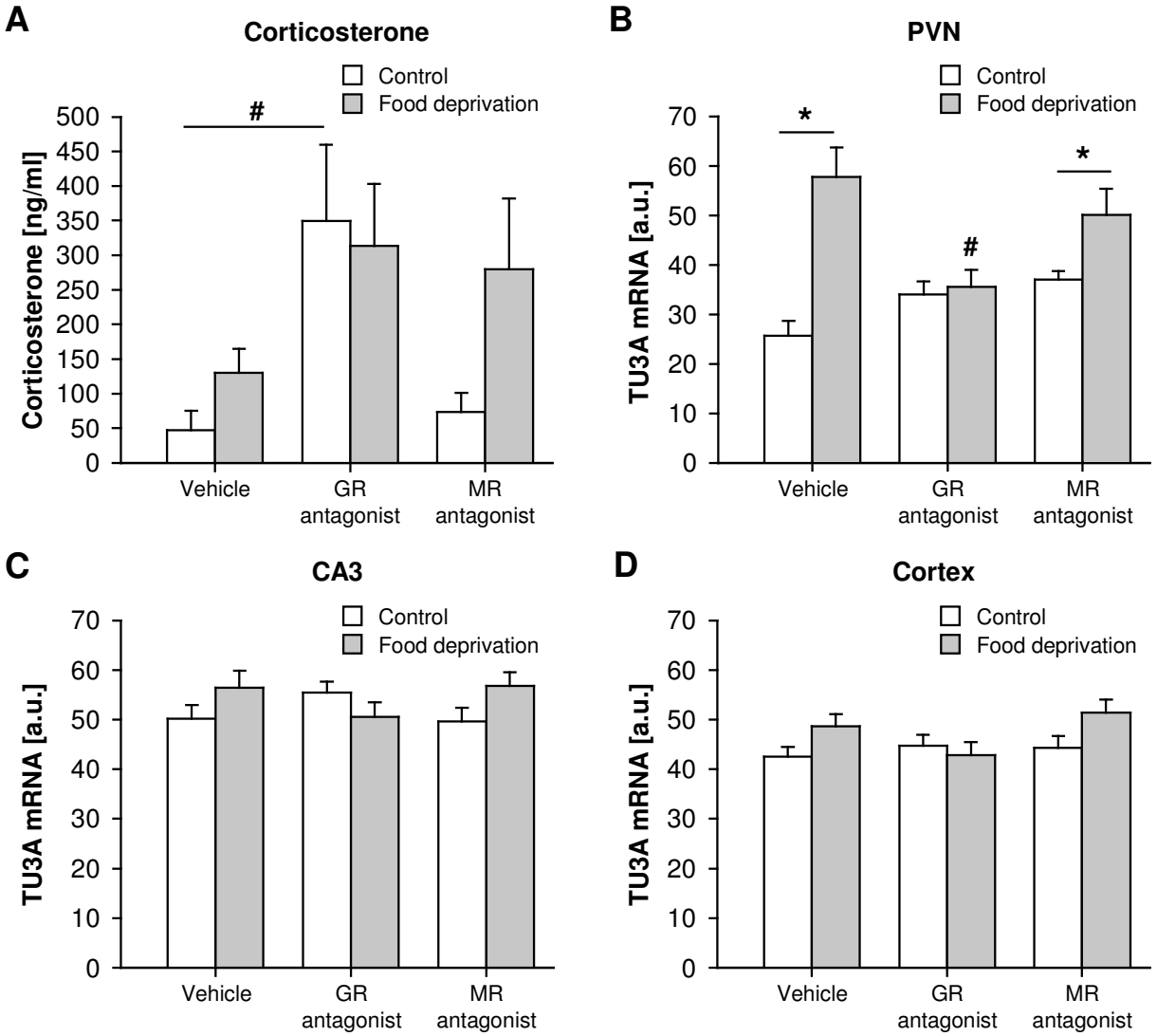

Figure 4.16 Effect of corticosteroid receptor antagonist treatment on TU3A mRNA expression in adult mice

*(**A**) Circulating plasma corticosterone levels. (**B**) TU3A mRNA expression in the PVN. (**C**) TU3A mRNA expression in the hippocampal CA3 region. (D) TU3A mRNA expression in the cortex. TU3A mRNA expression is displayed as arbitrary units [a. u.]; * significantly different from the control group, $p < 0.05$; # significantly different from the vehicle treated group, $p< 0.05$.*

4.4 Effects of TU3A overexpression in the hippocampus

To gain more insights into a potential function of TU3A in the modulation of hippocampus-dependent emotional and cognitive processes, TU3A was overexpressed in the hippocampus using an adeno-associated viral vector.

4.4.1 Verification of TU3A overexpression

First, the overexpression was confirmed in regard of effectiveness and persistence. In addition, the general physical condition of the animals was examined.

A strong and stable TU3A overexpression was present throughout the whole dorsal hippocampus, with a pronounced TU3A staining in the pyramidal neurons of the hippocampal CA3 region. Substantial TU3A overexpression was detectable 3 weeks after the injection and lasting at least up to 5 months following injection (Figure 4.17 A). Animals of both condition groups were healthy and continuously gained weight during the weeks following virus injection, with no significant differences between TU3A overexpressing animals and the control group. This data indicated, that both groups recovered equally well after the surgery.

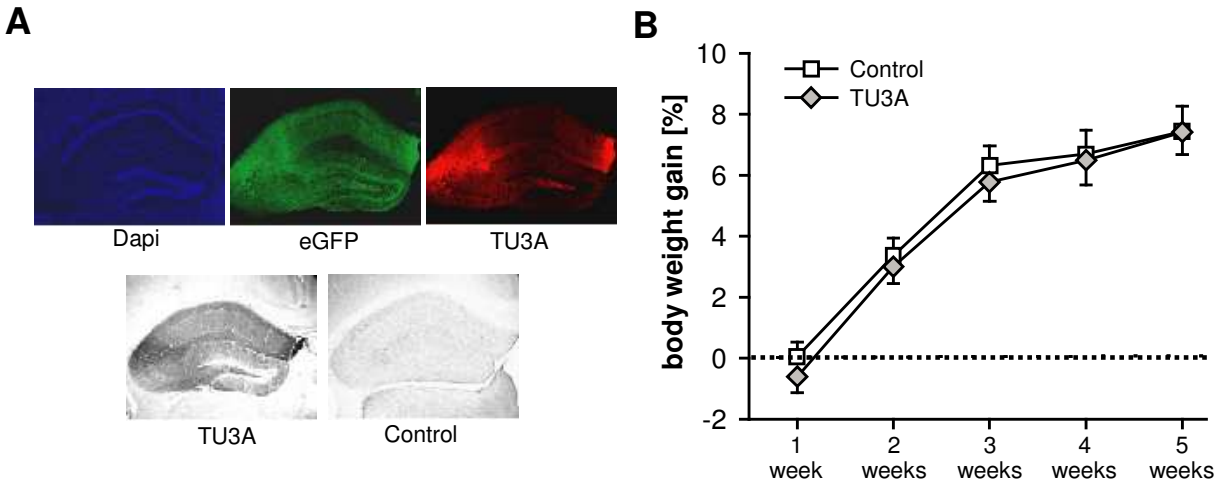

Figure 4.17 TU3A overexpression in the hippocampus

*(**A**) Representative photomicrographs of TU3A staining following overexpression and control virus injection in the hippocampus. In the upper part fluorescence eGFP and overexpressed TU3A staining are shown 3 weeks following the injection, while in the lower part the DAB stained TU3A signal is shown 5 months after viral injection in a control anmal and an overexpressing animal. (**B**) Body weight gain following viral injection into the hippocampus. Body weight gain is expressed as a percentage of increase in body weight from the day of the injection. Combined values of all injected animals are shown with n = 46 per group.*

4.4.2 Effects of TU3A overexpression on home cage activity and locomotion in the open field test

TU3A overexpressing animals were first examined with regard to their locomotor activity under basal conditions in the home cage as well as under mildly stressful conditions in the open field test.

Measurement of home cage activity showed no significant difference between TU3A overexpressing animals and the control group in locomotor activity. Both groups showed low activity during the light phase and an increased activity during the dark phase, which is characteristic for nocturnal rodents.

