Dissection of Forebrain Corticotropin-Releasing Hormone Receptor 1 Signaling in Stress-Induced Cognitive Deficits — The Role of Synaptic Cell Adhesion Molecules

Dissertation

an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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April 2011

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Tag der mündlichen Prüfung: 19.10.2011

“Research … is much more like paddling a small canoe on a mountain river. The river which is fed by many distant springs carries you along all right though often in a peculiar direction. You have to paddle quite hard to keep afloat. And sooner or later some of your ideas are upset and are carried downstream like an upturned canoe.”

Sir Alan Lloyd Hodgkin (1914-1998)

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Summary

Recurrent exposure to severely stressful events either early in life or in adulthood may negatively influence brain function, learning and memory. It has been proposed that central corticotropin-releasing hormone (CRH), one of the key stress factors, mediates the physiological and behavioral effects of repeated stress exposure in different life stages through the corticotropin-releasing hormone receptor 1 (CRHR1). However, direct evidence linking augmented forebrain CRH-CRHR1 signaling to stress-induced cognitive impairments and structural remodeling is lacking. In the studies presented in this thesis, transgenic mice with conditional forebrain CRHR1 inactivation or CRH overexpression are used. The involvement of forebrain CRHR1 signaling in modulating the consequences of early-life stress or chronic stress in adulthood on hippocampus-dependent learning and memory as well as hippocampal integrity is investigated. Moreover, the potential roles of several synaptic cell adhesion molecules — specifically, neurexins, neuroligins and nectin-3 — in these processes and their interactions with the CRH-CRHR1 system are examined. The results demonstrate that in adult mice exposed to either early-life stress or chronic social defeat stress, hippocampus-dependent spatial learning and memory are impaired in a CRHR1-dependent manner. These stress-induced cognitive deficits are associated with abnormal hippocampal cytoarchitecture and altered expression levels of synaptic cell adhesion molecules, which can be partially normalized by inactivating forebrain CRHR1. Furthermore, neurexins and nectin-3 colocalize with CRHR1 in specific subcellular compartments of hippocampal principal neurons. Interestingly, the loss of hippocampal nectin-3 function reproduces the effects of stress on hippocampal structural plasticity, spatial learning and long-term spatial memory, highlighting the importance of nectin-3 in forebrain CRHR1-mediated stress effects. In conclusion, the forebrain CRH-CRHR1 system interacts with synaptic cell adhesion molecules to mediate the effects of stress on cognition and hippocampal integrity.

Chapter 1

General introduction

- Stress response and stress mediators
- The CRH family
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- Animal models for early-life stress and chronic stress in adulthood
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"Everybody knows what stress is and nobody knows what it is."

Hans Selye (1907-1982)

Stress response and stress mediators

Exposure to actually or potentially threatening events (termed "stressors") evokes a stress response in living animals. Once an aversive stimulus has been detected, specific brain circuits and peripheral systems are activated, finally resulting in the release of various molecules that mediate physiological and behavioral adaptations. These "stress mediators" include neurotransmitters (noradrenaline, dopamine and serotonin), neuropeptides (corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP)), and adrenal corticosteroids (cortisol in humans and corticosterone in both humans and rodents), which act in concert to help the animal respond and adapt to the changing environment (de Kloet et al., 2005a; Joëls and Baram, 2009).

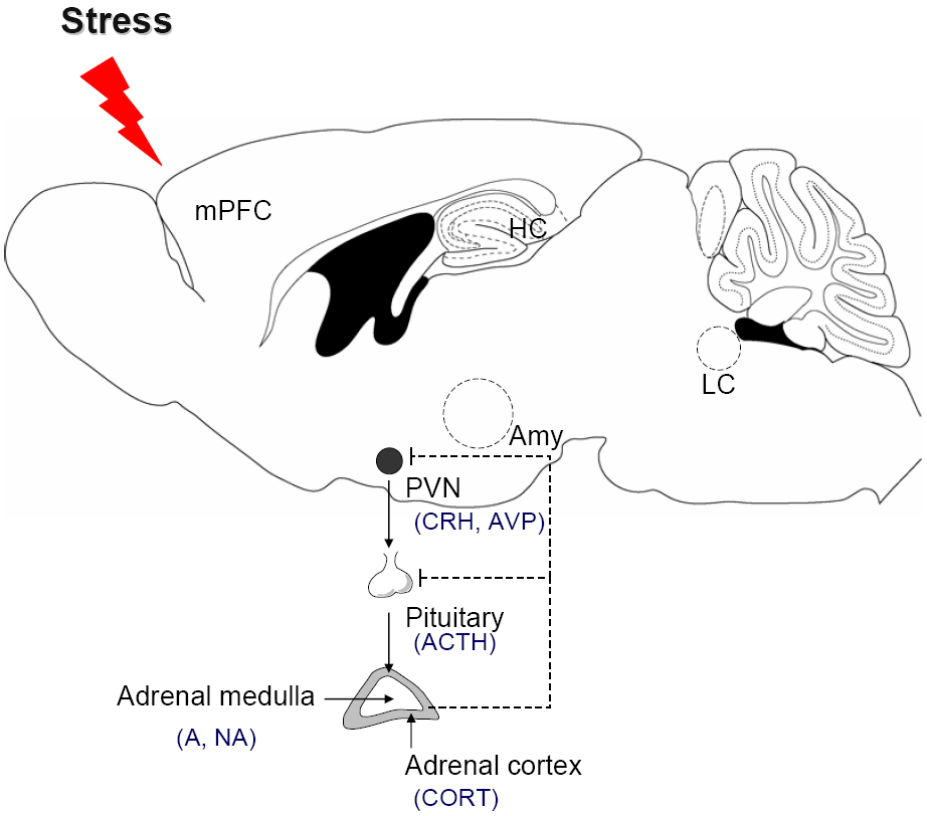

Figure 1. An overview of the stress system in rodents. A, adrenaline; ACTH, adrenocorticotropic hormone; Amy, amygdala; CORT, corticosterone; HC, hippocampus; LC, locus coeruleus; mPFC, medial prefrontal cortex; NA, noradrenaline; PVN, paraventricular nucleus. See text for details.

Two main systems, the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis, are activated upon perceiving the stressor and orchestrate a coordinated response to the threat (Figure 1). The activation of SNS stimulates a rapid release of adrenaline and noradrenaline from the adrenal medulla. Almost simultaneously, monoaminergic neurons in certain brain regions are also activated, including noradrenergic neurons in the locus coeruleus (Valentino and Van Bockstaele, 2008). Peripherally and centrally released monoamines exert rapid (within seconds after the onset of stress) but generally short-lasting effects on neuronal activity and brain function.

In the HPA axis, stressful events lead to the release of CRH and AVP in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN). Both neuropeptides act on respective receptors in the pituitary and trigger the processing and release of adrenocorticotropic hormone (ACTH), resulting in the secretion of corticosteroids from the adrenal cortex. CRH is also released in brain regions other than the PVN, including the locus coeruleus, the amygdala, the hippocampus, and the medial prefrontal cortex (Cummings et al., 1983; De Souza et al., 1985). The effects of CRH can start within seconds, whereas corticosteroids (mainly glucocorticoids), the end product of the HPA axis, largely take effect slower (from minutes to hours) than monoamines and neuropeptides. Glucocorticoids bind to the receptors in the pituitary, PVN and other sites to inhibit HPA activity (negative feedback regulation). In brief, the spatial convergence and temporal overlap of monoamines, neuropeptides and glucocorticoids permit intricate interactions to fine-tune physiological and behavioral responses to stress (Joëls et al., 2006; Joëls and Baram, 2009).

The patterns of stress responsiveness are dependent on multiple factors, such as the nature (physiological, psychological, or both) and intensity (mild, moderate, or severe) of the stressor, the duration of stress exposure (episodic or continuous), and the life stage of the animal (newborn, adolescent, adult, or aged). Whereas the impact of a single stress challenge usually diminishes over a short period of time, sustained stress exposure exerts profound and long-term effects and may increase the risk to develop (psychiatric) diseases in genetically predisposed individuals (Feder et al., 2009). In newborn animals, repeated exposure to stress can lead to persistent changes ("programming" effects) manifested by behavioral alterations later in life. In contrast, in adult animals, the prominent alterations induced by chronic stress may return to basal levels within several days after the termination of stress (Conrad, 2006; Lupien et al., 2009).

The CRH family

CRH (also known as corticotropin-releasing factor, CRF) is a 41 amino acid peptide first characterized in 1981 (Vale et al., 1981). Three other peptides of the mammalian CRH family, urocortin I, urocortin II (stresscopin-related peptide) and urocortin III (stresscopin) have been identified later on (Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). These peptides serve as ligands and bind to two of the class B subtype of G protein-coupled receptors, CRHR1 and CRHR2 (also termed CRF_1 and CRF_2), which have ~70% identity at the amino acid levels. CRH has a tenfold higher affinity for CRHR1 than for CRHR2, whereas urocortin II and urocortin III are relatively selective agonists for CRHR2. The members of the CRH family differ in their tissue distribution and pharmacology (for review, see Arzt and Holsboer, 2006; Bale and Vale, 2004).

As key mediators of neuroendocrine and behavioral responses to stress, CRH and CRHR1 are widely distributed in the brain (for example, the cerebral cortex, hippocampus, amygdala, and hypothalamus) (Alon et al., 2009; Chen et al., 2000; Van Pett et al., 2000; Wong et al., 1994), while central CRHR2 is mainly restricted to sites in the lateral septum, amygdala, entorhinal cortex, and hypothalamus (Lovenberg et al., 1995; Van Pett et al., 2000). The differential distribution patterns of CRHR1 and CRHR2 are congruent with their functional differences in modulating the stress response and behavior. It has been suggested that CRHR1 and CRHR2 are involved in the acute phase (activation of the HPA axis) and the recovery phase (gradual reduction of HPA axis activation) of the stress response, respectively (Korosi and Baram, 2008; Reul and Holsboer, 2002). Moreover, forebrain CRHR1 mediates anxiety-related behavior (Müller et al., 2003), whereas central CRHR2 may exert anxiolytic effects (Bale et al., 2000; Kishimoto et al., 2000). In addition, central CRH function is modulated by a CRH binding protein (CRH-BP) (Ungless et al., 2003).

Learning, memory and stress

Learning and memory are closely related cognitive processes. Learning refers to the process by which new information is acquired, and memory to the ability to recall things that have been learned. Three main stages of memory are distinguished: acquisition (learning of new information), consolidation (storage of learned information) and retrieval (access to stored information) (Abel and Lattal, 2001). Memory can be short-term (lasting from seconds to hours)

or long-term (from days to years) (McGaugh, 2000), and is generally classified into two types: (1) declarative/explicit memory (memory for facts, episodes or events) that involves the hippocampus and other components of the medial temporal lobe system; (2) nondeclarative/implicit memory (memory for skills, habits, and emotional and motor responses) that involves brain regions including the striatum, amygdala and cerebellum (Ashby and O'Brien, 2005; Rolls, 2000).

Stress mediators such as glucocorticoids, CRH and noradrenaline are potent modulators of learning and memory (Chen et al., 2010; de Kloet et al., 1999; McGaugh and Roozendaal, 2002). The cognitive consequences of stress and stress mediators have been extensively reviewed (Joëls et al., 2006; Roozendaal et al., 2009; Sandi and Pinelo-Nava, 2007; Schwabe et al., 2010b). Generally, stress may facilitate or impair learning and memory depending on the source, intensity, controllability, duration, and timing of the stressor and the memory system(s) influenced (Sandi and Pinelo-Nava, 2007). A brief stress challenge around the time of learning may impair retrieval but facilitate memory consolidation for emotionally arousing experiences and declarative information. For instance, a graduate student taking the oral examination may have difficulties recalling recently memorized knowledge (impaired retrieval), but this embarrassing situation is simultaneously engraved in the memory (enhanced consolidation). Prolonged exposure to intense, uncontrollable stressors may also enhance emotional memory but impair declarative memory. For instance, clinical findings reveal that stress-related disorders such as post-traumatic stress disorder (PTSD) (Layton and Krikorian, 2002) and depression (Gotlib and Joormann, 2010) are associated with learning and memory disturbances. Moreover, recent studies in both humans and rodents indicate that chronic stress impairs the flexible, hippocampus-dependent “cognitive” memory, but may favor the rigid, neostriatum-dependent “habit” memory (Schwabe et al., 2010a; Schwabe et al., 2007). Therefore, as a major part of the behavioral response to stress, altered learning and memory processes prepare the individual for future challenges which in turn may increase survival probability and reproductive success. On the other hand, however, chronic stress exposure may increase the risk to develop cognitive disorders in susceptible individuals.

Stressed hippocampus and lost memories

The hippocampus (Figure 2) has received much attention in stress research, not only for its key role in declarative (in humans) and spatial (in both humans and rodents) learning and memory, but also as a main target of stress mediators. An enormous amount of studies has demonstrated

that acute severe or chronic stress impairs spatial learning and memory in rodents, associated with structural and functional alterations in the hippocampus, including dendritic shrinkage, spine loss and altered synaptic plasticity (Conrad, 2010; Fenoglio et al., 2006; Kim and Diamond, 2002; McEwen, 1999).

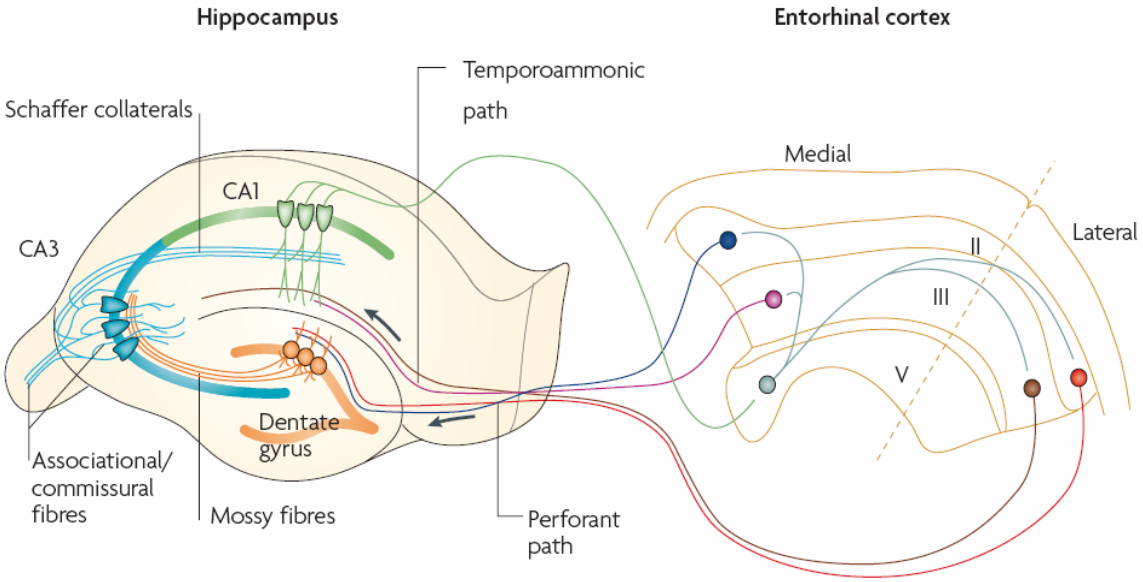

Figure 2. The neural circuitries in the rodent hippocampus. The hippocampus contains parallel and mostly uni-directional excitatory pathways referred to as the trisynaptic pathway and the monosynaptic pathway. (1) The traditional trisynaptic pathway carries information as follows: entorhinal cortex → dentate gyrus → CA3 → CA1 → entorhinal cortex. The perforant path, one of the major inputs to the hippocampus, is formed by axons arising from layer II of the entorhinal cortex and makes synaptic contacts with the apical dendrites of dentate granule cells. Granule cells project to the proximal apical dendrites of CA3 pyramidal neurons (through mossy fibers). Axons of CA3 pyramidal neurons then project to ipsilateral CA3 (through associational fibers) and CA1 neurons (through Schaffer collaterals), and to contralateral CA3 and CA1 neurons (through commissural fibers). (2) The monosynaptic pathway carries information as follows: entorhinal cortex → CA1 → entorhinal cortex. In this pathway, CA1 pyramidal neurons directly receive inputs from neurons in layer III of the entorhinal cortex (through temporoammonic path), and send back-projections to the deep-layer neurons of the entorhinal cortex. Both pathways are essential for learning and memory (Nakashiba et al., 2008; Neves et al., 2008). Adapted from Neves et al., 2008.

Dendritic spines receive excitatory synaptic inputs and are crucial for synaptic plasticity and memory formation (Lamprecht and LeDoux, 2004; Segal, 2005). Cognitive deficits evoked by stress are correlated with hippocampal dendritic spine loss (Chen et al., 2010). Early-life adversities lead to atrophy of apical dendrites in hippocampal principal neurons in adult rats (Ivy et al., 2010; Oomen et al., 2010), and also reduce the complexity of basal dendrites (Brunson et

al., 2005). In adulthood, acute (Chen et al., 2010; Chen et al., 2008; Kole et al., 2004) or chronic stress (Magariños and McEwen, 1995; Sousa et al., 2000; Watanabe et al., 1992) reduces both apical dendritic complexity and dendritic spine density in hippocampal neurons, but may increase the complexity of basal dendrites (Kole et al., 2004). Notably, chronic stress-induced dendritic shrinkage can be reversed following several days of withdrawal from stress, whereas early-life stress evokes persistent alterations in dendritic morphology and spine density. Hence, structural remodeling in the hippocampus may account for stress-induced cognitive impairments.

Hippocampal long-term potentiation (LTP) provides another potential neural basis for the effects of stress on hippocampus-dependent learning and memory. LTP is an activity-dependent and sustained enhancement of synaptic efficacy, which was first described in the rabbit perforant path (Bliss and Lomo, 1973). In this classic experiment, Bliss and Lomo found that trains of high-frequency stimulation to the rabbit perforant path elicited a long-lasting facilitation of excitatory postsynaptic potentials in the dentate granule cells. LTP can be induced at glutamatergic synapses in the hippocampus and many other brain regions. A considerable body of evidence indicates that LTP may be fundamental for learning and memory storage (for review, see Lynch, 2004), some of which include: (1) hippocampal LTP can be induced by learning (Whitlock et al., 2006), (2) inhibition of hippocampal LTP can impair spatial learning and memory (Morris et al., 1986), (3) the induction and maintenance of LTP are closely associated with dendritic spine expansion (Yang and Zhou, 2009). Interestingly, LTP can be modulated by several stress mediators such as glucocorticoids and CRH. For example, CRH can facilitate the induction and stability of LTP in CA1 (Blank et al., 2002) and DG (Wang et al., 2000). Moreover, postnatal stress (Bagot et al., 2009; Brunson et al., 2005; Oomen et al., 2010) or stress in adulthood (Foy et al., 1987; Kim et al., 2007a) impairs hippocampal LTP.

Molecular mechanisms of stress-induced cognitive impairments

As discussed above, repeated stress exposure exerts deleterious effects on cognition in different life stages (Lupien et al., 2009), but the underlying molecular mechanisms have not been fully addressed. Stress triggers a surge in circulating glucocorticoids through activation of the HPA axis. Glucocorticoids enter the brain and bind to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) that are enriched in the hippocampus (Figure 3), and modulate information processing. Because of the lower affinity than MRs, GRs are mostly occupied after

stress when glucocorticoids levels are significantly elevated. Glucocorticoids, MRs and GRs are important for optimal cognitive performance (Oitzl et al., 2001; Oitzl and de Kloet, 1992), but chronically elevated levels of glucocorticoids may reduce dendritic complexity (Conrad et al., 2007; Woolley et al., 1990) and impair spatial memory (Coburn-Litvak et al., 2003). It has been suggested that an imbalance in MR- and GR-mediated function contributes to chronic stress-induced cognitive deficits (de Kloet et al., 1999; Joëls et al., 2006). The glucocorticoids – MR/GR hypothesis is supported by studies using transgenic mouse lines, which show that hippocampal MR deficiency or GR overactivation impairs learning and memory (Berger et al., 2006; Kim et al., 2008; Lai et al., 2007). However, this hypothesis is limited by several findings. Some studies in rodents reported that repeated and systemic postnatal GR activation or chronic glucocorticoids administration in adulthood fails to impair spatial learning and memory (Conrad, 2010; Kamphuis et al., 2003; Lin et al., 2006; Schmidt et al., 2009). Furthermore, in patients suffering from PTSD, declarative memory is impaired (Bremner et al., 2003) yet basal cortisol levels are reduced (Yehuda et al., 2005). Therefore, additional mechanisms are likely involved in this process.

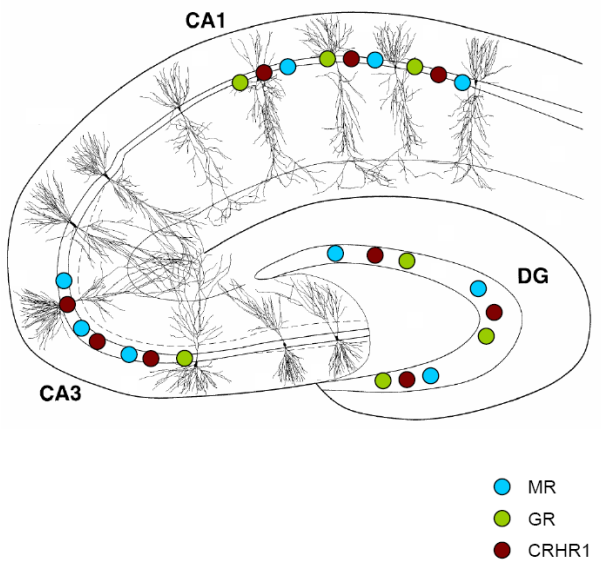

Figure 3. Distribution of three receptors (MR, GR and CRHR1) for the key stress mediators (glucocorticoids and CRH, respectively) in the mouse hippocampus. Relative abundance of the receptors is indicated by the number of colored circles. Pyramidal neurons with apical and basal dendrites in CA1 and CA3 regions are shown. Adapted from Ishizuka et al., 1995; Joëls and Baram, 2009.

Stress early in life (Ivy et al., 2010) or in adulthood (Chen et al., 2010) augments the release of CRH from hippocampal interneurons, which primarily binds to CRHR1 abundant in the spines of pyramidal neurons (Figure 4) to modulate synaptic plasticity and cognition (Arzt and Holsboer, 2006; Korosi and Baram, 2008). Interestingly, the expression levels of hippocampal CRH and CRHR1 are much higher during the second and third weeks after birth compared to those in adulthood (Avishai-Eliner et al., 1996; Chen et al., 2001), pointing to their unique roles in sculpting hippocampal development and plasticity (Brunson et al., 2001; Fenoglio et al., 2006). Prolonged exposure to elevated CRH impairs spatial memory (Heinrichs et al., 1996), while central administration of CRH to neonatal rats recapitulates the effects of early-life stress on cognitive performance (Brunson et al., 2001; Brunson et al., 2005) through CRHR1 (Ivy et al., 2010). These findings suggest that the hippocampal CRH-CRHR1 system is essential in mediating stress-induced cognitive decline.

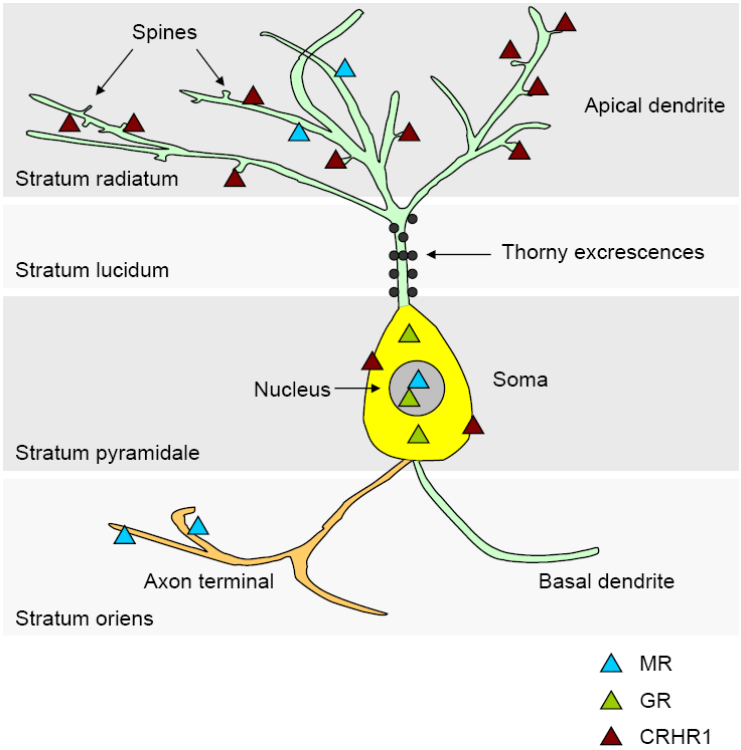

Figure 4. Subcellular localization of MR, GR and CRHR1 in a CA3 pyramidal neuron. Note that CRHR1 resides on the soma, dendrite and dendritic spines. Adapted from Joëls and Baram, 2009.

Synaptic cell adhesion molecules, cognition and stress

A number of cell adhesion molecules (CAMs) are found at or adjacent to synaptic sites in neuronal axons and dendrites. These synaptic CAMs (some are shown in Figure 5) include the

immunoglobulin superfamily (for example, neural cell adhesion molecule (NCAM), nectin 1-4 and SynCAM 1-4), the cadherin superfamily (for example, N (neural)-cadherin and protocadherins), neurexins-neuroligins (neurexin 1-3 and neuroligin 1-4), and integrins (Shapiro et al., 2007; Yamagata et al., 2003). Synaptic CAMs connect pre- and post-synaptic specializations and dynamically regulate synaptic formation, synaptic activity, and dendritic spine morphology (Giagtzoglou et al., 2009).

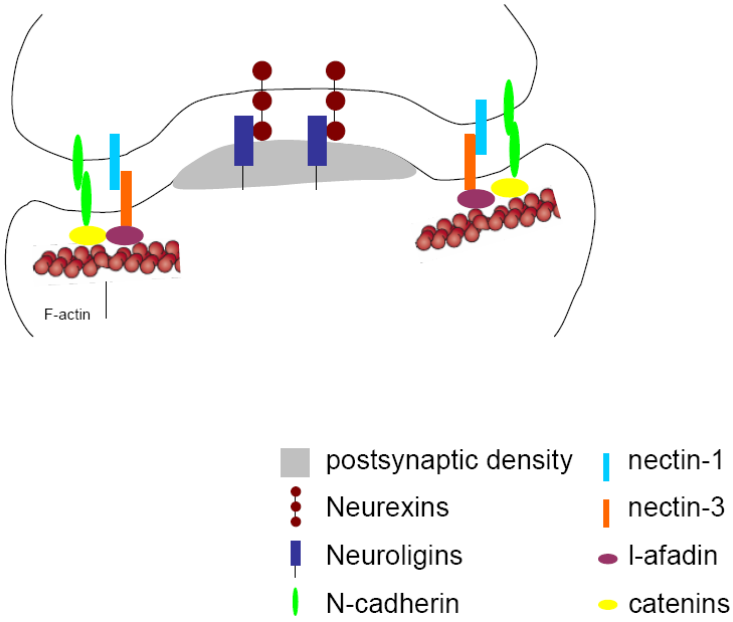

Figure 5. Localization of the neurexin-neuroligin, nectin-afadin, and cadherin-catenin complexes at an excitatory synapse. Neurexins participate in the organization of presynaptic apparatus and bind to neuroligins, which aggregate at the postsynaptic density, to specify synaptic function and modulate excitatory and inhibitory neurotransmission. The nectin-afadin complex colocalizes with the cadherin-catenin complex at adherens junctions (also named *puncta adherentia* junctions), the areas adjacent to synaptic sites. The nectin-afadin and cadherin-catenin complexes are connected to actin cytoskeleton, and cooperate to organize adherens junctions and participate in synaptic remodeling.

In nascent synapses, synaptic CAMs stabilize axodendritic/dendrodendritic contacts and facilitate synaptic maturation. In mature synapses, they interact with other synaptic proteins and signaling pathways to modulate synaptic function and plasticity, which are thought to underlie learning and memory (Dalva et al., 2007). Therefore, a disruption in the expression and/or function of synaptic CAMs may impair learning and memory. For instance, the neurexin-neuroligin complex has been implicated in cognitive diseases such as schizophrenia and autism spectrum disorders (Jamain et al., 2003; Kim et al., 2008; Rujescu et al., 2009; Yan et al., 2008).

The phenotypes of neurexins or neuroligins mutant mice pinpoint their importance in synaptic transmission and cognitive function. Deletion of the neurexin-1α isoforms attenuates excitatory synaptic transmission and several aspects of cognition (Etherton et al., 2009), whereas mutant mice with either deficiency or overexpression of neuroligin-1 exhibit impaired hippocampal LTP and similar deficits in hippocampus-dependent learning and memory (Blundell et al., 2010; Dahlhaus et al., 2010).