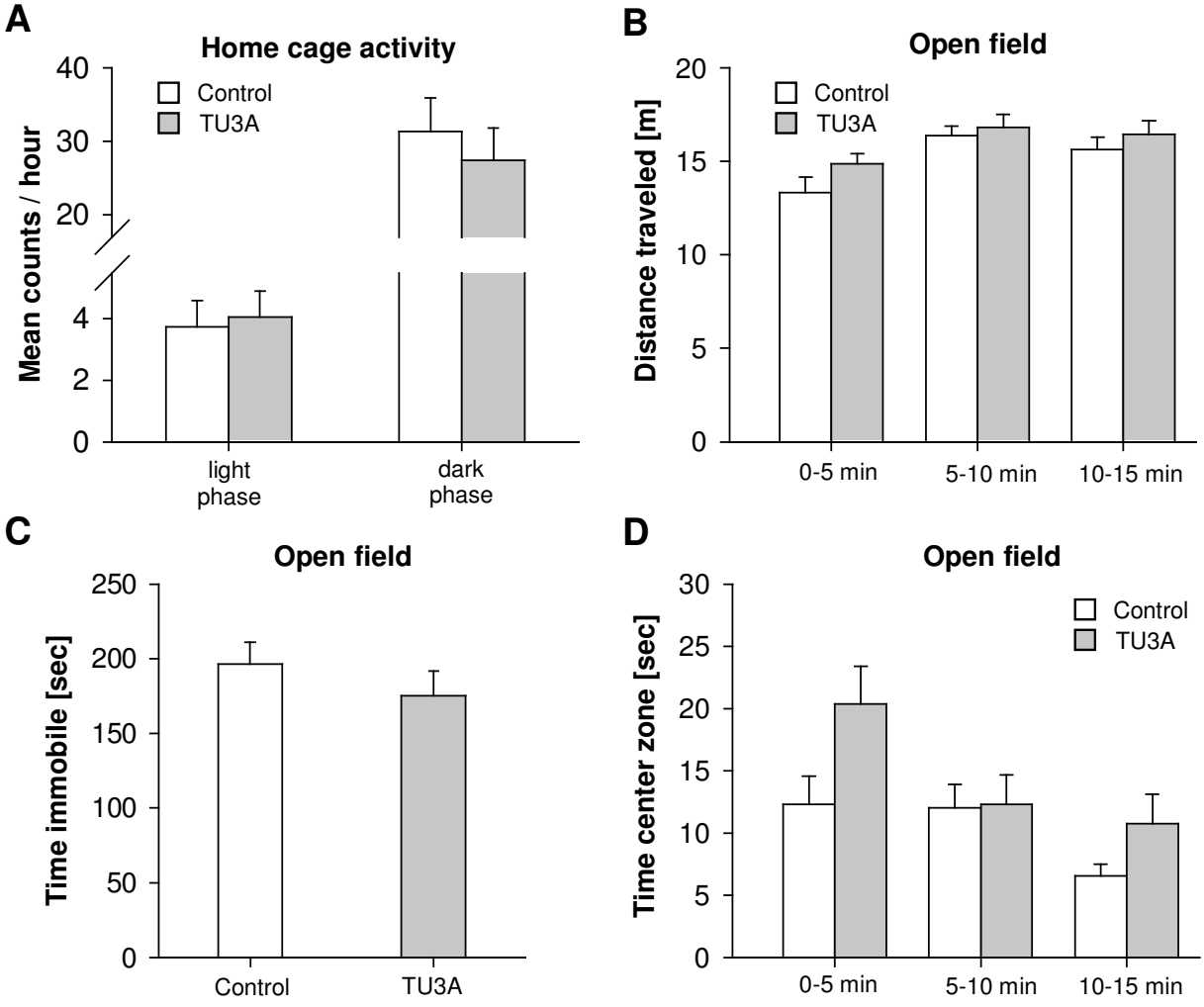

Figure 4.18 Effects of hippocampal TU3A overexpression on home cage activity and locomotion in the open field test

*(**A**) 24 hours home cage activity measurement segmented in 12 hours light phase and 12 hours dark phase. (**B**) Distance traveled in a 15 minutes open field exposure, split into three segments. (**C**) Time the animals spent immobile during the 15*

*minutes open field testing. (**D**) Time the animals spent in the adverse center zone of the open field apparatus, split into three segments.*

Locomotor activity was subsequently tested in the novel environment of an open field arena, which represents a mild stressor for small rodents. The total distance traveled in the novel environment of the open field test revealed no differences between both groups at any time segment of the test. Additionally, there was no difference found between both groups in the total time spent immobile during the open field testing. Furthermore, both treatment groups spent a similar amount of time during each time segment of the test in the more aversive center zone of the open field arena.

4.4.3 Effects of TU3A ove expression on anxiety-related behavior

To assess anxiety-related behavior, two different behavioral tests were conducted in TU3A overexpressing animals.

In the light-dark box test, both groups spent more time exploring the secure dark compartment with no significant difference. Furthermore, no significant differences between TU3A overexpressing mice and the control group were visible concerning neither the latency to first entry into the light compartment, nor in the total number of entries into the light compartment. Hippocampal TU3A overexpression did not alter anxiety-related behavior in the light dark box test. This finding was confirmed by the results of the elevated plus-maze test. TU3A overexpressing mice and the control group spent more time in the closed arms of the apparatus with no significant differences between both groups.

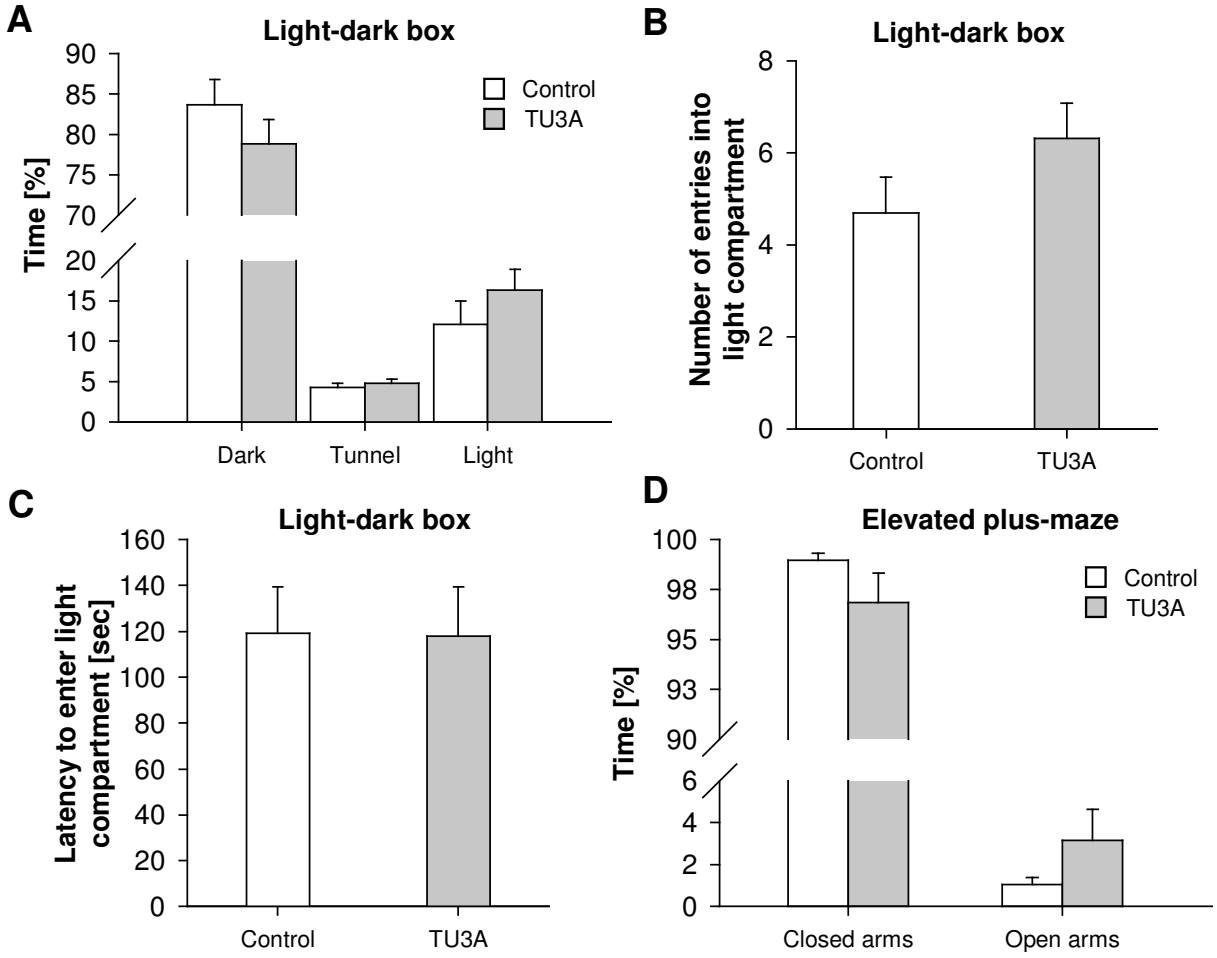

Figure 4.19 Effects of TU3A overexpression on anxiety-related behavior in the light-dark box test and the elevated plus-maze

*(**A**) The percentage of total time the animals spent in the different compartments of the light-dark box apparatus. (**B**) The number of entries into the aversive light compartment of the light-dark box. (**C**) The latency of the first entry into the light compartment of the light-dark box. (**D**) The percentage of time the animals spent in the open and closed arms of the elevated plus-maze.*

4.4.4 Effects of TU3A overexpression on cognitive function in the Y-maze test

To test whether hippocampal TU3A overexpression affects cognitive abilities, the Y-maze test was applied.

When conducting the Y-maze with an inter-trial interval of 15 minutes between acquisition phase and retrieval phase both, the control group and the TU3A overexpressing group spent significantly more time exploring the novel arm of the Y-maze (T_{30} =2.804, $p < 0.01$ and T_{30} = 3.81, $p < 0.01$ respectively). However, when prolonging the inter-trial interval up to 30 minutes, TU3A overexpressing animals still spent significantly more time exploring the novel, unfamiliar arm of the apparatus (T_{26} = 2.957, $p < 0.01$), while the

animals of the control group explored all three arms at chance level. Once the inter-trial interval was extended to 60 minutes, both groups spent a similar amount of time exploring the novel arm and the familiar arms. This finding indicated that TU3A overexpression in the hippocampus enhanced hippocampus-dependent spatial memory in mice.

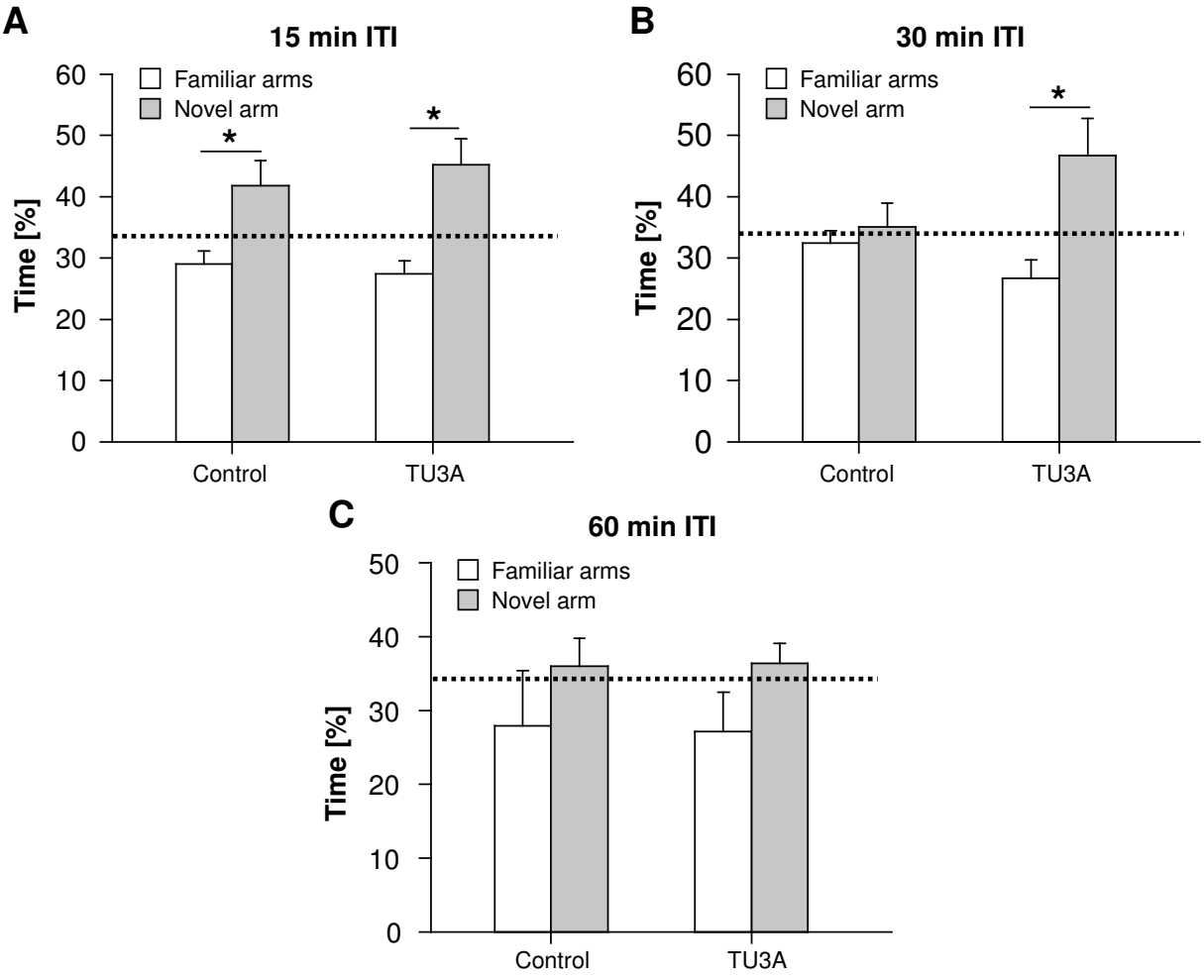

Figure 4.20 Effects of TU3A overexpression on cognitive function in the Y-maze test

*The time spent in the novel arm compared to time spent in familiar arms with (**A**) 15 minutes inter- trial interval (ITI), (**B**) 30 minutes ITI and (**C**) 60 minutes ITI. Time is expressed in percent of total time spent in the three arms; time for the familiar arms is represented as average value of both arms; dashed line represents the chance level of 33%; * significantly different from the familiar arms, $p < 0.05$.*

4.4.5 Effects of TU3A overexpression on cognitive function in the Morris water maze test

To confirm the intriguing finding of the Y-maze, the Morris water maze, a commonly used test paradigm to assess spatial learning performance and memory, was applied.

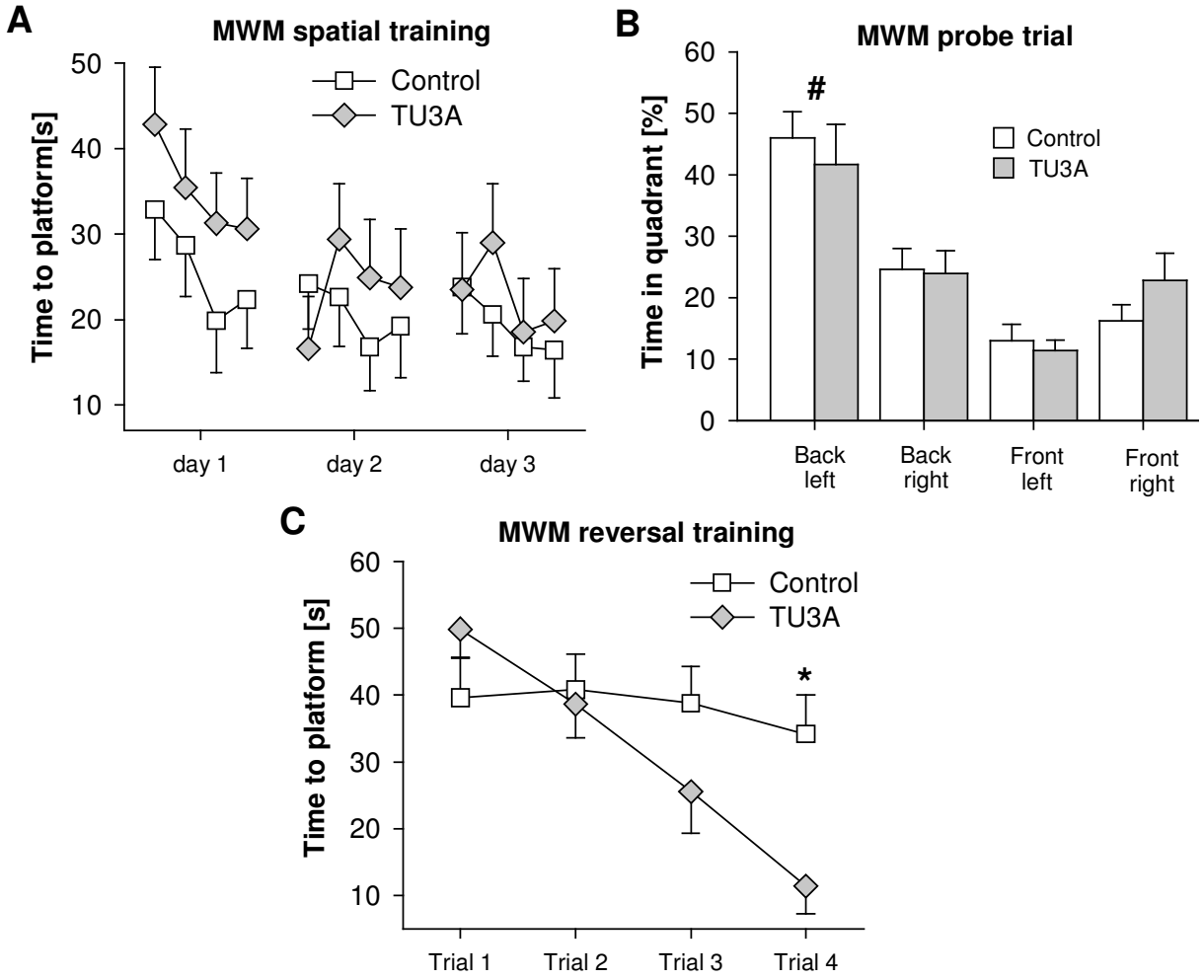

Figure 4.21 Effects of TU3A overexpression on cognitive function in the Morris water maze test

*(**A**) Time the animals needed to find the hidden platform at 3 consecutive days with four trials during the spatial training of the Morris water maze (MWM). (**B**) Time the mice spent in the different quadrants of the MWM during the probe trial, where the platform is absent. Back left is the former platform quadrant. (**C**) Time the animals needed to find the new position of the hidden platform during the reversal training of the MWM. * significantly different from the control group, $p < 0.05$, # significantly different from all other quadrants for both groups, $p < 0.001$.*

Animals of both groups behaved normally during testing. TU3A overexpressing mice and the control group were able to successfully locate the platform and improved during the spatial training with no significant differences between the groups. For the probe trial, repeated measurements ANOVA revealed a significant effect of the quadrant ($F_{119,3}$ = 24.299, p< 0.001), while there was no effect of treatment or quadrant*treatment interaction. After removing the platform, both groups spent significantly more time exploring the former platform quadrant compared to all other quadrants ($F_{119,3}$ = 24.691,

$p < 0.001$), indicating that both groups remembered the position of the platform with no differences between the groups.

However, during the reversal training, TU3A overexpressing animals improved significantly in locating the new position of the platform compared to the mice of the control group. Repeated measurements ANOVA revealed a significant effect of time ($F_{1,28} = 33.653$, $p < 0.001$) and a treatment*time interaction ($F_{1,28} = 18.957$, $p < 0.001$). Hence, TU3A overexpressing animals demonstrated an improved cognitive flexibility in comparison with the control group.

4.4.6 Effect of TU3A overexpression on the forced swimming test performance

The influence of TU3A overexpression on stress-coping behavior in the forced swimming test was examined. Due to the fact that the forced swimming test represents a strong stressor for mice, the corticosterone response after testing was additionally measured.