Intriguingly, recent evidence implies synaptic CAMs as important mediators of the effects of stress on hippocampal plasticity and spatial learning and memory (Sandi, 2004). NCAM is one of the well-characterized synaptic CAMs that modulate such effects. Chronic stress in adulthood decreases NCAM mRNA and protein expression levels in the rat hippocampus, associated with alterations in hippocampus-dependent cognitive performance (Sandi et al., 2001; Venero et al., 2002). Similar findings have been reported in adult rats with perinatal (Koo et al., 2003) or acute severe stress exposure (Sandi et al., 2005). Consistent with these results, hippocampal NCAM levels are increased by spatial learning experiences, whereas disrupting NCAM function using pharmacological approaches impairs spatial memory (Venero et al., 2006). Moreover, FGL, a mimetic peptide for NCAM, can prevent spatial memory impairments induced by chronic stress in aging rats (Borcel et al., 2008). Recently, it has been shown that chronic stress also decreases NCAM expression and attenuates cognitive performance in mice, which is mirrored in conditional NCAM-deficient mice and improved by FGL treatment (Bisaz et al., 2011), highlighting the role of NCAM in chronic stress-induced cognitive alterations.

Because of the functional importance, some synaptic CAMs other than NCAM may also modulate the cognitive effects of stress. In addition, considering their subcellular distribution patterns, synaptic CAMs may link CRH-CRHR1 signaling or MR/GR function to synaptic plasticity and cognition.

Animal models for early-life stress and chronic stress in adulthood

Early-life stress and chronic stress in adulthood are well-recognized environmental risk factors for depression and cognitive deficits. Animal models for recurrent stress in different life stages provide useful tools for studying the neural substrates of stress-related disorders and developing new therapeutic strategies.

In rodents, the postnatal period is relatively hyporesponsive to stress, characterized by very low circulating corticosterone levels and a blunted HPA response to mild stressors (Levine, 1994). However, severe stressors such as separation from the dam activate the pups' HPA activity. A large body of evidence has convincingly shown that the quantity and quality of maternal care as well as the mother-pup interaction are crucial for the programming of the stress response and behavior in the offspring (Korosi and Baram, 2009; Schmidt, 2010). Based on the "maternal mediation" hypothesis, several "classical" early-life stress paradigms are established, including early handling (pups separated from the mother for 3-15 min), repeated maternal separation (for 1-8 h) and single prolonged maternal separation (for 24 h). Recently, a novel model for early-life stress has been established in rats (Avishai-Eliner et al., 2001) and mice (Rice et al., 2008), in which the quality of maternal care and mother-pup interaction is reduced by an impoverished postnatal environment (Figure 6). Adult offspring experiencing such early-life adversities show cognitive decline and impaired hippocampal integrity (Brunson et al., 2005; Ivy et al., 2010; Rice et al., 2008), making it a suitable model to study the long-lasting consequences of early-life stress and the neurobiological underpinnings.

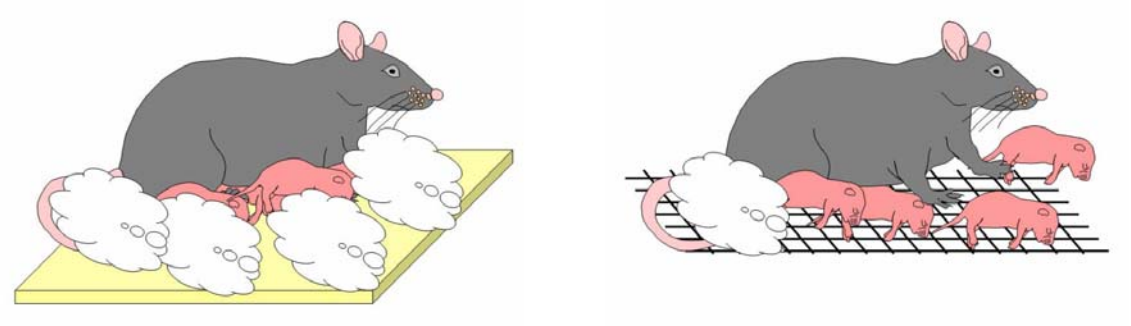

Figure 6. The limited nesting and bedding material paradigm. This paradigm starts on postnatal day 2 and ends on postnatal day 9. **(Left)** In the control cage, the dam is supplied with sufficient nesting (shown in white) and bedding (shown in yellow) material. **(Right)** In the stress cage, an impoverished postnatal environment is made by providing the dam with a very limited amount of nesting material (25% of that for the control condition). Moreover, normal sawdust bedding is replaced by an aluminum mesh platform, which is placed above the cage floor so that droppings are filtered without trapping the pups.

As mentioned above, early-life experience exerts programming effects on the development of HPA axis and cognition. In comparison, recurrent stress exposure in adulthood evokes dramatic but relatively short-lasting changes (Conrad et al., 1999; Luine et al., 1994), which may be due to the differences in the developmental stages of the hippocampus or in the properties of the stressor

to which newborn and adult animals are exposed. Though many chronic stress paradigms have been described, several commonly used strategies (for example, the chronic restraint stress paradigm) introduce a predictable stress procedure that is monotonous and may lead to habituation (Haile et al., 2001; Magariños and McEwen, 1995; Willner, 2005). Chronic social defeat provides a potent, unpredictable and naturalistic psychosocial stress (Figure 7). Susceptible mice exposed to such stress exhibit neuroendocrine and metabolic abnormalities (Bartolomucci et al., 2009; Keeney et al., 2006), social avoidance, and anxiety- and depression-like phenotypes (Keeney and Hogg, 1999; Krishnan et al., 2007; Tsankova et al., 2006; Wagner et al., 2011). Therefore, this paradigm shows a high validity for psychiatric disorders and is ideal for evaluating the effects of chronic stress on spatial memory and hippocampal dendritic integrity in adulthood.

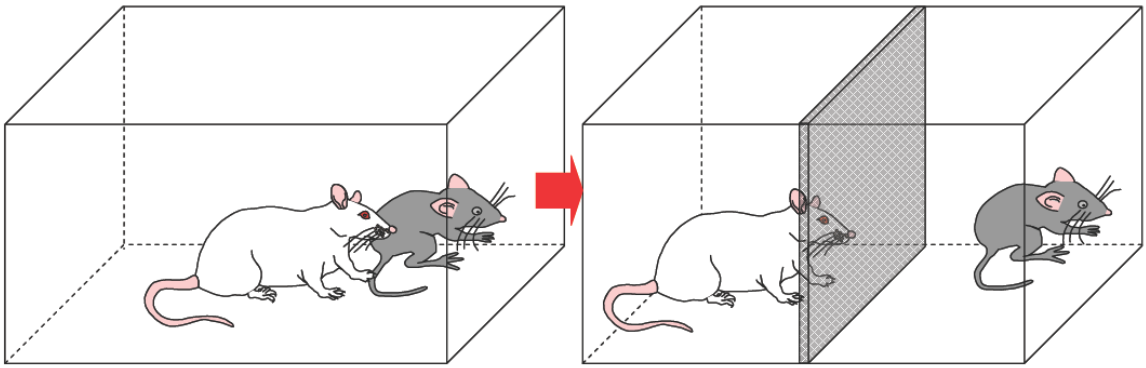

Figure 7. The chronic social defeat stress paradigm. Over a total of 21 days, the adult test mouse (colored in grey) is introduced into the home cage of a different dominant CD1 mouse (shown in white) each day. **(Left)** The heavier and aggressive CD1 resident rapidly recognizes and attacks the intruder. **(Right)** After the brief aggressive encounter (usually less than 2.5 min), the mice are separated by a holed metal partition, allowing the animals to keep continuous sensory but not physical contact for the next 24 hours. Control animals (not shown) are allowed to explore the defeat cages for 30 sec in the absence of dominant mice and are then returned to their home cages.

Dissecting the involvement of forebrain CRH-CRHR1 signaling in stress effects using conditional mouse mutants

Studies using genetically manipulated mice may provide insight into the effects of (a) specific gene(s) on brain and behavior. The neuroendocrine and behavioral phenotypes of CRH or CRHR1 mutant mice have been documented (for review, see Bale and Vale, 2004; Müller and Holsboer, 2006). Conventional CRH knockout mice are viable and show normal fertility and longevity but impaired neuroendocrine response to stress (Jacobson et al., 2000; Muglia et al., 1995). No apparent behavioral abnormalities are observed (Weninger et al., 1999). Transgenic

mice overexpressing CRH under the control of either the broadly active metallothionein promoter (Stenzel-Poore et al., 1992) or centrally restricted Thy-1.2 promoter (Groenink et al., 2002) exhibit elevated ACTH and corticosterone levels accompanied by symptoms of Cushing-like syndrome, complicating the interpretation of behavioral results (Heinrichs et al., 1996; van Gaalen et al., 2002). Two conventional CRHR1 knockout mouse lines display reduced anxiety-like behavior (Smith et al., 1998; Timpl et al., 1998), but the results are again confounded by prominent glucocorticoid deficiency and severe impairments in the neuroendocrine response to stress. Because the conventional approaches to manipulate the activity of the CRH-CRHR1 system are limited by prominent neuroendocrine and physiological alterations, it is essential to apply novel techniques to dissect CRH-CRHR1 function in a temporally and spatially restricted manner (Lewandoski, 2001).

Recently, transgenic mouse lines with conditional inactivation of CRHR1 (Müller et al., 2003) and overexpression of CRH (Lu et al., 2008) in forebrain neurons have been generated. Using a region- and cell type-specific promoter (calcium/calmodulin kinase IIα), forebrain CRH-CRHR1 signaling can be postnatally reduced or enhanced without affecting the development and activity of the HPA axis. Forebrain CRHR1 deficient mice show reduced anxiety and a moderately blunted HPA axis feedback to an acute stress challenge (Müller et al., 2003), while forebrain CRH overexpressing mice exhibit normal stress-coping behavior and HPA activity (Lu et al., 2008). With these conditional mouse mutants, it is possible to investigate the interactions between forebrain CRH-CRHR1 signaling and environmental risk factors in shaping complex behaviors (such as anxiety-like behavior and cognition) and the underlying neurobiological mechanisms.

Scope and aims of the thesis

To investigate whether the hippocampal CRH-CRHR1 system mediates cognitive impairments induced by early-life adversities or repeated stress exposure in adulthood, male mice with conditional CRHR1 inactivation (CRHR1-CKO mice) or CRH overexpression (CRH-COE mice) in forebrain neurons are used. Furthermore, the potential involvement of several synaptic CAMs (specifically, nectin-3 and the neurexin-neuroligin complex) in spatial learning and memory is explored.

In **Chapter 2**, findings concerning early-life stress as a risk factor for the development of depression-like symptoms in rodents are reviewed. In **Chapter 3**, the limited nesting and bedding material paradigm is applied in wild-type and CRHR1-CKO mice to study the role of CRH-CRHR1 signaling and the neurexin-neuroligin complex in early adversity-programmed later-life cognitive dysfunction. In addition, adult CRH-COE mice are used to evaluate the effects of postnatal forebrain CRHR1 overactivation on cognition. In **Chapter 4**, the chronic social defeat stress paradigm is applied in adult wild-type and CRHR1-CKO mice, and their hippocampus-dependent spatial memory is tested. Moreover, the potential role of nectin-3 in chronic stress-modulated cognition and structural changes is examined. In **Chapter 5**, the importance of nectin-3 in hippocampal structural remodeling and spatial cognition is investigated using viral-mediated RNA interference to suppress hippocampal nectin-3 expression in adult C57BL/6 mice. The data presented in this thesis are discussed in **Chapter 6**.

Chapter 2

Early-life stress paradigms in rodents: potential animal models of depression?

Psychopharmacology (Berl) 214 (2011): 131-140.

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Abstract

While human depressive illness is indeed uniquely human, many of its symptoms may be modeled in rodents. Based on human etiology, the assumption has been made that depression-like behavior in rats and mice can be modulated by some of the powerful early-life programming effects that are known to occur after manipulations in the first weeks of life. Here we review the evidence that is available in literature for early-life manipulation as risk factors for the development of depression-like symptoms such as anhedonia, passive coping strategies, and neuroendocrine changes. Early-life paradigms that were evaluated include early handling, separation, and deprivation protocols, as well as enriched and impoverished environments. We have also included a small number of stress-related pharmacological models. We find that for most early-life paradigms *per se*, the actual validity for depression is limited. A number of models have not been tested with respect to classical depression-like behaviors, while in many cases, the outcome of such experiments is variable and depends on strain and additional factors. Because programming effects confer vulnerability rather than disease, a number of paradigms hold promise for usefulness in depression research, in combination with the proper genetic background and adult life challenges.

Affective disorders

Affective disorders affect the life of millions of people worldwide and are estimated to be one of the leading causes of disability. Life time prevalence of depression was reported to range between 15 and 20%, with a twice-as-high risk for women than for men (Kendler et al., 2002; Wittchen and Jacobi, 2005). Core symptoms of depression include a depressed, irritable or apathetic mood, a loss of interest and enjoyment and reduced energy. These symptoms, which are often accompanied by reduced attention, feelings of guilt, disturbed sleep and loss of appetite, are present over a prolonged period of time with a minimum of 2 weeks and seem to be beyond personal control. Next to its devastating impact on the patient the disease is also associated with impairment at work and in personal or family relationships. In addition, depression negatively affects the outcome and prognosis of a number of other diseases, including coronary heart disease or diabetes (Paile-Hyvärinen et al., 2007). As a consequence, the direct and indirect costs associated with depression are enormous and are in Europe estimated at about 1% of the total gross economic product of the European Union.

Diagnosis of affective disorders is based on diagnostic rating scales as, for example, the Hamilton depression scale. Unfortunately, many of the patients may remain undiagnosed or are inadequately treated by their general practitioner and only a small percentage of patients are transferred to a psychiatrist. The standard pharmacological treatment options for affective disorders are selective serotonin reuptake inhibitors (SSRIs) or selective serotonin and noradrenaline reuptake inhibitors (SNRIs). These drugs are based on the serendipitous discovery of tricyclic antidepressants in the 1950s, which function as non-selective serotonin and noradrenalin reuptake inhibitors. While the refined successor drugs are in general safe and effective, there has been little improvement regarding efficacy. It was recently estimated that only about half of the patients treated with two sequential treatment interventions achieve remission, with relapse rates of more than 40% (Huynh and McIntyre, 2008). Some authors even claim based on meta-analyses that currently available antidepressants are not more effective than placebo treatment in mild or moderate depression (Kirsch et al., 2008). While these conclusions may be premature and need to be treated with caution, it is nonetheless clear that current treatment options for affective disorders are unsatisfactory. Therefore, the identification of alternative strategies (for example, new pharmacological targets) to treat the disease or to prevent its development would be of great benefit to many.

Early-life stress as a risk factor for depression

Depression is a multifactorial disease and has been shown to include a substantial heritable, thus genetic, portion. Kendler and colleagues estimated the heritability of depression to be in the range of 30-50%, depending on sex and symptom severity (Kendler et al., 2002). On the other hand, environmental challenges seem to have a decisive impact on the disease, either increasing or decreasing the individual disease risk. Among the best studied and validated environmental risk factors for depression are stressors or traumatic situations early in life (Heim et al., 2008), giving rise to a two-hit model for the susceptibility for depressive disease.

Child abuse and neglect is a major problem, with recently verified 1.2 Million incidents in the USA per year (Sedlack et al., 2010). A number of meta-analyses and large-scale studies indicated a significantly increased risk for depression in relation to early-life stress, for example, childhood abuse (Jumper, 1995; Molnar et al., 2001; Paolucci et al., 2001). Representative of many other studies, MacMillan found childhood abuse to be significantly associated with lifetime rate of depression in a community sample of 7016 individuals, with a bigger effect size in women (MacMillan et al., 2001). These findings are also supported by a number of more recent studies, also demonstrating a clear link between traumatic or stressful life events during childhood and adult psychopathology (Kim and Cicchetti, 2006; Larkin and Read, 2008; Weber et al., 2008).

However, it is also unquestionable that the majority of individuals exposed to early-life stress are resilient and do not develop a psychiatric pathology later in life. It was therefore hypothesized that early adverse experiences may impact on a preexisting genetic or epigenetic individual vulnerability, resulting in an individual with an increased risk for disease under certain environmental conditions. The first clear evidence for gene × environment interaction for depression came from Caspi and colleagues, demonstrating that a functional polymorphism in the promoter region of the serotonin transporter (5-HTT) gene influenced the individual risk to develop depression as a consequence of stressful life events in young adults (Caspi et al., 2003). This finding has now been replicated several times (Kim et al., 2007b; Wilhelm et al., 2006) and was also widely supported by findings in animal models (Suomi, 2006). However, while the failure of a recent meta-analysis to show an overall effect (Risch et al., 2009) needs to be interpreted with caution, it illustrates that one genetic risk factor can only account for a small overall effect.

A few other genetic risk factors have by now been identified (Binder et al., 2008; Binder et al., 2004; Bradley et al., 2008), including polymorphisms in the *Fkbp5* and *Crhr1* gene, which significantly interact with early-life events and thereby modulate adulthood risk of depression. It is therefore clear that animal models for depression need to encompass both genetic and environmental risk factors in order to match the situation in humans.

Modeling depression in rodents

A first important decision with regard to modeling depression is the choice of the model system, that is, the species. It seems obvious that complex psychiatric diseases are best modeled in animals that are closely related to humans, as for example, primates. While there are a number of outstanding researchers working with non-rodent models for depression (Barr et al., 2003; Fuchs, 2005; Pryce et al., 2005), there are many ethical, political and practical issues that prevent research with these models on a larger scale. Most of the scientific community therefore relies on rodent models for psychiatric disorders, which is also the focus of this review. A second important question is then whether or not a complex psychiatric disease as depression can actually be modeled in rats or mice. This is a critical issue given some core symptoms of depression, as for example, low self-esteem, feelings of guilt or suicidality. While some of these aspects may have their correlates in non-human primates (if hard to address), they cannot be modeled in rodents. However, a number of core symptoms of depression in humans do have an equivalent in animals (see Table 1). It is therefore possible to reach a certain level of face validity with animal models of depression, even though it has to be acknowledged that the available tests are often only a very crude approximation of the desired readout. For a detailed description and evaluation of the different available tests, please refer to other review articles (Pryce and Seifritz, 2011). In addition, many models have to settle for a subset of validity aspects, which moreover are not highly specific for depression (Veenema, 2009). However, the lack of specificity of symptoms also reflects the actual complexity of the depression syndrome, which is also becoming apparent from human polymorphisms that are associated with multiple diseases (Knight et al., 2009).

Face validity, thus the level of similarity in the disease symptoms, is one of the core validity criteria proposed by Willner and colleagues (McKinney and Bunney, 1969; Willner, 1984). Two other often considered validity criteria for a possible animal model of depression are construct validity and predictive validity. Construct validity implies that the theoretical rationale of the

model is matching the actual human situation. Thus, the more known risk factors of depression, both genetic and environmental, are incorporated in an animal model, the higher the validity of this model would be rated. Construct validity also demands that the actual psychopathological mechanisms are similar between disease and model. The demand for similar etiology of a condition in humans and in the animal model has also been defined as etiological validity (Geyer and Markou, 1995). Obviously, such validity is not perfect just because of "early-life" events, but would need to incorporate factors like emotional neglect. Predictive validity addresses the ability of successful treatment options in humans to improve the symptomatology in the animal model. These validity criteria, although sometimes limited in their applicability, are still very helpful in assessing the validity of a model and comparing the various model approaches with each other.

The main focus of the current review is animal models incorporating early-life stress as a risk factor for depression. This is obviously not the only described risk factor for depression and there are many other valid approaches, which have previously been summarized in a number of excellent reviews (El Yacoubi and Vaugeois, 2007; Frazer and Morilak, 2005; Fuchs and Flïugge, 2006; Kalueff et al., 2007; Müller and Holsboer, 2006; Willner and Mitchell, 2002). There are also already a number of outstanding reviews on the topic of early-life stress paradigms as potential models for depression, which should be used as complementary source of information (Kaufman et al., 2000; Ladd et al., 2000; Pryce et al., 2005). The term early-life stress has a broad range and can be roughly subdivided in the prenatal phase, the early postnatal phase (until P21), and the early adolescent phase (P21-P30), with the current review focusing primarily on models in the early postnatal phase. In the following paragraphs the most frequently used animal models of early-life stress will be described and discussed in terms of their potential validity as models for depression (see Figure 1). Other approaches may be mentioned, but will not be discussed in detail, mainly due to the lack of available data that would make an evaluation of these models possible.

depression

core symptoms in humans	analogous parameter in rodents
loss of enjoyment	anhedonia
loss of motivation	passive coping strategies; low locomotor activity
sleep disturbances	altered sleep / activity patterns
anxiety	anxiety-related behaviour
cognitive deficits	cognitive deficits
hypercortisolism	hyperactivity of the stress system

Table 1. Comparison of core symptoms of depression in humans with the possible analogous parameters assessable in rodents.

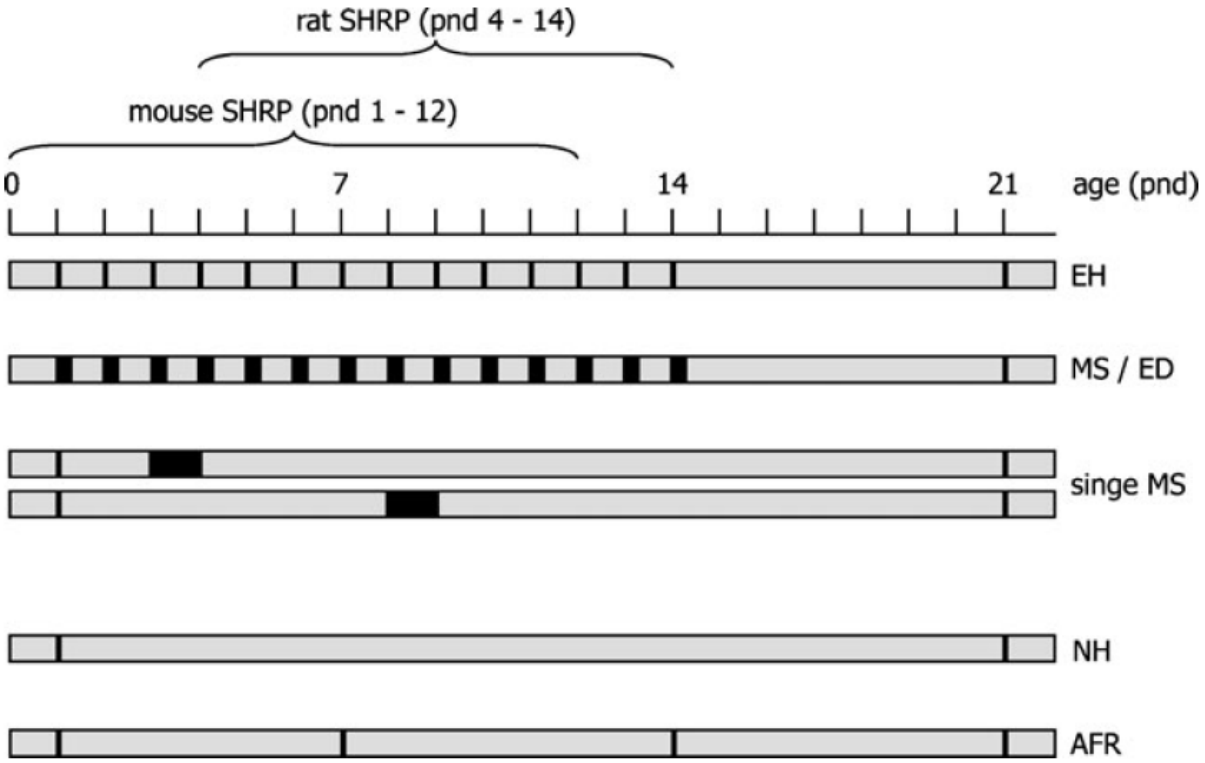

Figure 1: Illustration of the different types of maternal separation paradigms. Black bars indicate times of interference with mother-pup interaction (pnd, postnatal day).

Rodent models of early-life stress

Interventions in mother-pup interaction time periods

Early handling 3-15 min

One of the first experimental paradigms described in rodents that manipulate mother-pup interaction periods is the early handling (EH) paradigm developed by Levine (Levine, 1957). The central characteristic of this model is a daily physical manipulation of the litter, where the pups are separated from the mother for a short period of time (maximally 15 min). It has been shown that this procedure, which is carried out during the first 2-3 weeks of life, stimulates maternal care behavior towards the offspring (Liu et al., 1997) and elicits acute neuroendocrine responses from the pups (Meaney et al., 1991). A critical issue with regard to the EH paradigm is the choice of the appropriate control or comparison group. Historically, EH was compared to non-handled (NH) pups, thus litters that are not exposed to any physical human disturbance. However, this

procedure can be regarded as an experimental group as well, as the lack of any external stimulation also affects maternal care behavior. It was therefore suggested that both EH and NH groups should be compared to litters exposed to normal animal facility rearing, thus regular exposure to, for example, cage changes (Pryce and Feldon, 2003). This so-called animal facility rearing (AFR) group is therefore an intermediate group between EH and NH.

It can now be discussed whether any of the two extreme groups — NH or EH — display a depression-like phenotype as adults. Unfortunately, in spite of the long history of this model, the available data with regard to face validity for depression are sparse, which may also be due to the fact that this model was never intended to be a stand-alone model for depression. The available data are also often conflicting, with some authors reporting differential phenotypes, while others find no differences. In Sprague-Dawley rats EH has no effect on sucrose preference, a frequent measure of hedonic behavior, in comparison to NH (Maniam and Morris, 2010). In male Wistar or Fischer rats, no differences in the performance of EH or NH animals were observed in the forced swim test (FST) in recent studies (Papaioannou et al., 2002; Rüedi-Bettschen et al., 2006), while an older study in Wistar rats did report a shorter immobility time on the FST in EH animals (Hilakivi-Clarke et al., 1991). In Fischer rats, EH resulted in escape and avoidance deficits in a two-way shuttle box compared to NH animals (Rüedi-Bettschen et al., 2004), suggesting that under certain conditions and genetic backgrounds, EH can even be detrimental for the adult phenotype. In mice, Millstein and Holmes found no clear evidence of altered anxiety- or depression like behavior when comparing EH and AFR in different strains (Millstein and Holmes, 2007). The situation is somewhat different for anxiety-related behavior, where many authors observed a decreased anxiety in EH animals compared to NH (Durand et al., 1998). However, as there are no consistent indications of the EH/NH paradigm in terms of depression-like behavior in either rats or mice, this paradigm seems not suited to be used as a potential model for depression *per se*. The observed molecular, neuroendocrine and behavioral alterations observed with this model rather suggest that growing up in an AFR, EH or NH environment alters the degree of vulnerability to environmental challenges later in life.

Repeated maternal separation for 1-8 h

While EH stimulates maternal care, prolonged separations of the dam from the litter are meant to reduce the amount of maternal care for the pups, thereby modeling emotional as well as physical

neglect. The applied methodologies are highly variable not only in the separation time and duration but also with respect to temperature (warm or cold), type of separation (mother or litter removed from the home cage), or isolation (pups separated in isolation or as whole litter). As a consequence, the nomenclature of this paradigm is also highly variable. In the current review, we will apply the nomenclature suggested by Pryce and Feldon (Pryce and Feldon, 2003). Thus, maternal separation (MS) is used to describe the repeated separation of the intact litter from the dam for one or more hours per day across several days, while early deprivation (ED) is used to describe a separation of a pup from the dam as well as the litter for one or more hours across several days. Nonetheless, the technical variability of these paradigms makes the interpretation and comparison of the data obtained in different laboratories extremely difficult. One specific problem is the choice of the comparison group, which in some cases is NH, in others AFR.

One important aspect with regard to maternal separation paradigms is that they usually take place during the so-called stress hyporesponsive period (SHRP) in rodents, which lasts from postnatal day 4-14 in the rat and from postnatal day 1-12 in the mouse (Enthoven et al., 2010; Levine et al., 1967; Levine, 1970; Schmidt et al., 2003). First described by Schapiro and colleagues (Schapiro et al., 1962), this period is characterized by a low basal corticosterone secretion and the relative inability of mild stressors to elicit a corticosterone response (Levine, 2001).

As with the NH-EH paradigm, there are only few data available for the MS or ED models with regard to depression-like behavior. In rats, some authors report an increased immobility in the FST in MS (Lambas-Senas et al., 2009; Lee et al., 2007; Rüedi-Bettschen et al., 2005), while others find no effect (Marais et al., 2008). For mice, there are also no consistent effects of MS in the FST (Bhansali et al., 2007; MacQueen et al., 2003; Millstein and Holmes, 2007). Interestingly, marked strain differences in the response to MS indicate that lasting effects of this paradigm can only be expected in animals with a specific of genetic vulnerability (El Khoury et al., 2006), probably in combination with a specific adult environment. The same is true for learned helplessness, where ED Wistar rats show actually an improved escape behavior compared to NH, while Fisher rats subjected to ED display deficits in escape behavior compared to NH counterparts (Rüedi-Bettschen et al., 2005). The literature on anhedonia-like behavior, thus sucrose consumption and sucrose preference, is also not consistent. While most authors report no effects of MS on sucrose preference (Matthews and Robbins, 2003; Shalev and Kafkafi, 2002),

there are also reports of decreased sucrose preference compared to NH (Michaels and Holtzman, 2007). Interestingly, when sucrose is not freely available but requires effort in terms of bar presses on a progressive ratio schedule, ED male Wistar rats have been shown to consume significantly less sucrose (Leventopoulos et al., 2009; Rüedi-Bettschen et al., 2005). However, from the inconsistency in the literature with regard to the depression-like phenotype elicited by MS or ED, it can only be concluded that other — mostly not controlled — factors influence the outcome of the study. Thus, (epi)genetic predispositions carried by the individual animals of the different rat and mouse strains are likely determinants of the beneficial or detrimental effects of a disrupted maternal care. As with the EH/NH paradigm, repeated separations have been shown to clearly affect neuroendocrine and physiological parameters, which are likely to impact on the vulnerability or resilience of the individuals to subsequent challenges.