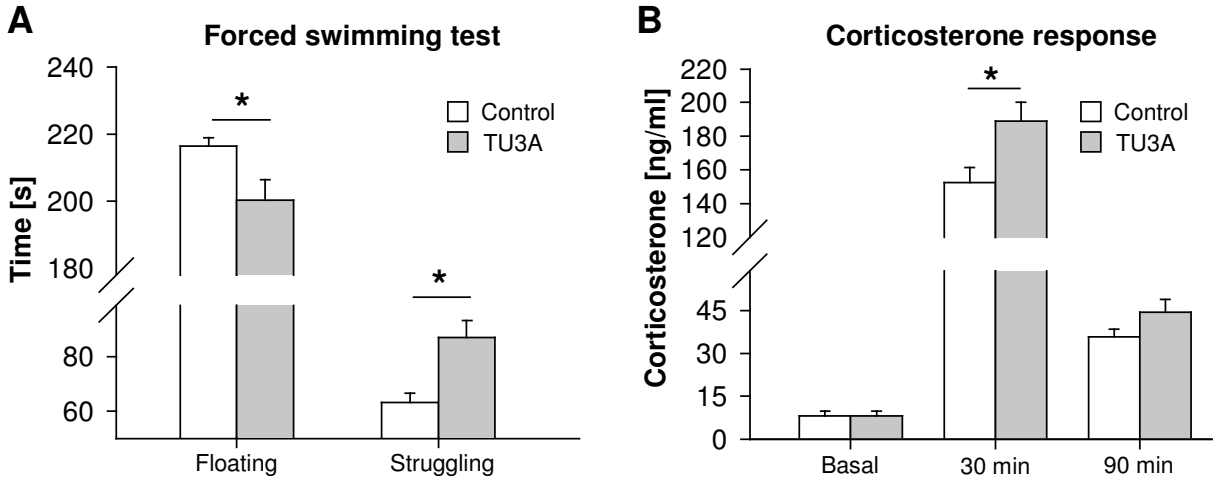

Figure 4.22 Effect of Tu3A overexpression on the forced swimming test performance

*(**A**) Time the animals spent floating or struggling during the five minutes testing period in the forced swimming test. (**B**) Circulating plasma corticosterone levels under basal conditions, 30 minutes and 90 minutes following the forced swimming test. * significantly different from control group p, < 0.05.*

The TU3A overexpressing animals showed a more active coping strategy in the forced swimming test compared to the control group. TU3A overexpressing animals spent significantly more time struggling ($T_{26} = 2.831$; $p < 0.05$) and significantly less time floating ($T_{26} = 2.302$; $p < 0.05$) compared to the control animals. Regarding the plasma corticosterone response after the forced swimming test ANOVA showed a significant effect of time ($F_{84,2} =$

390.804, $p < 0.001$) condition ($F_{84,1} = 8.919$, $p < 0.01$) and a time*condition interaction ($F_{84,2} = 4.778$, $p < 0.001$). While there were no significant differences detectable in basal morning plasma corticosterone levels, plasma corticosterone levels of TU3A overexpressing animals were significantly elevated 30 minutes after the forced swimming test compared to plasma corticosterone levels of the control group ($T_{26} = 2.579$, $p < 0.05$). However, 90 minutes after the forced swimming test the differences in plasma corticosterone levels between both groups were no longer apparent.

5 Discussion

Stressful environmental stimuli initiate multifactorial behavioral and physiological responses in order to enable the organism to cope optimally with the challenge. The HPA axis plays a central role in many of these processes mainly by activation of two receptor subtypes, the MR and the GR. Both receptors act as transcription factors and induce a plethora of transcriptional changes, leading to acute as well as persistent alterations in neuronal circuits. In this thesis, TU3A, a novel stress-induced gene, was characterized in the context of its potential function in the central stress system. Our analysis, ranging from molecular to behavioral approaches, could demonstrate that TU3A represents a novel molecular link between stress and complex behavior.

5.1 TU3A is region-specifically expressed in the postnatal and adult mouse brain

The fact that TU3A is highly expressed in the CNS (Yamato et al., 1999; Wang et al., 2000; Su et al., 2004) indicates an important role of this gene in brain function. We therefore investigated the regional distribution of TU3A expression at different developmental stages in the mouse brain, in order to obtain additional information on the processes in which TU3A might be involved.

The neuroanatomical expression analysis revealed an interesting spatial TU3A mRNA expression pattern in the mouse brain at both examined developmental stages: Apart from the prominent TU3A expression in specific cerebellar cell layers, the TU3A expression followed mostly an anterior-posterior gradient in the brain, with a more pronounced expression in regions of the *prosencephalon* and only very little TU3A expression in the *mesencephalon* and the *rhombencephalon*. This region-specific expression pattern is an indicator that the function of TU3A is specific for distinct brain regions and cell populations, rather than exerting a ubiquitous functional relevance in the brain. Interestingly, the most prominent TU3A mRNA and protein expression was observed in distinct neuronal cell types: the pyramidal neurons of the hippocampal CA3 region and the Purkinje cells in the cerebellum. While TU3A expression is certainly not exclusively restricted to neurons, since TU3A mRNA expression has been demonstrated for other tissues and cell types as well

(Wang et al., 2000; van den Boom J. et al., 2006), our findings suggest a distinct role of TU3A in specific neuronal populations of the brain. Noticeably, a pronounced TU3A expression is present in several limbic structures, including the amygdala, the prefrontal cortex, the lateral septum and the hippocampus. Limbic brain structures are strongly involved in emotional, autonomic, motor and cognitive processes (McLachlan, 2009) and are pivotal for HPA axis response integration and regulation (Herman et al., 2005). In particular the CA3 region of the hippocampus and the lateral septum showed the strongest TU3A expression in the *prosencephalon*. Both structures are part of a neuronal regulatory circuit mediating the negative feedback inhibition of the HPA axis (Dobrakovova et al., 1982; Sapolsky et al., 1991). Therefore, the pronounced TU3A expression in limbic structures points towards a potential involvement of TU3A in stress-related neuronal circuits and strongly encouraged us to further study its involvement in the central stress physiology.

Strikingly, the regional expression pattern of TU3A was very similar in the neonatal and the adult mouse brain. However, there were also some marked region-specific differences in expression intensity between both ages, suggesting a specific role of TU3A during development in some, but not all brain regions. Examples are the hippocampal CA1 and CA2 regions where TU3A mRNA was moderately expressed in the neonatal brain, while it was only weakly expressed in the adult brain. Additionally, the horizontal cortical layers IV and V showed a more pronounced TU3A mRNA expression at postnatal day 9 compared to the adult mouse brain. Conversely, TU3A mRNA was only weakly expressed in the hypothalamic mammillary nuclei of neonatal animals, but was strongly expressed in the adult mouse brain. Our findings are supported by previous reports, which suggested a putative role of TU3A during early brain development in *Xenopus laevis* (Zhao et al., 2007) and in a neural differentiation system of human embryonic stem cells (Pankratz et al., 2007).Taken together, these findings emphasize a region-specific role of TU3A at different aging stages.

5.2 TU3A mRNA shows a distinct spatiotemporal pattern during postnatal ontogeny

The intriguing finding that there are region-specific variations of TU3A mRNA expression between the neonatal and the adult mouse brain motivated a detailed TU3A expression comparison during postnatal ontogeny in specific brain regions.

During the first weeks following birth, extensive maturation of the murine brain takes place, including increase in size, neuronal outgrowth and differentiation (Hahn et al., 1983; Verma et al., 2005). The maturation of distinct brain regions follows different spatiotemporal patterns. For instance, in the cortex neurogenesis is almost complete at birth and only migration and differentiation are still in progress (Nadarajah et al., 2003), while in the hippocampal dentate gyrus a peak in neurogenesis arises postnatally (Li and Pleasure, 2005). Severe mental disorders like schizophrenia strongly correlate with environmental insults during critical developmental periods (Buka and Fan, 1999).Therefore, an undisturbed brain development, directed by induction and termination of specific transcripts at distinct time windows, is essential for the normal function of the organism during adulthood. For TU3A we found a distinct spatiotemporal expression pattern throughout postnatal ontogeny. In the amygdala and the hypothalamic PVN, moderate TU3A expression levels remained almost unchanged from birth up to adulthood, suggesting an age-independent continued relevance for TU3A in both regions under basal conditions. In contrast, TU3A expression strongly increased at postnatal day 12 in the hippocampal CA3 and the cortex, indicating an enhanced importance of TU3A in those regions at later developmental stages up to adulthood. Interestingly, we detected the lowest TU3A expression in the PVN at postnatal day 12, while at this point of time the highest TU3A expression was present in the hippocampal CA3 region. Postnatal day 12 is a crucial point of time during brain development and marks the end of the SHRP in mice (Schmidt et al., 2003). During this developmental period, mice exhibit very low plasma corticosterone level and are protected from mild stressors. Additionally, several central marker of the HPA axis, like CRH or the GR, show a specific spatiotemporal expression pattern with significant changes at the end of the SHRP (Schmidt et al., 2003). Our findings therefore support a varying region-specific importance of TU3A at this critical point of brain maturation.

Moreover, the region-specific induction of a transcript at a distinct time window can hint at a potential function for the gene. Mody et al. analyzed the gene expression pattern in

the hippocampus during ontogeny and found several distinct functional gene clusters, which are specifically expressed at defined time windows (Mody et al., 2001). The early postnatal developmental stage, specifically the first week after birth, is characterized by rapid morphological changes, cellular differentiation and synapse formation. Hence, predominantly cytoskeletal and structural proteins are strongly expressed during this period (Bradke and Dotti, 2000; Mody et al., 2001). By the second postnatal week hippocampal synapses and circuits are well established and become more active. This is reflected by a pronounced increase of genes involved in synaptic function and signal transduction (Mody et al., 2001). A similar functional gene expression pattern was demonstrated during the postnatal cortical development in rats (Lee et al., 2009a). Intriguingly, TU3A mRNA expression strongly increased in the hippocampus and the cortex during the second postnatal week, suggesting a putative role of TU3A in modulating synaptic function. This is further supported by an electron microscopy study in the adult hippocampus, where TU3A was predominantly localized in the presynaptic terminals and to a lesser extent also to the postsynaptic site of hippocampal pyramidal neurons (Michael Stewart, The Open University, Department of Life Sciences, unpublished data). Taken together, we could show an interesting spatiotemporal expression pattern of TU3A during postnatal ontogeny, which points towards a possible role of TU3A in processes related to synaptic function.

5.3 TU3A is stress-inducible in the neonatal and adult mouse brain

The ability of environmental challenges to induce the expression of a specific transcript can be strongly dependent on the developmental stage of the individual. In rodents, the HPA axis is characterized by a period of relative quiescence during the postnatal development, the so-called SHRP (Levine, 2001). This period has been shown to be of particular importance for the individual's stress responsiveness later in life (Gillespie et al., 2009). Disturbances of the SHRP have a programming influence on adult HPA axis activity and are associated with increased vulnerability to stress-related disorders like major depression (Neigh et al., 2009). Genes that are stress-responsive during this particular period are therefore interesting candidates, as they might represent potential mediators of these programming effects. Confirming the result of the initial microarray study in neonatal mice (Liebl et al., 2009) and the subsequent validation during the author's diploma thesis (Liebl, 2006), we found TU3A to be increased in the PVN after maternal separation. In addition, we found TU3A mRNA

expression to be stress-regulated in the CA3 region of the hippocampus and in the amygdala. Interestingly, TU3A is stress-inducible in a region-specific manner in neonates. We found TU3A neither to be stress-regulated in the cortex nor in the cerebellum, areas where the transcript is strongly expressed under basal conditions. Taken together, we could demonstrate that TU3A mRNA expression is stress-sensitive in neonates in HPA axis relevant regions. These findings imply that TU3A may play an important role in mediating the consequences of robust HPA axis activity during the postnatal period.

While stress-regulation of TU3A during the postnatal period points to an important function during development, it is of further interest whether TU3A may also play a decisive role in adult stress physiology. Therefore, we examined TU3A mRNA expression with regard to three different stress paradigms in the adult mouse brain: 24 hours of food deprivation, 30 minutes of restraint stress, and chronic social stress. Food deprivation, like maternal separation in neonates, represents a very potent and stressful stimulus mimicking a potentially life-threatening situation for the animals. This is reflected in a profound and sustained plasma corticosterone increase (Kiss et al., 1994). Restraint stress is a commonly used stress paradigm representing a single, moderately stressful stimulus for rodents with a peak of plasma corticosterone after 30 minutes, which gradually decreases to basal levels during the next hour (Paskitti et al., 2000; Schmidt et al., 2009). Chronic social stress, like restrained stress, results in a comparably lower corticosterone release than food deprivation, but induces a HPA axis activation over a prolonged period (Schmidt et al., 2007). Intriguingly, only the stress paradigm that evoked a very pronounced corticosterone response was able to increase TU3A mRNA expression, while less profound or shorter stress paradigms did not significantly increase TU3A mRNA expression in any of the examined regions. This is in contrast to earlier findings during the author's diploma thesis (Liebl, 2006), where restraint stress induced TU3A mRNA expression 4 hours after the stress. This discrepancy may be due to procedural differences, which resulted in a more pronounced HPA activation in the former study. To assess a possible temporal TU3A induction at other time points after restraint stress, the time course of gene expression was examined. However, no differences in TU3A expression were evident at any time point after restraint stress, supporting our finding that comparably high corticosterone levels are necessary to induce TU3A expression. These findings imply that, in addition to the neonatal situation, TU3A may play an important role in mediating the consequences of pronounced and

prolonged HPA axis activity in the adult brain, while probably being less important for moderate stress situations or e.g. variations due to circadian rhythmicity.

The impact of stress in humans is highly dependent on the genetic background and the gender of the individual (McEwen and Stellar, 1993; Kudielka and Kirschbaum, 2005). Women are generally more prone to develop stress-related disorders and there is a strong sexual divergence in the susceptibility to adverse life events (Kendler et al., 2003). In rodents, females display higher corticosterone levels in response to stress than males (Kant et al., 1983; Frederic et al., 1993) and the expression of several central markers of HPA axis activity differs from the male situation (McCormick et al., 2005; Schmidt et al., 2010). Moreover, numerous studies demonstrated strong inter-strain variances in behavior and gene expression (Rossi-Arnaud and Ammassari-Teule, 1998; Sandberg et al., 2000; Brown and Wong, 2007). To prove the stress-related relevance of TU3A to be largely independent of strain and gender-specific factors, we examined the TU3A mRNA expression in a different, genetically more heterogonous outbred CD1 strain and in female mice following food deprivation. We found that TU3A is also stress-inducible in the outbred mouse strain and in female mice in all examined regions. As induction of TU3A seems largely strain- and gender-independent, these findings further support a function of this gene in general stress physiology.

Specific brain regions have distinct activating or inhibitory impact on HPA axis regulation and several regions form hierarchical networks to convey the organism's response to stress (Herman et al., 2003). In the rodent brain, a considerable amount of morphological development, neuronal differentiation and outgrowth as well as the establishing of functional networks takes place during the postnatal period (Hagberg et al., 2002). This maturation of the brain is reflected in dynamic temporal changes of gene expression during development. Interestingly, TU3A expression is stress-inducible in different regions in the adult and neonatal brain. While TU3A is age-independently inducible in the hypothalamic PVN and the hippocampal CA3 region, induction of TU3A is age-dependent in the cortex and the amygdala. In the cortex, where TU3A expression increased during postnatal development, TU3A mRNA expression was only stress-regulated in adult mice. In the amygdala, a continued moderate TU3A mRNA expression is present at all ages, although TU3A mRNA expression is only stress-inducible during the postnatal period. Thus, the stress related function of TU3A seems to be necessary in distinct regions at different points of

time. One possible explanation for this varying stress sensitivity in gene expression could be a different epigenetic programming of the promoter region. Awakura et al. demonstrate that a promoter hypermethylation is causal for the TU3A down-regulation in several renal cell carcinoma lines (Awakura et al., 2008). Another reason could be the different expression of transcription factors and their co-regulators at different points of time in a region-specific manner (Kino, 2007). These findings imply a prominent role of TU3A in stress physiology at a distinct region-specific time window as well as the persistent importance of TU3A in central regions of the HPA axis.

5.4 The stress-induced regulation of TU3A is mediated by the GR

The main mediators of glucocorticoid action in the brain are the GR and the MR (de Kloet et al., 1998). Both receptors act as transcription factors and modulate gene expression by binding to GREs (Arriza et al., 1987). While glucocorticoids bind to both receptors, the MR exhibits a 10-fold higher affinity for glucocorticoids than the GR (Reul and de Kloet, 1985). The MR is already active at basal glucocorticoid levels, while the GR responds to increased stress-induced levels of glucocorticoids indicating a different functional relevance of both receptors (de Kloet et al., 1999). Bioinformatical analysis identified highly conserved GREs in the promoter region of the TU3A gene (Dietrich Trümbach, Helmholtz Zentrum München, Institute for Developmental Genetics, unpublished data). In a subsequent receptor binding assay, those GREs proved to be functionally active *in vitro* (Gabi Wochnik, Max Planck Institute of Psychiatry, RG Rein, Chaperones, unpublished data). We could show that TU3A expression is inducible in the neonatal and adult mouse brain by a stressor, which evoked a pronounced corticosterone response. We therefore addressed the question, whether the increased TU3A expression following stress is mediated via a GR- or MR-dependent mechanism. Treatment with dexamethasone, a potent synthetic GR agonist strongly activates the GR but not the MR (Veldhuis et al., 1982; de Kloet et al., 1984; Rupprecht et al., 1993), and caused a pronounced elevation of TU3A mRNA expression in all brain regions where we previously found TU3A to be up-regulated by food deprivation in the adult mouse. The TU3A increase is also reflected in elevated protein levels in the examined brain regions, further supporting a functional relevance of the gene product in the context of stress. Dexamethasone strongly induces the GR-mediated negative feedback mechanism of the HPA axis, which results in the suppression of endogenous corticosterone (Britton et al., 1986).

Therefore, observed gene expression differences are clearly mediated by GR stimulation via dexamethasone. Supporting our findings, other studies demonstrated a dexamethasone-induced TU3A increase in human lens tissue (Gupta et al., 2005; Rozsa et al., 2006). This suggests a regulatory role of the GR in TU3A expression in tissues other than the brain. Interestingly, in the cerebellum, where TU3A is strongly expressed under basal conditions in Purkinje cells, we found no effect of dexamethasone on the TU3A protein concentration. This finding suggests additional GR-independent regulatory mechanisms for TU3A expression under basal conditions, which is possibly region- or cell type-specific. Our findings that dexamethasone strongly induces TU3A gene expression clearly identifies the GR to be the main regulator of TU3A gene expression in response to stress.