Single maternal separation for 24 h

First developed by Levine and colleagues, this paradigm consists of a single separation period of mother and pups for 24 hours, which can be applied at different time points during postnatal development (Stanton et al., 1988). While it has been extensively used to study the neuroendocrine function of the developing rat or mouse pup (Dent et al., 2000; Dent et al., 2001; Liebl et al., 2009) and a number of neuroendocrine effects in adult animals were reported (Ladd et al., 1996; Rots et al., 1996; Suchecki et al., 2000; Sutanto et al., 1996; Workel et al., 1997; Workel et al., 2001), there are only a few reports addressing the influence of single MS on depression-like behavior. CD1 mice subjected to 24 hours maternal separation at postnatal day 12 showed no differences in floating or struggling time in the FST at adulthood (Macri and Laviola, 2004). Similarly, 24-h maternal separation at postnatal day 9 resulted in no clear FST phenotype during adolescence (Marco et al., 2009). Thus, while single MS has been proposed as a valid model for other psychiatric diseases as, for example, schizophrenia (Ellenbroek and Cools, 2000), there is no apparent validity for the study of depression-like phenotypes. Again, a combination of early experience with other risk or triggering factors is needed to have a useful model for depression. As with the other paradigms discussed so far, it becomes apparent that the crucial experiments with regard to depression have not yet been performed. Thus, animals with a history of early-life stress should be combined with additional genetic or environmental risk factors, for

example, a specific genetic knockout or a second stress exposure during adulthood. This critical issue will also be discussed at the end of this review.

Models based on the quantity and/or quality of maternal care

Naturally occurring differences in maternal care

First reported by the group of Meaney and colleagues, this model is based on the hypothesis that early handling mirrors naturally occurring differences in maternal care (Liu et al., 1997). During an observation period of postnatal day 0 to 8, high or low licking and grooming/arched-back nursing mothers are identified as those where both measures are one standard deviation above or below the mean of the cohort, respectively. This model, which is routinely used with Long-Evans rats, lacks a control group problem in relation to the experimentalist's influence, as present in the EH/NH paradigm (de Kloet et al., 2005b). While this paradigm has been extensively studied with respect to neuroendocrine regulation (Liu et al., 1997), hippocampal function (Champagne et al., 2008; Liu et al., 2000) and anxiety-related behavior (Caldji et al., 1998), little or no data are available suggesting a depression-like phenotype.

Enriched postnatal social environment

Another way to manipulate maternal care behavior in a naturalistic way is the communal nesting paradigm (Branchi, 2009). In a communal nest (CN), three females breed and keep pups together, and share care-giving behavior in a single nest from birth to weaning (P1 to P25), representing natural ecologic condition of (altricial) rodents. This early social enrichment provides pups with high level of maternal care and peer interaction. Compared to mice reared in standard laboratory conditions (SN), CN mice display more passive behavior in the FST, which could be modulated by acute, but not chronic, fluoxetine treatment (Branchi et al., 2006; Branchi et al., 2010). However, the finding that chronic fluoxetine treatment does not affect the duration of immobility in CN mice while increasing immobility in SN mice may seem counterintuitive and warrants further investigation. Further, adult CN mice display increased anxiety-related behavior (Branchi et al., 2006), which can be ameliorated when the test is performed in a social context. Adult CN mice also show greater sucrose preference under both basal and stressful conditions, and display a lower corticosterone response following an acute stressor. In addition, CN mice have elevated brain NGF and BDNF levels and increased survival of adult-born new neurons in the

hippocampus (Branchi et al., 2006). Taken together, these studies point to the possibility that standard nesting conditions may represent an impoverished environment and communal nesting could represent a model for resilience to depression later in life under specific environmental circumstances.

Impoverished postnatal environment

An opposite effect to the CN paradigm is achieved by the limited nesting material model recently established by the group of Baram in rats and mice (Brunson et al., 2005; Ivy et al., 2008; Ivy et al., 2010; Rice et al., 2008), where mothers are provided with reduced nesting and bedding material from postnatal days 2 to 9 of their litter. This manipulation results in frequent changes of maternal behavior and inconsistent or fragmented maternal care, resulting in a higher stress exposure of the offspring. So far, this promising model has only been investigated in terms of anxiety-related behavior and cognitive performance, so future studies will reveal whether this paradigm also results in alterations of depression-like phenotypes.

Pharmacological models

Effects of early-life environment may be mediated by any of a number of hormones, neurotransmitters/peptides, or inflammatory mediators. Signaling by these mediators can be modulated directly during early-life by classical pharmacology. While a review of all the pharmacological approaches to mimic an early-life stress exposure would be beyond the scope of the current review, we will mention and discuss the most common ones in the next paragraphs.

Postnatal glucocorticoids

Much work with glucocorticoids (GCs) has focused on the use of high doses of the synthetic GC dexamethasone. This surely models the treatment of premature children and the many associated programming effects, that is, long-term effects that emerge at a later age, such as impaired neuromotor skills, cognitive deficits, and disrupted HPA axis activity at school age (Karemaker et al., 2008; Yeh et al., 2004). However, dexamethasone may display quite different pharmacodynamic characteristics from corticosterone, and its superagonistic properties probably affect development in a manner that is quite different from the endogenous corticosteroids that are associated with actual early-life circumstances. Postnatal dexamethasone treatment has been

shown to increase anxiety-related behavior and the time immobile in the FST in adult Wistar rats (Felszeghy et al., 1993; Neal et al., 2004). Adolescent and adult rats with early-life dexamethasone exposure also show impaired hormonal response to stress (Flagel et al., 2002; Neal et al., 2004). On the other hand, mice exposed to excessive corticosterone levels during the SHRP due to a conditional knockout of pituitary GR receptors display no depression- or anxiety-related phenotype as adults (Schmidt et al., 2009). On the contrary, it was recently demonstrated that those mice are resilient to the behavioral effects of chronic social defeat stress in adulthood (Wagner et al., 2011). Thus, while early postnatal GCs treatment can strongly program development, it is far from proven that these effects mimic those of endogenous hormones. These approaches are of little use when developing animal models of depression based on early-life stress.

Postnatal lipopolysaccharide

The immune system has been implicated in the development of psychopathologies, including depression (Dantzer et al., 2008). It has been argued that in adult animals, chronic exposure to lipopolysaccharide (LPS) can be useful to model aspects of depression (De La Garza, 2005). Early-life exposure to such endotoxins is common and represents a prominent environmental challenge due to the fragile immune system of the newborn (Shanks and Meaney, 1994). Thus, postnatal LPS treatment in rodents would mimic a mild (gram-negative) bacterial infection of the human infants.

It is clear that LPS administration in early life has immediate effects on the HPA axis, disrupts the development of stress system, and results in changed neuroendocrine and neuroimmune responses in adulthood (Kohman et al., 2008; Shanks et al., 2000; Shanks et al., 1995; Walker et al., 2008). Behavioral changes occur in several domains including social behavior, cognitive capacity and anxiety (Granger et al., 2001; Harre et al., 2008). Effects on cognition and anxiety both are relevant for depression but are not regarded as core features of the disease. Kentner and colleagues observed no effect of postnatal LPS treatment on sucrose preference (Kentner et al., 2010). Further, postnatal LPS treatment did not affect adult behavior in the FST in either male or female mice (Lucchina et al., 2010). Thus, a combination with additional risk factors would be a prerequisite when using postnatal LPS treatment as a model for depression.

Conclusions

Many parameters in early life determine the stress- and behavioral responsiveness in adulthood, and many of these potentially qualify for a "second hit" in a vulnerability model consisting of genomic background, predisposing events and precipitating events. Obviously, these parameters are not independent, for example because maternal responsiveness to stressed neonates can ameliorate the social-developmental effects of early illness (Hood et al., 2003). A major question obviously is *how* changes in early life can increase the risk to develop depression. As will be clear from this volume, a vast number of changes in neurochemistry can be the consequence of early-life stress, and the strongest explanation for such enduring changes is that they are mediated through epigenetic modification of the DNA and/or chromatin that are triggered by any of the neuronal and hormonal mediators associated with the early-life event. The review of the available literature on the different early-life stress models underscores the importance of the genetic predisposition as well as the later (adult) environment. When studied out of the genetic and environmental context, none of the established models of early-life stress can be regarded as robust model of depression. Therefore, more studies are needed that actually address this complex interplay of genetic vulnerability or resilience to postnatal stressors. In addition, the adult environment will be decisive for the outcome, where an early-life stress event may have adaptive consequences in aversive adult environments, but may be maladaptive in non-aversive environments (Schmidt, 2011).

Chapter 3

Forebrain corticotropin-releasing hormone receptor 1 is required for early life stress-programmed cognitive deficits

Manuscript in preparation

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Abstract

Childhood traumatic events hamper the development of the hippocampus and impair declarative memory in susceptible individuals. Persistent elevations of hippocampal corticotropin-releasing hormone (CRH) in experimental models of early-life stress have suggested a role for this endogenous stress hormone in the resulting structural modifications and cognitive dysfunction, but direct testing of this possibility has been difficult. In the current study, we subjected conditional forebrain CRHR1 knockout (CRHR1-CKO) mice to an impoverished postnatal environment and examined the role of forebrain CRHR1 in the long-lasting effects of early-life stress on learning and memory. Early-life stress impaired spatial learning and memory in wild-type mice, and postnatal forebrain CRH overexpression reproduced these deleterious effects. Cognitive deficits in stressed wild-type mice were associated with disrupted long-term potentiation (LTP) and a reduced number of dendritic spines in area CA3 but not in CA1. Forebrain CRHR1 deficiency restored cognitive function, LTP and spine density in area CA3, and augmented CA1 LTP and spine density in stressed mice. In addition, early-life stress differentially regulated the amount of hippocampal excitatory and inhibitory synapses in wild-type and CRHR1-CKO mice, accompanied by alterations in the neurexin-neuroligin complex. These data suggest that the functional, structural and molecular changes evoked by early-life stress are at least partly dependent on persistent forebrain CRH-CRHR1 signaling, providing a molecular target for the prevention of cognitive deficits in adults with a history of early-life adversity.

Introduction

Early-life adverse events increase the danger of developing psychopathologies (Evans and Schamberg, 2009; Sadowski et al., 1999; Schenkel et al., 2005) in adult individuals with genetic risk factors (Feder et al., 2009), including specific polymorphisms and haplotypes of the corticotropin-releasing hormone (CRH) gene (Smoller et al., 2005) and the CRH receptor 1 (CRHR1) gene (Bradley et al., 2008; Tyrka et al., 2009). The hippocampus, a region essential for the regulation of the hypothalamic-pituitary-adrenal (HPA) axis and processing of spatial information, undergoes critical development early in life and is vulnerable to stress (Avishai-Eliner et al., 2002; Kim and Diamond, 2002; Lupien et al., 2009). In rodents, psychological stress during the first 2 weeks of life impairs hippocampus-dependent spatial learning and memory (Aisa et al., 2007; Oitzl et al., 2000; Rice et al., 2008), disrupts hippocampal long-term potentiation (LTP) (Bagot et al., 2009; Champagne et al., 2008; Ivy et al., 2010), and reduces dendritic complexity in hippocampal neurons later on (Brunson et al., 2005; Oomen et al., 2010).

In the hippocampus, CRH is released from inhibitory interneurons (Chen et al., 2001), binds with high affinity to CRHR1 abundant in dendritic spines of pyramidal neurons (Chen et al., 2004b), and modulates neuronal function (Aldenhoff et al., 1983; Sheng et al., 2008) and cognition (Radulovic et al., 1999; Row and Dohanich, 2008). Interestingly, the levels of hippocampal CRH and CRHR1 are much higher during the second and third weeks after birth compared to those in adulthood (Avishai-Eliner et al., 1996; Chen et al., 2001). Acute stress differentially activates hippocampal neurons in immature and adult brains, which is dependent on CRHR1 (Chen et al., 2006). Early-life stress evokes enduring elevations of hippocampal CRH (Ivy et al., 2010) and may disrupt hippocampal CRHR1 expression (O'Malley et al., 2011). Moreover, central administration of CRH to neonatal rats recapitulates the effects of early-life stress on cognition and hippocampal morphology (Brunson et al., 2001), whereas postnatal CRHR1 antagonism prevents these effects (Ivy et al., 2010) and enhances spatial performance (Fenoglio et al., 2005) in adult rats. Hence, hippocampal CRH-CRHR1 signaling may play an essential role in modulating the persistent programming effects of early-life stress on cognition.

While there is already some evidence for the involvement of the CRH-CRHR1 system in mediating the effects of early-life stress on cognition, previous pharmacological approaches were limited with regard to regional specificity. Therefore, we here used transgenic mouse lines with conditional CRHR1 deficiency (Müller et al., 2003) or CRH overexpression (Lu et al., 2008)

specifically in forebrain regions to investigate the role of hippocampal CRH-CRHR1 signaling in early-life stress-induced later-life cognitive impairments. A novel mouse model of early-life stress was utilized (Rice et al., 2008), in which the mother-pup interaction is disrupted by an impoverished postnatal environment. We examined whether forebrain CRHR1 overactivation would reproduce the effects of early-life stress on spatial learning and memory during adulthood, and whether forebrain CRHR1 inactivation would prevent the functional, structural, and molecular abnormalities induced by early-life stress.

Materials and methods

Animals

Male transgenic mice with postnatal inactivation of the *Crhr1* gene in forebrain neurons (referred as CRHR1-CKO hereafter) were generated as described previously (Müller et al., 2003; Wang et al., 2011). To generate a mouse line with forebrain-restricted overexpression of CRH in principal neurons, $R26^{+/flopCrh}$ mice were crossed to Camk2a-Cre mice (Lu et al., 2008). Male $R26^{flopCrh/flopCrh}$ Camk2a-cre mice (referred as CRH-COE hereafter) were obtained in the F2 generation.

Adult female CRHR1-EGFP reporter mice were used to test the colocalization of CRHR1 and neurexins. The detailed step-by-step targeting procedure will be published elsewhere or is available upon request (to Dr. Deussing). Briefly, the endogenous *Crhr1* locus was modified via homologous recombination in embryonic stem cells. Gene targeting resulted in a *Crhr1* knock-in allele where EGFP is inserted in frame into exon 2 of the *Crhr1* gene, concomitantly a selection cassette was introduced into intron 2 harboring a strong splice acceptor. In this configuration exon 2 is spliced to the selection cassette resulting in a *Crhr1*-EGFP reporter allele which is at the same time a *Crhr1* null allele due to an immediate stop codon.

All animals were housed under a 12-hour light/dark cycle (lights on at 6 am) and constant temperature (22 ± 1°C) conditions with free access to both food and water. At 7-8 months of age, all mice were sacrificed. The experiments were carried out in accordance with European Communities Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Early-life stress procedure

The limited nesting and bedding material paradigm was carried out as described previously (Rice et al., 2008). Briefly, the day of birth was designated postnatal day 0 (P0). On the morning of P2, control dams were provided with sufficient amount of nesting material (2 squares of Nestlets, Indulab, Gams, Switzerland) and standard sawdust bedding. In the "stress" cages, dams were provided with limited quantity of nesting material (1/2 square of Nestlets), which was placed on a fine-gauge aluminum mesh platform (McNichols, Tampa, Florida, USA). All litters remained undisturbed during P2-P9. On P9, all dams were provided with standard nesting and bedding material. Male offspring were weaned on P28 and group housed in 4-5 per cage. Tail tips were collected and genotyped upon weaning when appropriate.

Behavioral and cognitive testing

At 1 month before the behavioral tests, all mice were single housed. Mice were tested at 6 months of age, and the tests were always performed between 8 am and 12 noon and scored by the ANY-maze software (ANY-maze 4.50, Stoelting, Wood Dale, Illinois, USA). To assess the effects of early-life stress on spatial learning and memory in wild-type and CRHR1-CKO mice, two successive batches of mice were tested under the same conditions and results were pooled.

Y-maze

The Y-maze apparatus was made of grey polyvinyl chloride with three symmetrical arms (30 × 10 × 15 cm^3) marked by triangle-, bar- and plus-signs, respectively, as intra-maze spatial cues, and was evenly illuminated (30 lux) (Sterlemann et al., 2010). Prominent extra-maze spatial cues were attached to the walls at a distance of ~25 cm from the apparatus. During the first trial (acquisition phase; 10 min), the mice were allowed to explore two of the three arms with the third arm blocked. After a 30 min intertrial interval, the mice were placed in the center of the Y-maze and allowed to explore all arms freely (retrieval phase; 5 min). An arm entry was counted when all four limbs of the mouse were within an arm. The percentage of time spent in the novel arm and the two familiar arms was calculated, with a higher preference for the novel arm being rated as intact spatial recognition memory.

Morris water maze

The Morris water maze test was carried out as described previously (Sterlemann et al., 2010). A circular tank (110 cm in diameter) was filled with opaque colored water (22 ± 1°C), and prominent extra-maze visual cues were attached to the walls at a distance of ~50 cm from the pool. After day 1 with a 60-s free swim trial, mice were trained to locate a visible platform (10 cm in diameter) above the surface of the water for 4 trials (visual training). In the following spatial training sessions, mice received 4 trials per day to locate the submerged platform in a fixed position over 3 consecutive days. The order of starting locations was varied throughout trials. Next day, the reference memory was assessed in a 60-s probe trial with platform removed, and the latency to reach the platform area and the time spent in each quadrant were calculated. After 4 days of rest, mice received 4 trials to locate the hidden platform placed in the quadrant opposite to that in the spatial training sessions (reversal learning). The trials in visual, spatial and reversal training sessions were terminated once the mouse found the platform or 60 s had elapsed, and the latency to reach the platform was recorded for each trial. The intertrial interval was 10 min.

Brain slice preparation and electrophysiological recordings

Mice (7-8 months of age) were anesthetized with isoflurane and decapitated, and brains were quickly removed. Brain slices were prepared using a vibrating microtome in ice-cold Ringer solution (124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-glucose, and 1.25 mM NaH_2PO_4; pH 7.3) bubbled with a 95% O_2-5% CO_2 mixture. All slices were placed in a holding chamber for at least 60 min and were then transferred to a superfusing chamber for extracellular recordings. The flow rate of the solution through the chamber was 1.5 ml/min. Extracellular recordings were made using glass microelectrodes (2-3 MΩ) filled with bath solution. All experiments were performed at room temperature (RT).

Field excitatory postsynaptic potentials (fEPSPs) at synapses between mossy fibers and CA3 pyramidal neurons or Schaffer collateral-commissural pathway (SCCP) and CA1 pyramidal cells were recorded extracellularly in the stratum lucidum of the CA3 or the stratum radiatum of CA1 and evoked by test stimuli (0.066 Hz, 4-5 V, 20 ms) delivered via a bipolar tungsten electrode insulated to the tip (50 μm in diameter) placed in either the granule cell layer of dentate gyrus (DG) or SCCP, respectively. High-frequency stimulation (HFS) of 3 × 100 Hz/100 pulses with 10

s interstimulus intervals to mossy fibers or 1 × 100 Hz/100 pulses to the SCCP were delivered to induce LTP.

The recordings were amplified, filtered (3 kHz) and digitized (9 kHz) using a laboratory interface board (ITC-16, Instrutech, New York, USA), and stored with the acquisition program Pulse, version 8.5 (Heka electronic, Lambrecht, Germany). Data were analyzed offline with the analysis program IgorPro v.6 (Wavemetrics, Lake Oswego, Oregon, USA) software. Measurements of the amplitude of the fEPSP were taken and normalized with respect to the 30-min control period before tetanic stimulation.

Golgi impregnation and the analysis of spine density

Mice (7 months of age) were anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneally) and transcardially perfused with 0.9% saline/heparin followed by 3% paraformaldehyde containing 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were stored in the same fixative overnight. Coronal sections (100 µm thick) from the dorsal hippocampus were cut on a vibrating microtome (VT1000, Leica, Milton Keynes, United Kingdom) and washed. Sections were equilibrated in 0.1 M PB, postfixed in 1% osmium tetroxide in 0.1 M PB for 30 min, and further washed before trimming with a razor blade to maximize the diffusion of Golgi labeling solutions. The single-section Golgi-impregnation technique was carried out as previously described (Gabbott and Somogyi, 1984). Areas where apical dendrites of Golgi-impregnated neurons were examined included the stratum radiatum of area CA3 and CA1 and the middle molecular layer of DG.

Spines were counted using Neurolucida software (MicroBrightField, Magdeburg, Germany) on a Nikon 80i microscope equipped with a 100×, 1.25 numerical aperture oil-immersion lens. For each area examined, 10 segments of dendrites of similar diameter and a length of > 30 µm were chosen. Spine density was calculated as the number of spines per 1 µm of dendrite segment.

In situ hybridization

Mice were anesthetized with isoflurane and sacrificed at 1 week after the behavioral tests. Brains were removed, snap-frozen and sectioned coronally at 16 µm through the dorsal hippocampus (bregma -1.58 to -2.18) (Paxinos and Watson, 2001) at -20°C in a cryotome (Microm HM 560, Thermo Fisher Scientific, Germany). The sections were thaw-mounted on superfrost slides, dried,

and kept at -80°C. *In situ* hybridization using ^{35}S UTP labeled ribonucleotide probes was performed as previously described (Schmidt et al., 2007). The following primers were used to generate antisense RNA hybridization probes that recognize neurexin-1 (469 base pairs), neuroligin-1 (461 base pairs), neuroligin-2 (401 base pairs), and neuroligin-3 (511 base pairs), respectively: (1) neurexin-1, AGTTGTACCTGGGTGGCTTG (forward primer) and TCACACGTCCTGCATCTAGC (reverse primer); (2) neuroligin-1, GGGGATGAGGTTCCCTA TGT (forward primer) and GGATCATCCTGTTTGGCAGT (reverse primer); (3) neuroligin-2, TGTGTGGTTCACCGACAACT (forward primer) and CTCCAAAGTGGGCAATGTTT (reverse primer); (4) neuroligin-3, CCATCATCCAAAGTGGCTCT (forward primer) and TCAGTGAAGAGTGCCACCAG (reverse primer). The slides were apposed to Kodak Biomax MR films (Eastman Kodak, Rochester, New York, USA) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion, Frederick, Maryland, USA).

Double-fluorescence immunohistochemistry, image acquisition and quantification

Mice were anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline/heparin followed by 4% buffered paraformaldehyde at 1 week after the behavioral tests. Double-labeling immunofluorescence was performed on free-floating coronal sections (25 µm thick) obtained from postfixed and cryoprotected brains as described previously (Chen et al., 2004b). The following primary antibodies were used: goat anti-EGFP (1:2000, Abcam, Cambridge, United Kingdom), mouse anti-vesicular glutamate transporter 1 (VGLUT1; 1:1000, Synaptic Systems, Göttingen, Germany), goat anti-vesicular gamma-aminobutyric acid transporter (VGAT; 1:1000, Synaptic Systems), and rabbit anti-neurexins (detects most isoforms and corresponding splice-variants of neurexins; 1:500, Synaptic Systems). After incubation with primary antibodies diluted in 1% donkey serum and 0.3% Triton X-100 in 0.1 M PB at 4°C for 40 hr, sections were rinsed and incubated with Alexa Fluor 488- or 647-conjugated donkey secondary antibodies (1:500, Invitrogen, Karlsruhe, Germany) for 2 hr at RT. After rinsing, sections were transferred onto slides, dried, and coverslipped with Vectashield containing 4',6-diamidino-2-phenylindole (Vector laboratories, Burlingame, California, USA).

Fluorescent images (1600 × 1600 pixels) were obtained with an Olympus IX81 confocal microscope and a 40× water-immersion objective (Olympus, Tokyo, Japan) using the Kalman

filter and sequential scanning mode under identical settings for laser power, photomultiplier gain and offset. For the colocalization assessment of CRHR1 and neurexins, images were adjusted for better brightness and contrast using the FV10-ASW 1.7 software (Olympus). For the comparison of fluorescent signals among groups, images were imported into the NIH ImageJ software, converted to 8-bit grayscale and thresholded uniformly. The density of synaptic puncta was quantified using the "analyze particle" module of the ImageJ program.

Statistical analysis

For the analyses of spine density and VGLUT1- and VGAT-immunoreactive puncta density, data were normalized by taking the value of the control wild-type group as 100%. Data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* test as necessary. Three-way ANOVA with condition and genotype as between-subjects factors and trial as a within-subject factor was performed on the Morris water maze data of each spatial training day and the reversal learning day, followed by two-way ANOVA with either condition or genotype as a between-subjects factor and trial as a within-subject factor when applicable. Student's *t*-test was used to compare pairs of means. The level of statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

Results

Forebrain CRH-CRHR1 signaling mediates early-life stress-impaired spatial learning and memory

Hippocampus-dependent spatial memory in adult wild-type and CRHR1-CKO mice was first evaluated by the Y-maze test (Figure 1A). A significant main effect of stress [$F(1, 61) = 4.482$, $p < 0.05$] on time spent in the novel arm was observed. *Post hoc* analysis showed that stressed wild-type mice, while able to discriminate the novel arm from the familiar, performed significantly worse than wild-type controls ($p < 0.05$, Bonferroni's test). In contrast, the performance of stressed CRHR1-CKO mice was similar to that of the controls.

To further assess spatial learning and memory, mice were tested in the Morris water maze task (Figure 1B). On the first day of spatial training, a significant stress × genotype interaction [three-way ANOVA, $F(1, 57) = 4.028$, $p < 0.05$] effect on escape latency was noticed. Spatial acquisition was hampered by early-life stress in wild-type mice, as shown by a significant

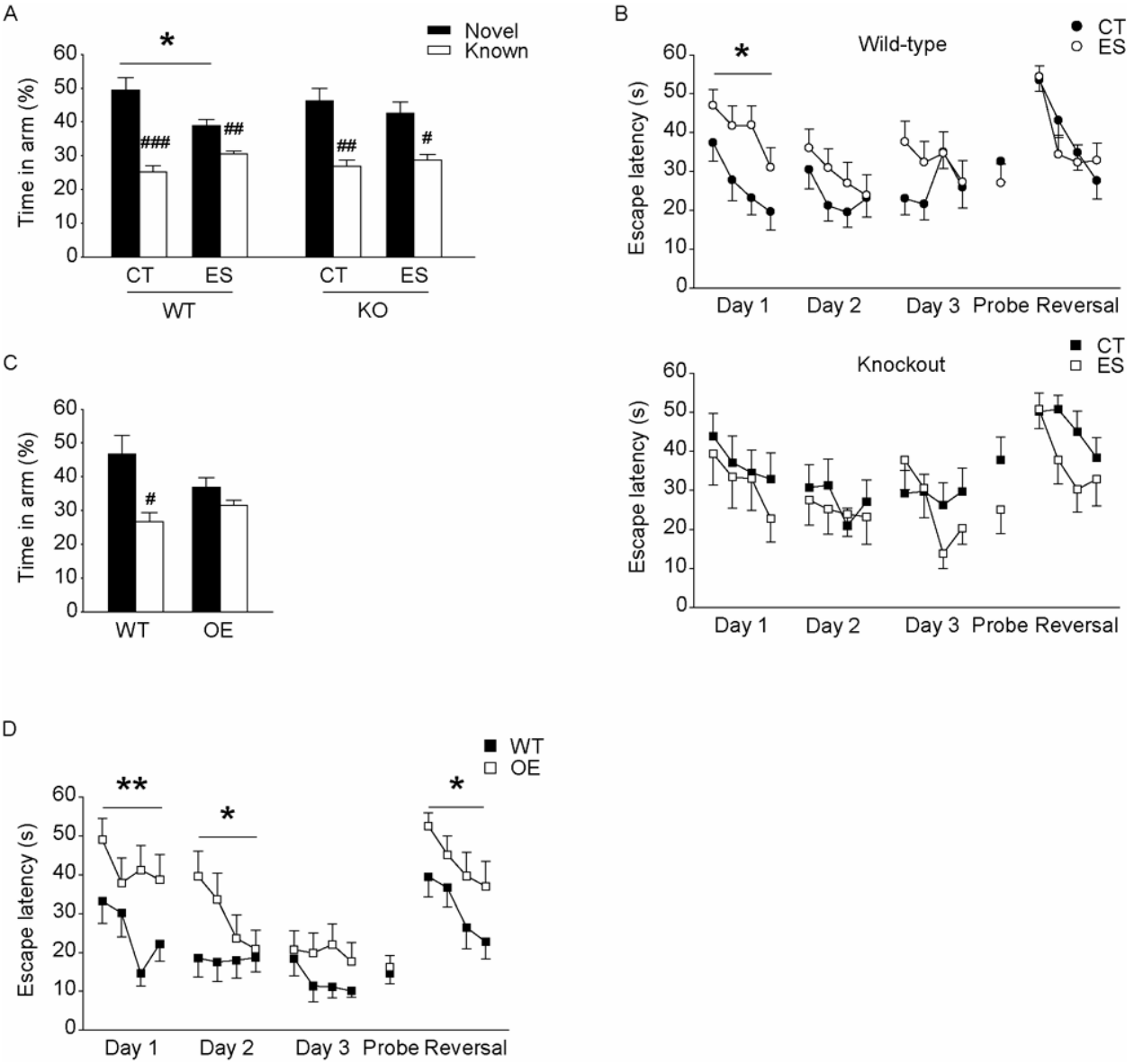


Figure 1. (A, B) Spatial learning and memory in adult wild-type (WT) and CRHR1-CKO (KO) mice that were exposed to either control condition (CT) or early-life stress (ES). **(A)** In the Y-maze test, all groups of animals spent more time exploring the novel arm versus the known ones. However, stressed wild-type mice performed significantly worse than wild-type controls, while stressed CRHR1-CKO mice displayed comparable performance to the controls. **(B)** In the Morris water maze test, stressed wild-type mice took significantly longer to locate the hidden platform in the first spatial training day, indicating impaired acquisition of spatial information. In contrast, stressed CRHR1-CKO mice spent similar time to reach the platform compared to the controls. **(C, D)** Spatial learning and memory in adult wild-type (WT) and CRH-COE (OE) mice. **(C)** In the Y-maze test, wild-type mice showed preference to the novel arm, whereas CRH-COE mice visited the novel and familiar arms similarly. **(D)** In the Morris water maze test, CRH-COE mice exhibited profound spatial learning impairments in the first two spatial training days and reduced

cognitive flexibility in the reversal learning session. *, $p < 0.05$; **, $p < 0.01$ versus control wild-type group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ versus respective novel arm. Mice: **(A, B)** n = 10-20 per group; **(C, D)** n = 10-13 per group.

increase in the latency to locate the hidden platform compared to the controls [two-way ANOVA of condition, $F(1, 35) = 6.622$, $p < 0.05$]. This impairment was mostly evident in the third trial ($p < 0.01$, unpaired *t* test). In contrast, stressed CRHR1-CKO mice spent similar time to reach the platform compared to the controls. Notably, impaired spatial learning in stressed wild-type mice was not due to swimming ability or motivation as shown by similar swim speed to the controls (data not shown). Animals from all groups acquired the task similarly on the following two days. In addition, no difference was observed among groups in the retention of spatial information (probe trial, data not shown) and cognitive flexibility (reversal learning). Together, these data suggest that stressful early-life experience attenuates hippocampus-dependent learning and memory in adulthood, and that this effect is dependent on forebrain CRHR1.