We were able to further support this finding by treatment with a GR antagonist during stress. GR antagonist, but not MR antagonist treatment, completely prevented the stress-induced TU3A expression in the PVN and the hippocampal CA3 region of neonatal mice. These results verify and complement our previous findings during the author's diploma thesis, where only the PVN was examined (Liebl, 2006). Systemic blockade of the GR results in decreased feedback inhibition of the HPA axis and thereby causes elevated corticosterone levels (Schmidt et al., 2005), which was also observed in the present study. In addition to the neonatal situation, the inhibitory influence of the GR antagonist on stress-induced TU3A expression is also present in the PVN of adult mice, while treatment with a MR antagonist had no effect. This demonstrates an age-independent regulatory role of the GR in stress-induced TU3A mRNA expression. A clear picture on the influence of the GR antagonist treatment in the other brain regions was not possible due to confounding factors: In contrast to earlier findings TU3A expression was not elevated in the CA3 region of the hippocampus and the cortex of adult animals after food deprivation in this experiment. This is possibly due to the additional stress of the repeated injection, reflected in the relatively high morning levels of plasma corticosterone levels in the vehicle treated group. This corticosterone increase in the control animals is very likely masking the differences between the groups. Additionally, the ratio of the relative stress-induced TU3A increase is more pronounced in the PVN compared to other examined regions, which is possibly due to the high GR expression in this region (Reul and de Kloet, 1985). This facilitates the detection of expression differences in the PVN even under the influence of confounding variables like the additional stress of the injection procedure. This problem is not apparent in neonatal mice,

due to the protective effect of the SHRP that prevents HPA axis activation in response to mild stressors like injections (Levine, 1957). Thus, we demonstrated that blockade of the GR, but not the MR, abolished the stress-induced TU3A transcription. The GR, which is only active at peak levels of corticosterone (Reul et al., 1987), is a main mediator of HPA axis response to stress. Both the strong TU3A induction after GR agonist treatment and the inhibition of TU3A expression following stress by a GR antagonist demonstrate the GR as the main mediator of the stress-induced TU3A transcription. As a result these findings strengthen a role of TU3A in the physiology of stress.

The GR modulates the expression of numerous target genes in response to a stressful stimulus and thereby provides the molecular basis for physiological and behavioral processes of adaptation in response to a challenge. The transcriptional activity of the GR is highly fine-tuned in a spatial and temporal manner by numerous co-factors. Modification of target-gene transcription can occur as early as 15 to 30 minutes following receptor activation and may last from less than an hour up to several days (Datson et al., 2008; John et al., 2009). Several studies demonstrated a phased transcriptional response to GR-activation, with different clusters of transcribed genes at distinct points of time, with only very little overlap between the different time windows (Almon et al., 2003; Morsink et al., 2006; Tsolakidou et al., 2008). This phased transcription suggests that not only the inducibility per se, but also the time window of transcript induction is important. At earlier time windows genes are more likely to be directly GR-regulated and often involved in regulatory transcriptional functions, while at later points of time genes that are downstream of regulatory cascades predominate (Tsolakidou et al., 2008). Additionally, transcriptional rates for some genes are rapidly increased and remain continuously elevated over longer periods, suggesting a pronounced impact of these genes in mediating persistent stress effects. To examine the kinetic pattern of GR-induced TU3A expression, the time course after a single dexamethasone injection was determined. TU3A levels were increased 4 hours after dexamethasone treatment and were still elevated after 8 hours in all examined regions, suggesting a prolonged impact of TU3A function after GR stimulation. Our findings are in line with another study, showing a TU3A increase 4 and 16 hours following dexamethasone treatment in human lens tissue (Gupta et al., 2005). Remarkably, of the 222 genes regulated by dexamethasone, Gupta et al identified only 6 others which were continuously regulated at both points of time. Interestingly, in the PVN and the hippocampal CA3 region TU3A expression reverted to basal level after 24 hours,

while increased TU3A expression was still present in the cortex at this time. This continued TU3A increase is likely due to a different region-specific composition of GR co-regulators (Tetel, 2009) and suggests a different region-specific importance of TU3A. Thus, our findings indicate a long lasting effect of TU3A after induction and further suggest a possible functional relevance of TU3A in the mediation of the stress-response.

5.5 TU3A overexpression in the hippocampus influences behavior and synaptic function

In the previous chapters we demonstrated that TU3A is strongly inducible by profound stressful stimuli, coupled with pronounced plasma corticosterone elevations. This stress-induced TU3A increase could be shown to be mediated by the GR, one of the main regulatory effectors of the HPA axis. These findings imply a functional relevance of TU3A in stress-related processes. However, stress exposure affects a plethora of other factors, leaving the specific function of TU3A indistinct. Hence, we used a region-specific overexpression approach to investigate the function of TU3A *in vivo*. By use of an adeno-associated viral vector for gene transfection we were able to induce a stable TU3A overexpression in a distinct brain region. The hippocampus was targeted, since on the one hand strong TU3A expression was present at all ages under basal conditions and on the other hand TU3A was strongly stress-inducible in this brain region. Furthermore, the hippocampus is a well characterized brain region, which is crucially involved in cognitive performance and contributes to several aspects of emotional behavior like anxiety-related or stress coping behavior (File et al., 2000; Bannerman et al., 2004; Bishop, 2007), enabling the examination of a potential influence of TU3A in those systems.

The successful TU3A overexpression was confirmed by a pronounced and stable immunoreactive TU3A staining throughout the hippocampal formation. This TU3A overexpression had no obvious negative effects on the well-being of the animals, even after several months of continued overexpression. Additionally, we did not detect any alteration in locomotor activity of the animals neither under familiar home cage nor mild stressful conditions. These findings demonstrate that TU3A overexpression in the hippocampus had no potentially confounding effects on general behavioral parameters in mice.

As a next step, we characterized the effects of hippocampal TU3A overexpression on anxiety-related behavior using two different test paradigms: the light-dark box test (Crawley and Goodwin, 1980) and the elevated plus maze test (Handley and Mithani, 1984). Although, anxiety-related behavior is classically mediated by other brain regions, like the amygdala or the lateral septum, several studies also demonstrated an involvement of the hippocampus in anxiety-related behavior (Bannerman et al., 2004; Deacon and Rawlins, 2005). However, we did not find any effect on anxiety-related behavior due to TU3A overexpression in both tests. These results suggest that TU3A, at least in the hippocampus, seems to be unrelated to anxiety-related behavioral processes under basal conditions.

Next, we examined potential effects of TU3A overexpression on stress-coping behavior, applying another commonly used test paradigm, the forced swimming test. The forced swimming test is the most widely used animal test predictive of antidepressant action, because it models depression-like behavior in the form of “behavioral despair” in rodents (Petit-Demouliere et al., 2005). Antidepressants are known to induce a more active coping strategy in this test paradigm, reducing the “behavioral despair” expressed as immobile behavior (Porsolt et al., 1977).While the test paradigm does not model depression per se, it gives an indication of the animal’s coping strategy in an averse situation. We found, that TU3A overexpressing mice showed a more active coping strategy in the forced swimming test, suggesting a beneficial influence of TU3A comparable to antidepressant action. While the exact molecular mechanisms underlying this finding remain elusive, TU3A overexpression clearly induced specific changes in the emotional behavior of the animals, which might be founded in altered hippocampal structural characteristics as well as modified synaptic transmission and plasticity.

Apart from its role in anxiety-related and stress-coping behaviors, the hippocampus plays an important role in cognitive processes like learning and memory and is crucially involved in spatial learning tasks (Eichenbaum, 2004; McDonald et al., 2004). To investigate a possible impact of TU3A in cognitive processes, two commonly used test paradigms for hippocampus-dependent spatial memory were applied: the y-maze test (Dellu et al., 1992) and the Morris water maze test (Morris, 1984). Intriguingly, we found a stimulating effect of TU3A overexpression on cognitive performance in both test paradigms. In the y-maze, animals overexpressing TU3A remembered the familiar arms better than the controls after a prolonged inter-trial interval, demonstrating that TU3A may be important to enhance or

preserve memory over a longer time. This suggests an increased cognitive capacity in the mice, due to TU3A overexpression in the hippocampus. In the Morris water maze test, both groups learned the location of the platform equally well. This finding may be due to the short inter trial interval of 10 minutes between the different trials, since the cognitive improvement in the y-maze became only apparent after a prolonged inter-trial interval phase. Nonetheless, the overexpressing mice adapted better to the changed platform position in the reversal learning task. The ability to forsake a previously learned spatial navigation strategy in order to develop a novel strategy is attributed to increased cognitive flexibility (Clapcote and Roder, 2004). Therefore, this finding indicates an enhanced cognitive flexibility following TU3A overexpression.

Although, the effects of corticosterone on cognitive function are known to be very complex, there is evidence to suggest that the enhanced cognitive performance in both test paradigms might be partially mediated by an increase of corticosterone caused by the testing procedure. The Morris water maze represents a strong stressor for mice and causes elevated plasma corticosterone levels 30 minutes after testing (Harrison et al., 2009). Exposure to a novel environment, as in the y-maze paradigm, is also known to increase plasma corticosterone levels in rodents (Hennessy and Foy, 1987). Although the effect of corticosterone on cognition is time- and dose-dependent, elevated plasma corticosterone levels have previously been shown to act beneficial on cognitive performance e.g. in the Morris water maze (Sandi et al., 1997). TU3A overexpressing mice showed an enhanced corticosterone response following stress compared to the controls: 30 minutes after the forced swimming test, overexpressing animals displayed higher corticosterone levels than the control group. Those differences were no longer detectable 90 minutes after the test and were not apparent under basal conditions. This is an important factor, as elevated corticosterone levels manifested after the forced swimming test during the same time window when TU3A overexpressing mice displayed enhanced cognitive performance in both spatial learning paradigms. Taken together, we could show that TU3A overexpression in the hippocampus enhanced spatial memory and improved cognitive flexibility, thereby clearly exerting a beneficial effect on cognitive performance in mice.