To further test this possibility, we evaluated the cognitive performance in conditional forebrain CRH-overexpressing mice. In the Y-maze task (Figure 1C), wild-type but not CRH-COE mice distinguished the novel arm from the familiar ones ($p < 0.05$, paired *t* test). In the Morris water maze task (Figure 1D), the performance of CRH-COE mice was significantly poorer in the first two days of spatial training [$F(1, 24) = 11.043$, $p < 0.01$, and $F(1, 24) = 4.709$, $p < 0.05$, respectively] and the reversal learning session [$F(1, 24) = 5.302$, $p < 0.05$] compared to wild-type mice. No differences in swimming ability or reference memory were found between groups (data not shown).

Forebrain CRHR1 inactivation abolishes the impairment of CA3 LTP and enhances CA1 LTP in early-life stressed mice

LTP is considered a major cellular correlate for learning and memory (Lynch, 2004). In the rat it was previously shown that early-life stress impaired hippocampal LTP (Brunson et al., 2005) in a CRHR1-dependent manner (Ivy et al., 2010). To assess whether impaired spatial memory in stressed wild-type mice is associated with altered synaptic plasticity in hippocampal circuits, we examined both mossy fiber-CA3 LTP and SCCP-CA1 LTP in acute brain slices (Figure 2). A significant condition × genotype interaction [$F(1, 24) = 4.648$, $p < 0.05$] effect on mossy fiber-

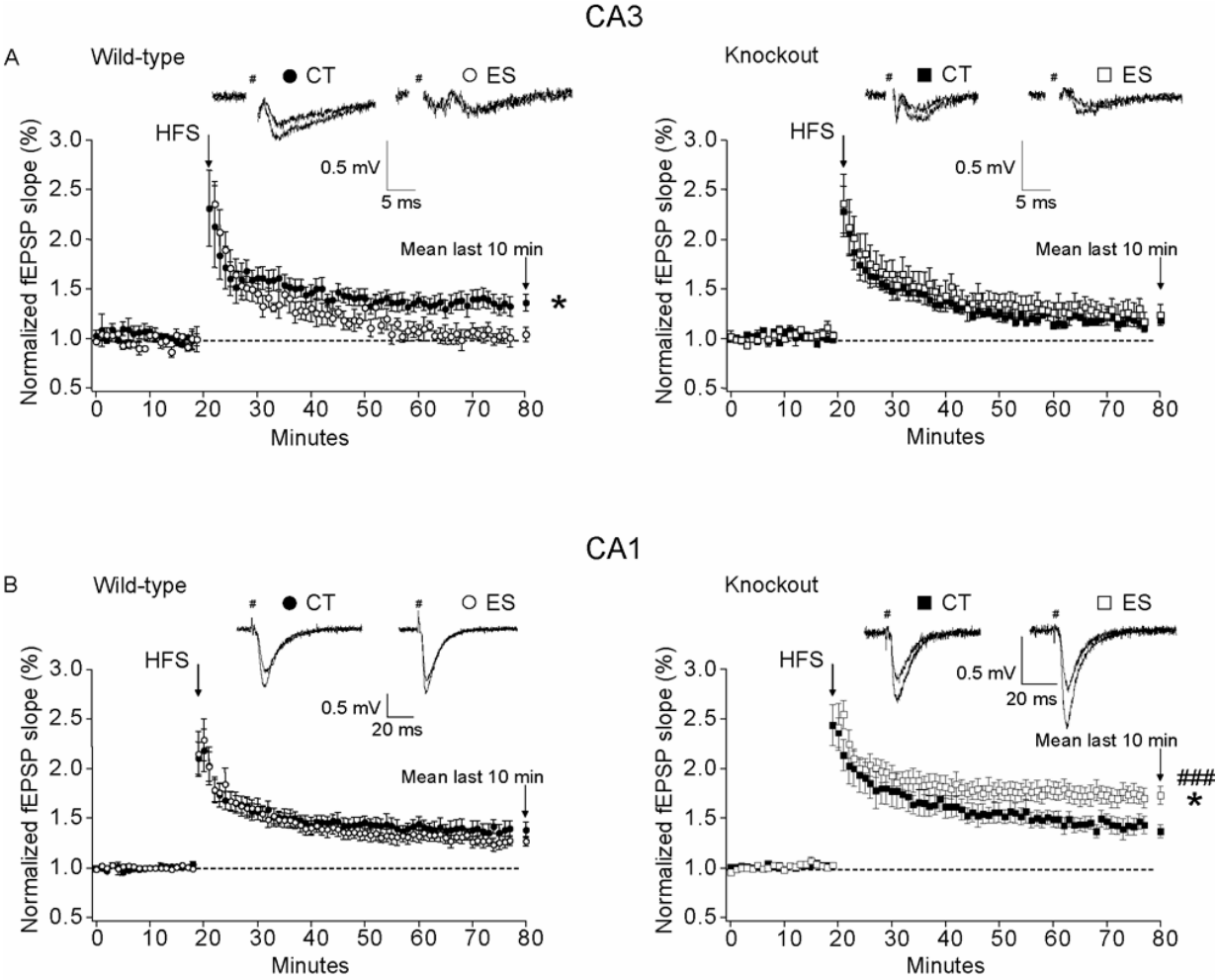


Figure 2. Effects of early-life stress on mossy fiber-CA3 LTP **(A)** and SCCP-CA1 LTP **(B)** in adult wild-type and CRHR1-CKO mice. Representative traces for control and LTP are shown. **(A)** After a HFS was delivered, mossy fiber-CA3 LTP was absent in stressed wild-type mice as indicated by significantly reduced amplitude of fEPSP in the last 10 minutes compared to wild-type controls. In contrast, CRHR1-CKO mice showed intermediate LTP. **(B)** In the CA1 region, no difference in fEPSP potentiation was noticed between control and stressed wild-type mice, whereas LTP was surprisingly enhanced in the slices of stressed CRHR1-CKO mice. *, $p < 0.05$ versus the control group. ###, $p < 0.001$ versus stressed wild-type group. n = 4-6 mice per group.

CA3 LTP was revealed. In the final 10 minutes, LTP was significantly impaired in stressed wild-type mice compared to the controls ($p < 0.05$, Bonferroni's test). LTP deficits seen in stressed wild-type mice were prevented by forebrain CRHR1 deficiency, as indicated by similarly prominent LTP in hippocampal CA3 neurons in both control and stressed CRHR1-CKO mice (Figure 2A). In the CA1 region, a significant interaction effect [$F(1, 30) = 8.457$, $p < 0.01$] and a main effect of genotype [$F(1, 30) = 12.850$, $p < 0.01$] on LTP were observed. Unlike findings in

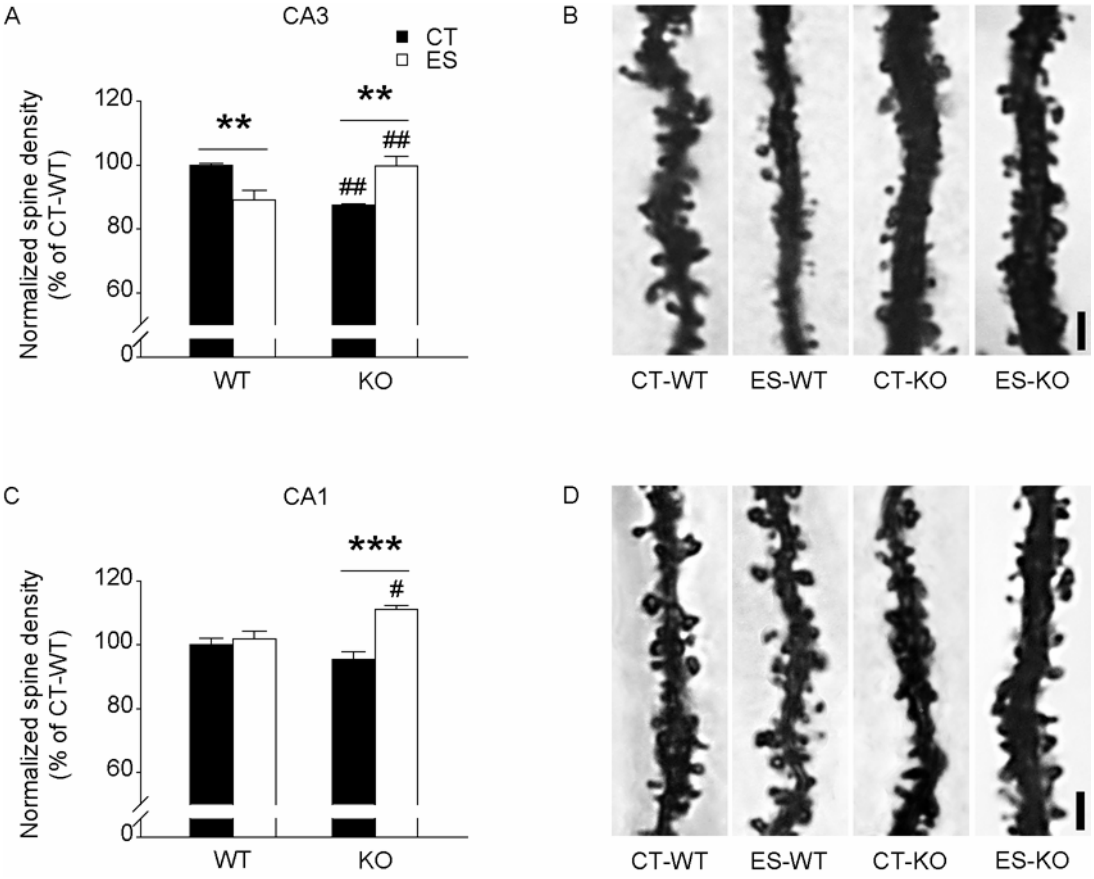


Figure 3. Effects of early-life stress on apical dendritic spine density in the stratum radiatum of CA3 and CA1 in adult wild-type and CRHR1-CKO mice. **(A)** In the CA3 region, spine density was reduced in stressed wild-type and control CRHR1-CKO mice, whereas stressed CRHR1-CKO mice had more spines than control and stressed counterparts. **(B)** Representative photomicrographs showing the apical dendrites and spines of Golgi-impregnated CA3 pyramidal neurons. **(C)** In the CA1 region, stressed CRHR1-CKO mice had more exuberant spines than the control CRHR1-CKO and stressed wild-type groups. **(D)** Representative photomicrographs showing the apical dendrites and spines of Golgi-impregnated CA1 pyramidal neurons. Scale bars = 2 μm. **, $p < 0.01$; ***, $p < 0.001$ versus the control group. #, $p < 0.05$; ##, $p < 0.01$ versus the group under the same condition. n = 4 mice per group.

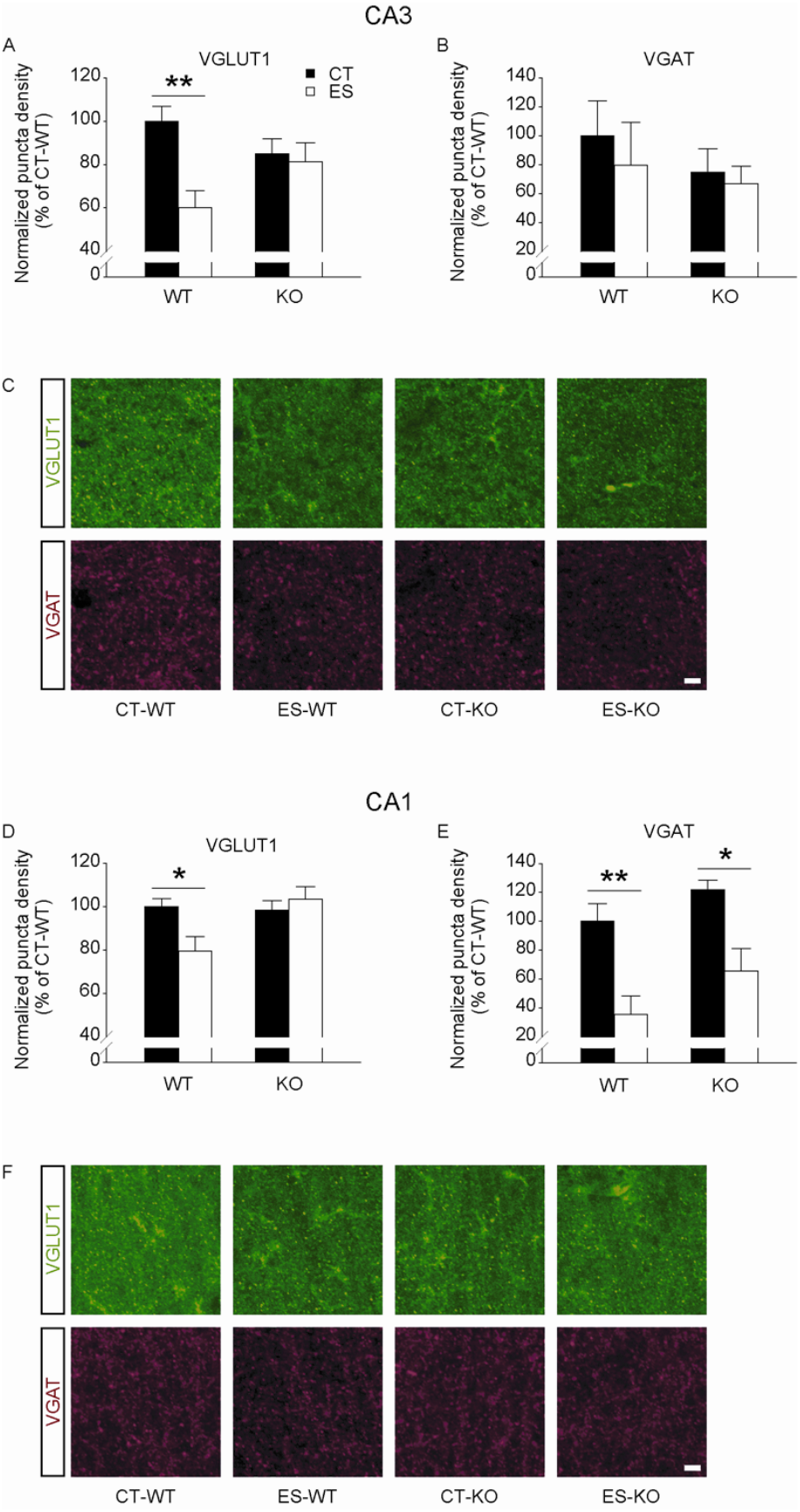
middle-aged rats (Brunson et al., 2005; Ivy et al., 2010), fEPSP potentiation was similar in control and stressed wild-type mice, whereas HFS-induced LTP was surprisingly augmented in stressed CRHR1-CKO mice compared to control and stressed counterparts ($p < 0.05$ and $p < 0.001$ respectively, Bonferroni's test; Figure 2B). These results point to the possibility that CRHR1 inactivation may overcompensate disrupted synaptic function in specific neuronal networks induced by early-life stress.

Forebrain CRHR1 deficiency attenuates structural alterations evoked by early-life stress

Stress-induced structural modifications such as dendritic atrophy (Brunson et al., 2005; Ivy et al., 2010) and loss of dendritic spines (Chen et al., 2008) are associated with impaired synaptic plasticity and memory. We therefore measured apical dendritic spine density in CA3 and CA1 pyramidal neurons in wild-type and CRHR1-CKO mice (Figure 3). In the stratum radiatum of area CA3, a significant effect of interaction [$F(1, 12) = 29.700$, $p < 0.001$] on spine density was revealed (Figure 3A and 3B). Stressed wild-type mice had fewer dendritic spines in CA3 stratum radiatum than the controls and stressed CRHR1-CKO mice (both $p < 0.01$, Bonferroni's test). The density of mature, Golgi-impregnated spines was lower in control CRHR1-CKO mice compared to control wild-type mice, and this was reversed by early-life stress (both $p < 0.01$, Bonferroni's test). In the stratum radiatum of area CA1, early-life stress did not influence spine density in wild-type mice. We found a significant effect of interaction [$F(1, 12) = 11.200$, $p < 0.01$] and a main effect of stress [$F(1, 12) = 17.920$, $p < 0.01$] on spine density (Figure 3C and 3D). Specifically, stressed CRHR1-CKO mice had higher spine density than control CRHR1-CKO and stressed wild-type mice ($p < 0.001$ and $p < 0.05$ respectively, Bonferroni's test). Notably, spine density in the middle molecular layer of DG and cell density of the hippocampus were similar among groups (data not shown).

Early-life stress interacts with forebrain CRHR1 to modulate excitatory and inhibitory synaptic networks in the hippocampus

To further investigate whether synaptic density in the apical dendritic region of hippocampal CA3 and CA1 is influenced by early-life stress and forebrain CRHR1 inactivation, VGLUT1 and VGAT were immunostained as markers for excitatory and inhibitory synaptic terminals, respectively (Figure 4). Two-way ANOVA revealed significant effects of interaction [$F(1, 10) = 5.593$, $p < 0.05$] and stress [$F(1, 10) = 8.091$, $p < 0.05$] on the number of VGLUT1-positive puncta in the stratum radiatum of CA3. As indicated by VGLUT1 immunostaining, there was no difference in excitatory synaptic density between control wild-type and control CRHR1-CKO mice. The number of excitatory synapses was significantly reduced in stressed wild-type ($p < 0.01$ versus wild-type controls, Bonferroni's test) but not stressed CRHR1-CKO mice (Figure 4A and 4C), while inhibitory synaptic density as shown by VGAT immunostaining in area CA3 remained unchanged among groups (Figure 4B). In the stratum radiatum of area CA1, a significant effect of


CA3
A
VGLUT1
CT
ES
**
Normalized puncta density (% of CT-WT)
120
100
80
60
40
0
WT
KO
B
VGAT
140
120
100
80
60
40
20
0
WT
KO
C
VGLUT1
VGAT
CT-WT
ES-WT
CT-KO
ES-KO
CA1
D
VGLUT1
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E
VGAT
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F
VGLUT1
VGAT
CT-WT
ES-WT
CT-KO
ES-KO

Figure 4. Effects of early-life stress on excitatory and inhibitory synaptic density in area CA3 and CA1 in adult wild-type and CRHR1-CKO mice. **(A)** The number of VGLUT1-positive synaptic puncta in CA3 stratum radiatum was significantly reduced in stressed wild-type but not stressed CRHR1-CKO mice. **(B)** VGAT puncta density in area CA3 remained unchanged among groups. **(C)** Representative confocal images taken from the stratum radiatum of CA3a (alongside stratum lucidum) showing VGLUT1- and VGAT-immunoreactive synaptic puncta. **(D)** The number of VGLUT1-positive puncta in CA1 stratum radiatum was also decreased in stressed wild-type but not stressed CRHR1-CKO mice. **(E)** VGAT puncta density in area CA1 was reduced by early-life stress in both wild-type and CRHR1-CKO mice. **(F)** Representative confocal images taken from the stratum radiatum of CA1b (alongside stratum pyramidale) showing VGLUT1- and VGAT-immunoreactive synaptic puncta. Scale bars = 5 µm. *, $p < 0.05$; **, $p < 0.01$ versus the control group. n = 3-4 mice per group.

interaction [$F(1, 10) = 5.427$, $p < 0.05$] on VGLUT1 puncta density and a significant main effect of stress [$F(1, 10) = 22.950$, $p < 0.001$] on VGAT puncta density were observed. The number of VGLUT1-positive puncta was reduced in stressed wild-type ($p < 0.05$ versus wild-type controls, Bonferroni's test) but not stressed CRHR1-CKO mice, whereas VGAT-immunoreactive puncta density was significantly decreased by early-life stress in both wild-type and CRHR1-CKO mice ($p < 0.01$ and $p < 0.05$ respectively, Bonferroni's test; Figure 4D-4F). Together with the electrophysiological and morphological data, these findings suggest that early-life stress interacts with forebrain CRHR1 to differentially regulate synaptic transmission and plasticity in CA3 and CA1.

Hippocampal neurexin-1 and neuroligin-3 are differentially altered by early-life stress and forebrain CRHR1

The trans-synaptic cell adhesion molecules neurexins and neuroligins specify synaptic function of excitatory and inhibitory networks, and are implicated in synaptic plasticity and cognitive function (Südhof, 2008). Therefore, we evaluated gene expression levels of hippocampal neurexins and neuroligins in wild-type and CRHR1-CKO mice (Figure 5). A significant main effect of stress [$F(1, 29) = 4.429$, $p < 0.05$] on CA3 neurexin-1 mRNA levels was revealed (Figure 5A and 5B). Compared to the controls, stressed wild-type but not CRHR1-CKO mice showed a significant decrease in neurexin-1 mRNA levels ($p < 0.05$, Bonferroni's test). Moreover, the mRNA levels of neuroligin-3 were reduced in CA1 by early-life stress [two way ANOVA of condition, $F(1, 27) = 14.360$, $p < 0.001$] in both wild-type and CRHR1-CKO mice (both $p < 0.05$,

Bonferroni's test; Figure 5C and 5D). Neuroligin-3 gene expression was also affected by stress in CA3 [two way ANOVA of condition, $F(1, 27) = 8.634$, $p < 0.01$] and DG [two way ANOVA of condition, $F(1, 27) = 12.420$, $p < 0.01$]. *Post hoc* test revealed that neuroligin-3 mRNA levels were significantly reduced in the DG of stressed wild-type mice ($p < 0.05$ versus wild-type controls, Bonferroni's test). In contrast, the gene expression levels of neurexin-1 in CA1 and DG and neuroligin-1 and neuroligin-2 in all hippocampal subregions remained unaltered among groups (data not shown).

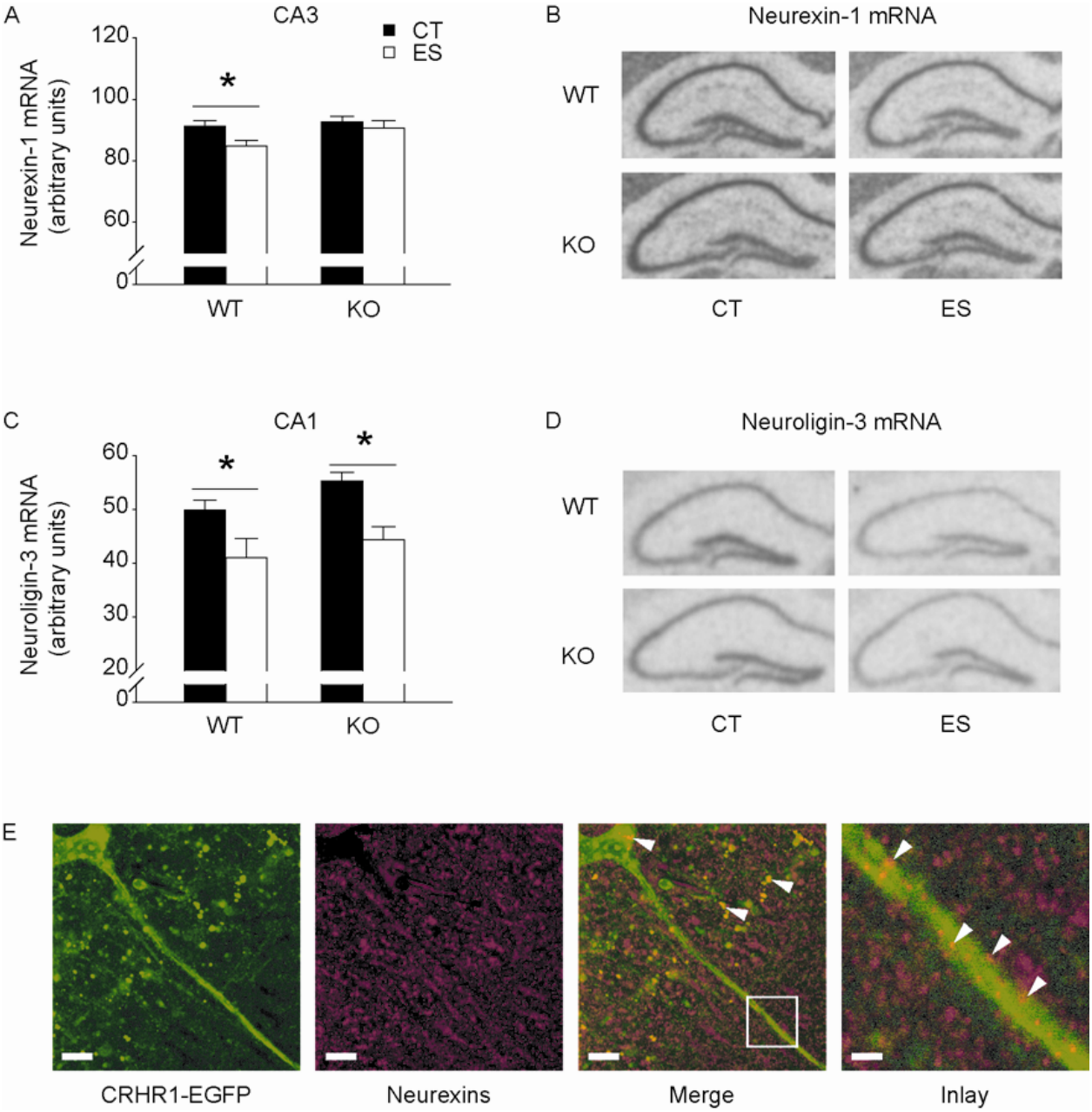

Figure 5. Effects of early-life stress on hippocampal neurexin-1 and neuroligin-3 gene expression in adult wild-type and CRHR1-CKO mice. **(A)** Neurexin-1 mRNA expression in area CA3 was reduced by early-life stress in wild-type but not CRHR1-CKO mice. **(B)** Representative *in situ* hybridization images showing neurexin-1 mRNA expression in the hippocampus. **(C)** Neuroligin-3 mRNA levels in area CA1 were reduced by early-life stress in both wild-type and CRHR1-CKO mice. **(D)** Representative *in situ* hybridization images showing neuroligin-3 mRNA expression in the hippocampus. **(E)** CRHR1 and neurexins partially colocalized in the perisomatic terminals and dendrites of CA1 pyramidal neurons. Arrows indicate CRHR1-EGFP and neurexins colocalized puncta. Scale bars: left and middle two panels, 10 μm; right panel, 2 μm. *, $p < 0.05$ versus the control group. n = 6-11 mice per group.

Additionally, we found that CRHR1 was in close proximity with neurexins in specific subcellular compartments of hippocampal pyramidal neurons (Figure 5 E). This partial colocalization was prominent in neuronal soma and dendrites, suggestive of potential functional interactions between CRHR1 and neurexins.