These findings are particularly interesting since we demonstrated that a stress-inducible factor, when overexpressed in the hippocampus, enhanced cognitive performance under basal conditions. Numerous studies have demonstrated that stress has a strong

impact on cognitive function (Sandi and Pinelo-Nava, 2007). While prolonged chronic stressors have a detrimental effect on cognition (Conrad et al., 1996; Radecki et al., 2005), acute stress often facilitates cognition (de Kloet et al., 1999; Das et al., 2000). However, the specific molecular mediators of the profound effects of stress on hippocampus-dependent cognitive functions are still unclear. The improved cognitive performance following TU3A overexpression suggests TU3A as a factor that could be involved in mediating particularly the beneficial and adaptive consequences of stress with regard to cognition.

Cognitive processes are strongly interrelated with hippocampal synaptic transmission and plasticity (Silva, 2003), which are partly reflected in the pre- and postsynaptic electrophysiological properties of hippocampal synapses. Another group at our institute (Charilaos Avrabos, Max Planck Institute of Psychiatry, RG Eder, neuronal network dynamics, unpublished data) performed field excitatory postsynaptic potential (fEPSP) recordings in CA3-CA1 synapses of acute brain slices from TU3A overexpressing mice as possible indicators for alterations in synaptic transmission and plasticity. Paired-pulse facilitation was examined as a marker for presynaptic short-term plasticity. In addition, input-output relationships as a marker for synaptic transmission as well as long-term potentiation (LTP) were investigated. They were able to demonstrate that overexpression of TU3A in the hippocampus increased paired-pulse facilitation in CA3-CA1 synapses along with a shift of input-output curves towards smaller fEPSP amplitudes and a reduced magnitude of LTP. The increase in paired-pulse facilitation is thought to be caused presynaptically (Zucker and Regehr, 2002), thereby strongly supporting a presynaptic function of TU3A. This will be discussed more detailed in the following chapter. While those findings clearly attest altered electrophysiological properties in the hippocampus following TU3A overexpression, the decreased LTP seems to be conflicting, as improved cognition is often associated with facilitated LTP (Gruart and gado-Garcia, 2007). However, there is also evidence that hippocampal LTP does not model the actual *in-vivo* processes of learning and memory (Hölscher, 1997). Indeed, several studies observed functional spatial memory under conditions where LTP is blocked (Saucier and Cain, 1995; Reisel et al., 2002). Thus, while the decreased LTP in TU3A overexpressing mice indicates an impact of TU3A on hippocampal plasticity and function, the data can not be directly correlated to the behavioral phenotype of the animals.

Taken together, we could demonstrate profound changes in hippocampal synaptic plasticity following TU3A overexpression, which were also evident at a behavioral level in the

animals. These results are further appealing, since cognitive dysfunction is known to play a pivotal role in a variety of diseases, including major depression and schizophrenia and there is evidence that cognitive impairment may in fact underlie many of the affective symptoms (Van Snellenberg, 2009; Geerts and Brune, 2009; Spijker and van Rossum, 2009). While there are numerous ways to disrupt memory, enhancing cognition proves to be more difficult (Lee and Silva, 2009). The identification of factors which improve cognitive aspects, like TU3A, may therefore contribute to the development of novel treatment strategies for diseases associated with cognitive dysfunction.

5.6 TU3A interacts with actin: a potential mechanism of modulating synaptic reorganization

Our study, in cooperation with several other groups, presents TU3A for the first time as a protein that unites the characteristics of being stress- and in particular glucocorticoid–responsive, directing actin formation as well as influencing synaptic plasticity and complex behavior. We could show that TU3A is strongly expressed in the pyramidal neurons of the hippocampal CA3 region. In more detail, an electron microscopy study located TU3A predominantly in the presynaptic terminals and to a lesser extent also in the postsynaptic density of hippocampal synapses (Michael Stewart, The Open University, Department of Life Sciences, unpublished data). This specific location of the protein in synapses strongly implies a role of TU3A in synaptic function.

In addition, the electrophysiological data, especially the increase of the paired-pulse facilitation ratio following TU3A overexpression further supports a presynaptic relevance of TU3A. Paired-pulse facilitation is an indicator for the release probability of fusion-competent synaptic vesicles in the presynaptic terminal (Thomson, 2000), therefore increased paired-pulse facilitation is characteristic for a low release probability of the synapse: The first stimulus induces exocytosis of fusion-competent vesicles in the active zone of a synapse, causing neurotransmitter release into the synaptic cleft. Synapses, which still have fusion-competent vesicles available after the first stimulus, are again able to release neurotransmitters when a second stimulus is applied. This results in an increase of paired-pulse facilitation, due to the initial low release probability. In contrast, synapses which

readily release the majority of fusion-competent vesicles during the first stimulus show a decrease in paired pulse facilitation.

Furthermore, TU3A is not only an interaction partner of actin, but promotes the polymerization of G-actin towards its helical filamentous form (F-actin) and exerts a stabilizing effect on F-actin by induction of actin bundles (Schülke JP, 2009). This finding is of particular interest, since actin is the major cytoskeletal element in presynaptic nerve terminals (Cingolani and Goda, 2008) and is believed to be crucially involved in regulating vesicle availability in the presynaptic nerve terminal (Landis et al., 1988). Synaptic vesicles are organized into three distinct functional pools: the readily releasable pool consisting of fusion-competent vesicles, the recycling pool and the reserve pool. Actin, due to its highly dynamic turnover and by interaction with several actin-binding proteins is believed to maintain and regulate those vesicle pools and contribute to the replenishment of fusion-competent vesicles (Evergren et al., 2007). Interestingly, another study demonstrated that changes in presynaptic actin dynamics directly affected the efficacy of neurotransmitter release (Morales et al., 2000). In particular, this study showed that latrunculin A, a factor that opposite to TU3A, promotes actin depolymerization, also caused the opposite electrophysiological effects, i.e. a decreased paired-pulse ratio and increased input-output relationship. In conclusion, we propose that the stress-induced increase of TU3A shifts actin dynamics towards a more inflexible state, due to an increase of condensed actin-bundles, which in turn influences the availability of fusion-competent vesicles. This results in decreased neurotransmitter release probability of the synapse (See Figure 5.1).

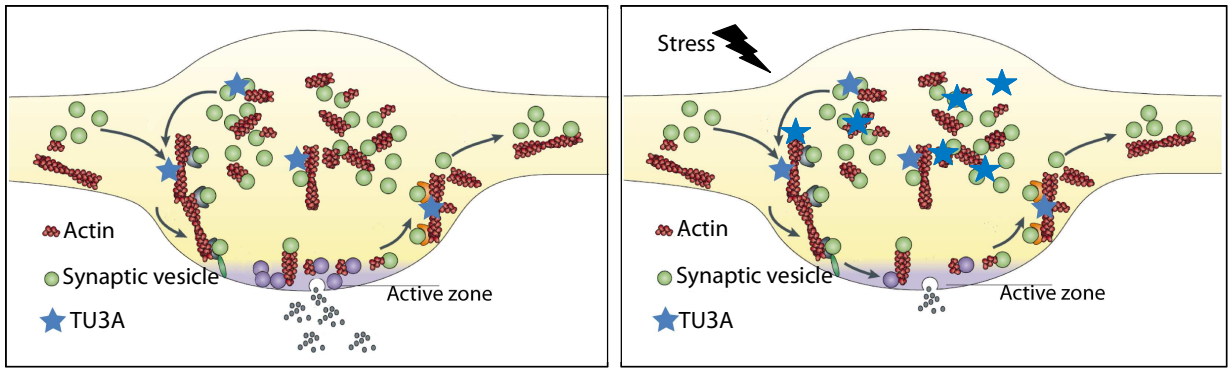

Figure 5.1 Schematic outline of the potential modulation of transmitter release by TU3A interaction with actin

Vesicles in the synaptic terminal are mobilized or suspended by actin dependent-processes and transported towards the active zone, where fusion-competent vesicles release neurotransmitter into the synaptic cleft by exocytosis. (A) Under

basal conditions TU3A is already present in the synaptic terminal and excerts a stabilizing effect on F-actin. (B) Following stress, TU3A is increased, which reduces actin dynamics in the presynaptic terminal probably resulting in less fusion-competent vesicles and thereby less neurotransmitter release. Adapted from (Cingolani and Goda, 2008).

In the last decades, a large body of evidence has accumulated revealing significant effects of stress exposure on synaptic plasticity (Jay et al., 2004; Kim et al., 2006). Actin-interacting proteins, in turn, have been identified as important modulators of synaptic structure and function (Evergren et al., 2007; Cingolani and Goda, 2008). However, to our knowledge, none of the previously described actin-binding proteins have been shown to be directly involved in modulating stress-related cellular mechanisms and the modulation of behavior. By virtue of its interaction with actin and the ensuing involvement in stress-associated synaptic reorganization, we propose TU3A as a possible novel candidate in bridging the gap between the experience of stressful life events, cellular adaptive mechanisms and the modulation of complex behavioral traits. Such a conceptually novel molecular link not only significantly adds to our understanding of the neurobiological mechanisms underlying stress-related human diseases, but opens up the intriguing possibility of designing improved and more specific treatment modalities in the future.