Discussion

In the experiments described here, we demonstrate that early-life stress impairs spatial learning and memory in adult mice, and is associated with physiological, morphological and molecular abnormalities in the hippocampus. Impairments of spatial learning and memory by early-life stress are recapitulated by forebrain CRH overexpression and attenuated by forebrain CRHR1 inactivation, suggesting that forebrain CRH-CRHR1 signaling is required for the programming of cognitive function by early-life stress.

Early-life experience and later-life cognition

Early experiences shape brain development and cognitive function (Korosi and Baram, 2009; Lupien et al., 2009). In rodents, postnatal exposure to an enriched environment or mild stress predicts improved adaptation and cognitive performance in adulthood (Fenoglio et al., 2005; Tang, 2001; Tang et al., 2006). Conversely, an impoverished postnatal environment, which disrupts maternal behavior and mother-pup interaction, impairs hippocampal integrity and cognition of adult offspring (Fenoglio et al., 2006).

Consistent with findings using the same stress paradigm (Rice et al., 2008), stressed wild-type mice exhibited impaired performance in hippocampus-dependent spatial tasks. Intriguingly, the cognitive impairments in adult stressed animals were abolished by forebrain CRHR1 inactivation, extending the findings using postnatal treatment of a selective CRHR1 antagonist (NBI-30775)

(Ivy et al., 2010). These data support the concept that the interactions between environmental risk factors and genetic predispositions are decisive in sculpting brain function and the expression of psychopathology (Charney and Manji, 2004; Sanchez et al., 2001; Schmidt, 2010).

Stress-induced late-onset cognitive deficits, forebrain CRH-CRHR1 signaling, and glucocorticoids

The involvement of CRH and CRHR1 in stress-induced cognitive decline has been investigated in previous studies. Transgenic CRH overexpression (Heinrichs et al., 1996) or postnatal CRH administration (Brunson et al., 2001) impaired spatial learning and memory in adult rodents, whereas postnatal administration of NBI-30775 prevented these effects (Ivy et al., 2010) and improved spatial performance in adult rats (Fenoglio et al., 2005). However, these approaches manipulated multiple brain regions, thus leave the neuroanatomical sites of action unclear. We showed that forebrain CRH-overexpressing mice exhibited impaired spatial performance, mirroring the cognitive phenotype of stressed wild-type mice, while stressed CRHR1-CKO mice performed similarly to the controls. These findings pinpoint the importance of forebrain CRHR1 in modulating cognitive function after postnatal stress exposure.

Notably, the deletion of the *Crhr1* gene in CRHR1-CKO mice occurred after the epoch of early-life stress. We imposed the stress on P2-P9 (Rice et al., 2008), whereas the calcium/calmodulin kinase IIα driven suppression of CRHR1 did not take place until the end of the second postnatal week (Wang et al., 2011). Thus, although the CRHR1-CKO mice were likely capable of CRH-CRHR1 signaling in the forebrain regions during the stress, its absence in the critical weeks after the stress protected them from structural and functional disturbances of the hippocampus. This indicates that following early-life stress is a window of opportunity where hippocampal plasticity is still present, and where intervention might rescue from the adverse effects of early-life stress (Ivy et al., 2010).

Glucocorticoids, acting via mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), are other key stress mediators affected by early-life stress (Joëls and Baram, 2009). Glucocorticoid excess during postnatal stress exposure, the resultant disruption of glucocorticoid feedback and the imbalance between hippocampal MR and GR have been postulated as a leading molecular basis of stress-induced cognitive deficits (Aisa et al., 2007; de Kloet et al., 1999; Joëls et al., 2006; Oitzl et al., 2010). However, neonatal dexamethasone (a synthetic glucocorticoid)

treatment failed to consistently impair cognition in adult rats (Kamphuis et al., 2003; Lin et al., 2006). Recent evidence indicates that the alterations of hippocampal GR after early-life experience may be secondary to changes of CRH-CRHR1 signaling in the paraventricular nucleus (Korosi et al., 2010; Korosi and Baram, 2009) and likely the hippocampus (Fenoglio et al., 2005), and may be paralleled with, instead of being causally related to, cognitive changes. In addition, basal corticosterone levels and stress response were unaltered by chronic stress in adult wild-type and CRHR1-CKO mice (Wang et al., 2011) or by forebrain CRH overexpression (Lu et al., 2008). Therefore, our data further support the hypothesis that abnormal hippocampal CRH-CRHR1 signaling largely accounts for the cognitive deficits in adult mice experienced early adversities.

Early-life stress-induced synaptic dysfunction and dendritic spine loss are attenuated by forebrain CRHR1 inactivation

LTP, an activity-dependent enhancement of synaptic efficacy assumed to underlie memory storage (Lynch, 2004), is disrupted in adult rodents by early-life stress in CA3 (Brunson et al., 2005), CA1 (Brunson et al., 2005; Champagne et al., 2008; Ivy et al., 2010) and DG (Bagot et al., 2009) in a CRHR1-dependent manner (Ivy et al., 2010). Whereas the commissural/associational LTP was examined in rats, we also found disturbed mossy fiber-CA3 LTP in stressed wild-type mice. In area CA1, LTP in SCCP-CA1 synapses was normal in stressed wild-type mice, whereas deficits were observed in stressed rats. This disparity may arise from species- and age- differences and the sensitivity of the procedure, as there was a reduction in the number of excitatory synapses in CA1 in both species (dendritic atrophy in stressed rats and reduced VGLUT1 immunoreactivity in stressed mice). Interestingly, forebrain CRHR1 inactivation not only abolished the effects of early-life adversity on CA3 LTP maintenance, but facilitated SCCP-CA1 LTP in stressed mice, indicating an important involvement of CRH-CRHR1 signaling in both neural circuitries.

Acute stress initiates the release of CRH (Chen et al., 2004b) that promotes rapid reduction in CA3 dendritic spine density (Chen et al., 2008), which is dependent on CRHR1 and correlates with cognitive defects and LTP attenuation (Chen et al., 2010); whereas recurrent exposure to high “stress levels” of CRH results in dendritic atrophy (Chen et al., 2004a). We found an overt reduction in CA3 spine density and the total number of excitatory synapses, coupled with attenuated CA3 LTP and spatial performance, in stressed wild-type but not stressed CRHR1-CKO mice. In area CA1, however, more subtle loss of excitatory synapses took place in stressed wild-

type mice. Because the relative number of spines on each dendrite remained unchanged in these animals, the loss of excitatory terminals in CA1 likely reflects the shrinkage of dendritic branches. Together with published data in rats (Brunson et al., 2005; Ivy et al., 2010), these results suggest that early-life stress may hamper the development of CA3 neurons, which in turn remodels structure and function in CA1.

We observed that forebrain CRHR1 inactivation *per se* reduced spine density in the apical dendrites of CA3 pyramidal neurons, and this defect was not apparent after early-life stress. Considering that cognition and synaptic plasticity in control CRHR1-CKO mice remained intact, mechanisms such as increased complexity of proximal dendrites in CA3 neurons (Chen et al., 2004a) likely compensate these morphological changes. It should be noted, however, that the predominant thin and filopodia-like spines previously observed in YFP-expressing CRHR1 mutant mice (Chen et al., 2008) may be poorly impregnated by the Golgi method employed here, resulting in a potential underestimation of the total number of spines in control CRHR1-CKO mice. Moreover, stressed CRHR1-CKO mice had more spines in CA1 neurons, which may account for enhanced SCCP-CA1 LTP.

In the hippocampus, excitatory synapses are found on dendritic spines whereas inhibitory synapses are primarily perisomatic. As CRHR1 resides on spines (Chen et al., 2004b), it is not surprising that the major contribution of CRHR1 deficiency on the effects of early-life stress involved more excitatory than inhibitory synapses. The modulation of excitatory and inhibitory network by the interaction between CRHR1 and early-life stress may be responsible for the observed functional alterations.

Potential link between CRH-CRHR1 and neurexin-neuroligin may modulate the effects of stress on synaptic plasticity, learning and memory

Excitatory and inhibitory synapses are modulated by the neurexin-neuroligin complex (Südhof, 2008). Neurexins are a family of synaptic cell adhesion molecules that primarily localize at presynaptic sites, while postsynaptic neuroligins abound in and act on excitatory and inhibitory synapses (Chubykin et al., 2007). Recently, the neurexin-neuroligin complex has been implicated in cognitive diseases (Jamain et al., 2003; Kim et al., 2008; Rujescu et al., 2009), and studies in mutant mice highlight their importance in synaptic transmission and cognition (Blundell et al., 2010; Dahlhaus et al., 2010; Etherton et al., 2009). In stressed wild-type mice, both neurexin-1

and neuroligin-3 mRNA levels were reduced, whereas only neuroligin-3 was decreased in stressed CRHR1-CKO mice. Hence, neurexins and neuroligins are potential molecular substrates that may partly mediate the effects of early-life stress on synaptic plasticity and cognition. Furthermore, the colocalization of CRHR1 with neurexins raises the possibility that the CRH-CRHR1 system interacts with the neurexin-neuroligin complex to modulate synaptic activity.

In conclusion, we provide multiple levels of evidence to show that forebrain CRH-CRHR1 signaling mediates, at least in part, the programming effects of early-life stress on cognition. Intriguingly, forebrain CRHR1 inactivation appears to enhance the function of specific neuronal networks after postnatal stress exposure, such as LTP and spine density in area CA1. Manipulation of forebrain CRHR1 may be a promising therapeutic strategy to abate the deleterious consequences of early-life stress on cognition and conceivably, to prevent or delay the onset of early-life stress-related psychiatric disorders.

Chapter 4

Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling

Neurobiology of disease: in press.

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Abstract

Chronic stress evokes profound structural and molecular changes in the hippocampus, which may underlie spatial memory deficits. Corticotropin-releasing hormone (CRH) and CRH receptor 1 (CRHR1) mediate some of the rapid effects of stress on dendritic spine morphology and modulate learning and memory, thus providing a potential molecular basis for impaired synaptic plasticity and spatial memory by repeated stress exposure. Using adult male mice with CRHR1 conditionally inactivated in the forebrain regions, we investigated the role of CRH-CRHR1 signaling in the effects of chronic social defeat stress on spatial memory, the dendritic morphology of hippocampal CA3 pyramidal neurons, and the hippocampal expression of nectin-3, a synaptic cell adhesion molecule important in synaptic remodeling. In chronically stressed wild-type mice, spatial memory was disrupted, and the complexity of apical dendrites of CA3 neurons reduced. In contrast, stressed mice with forebrain CRHR1 deficiency exhibited normal dendritic morphology of CA3 neurons and mild impairments in spatial memory. Additionally, we showed that the expression of nectin-3 in the CA3 area was regulated by chronic stress in a CRHR1-dependent fashion and associated with spatial memory and dendritic complexity. Moreover, forebrain CRHR1 deficiency prevented the down-regulation of hippocampal glucocorticoid receptor expression by chronic stress but induced increased body weight gain during persistent stress exposure. These findings underscore the important role of forebrain CRH-CRHR1 signaling in modulating chronic stress-induced cognitive, structural and molecular adaptations, with implications for stress-related psychiatric disorders.

Introduction

Chronic psychosocial stress in adulthood modulates brain structure and function, resulting in cognitive deficits and an increased risk for psychiatric disorders (de Kloet et al., 2005a; Lupien et al., 2009). The hippocampus, a limbic brain region critically involved in neuroendocrine regulation of stress hormones (Kim and Diamond, 2002) and spatial memory processing (Squire et al., 2007), is particularly vulnerable to uncontrollable stress (Chen et al., 2010; Joëls and Baram, 2009; Kim and Diamond, 2002). Various forms of chronic stress lead to reversible but long-lasting spatial memory impairments in adult male rodents (Bisaz et al., 2011; Conrad et al., 1996; Wright and Conrad, 2005). These effects are associated with atrophy of apical dendrites of CA3 pyramidal neurons (Kole et al., 2004; Magariños and McEwen, 1995; McLaughlin et al., 2007; Watanabe et al., 1992), suppression of hippocampal synaptic plasticity (Joëls et al., 2004; Kole et al., 2004; Pavlides et al., 2002), and altered expression of synaptic cell adhesion molecules in the hippocampus (Bisaz et al., 2011; Sandi, 2004).

The mechanisms of chronic stress-induced spatial memory impairments remain to be understood. Chronic exposure to elevated glucocorticoids has been shown to induce apical dendritic retraction of CA3 pyramidal neurons (Conrad et al., 2007; Woolley et al., 1990) and impair spatial memory (Coburn-Litvak et al., 2003), similar to the effects caused by chronic stress. Moreover, in some studies, inhibition of glucocorticoid elevations prevents spatial memory retrieval deficits due to CA3 damage (Roozendaal et al., 2001) and impairments of spatial recognition memory by chronic stress (Wright et al., 2006). It is therefore proposed that chronic stress reduces dendritic complexity of CA3 neurons, which in turn disrupts hypothalamic-pituitary-adrenal (HPA) axis function and elevates glucocorticoid levels, leading to spatial memory deficits (Conrad, 2006). However, discrepant findings have also been reported. Repeated exposure to high levels of glucocorticoids and stress produced CA3 dendritic atrophy, but failed to impair spatial memory function (Conrad et al., 2007) and even facilitated spatial memory in ovariectomized female rats (McLaughlin et al., 2005) and hippocampus-involved contextual fear conditioning (Conrad et al., 1999). Hence, factors other than elevated glucocorticoids and CA3 dendritic retraction must be responsible for chronic stress-induced spatial memory impairments.

Corticotropin-releasing hormone (CRH) and its receptors are not only key mediators of neuroendocrine and behavioral responses to stress, but are also involved in learning and memory (Arzt and Holsboer, 2006; Heinrichs and Koob, 2004; Joëls and Baram, 2009). Transient increase

in CRH facilitates hippocampus-dependent learning and memory (Lee et al., 1993; Radulovic et al., 1999; Row and Dohanich, 2008), whereas prolonged exposure to elevated CRH impairs spatial memory performance (Heinrichs et al., 1996). In the hippocampus, CRH is expressed in inhibitory interneurons (Chen et al., 2001) and interacts primarily with CRH receptor 1 (CRHR1), which is enriched in dendritic spines of pyramidal neurons (Chen et al., 2004b; Van Pett et al., 2000). Acute psychological stress induces release of hippocampal CRH (Chen et al., 2004b), which activates hippocampal neurons through CRHR1 (Chen et al., 2006; Refojo et al., 2005), and leads to rapid but reversible reduction in dendritic spines of CA3 neurons (Chen et al., 2008). Additionally, chronic exposure to CRH *in vitro* results in atrophy of dendrites of hippocampal neurons (Chen et al., 2004a). These findings point to the possibility that a sustained elevation of endogenous CRH during chronic stress contributes to dendritic remodeling.

Chronic stress paradigms in rodents typically involve intermittent restraint for 6 hours per day (Hains et al., 2009; Watanabe et al., 1992; Wright and Conrad, 2005), a monotonous procedure that may lead to habituation (Haile et al., 2001; Willner, 2005). Chronic social defeat provides a naturalistic and complex chronic stress in male mice that is relevant to human social interactions, and has a high validity for psychiatric disorders (Krishnan et al., 2007; Tsankova et al., 2006). However, the effects of such stress on dendritic arborization, specifically the role of CRH and CRHR1 in dendritic integrity and spatial memory during recurrent social defeat, have remained unclear.

To address these issues, we subjected conditional forebrain CRHR1-deficient mice and wild-type controls to chronic social defeat stress. The effects of chronic stress were evaluated, with a focus on cognitive function and its structural and molecular substrates. We hypothesized that chronic social defeat stress would impair spatial memory in a CRHR1-dependent manner, so that forebrain CRHR1 deficiency would protect from the memory impairments provoked by chronic stress.

Materials and methods

Animals

Mice with a homozygous mutation of the *Crhr1* gene, where exons 9-13 are flanked by two loxP sites, were generated as described previously (Müller et al., 2003). The *Crhr1*$^{loxP/loxP}$ mouse line was crossed with an effector mouse line that expresses Cre recombinase in a region- and cell

type-specific manner under the control of the calcium/calmodulin kinase IIα promoter. Cre recombinase catalyzes the site-specific recombination between the two loxP sites of the *Crhr1* gene and thus inactivates *Crhr1* in cells expressing Cre recombinase. In the resulting mouse line (CRHR1-CKO mice), the *Crhr1* gene is postnatally inactivated in forebrain neurons (Minichiello et al., 1999; Schmidt et al., 2006). The deletion of the *Crhr1* gene starts at the end of the second postnatal week and is completed before 8 weeks of age (personal communication by Dr. Deussing).

All animals were housed under a 12-hour light/dark cycle (lights on at 6 AM) and constant temperature (22 ± 1°C) with free access to both food and water. The experiments were carried out in accordance with European Communities Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Chronic social defeat paradigm

The chronic social defeat paradigm was carried out as described previously (Krishnan et al., 2007; Tsankova et al., 2006) with minor modifications. Adult male CD1 mice with a body weight over 40 g were used as aggressive residents. Adult male wild-type and CRHR1-CKO mice (2.5-4 months old) were single-housed 2 weeks before the start of the experiment, when the resident mice were housed in the defeat cages and their dominant behavior was trained in 3 sessions (5-10 min each) with young C57BL/6 males. CD1 males with attack latencies of more than 5 min in the last training session were not included in the experiment. The chronic social defeat stress procedure was carried out between 12 noon and 4 PM (6-10 hours after lights on). Over a total of 21 days, wild-type or CRHR1-CKO mice were introduced into the home cage of a different dominant CD1 mouse each day. All CD1 residents rapidly recognized and attacked the intruders within 2 min. To avoid serious injuries, the subordinate mouse was exposed to the CD1 aggressor for a maximum of 30 sec after being defeated. After the aggressive encounter, the mice were separated by a holed metal partition, allowing the animals to keep continuous sensory, but not physical contact for the next 24 hours. Control animals were single-housed. During the experiment, the controls were allowed to explore the defeat cages for 30 sec in the absence of dominant mice and then returned to their home cages.

Experimental design

The body weight of the first batch of adult male mice (3-4.5 months old; control wild-type, n = 13; control CRHR1-CKO, n = 12; stressed wild-type, n = 13; stressed CRHR1-CKO, n = 10) was monitored before and during the chronic stress procedure. Mice were tested in the object recognition task and the Y-maze task during the last week of the chronic stress paradigm. At 20 hours after the last aggressive encounter, mice were rapidly anesthetized by isoflurane and decapitated. Trunk blood was collected for plasma corticosterone determination by radioimmunoassay as described previously (Schmidt et al., 2007). Hippocampi were quickly dissected from the right brain halves and immediately frozen on dry ice for Western blot analysis. The left brain halves were snap-frozen and stored at -80°C for *in situ* hybridization. Adrenals were dissected and weighed.

To further assess the effects of chronic stress on spatial memory, a second batch of mice (control wild-type, n = 8; control CRHR1-CKO, n = 8; stressed wild-type, n = 7; stressed CRHR1-CKO, n = 7) were tested in the Y-maze task during the last week of the chronic stress paradigm. Peripheral blood was collected before and at 5 min, 30 min and 90 min after a 5-min restraint stress as described previously (Müller et al., 2003) on day 19 of the chronic social defeat paradigm to evaluate the endocrine response to acute stress. Plasma corticosterone levels were measured by radioimmunoassay (Schmidt et al., 2007). Mice were anesthetized and decapitated at 20 hours after the last aggressive encounter. The left brain halves were stored at -80°C until use, and the right halves were processed for Golgi staining (Gibb and Kolb, 1998). Another set of mice (control wild-type, n = 3; control CRHR1-CKO, n = 3; stressed wild-type, n = 3; stressed CRHR1-CKO, n = 2) were anesthetized with sodium pentobarbital (200 mg/kg of body weight, intraperitoneally) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were postfixed in the same fixative, cryoprotected and stored at -80°C for double-fluorescence immunohistochemistry.

Behavioral and cognitive testing

All tests were performed between 8:00 AM and 12:00 noon and manually scored using the ANY-maze software (ANY-maze 4.50, Stoelting, Wood Dale, IL, USA).

Object recognition

The one-trial object recognition test was performed as described previously (Dere et al., 2007; Sterlemann et al., 2010) in an open field apparatus (50 × 50 × 50 cm^3) under low illumination (30 lux). The floor of the apparatus was covered with sawdust bedding which was mixed between trials. During the first trial (acquisition phase; 10 min), the mice were presented a glass object placed in the center of the arena and allowed to explore the object freely. After a 30 min intertrial interval (ITI), the mice were presented the relocated familiar object alongside a novel one similar in size (retrieval phase; 5 min). The exploration of the objects was considered when the mice touched the objects with the nose, forepaws or vibrissae. Object preference was calculated as a ratio of time spent with the novel compared with the familiar object (Rice et al., 2008). The percentage of the time exploring and the number of contacts with the novel and the known objects was also calculated, with a higher preference for the novel object being rated as intact object recognition memory.

Y-maze

The Y-maze test was run using two batches of animals under the same conditions and results were pooled. The Y-maze apparatus was made of grey polyvinyl chloride with three symmetrical arms (30 × 10 × 15 cm^3) marked by triangle-, bar- and plus-signs, respectively, as intra-maze spatial cues, and was evenly illuminated (30 lux). During the first trial (acquisition phase; 10 min), the mice were allowed to explore two of the three arms with the third arm blocked. After a 30 min ITI, the mice were placed in the center of the Y-maze and allowed to explore all arms freely (retrieval phase; 5 min). An arm entry was counted when all four limbs of the mouse were within an arm. Discrimination index was expressed as the percentage of time exploring (novel - familiar)/(novel + familiar) (Burgin et al., 2010). The percentage of time spent in and number of visits to the novel arm and the two familiar arms was also calculated, with a higher preference for the novel arm being rated as intact spatial recognition memory.

Golgi impregnation and the analysis of dendritic branches

After impregnation in Golgi-Cox solution for 21 days at room temperature, the brain tissues were kept in 30% sucrose for 2-7 days at 4°C in the dark. Transverse hippocampal sections (200 μm) were collected and processed in 10% ammonium hydroxide (NH_4OH) for 30 min, followed by 30

min of fixation in Kodak Fix (Eastman Kodak, Rochester, NY, USA). Golgi-impregnated pyramidal neurons in the CA3 area were reconstructed "blindly" using camera lucida. Neurons for analysis were chosen using unbiased, systematic sampling (8-12 neurons per animal) (Chen et al., 2004a) and included equal representation of long- and short-shaft neuronal populations. Dendritic branching was evaluated using Sholl analysis (Sholl, 1953), measuring total dendritic length and the number of intersections at concentric circles of increasing distance from the soma.

In situ hybridization

Brain halves were sectioned coronally at 16 µm through the hippocampus (bregma -1.46 to -2.18) (Paxinos and Watson, 2001) at -20°C in a cryotome. The sections were thaw-mounted on superfrost slides, dried, and kept at -80°C. *In situ* hybridization using ^{35}S UTP-labeled ribonucleotide probes was performed as previously described (Schmidt et al., 2007). The following primers were used to generate an antisense RNA hybridization probe (485 base pairs) that recognizes a shared sequence of alpha-, beta-, and gamma-transcript variants of nectin-3: AGCCGTTACATTCCCACTTG (forward primer) and ATTGTCCATCCAACCTGCTC (reverse primer). The riboprobes for MR (750 base pairs) and GR (1250 base pairs) were generated according to Sterlemann et al. (Sterlemann et al., 2008). The slides were apposed to Kodak Biomax MR films (Eastman Kodak, Rochester, NY, USA) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion, Frederick, MD, USA).

Western blot

Western blot was performed as previously described (Sterlemann et al., 2010). Hippocampi were homogenized in ice-cold lysis buffer and centrifuged (12000 rpm, 30 min) at 4°C. Protein concentrations were determined using a detergent compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 40 µg of protein were resolved by 10% sodium dodecyl sulphate-polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). Membranes were labeled with rabbit anti-nectin-3 (1:2000, Abcam, Cambridge, UK) or goat anti-actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4°C. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:2000, DAKO, Glostrup, Denmark) or horse anti-goat (1:2000, Jackson

ImmunoResearch Laboratories, West Grove, PA, USA) secondary antibodies for 2 hours, bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) and quantified by densitometry (Quantity One 4.2, Bio-Rad). Because samples from the wild-type groups and the knockout groups were examined in two experiments, respective data were analyzed separately.

Double-fluorescence immunohistochemistry

Serial coronal sections were cut through the hippocampus (bregma -1.46 to -2.18) at 30 µm thickness and 210 µm intervals in a cryotome. Double-labeling immunofluorescence was performed on free-floating sections (n = 3 per mouse) as described previously (Chen et al., 2004b). In short, after incubation with mouse anti-microtubule-associated protein 2 (MAP2; 1:1000, Abcam) and rabbit anti-nectin-3 (1:500, Abcam) antibodies overnight at 4°C, sections were rinsed and labeled with Alexa Fluor 488- and 647-conjugated donkey secondary antibodies (1:500, Invitrogen) for 2 hours at room temperature. After rinsing, sections were transferred onto slides and coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector laboratories, Burlingame, CA, USA).

The fluorescent images (1600 × 1600 pixels) of area CA3 were obtained with an Olympus IX81 confocal microscope (Olympus, Tokyo, Japan) at 40× magnification using the Kalman filter and sequential scanning mode under identical settings for laser power, photomultiplier gain and offset. Images were imported into the NIH ImageJ software, converted to 8-bit grayscale and thresholded uniformly. MAP2 and nectin-3 colocalized puncta were revealed using the Colocalization Highlighter plugin of the ImageJ program. A square cursor (500 × 500 pixels) was placed on the stratum radiatum, stratum lucidum, stratum pyramidale, or stratum oriens of area CA3 to define the region of interest (ROI). The number of nectin-3-positive puncta and the number of MAP2 and nectin-3 colocalized puncta were determined using the Analyze Particle module of ImageJ within each ROI. Data were normalized by taking the value of the control wild-type group as 100%.

Statistical analysis

In the retrieval phase of the cognitive tasks, mice that failed to explore either of the objects (control wild-type, n = 3; stressed wild-type, n = 5; stressed CRHR1-CKO, n = 4) or any of the

three arms (control CRHR1-CKO, n = 1; stressed wild-type, n = 4; stressed CRHR1-CKO, n = 5) were excluded from analysis.

Data were analyzed by two-way analysis of variance (ANOVA) (condition × genotype) followed by Bonferroni *post hoc* test as necessary. Three-way ANOVA with condition and genotype as between-subjects factors and day as a within-subject factor was performed on body weight followed by *post hoc* testing when appropriate. Student *t* test was used to compare pairs of means. The level of statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

Results

Forebrain CRHR1 deficiency attenuates object recognition and spatial memory impairments induced by chronic social defeat stress

We evaluated the cognitive performance of the animals using the object recognition test, which involves medial temporal lobe structures including the hippocampus (Dere et al., 2007; Squire et al., 2007). A significant condition × genotype interaction effect [$F(1,32) = 4.253$, $p < 0.05$] on object preference was observed. Control wild-type and control and stressed CRHR1-CKO mice displayed intact memory as shown by significantly more exploration of the novel object than the familiar one in the retrieval phase [control wild-type: $t(9) = 4.623$, $p < 0.01$; control CRHR1-CKO: $t(11) = 4.302$, $p < 0.01$; stressed CRHR1-CKO: $t(5) = 3.624$, $p < 0.05$; paired *t* test], while stressed wild-type mice failed to discriminate between the novel and familiar objects [$t(7) = 0.626$, $p = 0.551$; Figure 1A]. Additionally, control mice and stressed CRHR1-CKO mice made more visits to the novel object than the familiar one [control wild-type: $t(9) = 3.248$, $p < 0.05$; control CRHR1-CKO: $t(11) = 4.556$, $p < 0.001$; stressed CRHR1-CKO: $t(5) = 3.428$, $p < 0.05$]. In contrast, stressed wild-type mice visited both objects similarly [$t(7) = 0.379$, $p = 0.716$; Figure 1B].

Hippocampus-dependent spatial recognition memory was characterized by the Y-maze test. In the retrieval phase, a significant main effect of stress [$F(1,63) = 4.025$, $p < 0.05$] on discrimination index was revealed. Control wild-type and control CRHR1-CKO mice significantly distinguished the novel arm from the known ones [control wild-type: $t(20) = 2.394$, $p < 0.05$; control CRHR1-CKO: $t(18) = 2.215$, $p < 0.05$], while both stressed groups spent similar time in the novel and known arms [stressed wild-type: $t(14) = -1.197$, $p = 0.251$; stressed

CRHR1-CKO: $t(11) = 1.341$, $p = 0.207$; Figure 1C]. However, control wild-type, control and defeated CRHR1-CKO mice visited the novel arm more frequently than the known arms as shown by percentage of arm entries [control wild-type: $t(20) = 4.252$, $p < 0.001$; control CRHR1-CKO: $t(18) = 5.074$, $p < 0.001$; stressed CRHR1-CKO: $t(11) = 2.672$, $p < 0.05$], whereas defeated wild-type mice entered the novel and familiar arms similarly [$t(15) = 0.660$, $p = 0.519$; Figure 1D]. Poor performance of stressed wild-type mice suggests impaired hippocampus-dependent learning and memory by chronic stress, and this impairment was attenuated by CRHR1 deficiency in forebrain regions.

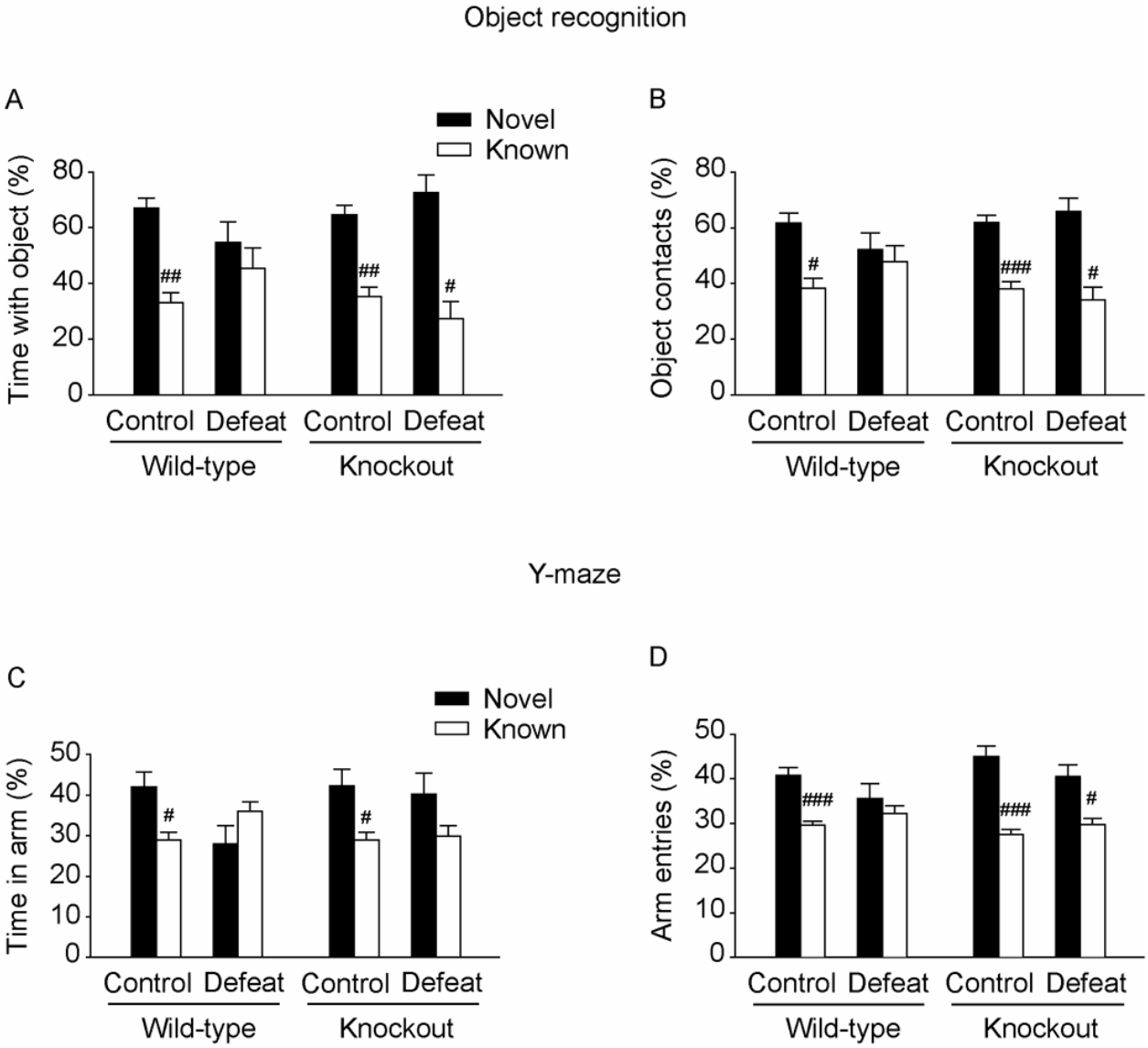

Figure 1. Object recognition and spatial recognition performance of wild-type and CRHR1-CKO mice on day 15 and day 16 of the chronic social defeat paradigm, respectively. **(A)** Stressed wild-type mice spent comparable time with the novel and known objects, suggesting impaired object recognition memory. **(B)** Stressed wild-type mice explored

the novel and known objects similarly. **(C)** Both stressed wild-type and CRHR1-CKO mice spent comparable time in the novel and known arms. **(D)** Stressed wild-type mice visited the novel and known arms similarly, indicating impaired spatial recognition memory. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ versus respective novel object or arm. Mice: object recognition test, n = 6-12 per group; Y-maze test, n = 12-21 per group.

Reduced apical dendritic complexity of hippocampal CA3 neurons by chronic stress in wild-type but not CRHR1-CKO mice

Apical dendrites of CA3 pyramidal neurons are highly vulnerable to stress-induced dendritic regression (McEwen, 1999). We therefore measured the dendritic length and the number of dendritic branching points from soma in both short- and long-shaft CA3 pyramidal neurons (Figure 2A-2D). A significant effect of stress [$F(1,18) = 6.793$, $p < 0.05$] on apical dendritic length of short-shaft neurons (data not shown) and a significant effect of stress [$F(1,18) = 4.445$, $p < 0.05$] and condition × genotype interaction [$F(1,18) = 4.931$, $p < 0.05$] on average dendritic length of both short- and long-shaft neurons (Figure 2B) were found. *Post hoc* analyses suggested that chronic stress induced atrophy of apical dendrites in short-shaft CA3 pyramidal neurons and all CA3 pyramidal neurons on average (both $p < 0.05$, Bonferroni's test) in wild-type but not CRHR1-CKO mice. Moreover, the number of branch intersections at 220-300 μm from the soma was significantly influenced by condition and/or condition × genotype interaction (Figure 2D). Compared to the controls, defeated wild-type mice had significantly fewer branching points at 240-300 μm from the soma. However, there was no difference between groups in dendritic length (data not shown) or the number of branch intersections (Figure 2C) of basal dendrites. These findings suggest that chronic stress reduces apical dendritic complexity of CA3 pyramidal neurons in a cell type-specific and dendritic segment-dependent manner, but could be abolished by hippocampal CRHR1 inactivation.

Dysregulated nectin-3 expression in hippocampal CA3 neurons in stressed wild-type but not CRHR1-CKO mice

Nectin-3 is highly enriched in hippocampal CA3 neurons and implicated in synaptic remodeling (Honda et al., 2006; Majima et al., 2009; Mizoguchi et al., 2002). Because the spatial distribution pattern of nectin-3 overlapped the sites of dendritic impoverishment observed here, we examined nectin-3 mRNA and protein expression in the hippocampus (Figure 3 and Figure 4).

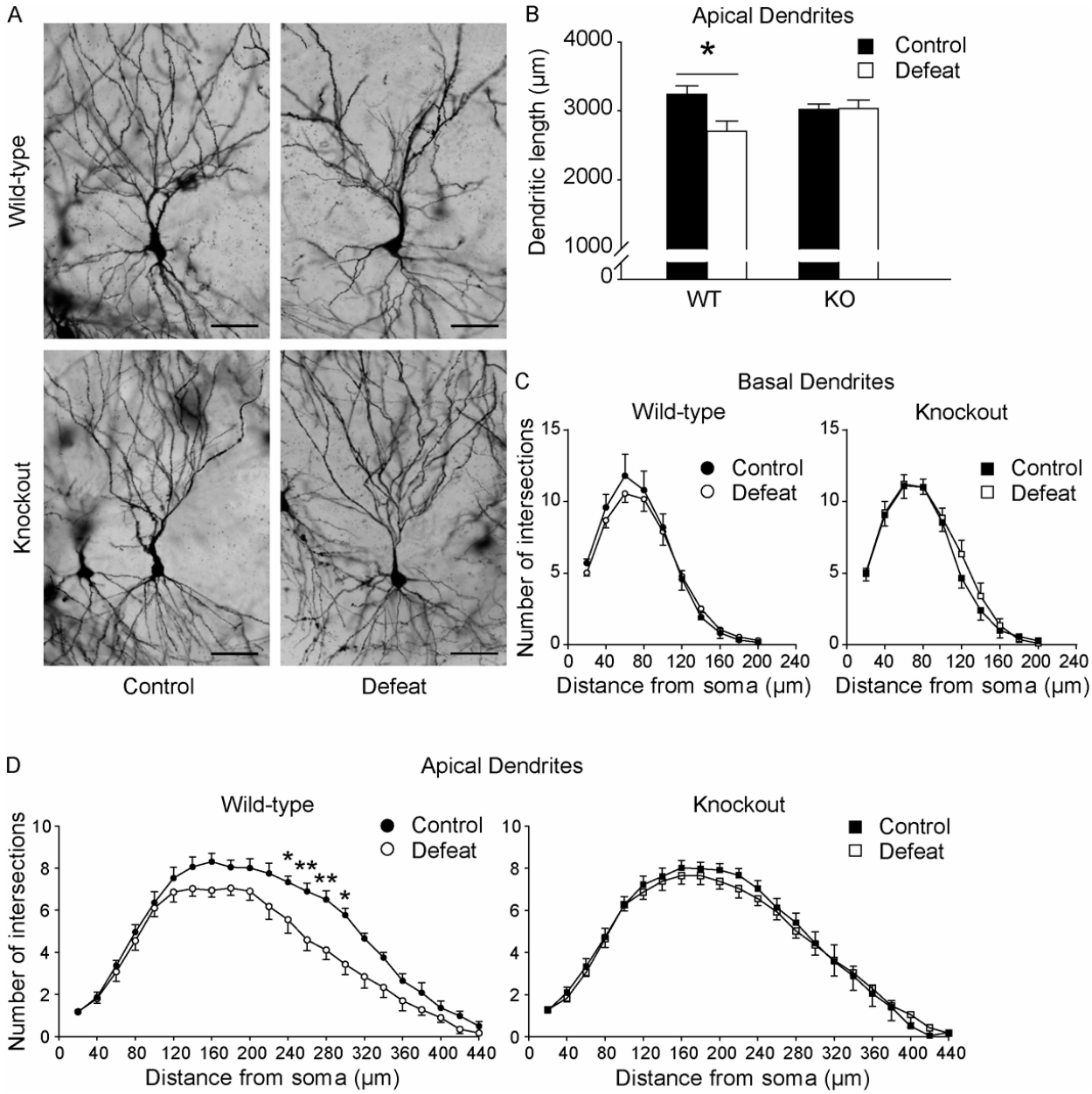

Figure 2. Effects of chronic social defeat stress on dendritic morphology of CA3 pyramidal neurons in wild-type (WT) and CRHR1-CKO (KO) mice. **(A)** Representative Golgi-impregnated CA3 pyramidal neurons illustrate the reduction in apical dendritic branching in neurons from stressed wild-type mice (upper right panel), and the normalization of dendritic arborization of neurons from stressed CRHR1-CKO mice (lower right panel). Scale bars = 70 µm. **(B)** Stressed wild-type mice showed reduced apical dendritic length in all CA3 pyramidal neurons on average compared to wild-type controls. **(C)** Basal dendritic complexity was not affected by chronic stress in wild-type and CRHR1-CKO mice. **(D)** Compared to the controls, stressed wild-type mice had significantly less apical dendrite branch points at 240-300 µm from the soma of CA3 pyramidal neurons. *, $p < 0.05$; **, $p < 0.01$ versus control wild-type group. Mice: control wild-type, n = 4; the other three groups, n = 6.

A significant effect of stress [$F(1,33) = 8.010$, $p < 0.01$] on nectin-3 mRNA expression was found (Figure 3A and 3B). Nectin-3 mRNA levels in stressed wild-type mice were significantly decreased in area CA3 ($p < 0.05$ versus wild-type controls, Bonferroni's test). The mRNA results were supported by reduced nectin-3 protein expression level in hippocampal lysates of stressed wild-type mice [$t(10) = 2.893$, $p < 0.05$, unpaired t test; Figure 3C and 3D]. Detailed analyses revealed that the number of nectin-3-positive puncta in area CA3 was significantly influenced by condition × genotype interaction [$F(1,7) = 13.370$, $p < 0.01$] in the stratum radiatum and by stress [$F(1,7) = 6.539$, $p < 0.05$] in the stratum pyramidale (Figure 4A). In stressed wild-type mice, decreased nectin-3 level was prominent in the stratum radiatum ($p < 0.05$, Bonferroni's test; Figure 4B). Moreover, an effect of stress [$F(1,7) = 12.800$, $p < 0.01$] was noticed on the number of MAP2 and nectin-3 colocalized puncta in the stratum radiatum, which was reduced in stressed wild-type mice ($p < 0.05$ versus the controls, Bonferroni's test; Figure 4C). No difference in either gene or protein expression of hippocampal nectin-3 between control and stressed CRHR1-CKO mice was found.

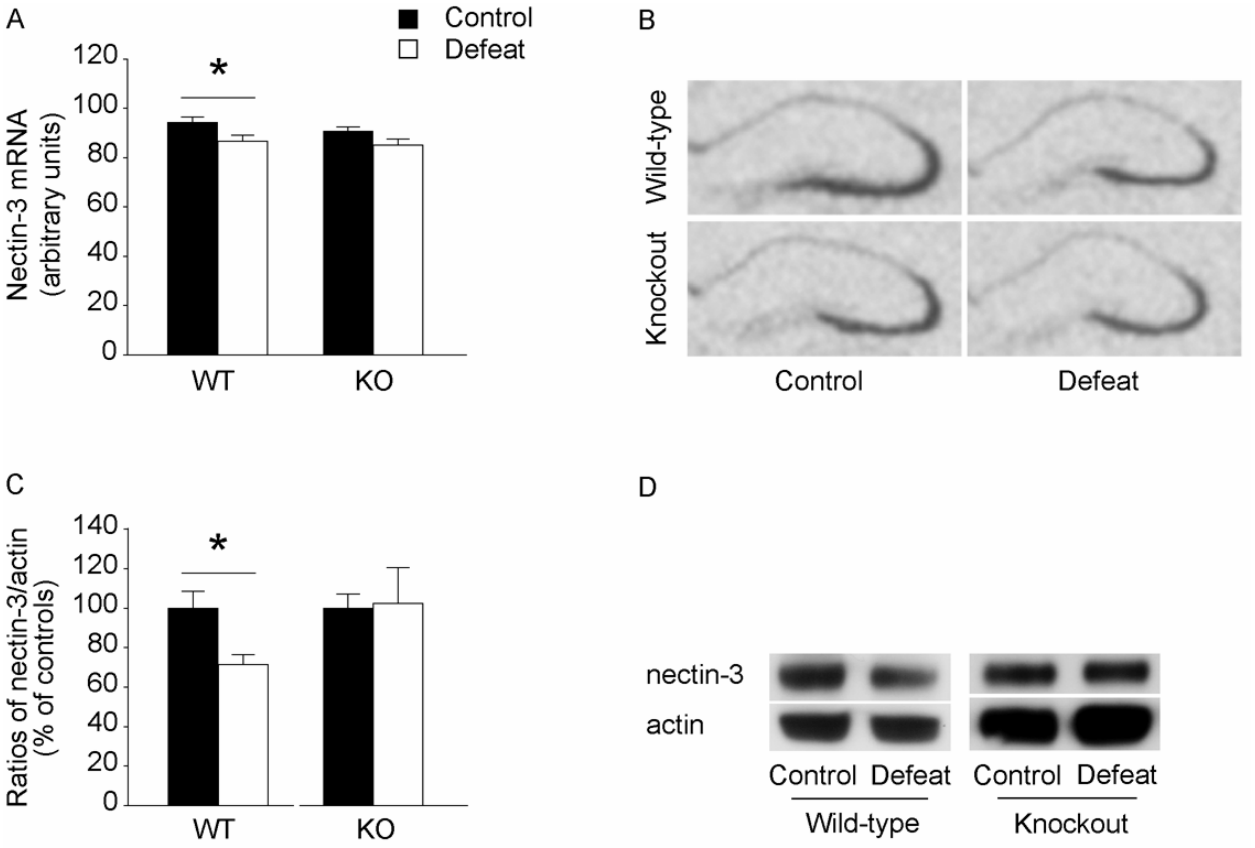

Figure 3. Chronic social defeat stress reduced hippocampal CA3 nectin-3 gene **(A)** and protein **(C)** expression in wild-type but not CRHR1-CKO mice. Representative *in situ* hybridization **(B)** and Western blot **(D)** images show nectin-3 mRNA and protein expression in the hippocampus, respectively. *, $p < 0.05$ versus control wild-type group. Mice: *in situ* hybridization, n = 8-11 per group; Western blot, n = 6 per group.

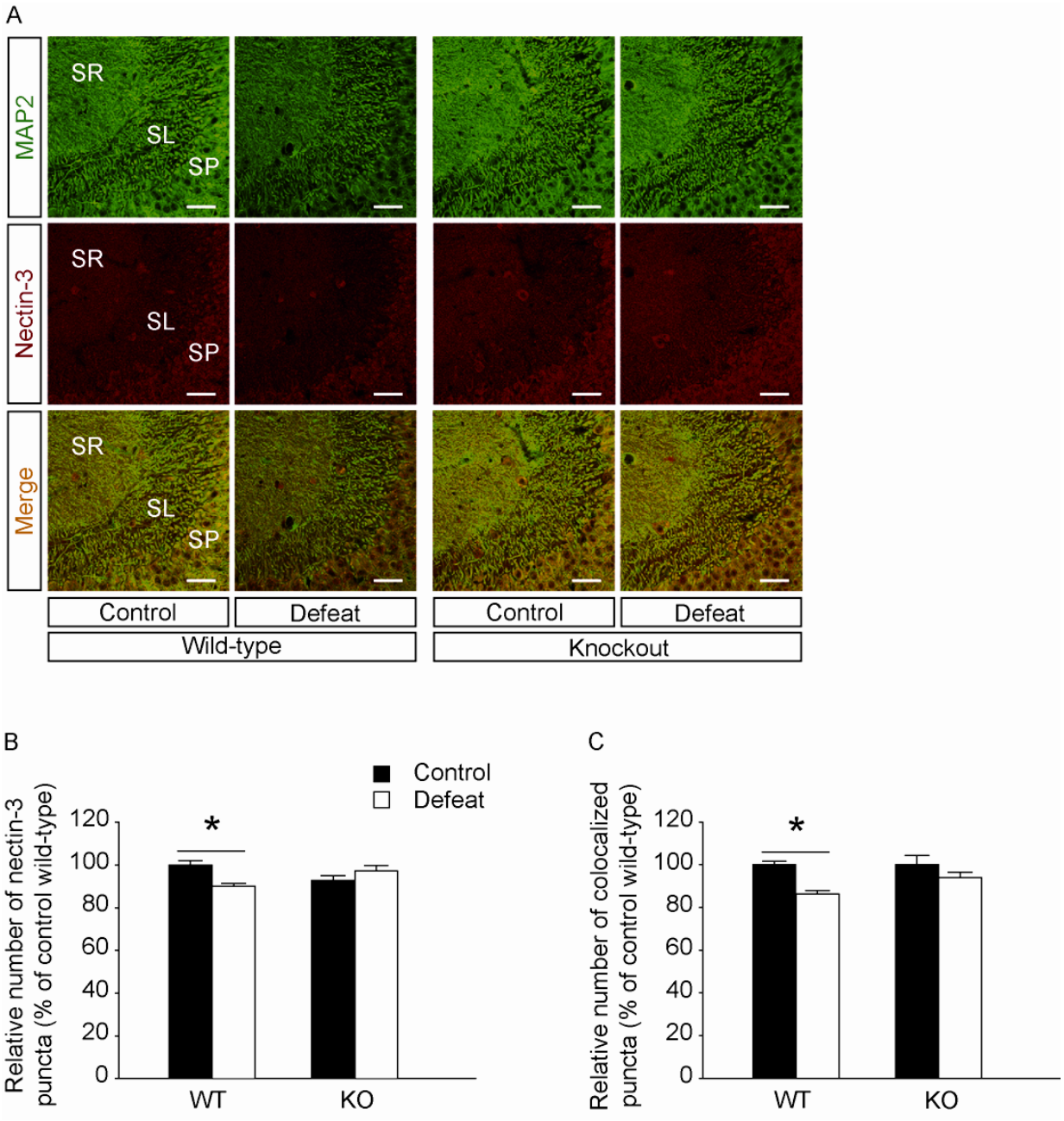

Figure 4. Effects of chronic social defeat stress on nectin-3 protein expression in area CA3 in wild-type and CRHR1-CKO mice. **(A)** Representative confocal images of area CA3 immunostained for MAP2 and nectin-3. SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale. Scale bars = 50 µm. Stressed wild-type mice had reduced number of nectin-3-positive puncta **(B)** and MAP2 and nectin-3 colocalized puncta **(C)** in the stratum radiatum of

area CA3. *, $p < 0.05$ versus control wild-type group. Mice: stressed CRHR1-CKO, n = 2; the other three groups, n = 3.

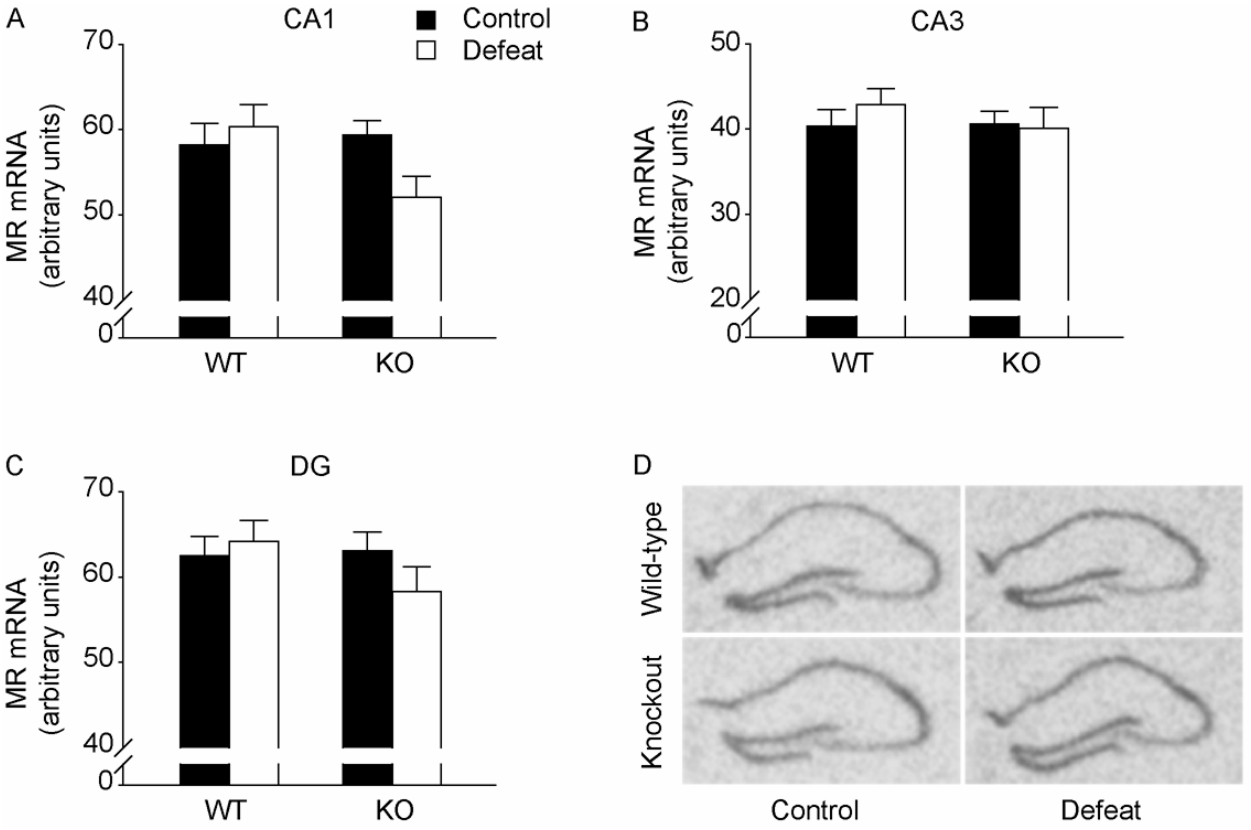

Figure 5. Effects of chronic social defeat stress on hippocampal MR gene expression in wild-type and CRHR1-CKO mice. MR mRNA levels in CA1 **(A)**, CA3 **(B)**, and dentate gyrus (DG) **(C)** were not altered by chronic stress in wild-type and CRHR1-CKO mice. **(D)** Representative *in situ* hybridization images showing MR mRNA expression in the hippocampus. Mice: n = 8-11 per group.

Altered hippocampal GR, but not MR, gene expression in stressed wild-type mice

To investigate whether the hippocampal dendritic loss that accompanied the cognitive deficits in stressed wild-type mice was related to genes involved in HPA axis regulation and neuroendocrine response to stress, mRNA levels of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the hippocampus were measured. MR gene expression in hippocampal CA1, CA3 and dentate gyrus regions was not altered by chronic stress in wild-type and CRHR1-CKO mice (Figure 5A-5D). A significant interaction effect [$F(1,33) = 4.380$, $p < 0.05$] on CA1 GR mRNA expression (Figure 6A) and a significant effect of stress [$F(1,33) = 7.314$, $p < 0.05$] on CA3 GR mRNA level (Figure 6B) were noticed. Similar to previous findings (Kitraki et al., 1999; Wright

et al., 2006), GR gene expression in CA1 and CA3 regions was significantly reduced by chronic stress in wild-type ($p < 0.05$ and $p < 0.01$ versus the controls, respectively, Bonferroni's test) but not in CRHR1-CKO mice. No difference in GR mRNA expression was observed in the dentate gyrus (Figure 6C and 6D).

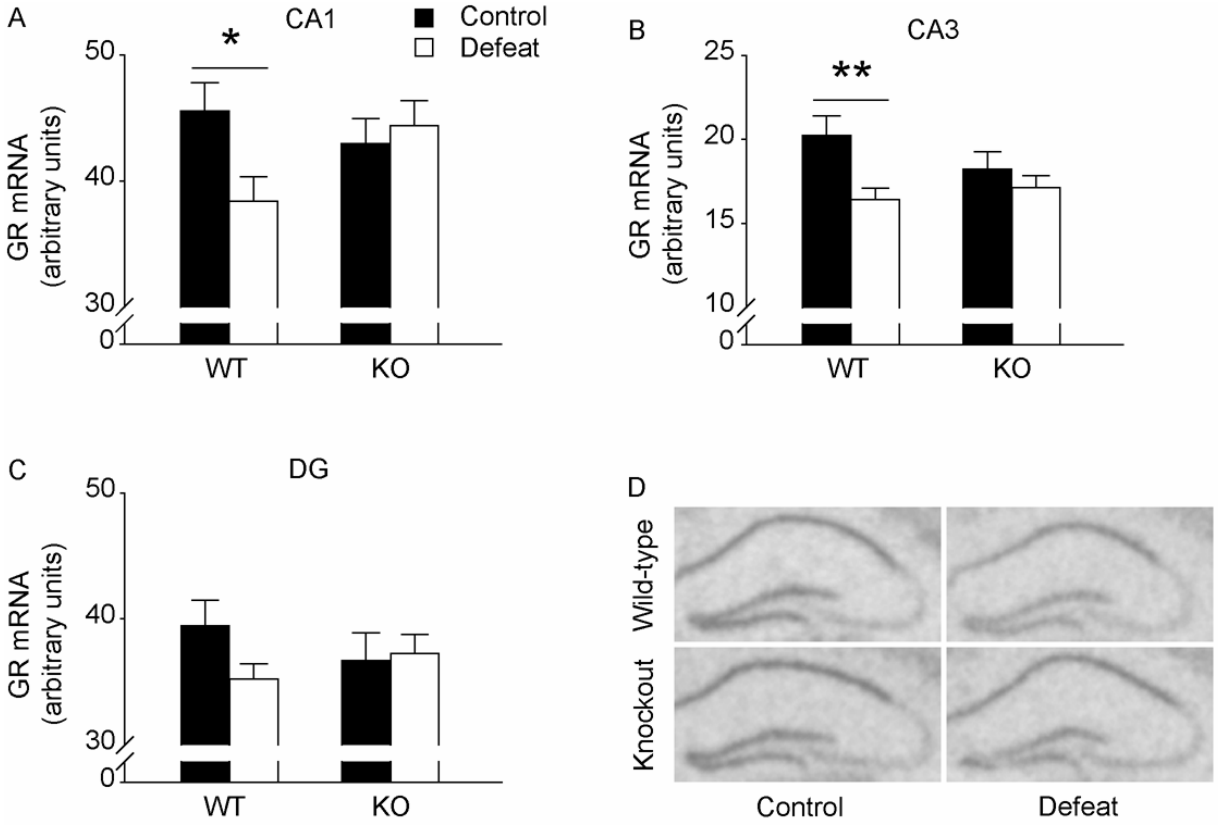

Figure 6. Effects of chronic social defeat stress on hippocampal GR gene expression in wild-type and CRHR1-CKO mice. GR mRNA signals were significantly decreased in CA1 **(A)** and CA3 **(B)**, but not DG **(C)**, in stressed wild-type mice versus wild-type controls. **(D)** Representative *in situ* hybridization images showing GR mRNA expression in the hippocampus. *, $p < 0.05$; **, $p < 0.01$ versus control wild-type group. Mice: n = 8-11 per group.