5.7 Perspectives

In this thesis, we characterized a novel stress-inducible transcript in the mouse brain and proposed TU3A as a potential beneficial factor mediating adaptive cellular and behavioral changes following stress. Furthermore, we suggested a potential mechanism for the action of TU3A in synaptic reorganization, but our knowledge about this novel gene and its actions in the brain is still far from complete.

Here, we used a gain-of-function approach and overexpressed TU3A region-specifically in the hippocampus. To confirm and substantiate our findings it will be necessary to additionally perform a loss-of-function study. Blocking of TU3A action in the brain under stressful conditions should lead to detectable alterations of synaptic functioning and behavioral changes and could strengthen the suggested role of TU3A as a link between stressful experiences and adaptive coping mechanisms. Possible strategies could be the generation of a conditional TU3A knockout mouse line or the down-regulation of the

transcript, e.g. via RNA interference (for methodological examples see (Boutros and Ahringer, 2008; Gondo et al., 2009)). Furthermore, this work focused on the hippocampus as a target for functional studies, but other brain regions like the lateral septum or the cerebellum have been found to strongly express TU3A and also represent interesting targets for elucidating potential region-specific functions of TU3A in the brain under basal as well as stressful conditions.

Additionally, we described an interesting spatiotemporal mRNA expression pattern of TU3A during postnatal development and an increase of TU3A mRNA expression after stress, indicating a functional role of TU3A in developmental processes. An interesting further point would be to examine if stressors during development permanently alter TU3A expression and thereby the stress-system function. Furthermore, region-specific, temporal modulation of TU3A expression during different developmental stages coupled with structural and behavioral analysis could provide further insights into the function of TU3A during critical periods of brain development. Transgenic mice with promoters, whose temporal activity can be directly manipulated, for instance the tetracycline-responsive system (Fedorov et al., 2001), could be a promising tool to investigate TU3A action during development. Such findings could contribute to a better understanding of the programming effects of stressful experiences during HPA axis system development.

Taken together, our study suggests TU3A as a novel molecular factor linking stressful experiences and complex behavior and opens up a multitude of intriguing future investigations concerning its function in the brain under basal and stressful conditions.

6 Summary

Stress, in form of constant challenges and changes, is an ever-present influence during the life of all organisms. In reaction to a challenge, the body initiates a complex repertoire of cellular, molecular and physiological responses to ideally adapt to the novel environmental demands. One central system involved in the stress response is the hypothalamic-pituitary-adrenal (HPA) axis. A short, controlled activation of the HPA axis is beneficial for an individual, while prolonged and unpredictable stress situations can have maladaptive and detrimental consequences and constitute a main risk factor for a variety of severe diseases, including psychiatric disorders like major depression or schizophrenia. The major hormonal mediator of the HPA axis in rodents is corticosterone, a steroid hormone secreted from the adrenal cortex. In the brain, corticosterone binds to two specific receptors, the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR), which act as transcription factors. Receptor activation induces a plethora of transcriptional changes, which in turn drive acute and persistent alterations in neuronal circuits and complex behavior. However, the detailed mechanisms of mediating adaptative and maladaptive consequences of stress during development and adulthood are not completely understood.

In this thesis TU3A, a novel gene originally identified as a putative tumor suppressor, was characterized in context of its potential function in the central stress system in mice. In a large scale gene expression analysis, TU3A was previously identified to be most strongly regulated by stress in the neonatal mouse brain and therefore presented an interesting candidate for further studies. Investigation of the neuroanatomical TU3A distribution revealed a distinct spatial TU3A expression pattern in the mouse brain, with prominent TU3A expression in structures of the limbic system under basal conditions. Limbic brain structures are crucially involved in emotional, autonomic, motor and cognitive processes and are important regulatory centers of the HPA axis response. Together with the region-specific induction of TU3A transcription during crucial periods of postnatal ontogeny, these findings suggested a role of TU3A in stress-related neuronal circuits.

To examine the consequences of stress on TU3A mRNA expression in the brain, different stress paradigms were applied in mice during development and adulthood. TU3A mRNA expression was found to be inducible in a region specific manner following maternal

separation in neonatal mice. In mature mice, food deprivation induced TU3A transcript up-regulation in HPA axis relevant regions. This was found to be independent of mouse strain and gender. These results indicated a possible role of TU3A in mediating the consequences of profound and prolonged HPA axis activity in neonates and during adulthood. In order to identify the transcriptional regulator of the stress-regulated TU3A increase, a GR agonist was applied and mice were treated with corticosteroid antagonists during maternal separation and food deprivation. GR agonist treatment induced pronounced TU3A mRNA up-regulation, while the GR antagonist prevented the stress-induced TU3A mRNA increase. Thereby, the GR was identified as the main regulator underlying the stress-induced TU3A mRNA up-regulation in neonate and adult mice. This finding further strengthened a possible role of TU3A in stress physiology.

To gain insights into a potential function of TU3A in the brain, a gain-of-function approach was performed using an adeno-associated viral vector for hippocampus-specific overexpression of TU3A. The behavioral phenotype of the animals was investigated in various test paradigms including cognitive performance as well as emotional behavior. Overexpression of TU3A caused a more active stress-coping strategy in the forced swimming test, enhanced cognitive performance in the Y-maze test and resulted in increased cognitive flexibility in the Morris water maze. These findings suggest TU3A as a novel molecular link in mediation of stressful experiences and beneficial behavioral adaptation.

In close cooperation with several other groups, TU3A was identified as a factor that unites the characteristics of being stress- and, in particular, glucocorticoid–responsive, directing actin formation as well as influencing synaptic plasticity and complex behavior. In conclusion, a novel role for TU3A in synaptic reorganization was suggested: the stress-regulated increase of TU3A induces formation of actin-bundles, thereby shifting actin dynamics towards a more inflexible state, which in turn influences the neurotransmitter release probability of the synapse. Such a conceptually novel molecular link significantly adds to our understanding of the neurobiological mechanisms underlying the complex responses to stressful situations.

7 Indices

7.1 Index of figures

7.2 Index of tables

7.3 List of abbrevations

aa	Amino acid
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginine-vasopressin
bgh-polyA	Bovine growth hormone polyadenylation signal sequence
CA1	Hippocampal cornu ammonis area 1
CA2	Hippocampal cornu ammonis area 2
CA3	Hippocampal cornu ammonis area 3
CCDS	Consensus coding sequence
cDNA	Complementary DNA
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRHR1	Corticotrophin-releasing hormone receptor type 1
cRNA	Complementary RNA
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPX	Distrene-80 Plasticizer Xylene
DRR1	Down-Regulated in Renal cell carcinoma 1
DUF1151	Domain of unknown function
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EPM	Elevated plus-maze
F-actin	Filamentous actin
FAM107A	Family with sequence similarity 107, member A
fEPSP	Field excitatory postsynaptic potential
FST	Forced swimming test
GABA	Gamma-aminobutyric acid

GL	Granular layer
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HCl	Hydrogen chloride
HPA	Hypothalamic-pituitary-adrenocortical
HUGO	Human genome organisation
IRES	Internal ribosome entry site
ITI	Inter-trial interval
ITR	Inverted terminal repeat
LDB	Light-dark box test
LTP	Long-term potentiation
ML	Molecular layer
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MWM	Morris water maze test
NCBI	National center for biotechnology information
NRI	Noradrenaline reuptake inhibitor
OF	Open field test
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
PTSD	Post-traumatic stress disorder
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
rAAV	Recombinant adeno-associated virus
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SAR	Scaffold attachment region
SCN	Suprachiasmatic nucleus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Standard error of the mean
SHRP	Stress hyporesponsive period
SNRI	Serotonin and norepinephrine reuptake inhibitor
SSC	Saline-sodium citrate
SSRI	Selective serotonin reuptake inhibitor
TBST	Tris-Buffered Saline and Tween 20
TCA	Tricyclic antidepressant
TEA	Triethanolamine
TU3A	Tohoku University cDNA clone A on chromosome 3
WPRE	Woodchuck post-transcriptional regulatory element

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9 Curriculum vitae

Name:	Claudia Liebl
Date of Birth:	April 11th, 1979
Place of Birth:	Munich
Nationality:	German

Education:

1985 – 1989	Grundschule an der Feldbergstr, Munich
1990 – 1999	Werner von Siemens-Gymnasium, Munich
Degree:	Abitur

University:

2000 – 2006	Ludwig Maximilians University, Munich
Degree:	Biology (Diploma)
May 2005 – February 2006	Diploma thesis at the Max Planck Institute of Psychiatry, Munich Department of Molecular Stressphysiology; Title of the thesis: Analyse der Expression und Regulation eines neuen, durch Stress induzierten Gens in der Maus.

Doctoral thesis:

April 2006 – March 2010	Doctoral thesis at the Max Planck Institute of Psychiatry, Munich Department of Molecular Stressphysiology; Title of the dissertation: TU3A is a novel stress-regulated protein involved in cognition and emotional behavior

10 Publications

Schmidt MV[+], Schülke J[+], **Liebl C**[+], Stiess M, Avrabos C, Wochnik G, Zimmermann N, Trümbach D, Wurst W, Turck C, Holsboer F, Stuart M, Bradke F, Eder M, Müller MB, Rein T
A novel Actin bundling factor links stress with synaptic plasticity and complex behaviour.
Manuscript in preparation. #
+ authors contributed equally to this work.

Liebl C, Scharf SH, Vogt Weisenhorn DM, Müller MB, Schmidt MV.
Characterization of TU3A expression in the developing and adult mouse brain.
Manuscript in preparation. #

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12 Assertion/Erklärung

Hiermit versichere ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 11.03.2010