Metabolic and endocrine consequences of chronic stress

To assess the effects of chronic stress on metabolic function, body weight was monitored (Figure 7A). All groups had similar baseline body weight (data not shown). A main effect of stress [$F(1,44) = 10.746$, $p < 0.01$] on body weight change was detected. CRHR1-CKO mice undergoing the stress paradigm showed a significantly higher body weight gain compared to un stressed controls (week 1, $p < 0.05$; week 2, $p < 0.001$; week 3, $p < 0.001$; Bonferroni's test). Moreover, during the last two weeks of the stress paradigm, CRHR1-CKO mice gained more

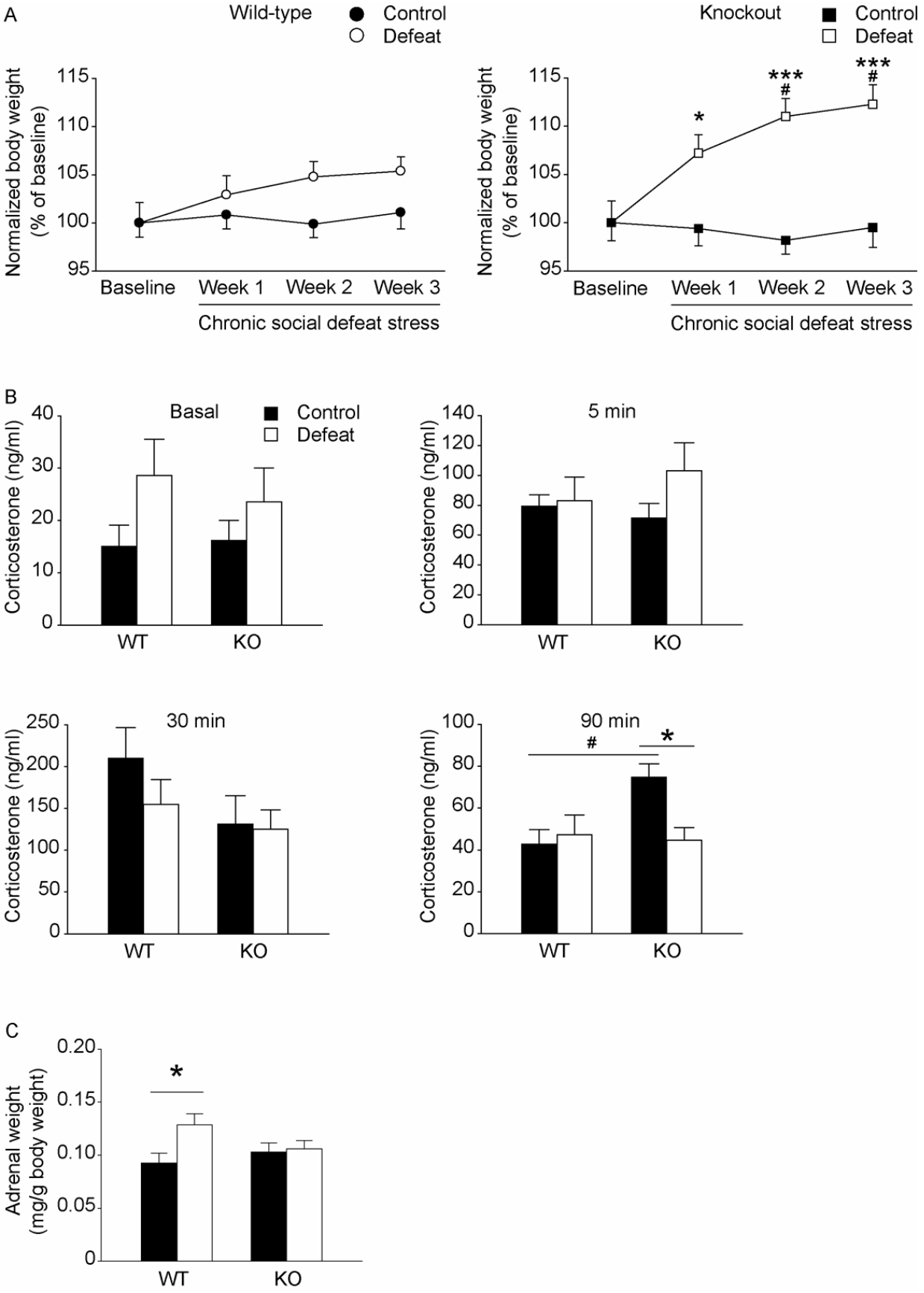
A
Wild-type
Control
Defeat
Normalized body weight (% of baseline)
115
110
105
100
95
Baseline
Week 1
Week 2
Week 3
Chronic social defeat stress
Knockout
Control
Defeat
*
***
#
***
#
B
Basal
Control
Defeat
Corticosterone (ng/ml)
40
30
20
10
0
WT
KO
5 min
140
120
100
80
60
40
20
0
30 min
250
200
150
100
50
0
90 min
#
*
C
Adrenal weight (mg/g body weight)
0.20
0.15
0.10
0.05
0

Figure 7. Effects of chronic social defeat stress on body weight, stress response and adrenal weight in wild-type and CRHR1-CKO mice. **(A)** Body weight at 1 day before the chronic stress procedure was set as baseline. Mice were again weighed on days 7, 14 and 21 of stress. Stressed CRHR1-CKO mice showed significantly increased body weight gain during the chronic stress paradigm compared to control CRHR1-CKO and stressed wild-type mice (n = 10-13 per group). **(B)** Plasma corticosterone levels at basal conditions and 5 min and 30 min after restraint stress were comparable between groups during the chronic stress procedure. Control CRHR1-CKO mice showed higher corticosterone levels than control wild-type and stressed CRHR1-CKO mice at 90 min after the acute stress (n = 4-7 per group). **(C)** Stressed wild-type mice had significantly enlarged adrenals compared to wild-type controls (n = 8-11 per group). *, $p < 0.05$; ***, $p < 0.001$ versus respective control group. #, $p < 0.05$ versus wild-type group under the same condition.

weight than wild-type mice under stressful situations ($p < 0.05$ for both weeks, Bonferroni's test). There was no significant effect of genotype or interaction on body weight.

Basal plasma corticosterone levels were comparable between groups during (Figure 7B) and immediately after (data not shown) chronic stress exposure. No difference in corticosterone levels was observed between groups at 5 min and 30 min after the restraint stress challenge. However, at 90 min after the acute stress, an interaction [$F(1,19) = 5.282$, $p < 0.05$] effect on corticosterone concentrations was found, and control CRHR1-CKO mice showed higher corticosterone levels than control wild-type and stressed CRHR1-CKO mice (both $p < 0.05$, Bonferroni's test). In addition, a significant main effect of stress [$F(1,34) = 4.248$, $p < 0.05$] on adrenal weight (Figure 7C) was noticed. *Post hoc* analysis showed that chronic stress increased adrenal weight in wild-type animals ($p < 0.05$ versus the controls, Bonferroni's test), while stressed and stress-naïve CRHR1-CKO mice had comparable adrenal weight. No other significant main or interaction effects on adrenal weight were detected.

Discussion

Chronic stress induces spatial memory impairments and dendritic remodeling. In the current study, we demonstrate that forebrain CRHR1 deficiency attenuates spatial memory deficits and prevents the dendritic regression of CA3 neurons and the loss of hippocampal nectin-3 expression induced by chronic social defeat stress. Moreover, the metabolic and neuroendocrine effects of chronic social defeat stress are also dependent on CRHR1 in the forebrain region. Together, our findings suggest that the forebrain CRH-CRHR1 system plays critical roles in the modulation of memory function under chronic stress.

Transient elevations of CRH in the hippocampus facilitate long-term potentiation under acute stress conditions (Blank et al., 2002), and enhance hippocampus-dependent learning and memory (Lee et al., 1993; Radulovic et al., 1999; Row and Dohanich, 2008). However, long-term consequences of hippocampal CRH and CRHR1 alterations and their involvement in chronic stress-induced cognitive deficits remain to be understood. Previous studies reported that both central CRH overexpressing (Heinrichs et al., 1996) and CRHR1 knockout mice (Contarino et al., 1999) showed impaired spatial memory, but the interpretation of the cognitive phenotype was limited by prominent physical and neuroendocrine dysfunctions in these mice. By using a mouse line in which CRHR1 is postnatally inactivated in forebrain neurons and basal HPA activity remains intact (Müller et al., 2003), we were able to dissect the involvement of hippocampal CRHR1 in chronic stress-modulated cognition. In the object recognition test, stressed wild-type mice, but not stressed CRHR1-CKO mice, exhibited impaired memory performance, indicating that forebrain CRHR1 activation may mediate chronic stress-induced cognitive dysfunction. Though the role of the hippocampus in this test remains controversial (Dere et al., 2007), the involvement of dorsal hippocampus has been recently demonstrated (Broadbent et al., 2010). Additionally, in the context of stress, memory defects in this test strongly correlated with spine loss in apical dendrites of CA3 neurons, which was dependent on CRH-CRHR1 signaling (Chen et al., 2010). In the Y-maze task that specifically requires hippocampal network function, stressed wild-type mice exhibited profound spatial memory deficits whereas stressed CRHR1-CKO mice displayed only mild cognitive impairments. Notably, cognition of unstressed CRHR1-CKO mice was intact in the object recognition and Y-maze tests, suggesting that lack of hippocampal CRHR1 signaling does not interfere with cognitive function assessed by these tests under basal conditions, but could ameliorate the deleterious effects of chronic stress on spatial memory. Furthermore, based on our results and previous reports (Blank et al., 2002; Chen et al., 2010; Heinrichs et al., 1996; Hogan et al., 2005; Row and Dohanich, 2008), hippocampal CRH may modulate spatial memory through CRHR1 in a biphasic pattern: short-term exposure to moderate levels of CRH facilitates, whereas prolonged elevations of CRH negatively influence, the spatial memory performance.

Neuronal structural changes are thought to underlie synaptic plasticity and memory. Chronic exposure to stress reversibly alters the dendritic length and complexity of CA3 pyramidal neurons (Kole et al., 2004; Magariños and McEwen, 1995; McLaughlin et al., 2007; Watanabe et al.,

1992), which is correlated with spatial memory deficits due to chronic stress (Conrad et al., 1996; Wright and Conrad, 2005). Repeated administration of glucocorticoids mimics the effects of chronic stress on dendritic morphology (Conrad et al., 2007; Woolley et al., 1990), but fails to impair spatial memory consistently (Coburn-Litvak et al., 2003; Conrad et al., 2007). Recently, CRH has been shown to regulate stress-induced spine loss and dendritic remodeling of CA3 pyramidal neurons via CRHR1 (Chen et al., 2008). Therefore, CRH and CRHR1 are promising molecular substrates for chronic stress-induced structural and cognitive changes. Consistent with previous findings (Kole et al., 2004), chronic social defeat stress induced atrophy of apical dendrites in a subpopulation of CA3 neurons in a segment-dependent manner. The loss of dendritic branches and their respective spines provides a plausible basis for some of the cognitive defects found in stressed wild-type mice. In contrast, forebrain CRHR1 deficiency prevented the effects of chronic stress on CA3 dendritic length and complexity in CRHR1-CKO mice. These results suggest that CRH-CRHR1 signaling plays a crucial role in chronic stress-evoked structural plasticity.

Mechanisms by which chronic stress promotes dendritic retraction are unclear. One possibility is via the dying-back of dendrites after loss of their synapses and spines. Synaptic cell adhesion molecules play essential roles in the growth, maintenance and retraction of synapse-carrying spines (Dalva et al., 2007), and have been implicated in chronic stress-induced structural changes and cognitive deficits (Bisaz et al., 2011; Sandi, 2004). Nectin-3, an immunoglobulin-like cell adhesion molecule highly enriched in CA3 neurons, primarily localizes at *puncta adherentia* junctions (PAJs) and participates in synaptogenesis and synaptic remodeling (Majima et al., 2009; Mizoguchi et al., 2002). Genetic deletion of nectin-3 destabilizes PAJs at the mossy fiber-CA3 synapses and leads to aberrant mossy fiber sprouting in the hippocampus, indicating its involvement in axodendritic adhesion and structural plasticity (Honda et al., 2006). Our results showed that the expression of hippocampal nectin-3, especially in the CA3 stratum radiatum where dendritic arborization was reduced by chronic stress, was down-regulated in stressed wild-type but not stressed CRHR1-CKO mice. These findings are inconclusive about the behavioral significance of nectin-3 and the casual relationship between nectin-3 dysregulation and dendritic shrinkage. However, we provide evidence that hippocampal nectin-3 expression is regulated by chronic stress and modulated by CRH-CRHR1 signaling, which is associated with dendritic remodeling and cognitive function. Additionally, based on its specific spatial distribution pattern,

nectin-3 is a potential molecular marker for compromised CA3 neurons in response to chronic stress.

Glucocorticoids bind to MR and GR, both abundantly expressed in the hippocampal neurons in rodents (Herman et al., 1989; van Steensel et al., 1996). Hippocampal MR and GR participate in the negative feedback inhibition of the HPA axis, and modulate learning and memory in a coordinated manner (de Kloet et al., 2005a). Studies using several transgenic mouse lines have assessed the effects of long-term alterations of forebrain MR or GR on cognitive performance. Forebrain MR overexpression enhances spatial memory retention (Lai et al., 2007), whereas forebrain MR deficiency (Berger et al., 2006) or GR overexpression (Wei et al., 2007) leads to cognitive deficits. Interestingly, considerable evidence shows that GR and/or MR are reduced in the hippocampus under chronic stress and this down-regulation could last for days (Kitraki et al., 1999; Wright et al., 2006). However, though the imbalance in MR- and GR-mediated actions is hypothesized to contribute to the memory impairments by chronic stress (de Kloet et al., 1999), our findings that hippocampal GR, but not MR, is down-regulated in stressed wild-type mice could be interpreted as being adaptive to protect hippocampal neurons from neurotoxic or metabolic challenges (Conrad, 2008) rather than being a substrate of cognitive impairments. It should be noted that we only examined hippocampal GR mRNA levels using *in situ* hybridization. As shown by previous studies (Nishi et al., 2007; Usuku et al., 2005), GR immunoreactivity is very weak in mouse CA3 region. Therefore, it may be difficult to detect any subtle alterations of CA3 GR protein expression induced by stress. Furthermore, corticosterone levels did not differ between control and stressed wild-type mice basally or in response to stress. Hence, it is unlikely that dysregulated hippocampal GR and the potential alterations of glucocorticoids account for the behavioral, structural and molecular changes observed in stressed wild-type mice.

Chronic social defeat stress resulted in a mild HPA axis hyperactivity in stressed wild-type mice, as indicated by increased adrenal weights and, to a lesser extent, reduced hippocampal GR mRNA expression. In contrast, stressed CRHR1-CKO mice had similar adrenal weights and hippocampal GR expression levels compared to the controls. Interestingly, the blunted HPA axis feedback to acute stress challenge in CRHR1-CKO mice, as indicated by elevated plasma corticosterone levels at 90 min after restraint stress, was restored under recurrent stress exposure. These findings highlight the importance of hippocampal CRH-CRHR1 function and its interaction with chronic stress in regulating HPA axis activity.

Under chronic psychosocial stress situations, subordinate male mice show increased food intake and gain more weight, leading to a positive energy balance and increased vulnerability to hyperphagia-associated obesity, whereas individually housed mice show reduced body weight gain (Bartolomucci et al., 2009; Moles et al., 2006). In line with these findings, our results further suggest that forebrain CRHR1 inactivation increases body weight gain in chronically stressed mice, which may not be surprising since activation of central CRH receptors suppresses food intake (Bell et al., 1998; Grill et al., 2000), and the *Crhr1* gene has been associated with obesity in humans (Challis et al., 2004).

In summary, the results demonstrate that forebrain CRHR1 inactivation ameliorates spatial memory deficits and prevents abnormal structural and molecular alterations in adult hippocampus that are induced by chronic stress. Though the involvement of CRHR1 in other forebrain regions in aforementioned changes needs to be delineated, hippocampal CRH-CRHR1 signaling may mediate the effects of chronic stress on cognition. These data highlight forebrain CRHR1 as an exciting molecular target for attenuating the cognitive effects of stress. However, our findings suggest that such inactivation may also increase the risk for metabolic disorders such as obesity.

Chapter 5

Nectin-3 mediates stress-induced cognitive decline and structural remodeling

Manuscript in preparation

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Abstract

Chronic stress exposure leads to impaired hippocampus-dependent cognition and structural rearrangements via corticotropin-releasing hormone (CRH) and CRH receptor 1 (CRHR1), and reduces hippocampal nectin-3 levels. However, it remains to be determined whether nectin-3 is causally related to cognitive deficits and structural changes induced by stress. In the current study, we found that the mRNA levels of nectin-3, which colocalized with CRHR1 in forebrain neurons, were also decreased by early-life stress and forebrain CRH overexpression in the adult hippocampus. Similar to findings in recurrently defeated adult mice, necin-3 levels were normalized by forebrain CRHR1 inactivation in adult mice experienced early-life adversities. Site-specific suppression of nectin-3 in the hippocampus disrupted spatial learning and long-term, but not short-term, memory in adult mice. Impaired cognitive performance in hippocampal nectin-3 knockdown mice was accompanied by the shrinkage of proximal dendritic trees and a loss of mushroom like dendritic spines in dentate granule cells. In addition, hippocampal l-afadin protein levels were also reduced in nectin-3 knockdown mice. Our findings provide evidence that nectin-3 is required to modulate the effects of stress on cognition and structural plasticity, and that the nectin-afadin complex may interact with the CRH-CRHR1 system to exert such effects.

Introduction

Synapses are specialized and asymmetric intercellular junctions that meditate the transmission of information between neurons. Glutamatergic excitatory synapses are established by presynaptic axonal terminals and postsynaptic dendritic spines, both of which anchor synaptic cell adhesion molecules (CAMs) (Giagtzoglou et al., 2009; Shapiro et al., 2007; Sheng and Hoogenraad, 2007; Waites et al., 2005). CAMs are not merely static constituents of synapses, but dynamic modulators of synaptic activity and plasticity. In the developing brain, synaptic CAMs are involved in neurite growth, synaptogenesis and synapse maturation; in the adult brain, CAMs interact with various synaptic proteins and receptors to shape synaptic function (Dalva et al., 2007; Parrish et al., 2007). Disruption of synaptic adhesion may lead to functional abnormalities, and an emerging body of evidence indicates that dysregulated synaptic CAMs contribute to structural modifications and cognitive deficits (Lin and Koleske, 2010; Südhof, 2008), including those induced by chronic stress (Bisaz et al., 2011; Borcel et al., 2008; Sandi, 2004).

Nectin-3 is an immunoglobulin-like cell adhesion molecule, which in adulthood primarily localizes at adherens junctions (also named *puncta adherentia* junctions), the sites adjacent to the presynaptic active zone and postsynaptic density (PSD) (Mizoguchi et al., 2002). Through heterophilic interaction with its presynaptic partner nectin-1 and homophilic adhesion, nectin-3 links indirectly to actin cytoskeleton via afadin. The nectin-afadin complex colocalizes and cooperates with the cadherin-catenin complex to organize adherens junctions, and participates in synaptic formation and remodeling (Honda et al., 2006; Majima et al., 2009; Mizoguchi et al., 2002; Togashi et al., 2006). Intriguingly, though expressed ubiquitously, nectin-3 is highly enriched in CA3 pyramidal neurons (Wang et al., 2011) that are vulnerable to both acute (Chen et al., 2010; Chen et al., 2008; Kole et al., 2004) and chronic (Conrad, 2006; Watanabe et al., 1992) stress exposure. Moreover, loss of nectin-3 leads to aberrant mossy fiber sprouting in the adult hippocampus and a reduced number of adherens junctions at the mossy fiber-CA3 synapses (Honda et al., 2006), which could be modulated by chronic stress (Magariños et al., 1997). Recently, we reported that chronic social defeat stress in adulthood decreased nectin-3 levels in area CA3, and that the spatial distribution patterns of nectin-3 partially overlapped the sites of dendritic shrinkage in CA3 pyramidal neurons (Wang et al., 2011). Additionally, we found that dysregulated hippocampal nectin-3 expression, associated with cognitive impairments and

dendritic regression, could be attenuated by inactivation of forebrain corticotropin-releasing hormone (CRH) receptor 1 (CRHR1).

In contrast to the dramatic but temporally limited consequences of chronic stress on cognition and hippocampal integrity in adulthood (Conrad et al., 1999; Luine et al., 1994), repeated stress exposure in newborn animals exerts persistent ("programming") effects (Korosi and Baram, 2009; Schmidt, 2010). It has been demonstrated that early-life stress induced later-life cognitive deficits (Rice et al., 2008) and atrophy of dendrites (Brunson et al., 2005) are mediated by forebrain CRH-CRHR1 signaling (Brunson et al., 2001; Ivy et al., 2010). However, whether nectin-3 modulates the long-lasting effects of early-life stress on hippocampal plasticity and cognition remains unknown.

In the current study, we first examined hippocampal nectin-3 levels in wild-type and conditional forebrain CRHR1 deficient mice with or without postnatal stress exposure, and assessed the potential interaction of CRHR1 with nectin-3. We hypothesized that the nectin-afadin complex would serve as a general mechanism linking stress-augmented CRH-CRHR1 signaling to stress-induced structural remodeling and cognitive decline. In the following experiments, the involvement of nectin-3 in spatial learning and memory and dendritic remodeling was investigated using viral-mediated RNA interference to suppress hippocampal nectin-3 expression *in vivo*.

Materials and methods

Animals

Male transgenic mice with postnatal inactivation of the *Crhr1* gene in forebrain neurons (CRHR1-CKO mice) were generated as described previously (Müller et al., 2003; Wang et al., 2011). Male $R26^{flopCrh/flopCrh}$ Camk2a-cre mice (CRH-COE mice) (Lu et al., 2008), female CRHR1-EGFP reporter mice and male C57BL/6N mice (Charles River, Maastricht, the Netherlands) were used.

All animals were individually housed in adulthood under a 12-hour light/dark cycle (lights on at 6 am) and constant temperature (22 ± 1°C) conditions with free access to both food and water. The experiments were carried out in accordance with European Communities Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Early-life stress procedure

The limited nesting and bedding material paradigm was carried out as described previously (Rice et al., 2008). Briefly, the day of birth was designated postnatal day 0 (P0). On the morning of P2, control dams were provided with sufficient amount of nesting material (2 squares of Nestlets; Indulab, Gams, Switzerland) and standard sawdust bedding. In the "stress" cages, dams were provided with limited quantity of nesting material (1/2 square of Nestlets), which was placed on a fine-gauge aluminum mesh platform (McNichols, Tampa, FL, USA). All litters remained undisturbed during P2-P9. On P9, all dams were provided with standard nesting and bedding material. Male offspring were weaned on P28 and group housed in 4-5 per cage. Tail tips were collected and genotyped upon weaning when appropriate.

Generation of recombinant adeno-associated viruses (AAVs) and intrahippocampal microinjection

Four short hairpin RNA (shRNA) sequences targeting human nectin-3 were screened *in vitro* for efficacy of knockdown. The two most effective sequences which reduced nectin-3 gene and protein expression in HEK293 cells were selected. U6-shRNA was cloned into a rAVE^{TM} construct (AAV1/2 vector) containing EGFP. The U6 promoter drives expression of the shRNA and the CAG promoter drives expression of EGFP. The CAG promoter consists of the chicken β-actin promoter hybridized with the CMV immediate early enhancer sequence and is highly efficient in most tissue types. The Woodchuck post-transcriptional regulatory element (WPRE) and the presence of a bovine growth hormone (BGH) polyadenylation sequence ensure high transcription following transduction. The vector was purified against immobilized heparin sulfate proteoglycan (GeneDetect, Auckland, New Zealand).

Mice were anesthetized with isoflurane. The anesthesia was maintained using isoflurane/O_2 (1~1.5:100) inhalation. Stereotaxic surgery was performed as previously described (Athos and Storm, 2001). Briefly, 1 μl of AAV (1.2~11 × 10^{12} viral genomes/ml) containing either EGFP gene (AAV1/2-U6-Empty/Null-terminator-CAG-EGFP-WPRE-BGH-polyA; AAV-empty), EGFP gene and scrambled shRNA (AAV1/2-U6-SCR shRNA-terminator-CAG-EGFP-WPRE-BGH-polyA; AAV-shSCR), or either of two nectin-3 shRNAs (AAV1/2-U6-Nectin-3 shRNA-terminator-CAG-EGFP-WPRE-BGH-polyA; AAV-shNEC and AAV-shNEC-2), was injected bilaterally aiming at the stratum radiatum of area CA3 in the dorsal hippocampus (coordinates:

1.9 mm posterior to bregma, 2.1 mm lateral from midline, 1.8 mm dorsoventral from dura) (Paxinos and Watson, 2001). The microinjection was always conducted during the light phase of the circadian cycle. The virus was delivered over a 15-min period followed by 5 min of rest. Mice were allowed to wake in the cage warmed up by a heating pad and were given drinking water with 2.5 mg/l Metacam (Boehringer Ingelheim, Ingelheim, Germany). All mice quickly recovered from surgery and were given a 4-week period prior to experimentation to allow sufficient viral infection in the hippocampus.

Experimental design

Experiment 1

To evaluate the effects of early-life stress and forebrain CRH-CRHR1 signaling on hippocampal nectin-3 expression, control or postnatally stressed wild-type and CRHR1-CKO mice (control wild-type: n = 11; control CRHR1-CKO, n = 7; stressed wild-type: n = 8; stressed CRHR1-CKO, n = 7) were sacrificed at 7-8 months of age. Adult CRH-COE and wild-type mice (7 months old; n = 9 per group) were also sacrificed. Brains were dissected, snap-frozen and stored at -80°C for *in situ* hybridization.

To examine the potential colocalization of CRHR1 with nectin-3, adult female CRHR1-EGFP reporter mice (3 months old) were anesthetized with sodium pentobarbital (200 mg/kg of body weight, intraperitoneally) and transcardially perfused with 0.9% saline/heparin followed by 4% buffered paraformaldehyde. Brains were postfixed in the same fixative, cryoprotected and stored at -80°C for immunostaining.

Experiment 2

To determine the effects of the four AAVs on nectin-3 protein expression *in vivo*, adult C57BL/6N mice (3 months old; n = 3 per group) were anesthetized and subjected to intrahippocampal viral injection. At 4 weeks after viral injection, mice were transcardially perfused, and brains were processed and stored at -80°C for immunostaining.

Experiment 3

To assess the effects of hippocampal nectin-3 knockdown on spatial learning and memory, two successive cohorts of C57BL/6N mice (3 months old; n = 12 and 15 per group, respectively)

injected with AAV-shSCR or AAV-shNEC were subjected to the Morris water maze test (both batches), Y-maze and spatial object recognition tests (only the second batch). At 1 week after behavior testing, mice were sacrificed. Brains were either dissected and cut into halves or perfused for *in situ* hybridization, Western blot and immunostaining.

Behavioral and cognitive testing

Mice were tested at 4.5 months of age. The tests were always performed between 8 am and 12 noon and scored by the ANY-maze software (ANY-maze 4.50, Stoelting, Wood Dale, IL, USA).

Y-maze

Short-term spatial working memory was tested by recording spontaneous alternation behavior in the Y-maze (Schmidt et al., 2010). The Y-maze apparatus was made of grey polyvinyl chloride with three symmetrical arms (30 × 10 × 15 cm^3) marked by triangle-, bar- and plus-signs, respectively, as intra-maze spatial cues, and was evenly illuminated (30 lux). Prominent extra-maze spatial cues were attached to the walls at a distance of ~25 cm from the apparatus. The mice were placed in the center of the maze and allowed to explore the arms freely for 5 min. Three consecutive choices of all three arms were counted as an alternation. Thus, the percentage of alternation was determined by dividing the total number of alterations by the total number of choices minus 2.

Spatial object recognition

The one-trial object-place recognition task was performed in an open field apparatus (50 × 50 × 50 cm^3) under low illumination (30 lux) as described previously (Dix and Aggleton, 1999). Prominent spatial cues were attached to the walls at a distance of ~25 cm from the apparatus. Mice were habituated to the apparatus and testing environment for two consecutive days (10 min per day). In the first sample phase (10 min), mice were presented two identical metal cubes (5 × 5 × 5 cm^3) placed in the arena and allowed to explore the objects freely. After a 15 min intertrial interval (ITI) when the objects were cleaned, mice were again introduced to the arena with both objects placed in previous locations for 10 min. After a 30 min ITI, mice were presented with a non-displaced object and a relocated one (retrieval phase; 5 min). The exploration of the objects was considered when the mice touched the objects with the nose, forepaws or vibrissae. The

percentage of the time exploring and the number of contacts with the displaced (novel) and the non-displaced (known) objects was calculated, with a higher preference for the novel object being rated as intact spatial recognition memory.

Morris water maze

The Morris water maze test was carried out as described previously (Sterlemann et al., 2010) in two cohorts of mice under the same conditions and data were pooled. A circular tank (110 cm in diameter) was filled with opaque colored water (22 ± 1°C), and prominent extra-maze visual cues were attached to the walls at a distance of ~50 cm from the pool. After day 1 with a 60-s free swim trial, mice were trained to locate a visible platform (10 cm in diameter) above the surface of the water for 4 trials (visual training). In the following spatial training sessions, mice received 4 trials per day to locate the submerged platform in a fixed position over 3 consecutive days. The order of starting locations was varied throughout trials. Next day, the reference memory was assessed in a 60-s probe trial with platform removed, and the latency to reach the platform area and the time spent in each quadrant were calculated. After 4 days of rest, mice received 4 trials to locate the hidden platform placed in the quadrant opposite to that in the spatial training sessions (reversal learning). The trials in visual, spatial and reversal training sessions were terminated once the mouse found the platform or 60 s had elapsed, and the latency to reach the platform was recorded for each trial. The ITI was 10 min.

Histological verification

The morphology of the hippocampus was evaluated by cresyl violet staining. Mice with unilateral or bilateral gliosis and neuronal loss in the hippocampus (possibly due to technical problems during the injection procedure) were excluded from analysis (AAV-shSCR, n = 4; AAV-shNEC, n = 5).

In situ hybridization

Brains were sectioned coronally at 20 µm through the hippocampus at -20°C in a cryotome. The sections were thaw-mounted on superfrost slides, dried, and kept at -80°C. *In situ* hybridization was performed as previously described (Schmidt et al., 2007; Wang et al., 2011). The following primers were used to generate an antisense RNA hybridization probe (485 base pairs) that

recognizes a shared sequence of alpha-, beta-, and gamma-transcript variants of nectin-3: AGCCGTTACATTCCCACTTG (forward primer) and ATTGTCCATCCAACCTGCTC (reverse primer). The slides were apposed to Kodak Biomax MR films (Eastman Kodak, Rochester, NY, USA) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion, Frederick, MD, USA).

Western blot

Western blot was performed as previously described (Wang et al., 2011). Hippocampi were homogenized in ice-cold lysis buffer and centrifuged (12000 rpm, 30 min) at 4°C. Protein concentrations were determined using a detergent compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 40 μg of protein were resolved by 10% sodium dodecyl sulphate-polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). Membranes were labeled with rabbit anti-nectin-3 (1:2000, Abcam, Cambridge, UK), rabbit anti-nectin-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-protein kinase RNA-activated (PKR; 1:1000, Santa Cruz Biotechnology), or goat anti-actin (1:2000, Santa Cruz Biotechnology) antibodies overnight at 4°C. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:2000, DAKO, Glostrup, Denmark) or horse anti-goat (1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) secondary antibodies for 2 hours, bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) and quantified by densitometry (Quantity One 4.2, Bio-Rad, Hercules, CA, USA).

Immunohistochemistry and image analysis

Serial coronal sections were cut through the hippocampus at 20 μm thickness and 160 μm intervals in a cryotome. Double-labeling immunofluorescence were performed on free-floating sections as described previously (Chen et al., 2004b). In short, after incubation with primary antibodies overnight at 4°C, sections were rinsed and labeled with Alexa Fluor 488- and 647-conjugated donkey secondary antibodies (1:500, Invitrogen) for 2 hours at room temperature. After rinsing, sections were transferred onto slides and coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector laboratories, Burlingame, CA, USA). For calbindin immunohistochemistry, after labeling with primary and biotinylated secondary

antibodies, sections were incubated with avidin-biotin-horseradish peroxidase (Vector laboratories) for 2 h at RT, developed by SIGMAFAST™ 3,3′-diaminobenzidine tablets (Sigma-Aldrich, Munich, Germany), dehydrated, and coverslipped (Wang et al., 2008). The primary antibodies used were goat anti-EGFP (1:2000, Abcam), rabbit anti-nectin-3 (1:500, Abcam), goat anti-calbindin (1:500, Frontier Science, Hokkaido, Japan), and rabbit anti-l-afadin (1:1000, Abcam).

Images (1600 × 1600 pixels) were obtained with an Olympus IX81 confocal microscope (Olympus, Tokyo, Japan) using the Kalman filter and sequential scanning mode under identical settings for laser power, photomultiplier gain and offset. A 40× water-immersion objective (NA 1.15, Olympus) and a 60× water-immersion objective (NA 1.20, Olympus) were used. For colocalization analysis, images were adjusted for better brightness and contrast using the FV10-ASW 1.7 software (Olympus). Images were imported into the NIH ImageJ software, converted to 8-bit grayscale and thresholded uniformly. The colocalized puncta were revealed using the Colocalization Highlighter plugin and analyzed by Analyze Particle module of the ImageJ program.

To measure dendritic complexity of dentate granule cells, images (4 per animal) were captured using the 40× objective without digital zoom. Regions of interest (ROIs; 100 × 250 pixels) were selected on the proximal, medial and distal segments (3 ROIs per segment) of EGFP-labeled apical dendrites, respectively. The area of EGFP-positive dendritic branches and the density of (colocalized) puncta were determined by ImageJ.

To quantify the dendritic spine density, apical dendrites (6 per animal) from the suprapyramidal blade of the dentate gyrus (DG) were randomly selected and scanned at 1 μm intervals along the z-axis using the 60× objective with a 2.5× digital zoom (3-4 planes per dendrite). Because the inner molecular layer (IML) of the DG was densely packed with EGFP-positive commissural/associational afferent fibers (Förster et al., 2006), only spines at 10-70 μm from the IML were quantified. Spines were classified as large-headed mushroom-like spines and small-headed thin/stubby spines, and manually counted in each 10-μm segment.

Calbindin immunoreactivity in the suprapyramidal and infrapyramidal blades of DG was analyzed by ImageJ (6 sections per animal). Relative calbindin level was expressed as the mean optical density ratio of the suprapyramidal or infrapyramidal blade to the corpus callosum, which was considered as background staining.

Statistical analysis

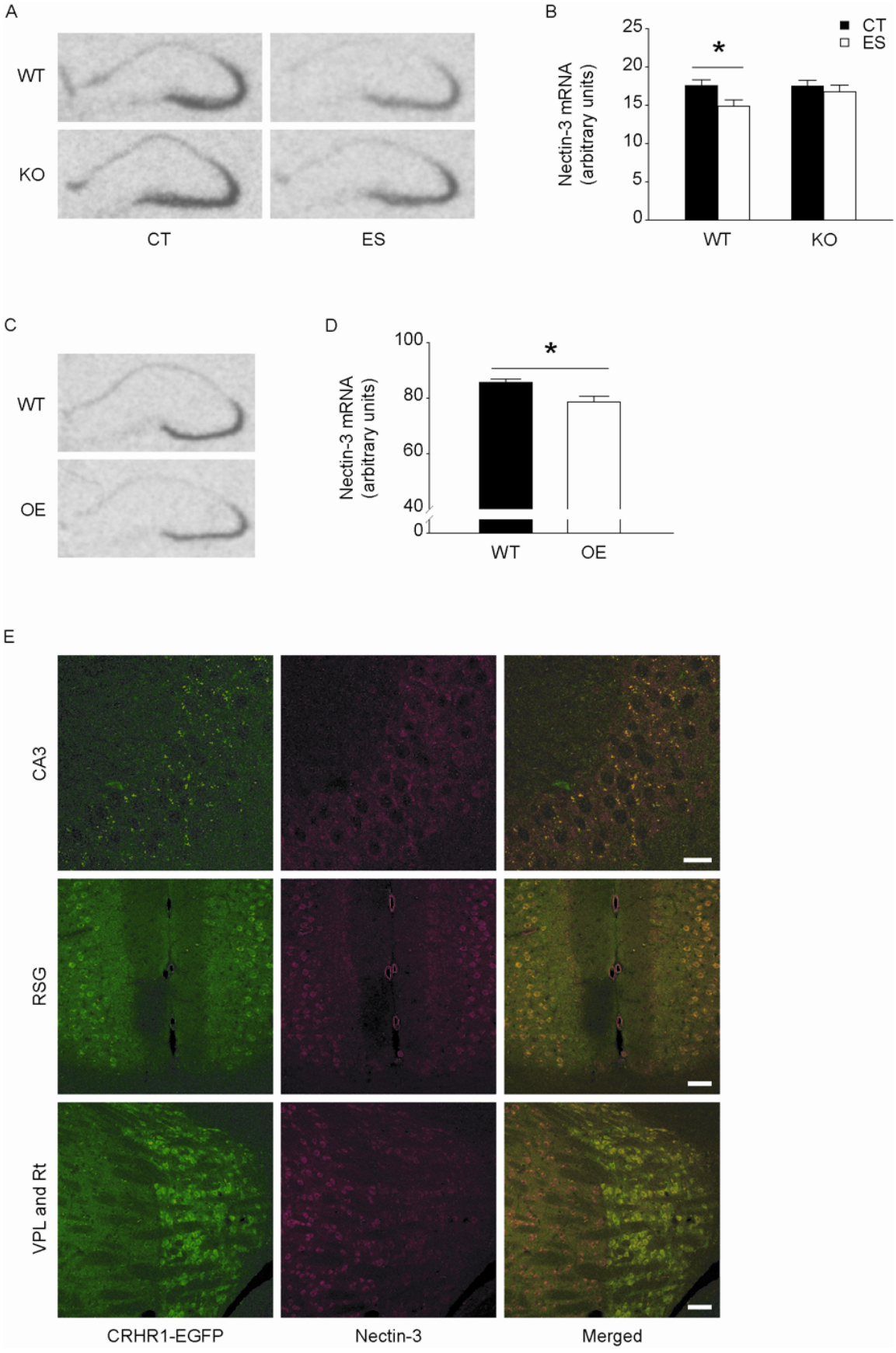
For the analysis of immunoblot signals and immunofluorescent images, data were normalized by taking the value of the control group as 100%. Data were analyzed by two-way or one-way ANOVA followed by Bonferroni *post hoc* test as necessary. Student's *t*-test was used to compare pairs of means. Two-way ANOVA with condition as the between-subjects factor and trial as a within-subject factor was performed on the Morris water maze data of each spatial training day and the reversal learning day. The level of statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

Results

Early-life stress-induced reduction of hippocampal nectin-3 gene expression is dependent on CRH-CRHR1 signaling

We examined nectin-3 gene expression in adult wild-type and CRHR1-CKO mice exposed to an impoverished or standard environment early in life (Figure 1A and 1B). A significant effect of condition [$F(1, 29) = 4.373$, $p < 0.05$, two-way ANOVA] on nectin-3 gene expression was revealed. Hippocampal nectin-3 mRNA levels were reduced in stressed wild-type ($p < 0.05$ versus the controls, Bonferroni's test) but not stressed CRHR1-CKO mice. To test whether reduced nectin-3 gene expression in stressed wild-type mice was due to persistently augmented forebrain CRH-CRHR1 signaling, we examined hippocampal nectin-3 mRNA levels in wild-type and CRH-COE mice (Figure 1C and 1D). Compared to wild-type mice, CRH-COE mice showed significantly lower nectin-3 mRNA levels in the hippocampus ($p < 0.05$, unpaired *t* test), indicating that hippocampal CRH-CRHR1 signaling modulates the effects of recurrent postnatal stress on nectin-3 expression.

Moreover, CRHR1-EGFP partially colocalized with nectin-3 in CA3 pyramidal neurons and neurons in various brain regions (Figure 1E), suggestive of functional interactions between CRHR1 and nectin-3.


A
WT
KO
CT
ES
B
Nectin-3 mRNA (arbitrary units)
25
20
15
10
5
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CT
ES
WT
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C
WT
OE
D
Nectin-3 mRNA (arbitrary units)
100
80
60
40
0
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WT
OE
E
CA3
RSG
VPL and Rt
CRHR1-EGFP
Nectin-3
Merged

Figure 1. Nectin-3 expression is regulated by forebrain CRH-CRHR1 signaling. **(A)** Representative *in situ* hybridization images showing nectin-3 mRNA expression in the hippocampus in adult wild-type (WT) or CRHR1-CKO (KO) mice exposed to either control condition (CT) or early-life stress (ES). **(B)** CA3 nectin-3 mRNA levels were reduced by postnatal stress in wild-type but not CRHR1-CKO mice. **(C)** Representative photomicrographs showing nectin-3 mRNA expression in the hippocampus in adult wild-type or CRH-COE (OE) mice. **(D)** CA3 nectin-3 mRNA levels were reduced in CRH-COE mice. **(E)** CRHR1-EGFP partially colocalized with nectin-3 in area CA3 (upper panels), retrosplenial granular cortex (RSG, middle panels), ventral posterolateral nucleus (VPL) and reticular nucleus (Rt) (lower panels). Scale bars: upper panels, 20 μm; middle and lower panels, 50 μm. * $p < 0.05$ versus the control group. n = 7-11 mice per group.

Suppression of nectin-3 expression in hippocampal neurons in vivo

Hippocampal nectin-3 expression levels were evaluated in mice received either sham injection or injections of the following viruses into the dorsal hippocampus: AAV-empty, AAV-shSCR, AAV-shNEC and AAV-shNEC-2 (Figure 2). One-way ANOVA revealed a significant effect of condition [$F(4, 14) = 47.106$, $p < 0.001$] on nectin-3 puncta density in area CA3. AAV-shNEC and AAV-shNEC-2 mice showed significantly reduced nectin-3 puncta density (both $p < 0.001$ compared to AAV-shSCR mice, Bonferroni's test) and reduced numbers of EGFP and nectin-3 colocalized neurons (both $p < 0.05$ compared to AAV-shSCR, Bonferroni's test). No difference was found in nectin-3 puncta density between sham-, AAV-empty- or AAV-shSCR-treated groups, indicating that AAV infection, EGFP and scrambled RNA sequences in the AAV vectors do not unspecifically affect nectin-3 expression. In the experiments described below, we used AAV-shSCR as the negative control and AAV-shNEC to suppress nectin-3 expression *in vivo*.

Specific knockdown of nectin-3 in the hippocampus by AAV-shNEC

To test the *in vivo* knockdown specificity of the AAV-shNEC vector, we first examined the protein levels of nectin-3 in hippocampal lysates (Figure 3A and 3B). Consistent with the immunostaining data, AAV-shNEC suppressed nectin-3 protein expression in the hippocampus ($p < 0.05$, unpaired t test). Next, the protein levels of hippocampal nectin-1, the presynaptic partner of nectin-3, were evaluated (Figure 3C and 3D). The 87 kDa- and 64 kDa- isoforms of nectin-1 remained unchanged between groups, indicating no off-target effect of AAV-shNEC.

Because shRNA vectors may activate the interferon system resulting in non-specific down-regulation of proteins (Bridge et al., 2003; Sledz et al., 2003), we examined the expression levels of PKR which mediates this effect in both groups (Figure 3E and 3F). No significant difference in

PKR protein levels was found between groups, further indicative of the knockdown specificity of AAV-shNEC.

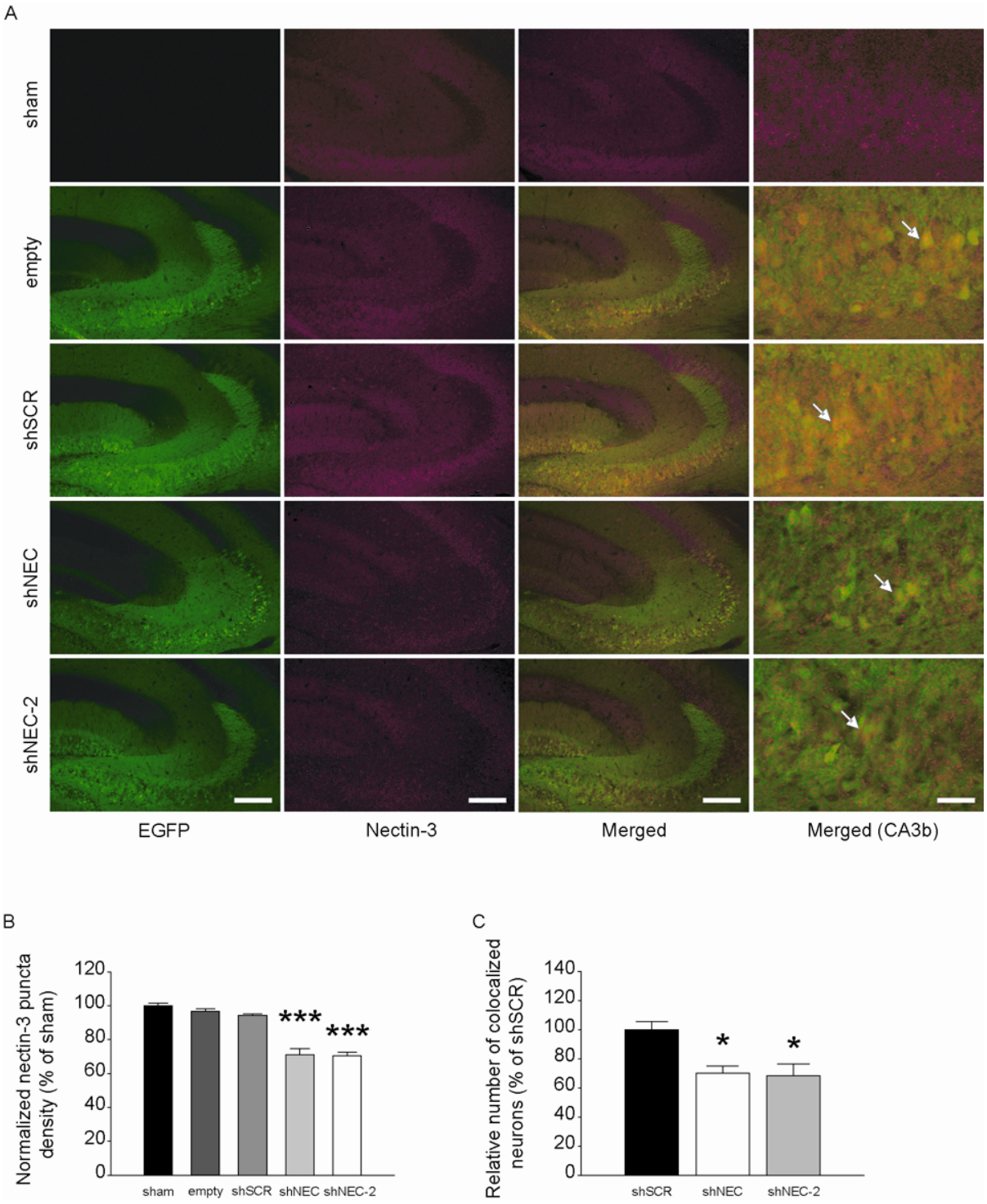

Figure 2. Selection of an AAV to suppress nectin-3 expression. **(A)** Representative images showing the expression of EGFP and nectin-3 in the hippocampus with sham injection, or injected with viruses containing EGFP sequence only

(empty), scrambled RNA sequence (shSCR), a shRNA sequence targeting nectin-3 (shNEC), or another shRNA sequence (shNEC-2). Arrows in the right panels indicate EGFP and nectin-3 double-labeled CA3b neurons. **(B)** Nectin-3 protein levels were reduced by AAV-shNEC or AAV-shNEC-2. **(C)** Hippocampus injected with either AAV-shNEC or AAV-shNEC-2 showed reduced number of EGFP and nectin-3 colocalized neurons. Scale bars: left and middle two panels, 200 μm; right panels, 40 μm. * $p < 0.05$; *** $p < 0.001$ versus AAV-shSCR group. n = 3 mice per group.

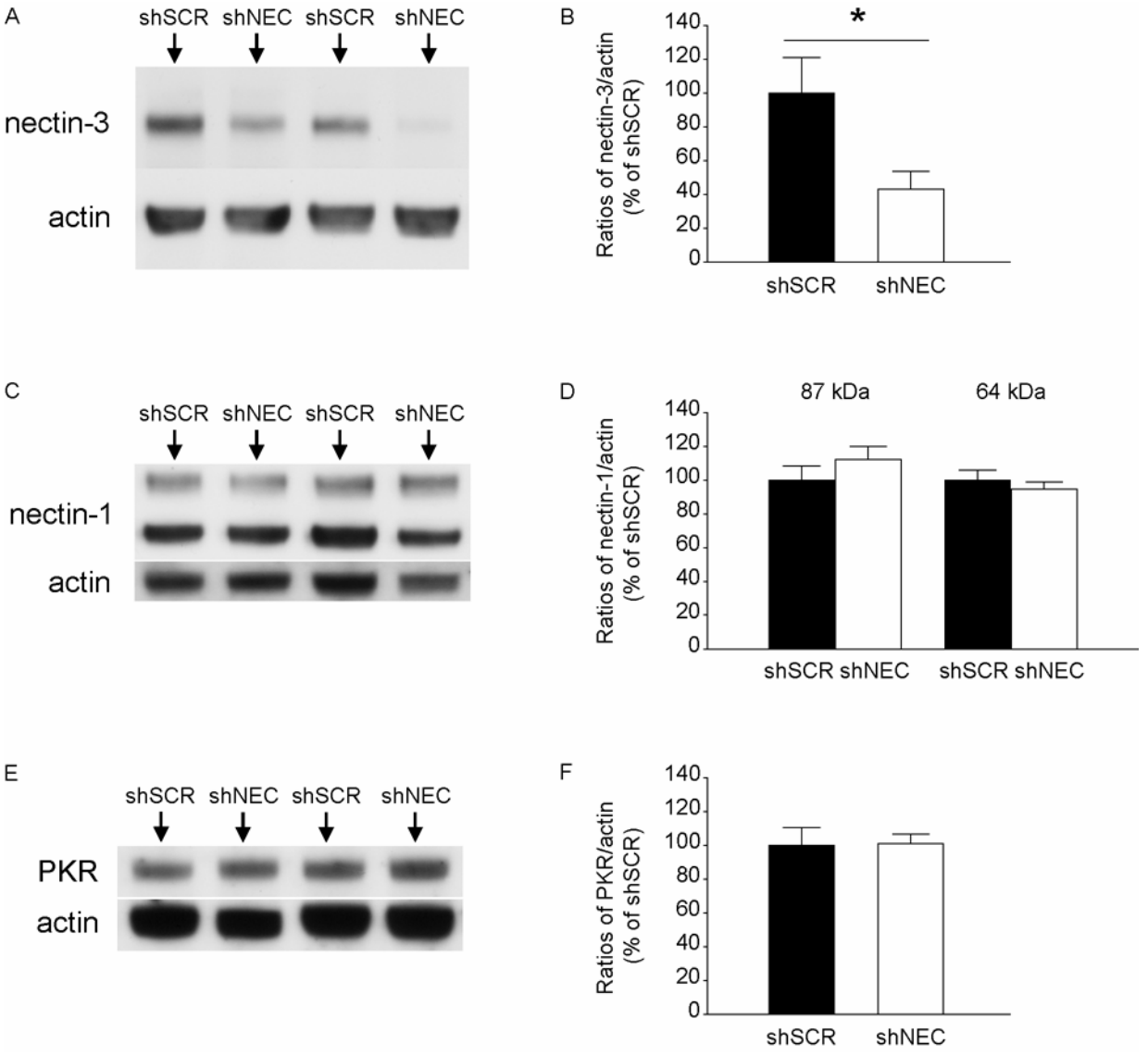

Figure 3. Specific *in vivo* knockdown of nectin-3 protein expression in the hippocampus. **(A, B)** Nectin-3 protein levels in the hippocampal lysates were reduced by AAV-shNEC. **(C, D)** The protein levels of the 87 kDa (upper bands) and 64 kDa (lower bands) nectin-1 isoforms were comparable between groups. **(E, F)** PKR protein levels were unaltered by AAV-shNEC. * $p < 0.05$ versus AAV-shSCR group. n = 6 mice per group.

Hippocampal nectin-3 knockdown attenuates spatial learning and memory in adulthood

It remains to be defined whether hippocampal nectin-3 is involved in learning and memory. Thus, we evaluated spatial learning and memory in control and AAV-shNEC mice. Short-term, spatial working memory was not affected by hippocampal nectin-3 knockdown, as shown by comparable spontaneous alternation behavior in performing the Y-maze task (Figure 4A). In the spatial object recognition test, control mice discriminated the displaced (novel) object from the non-displaced (familiar) one ($p < 0.001$, paired t test), whereas AAV-shNEC failed to show discrimination between the two objects. Additionally, control mice spent significantly more time visiting the novel object than AAV-shNEC mice ($p < 0.05$, unpaired t test; Figure 4B). Consistently, in the Morris water maze test, though no condition effect was revealed by two-way ANOVA, AAV-shNEC mice took significantly longer to reach the hidden platform in the third trial of spatial training day 1 and the second trial of spatial training day 2 compared to the controls (both $p < 0.01$, unpaired t test; Figure 4C), indicative of a mild impairment in spatial learning (Bisaz et al., 2011). In the probe trial, control but not AAV-shNEC mice searched the target quadrant (where

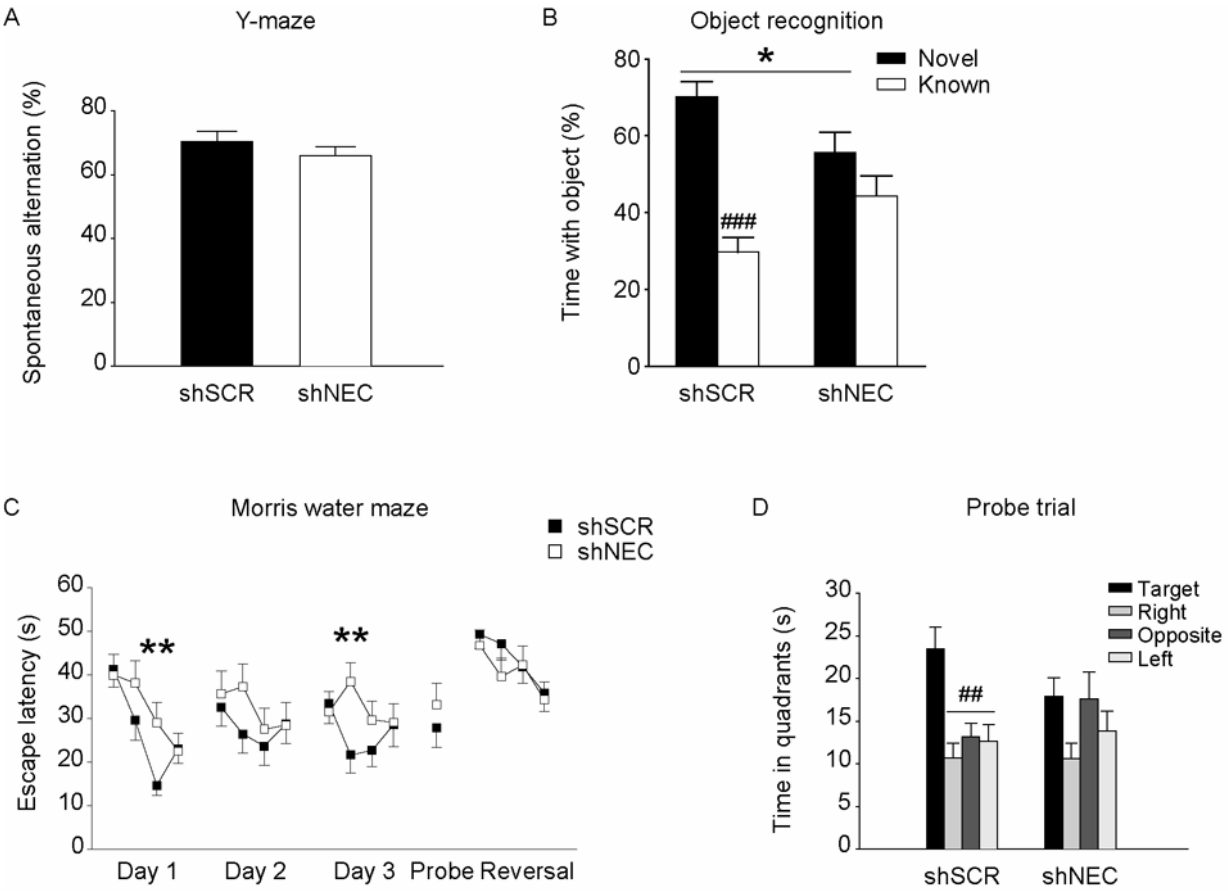

Figure 4. Spatial performance in adult AAV-shSCR and AAV-shNEC mice. **(A)** Spatial working memory was similar between groups. **(B)** In the spatial object recognition test, AAV-shSCR mice discriminated the novel object from the familiar, whereas AAV-shNEC failed to show object discrimination. **(C)** In the Morris water maze test, AAV-shNEC mice took significantly longer to reach the hidden platform in the third trial of spatial training day 1 and the second trial of spatial training day 2 compared to AAV-shSCR mice. **(D)** In the probe trial of the water maze test, AAV-shSCR but not AAV-shNEC mice spent more time searching the quadrant where the platform was previously placed than the other quadrants. * $p < 0.05$; ** $p < 0.01$ versus AAV-shSCR group. ## $p < 0.01$ versus the average time searching the three non-target quadrants. ### $p < 0.001$ versus the novel object. Mice: **(A, B)** n = 12 per group; **(C, D)** n = 22-23 per group.

the platform was placed in the spatial training sessions) more frequently than the other quadrants ($p < 0.01$ versus the average time in non-target quadrants, paired *t* test; Figure 4D).

Hippocampal nectin-3 knockdown in AAV-shNEC mice subjected to behavioral testing was validated by *in situ* hybridization (Figure 5). Nectin-3 mRNA levels were significantly decreased by AAV-shNEC in CA3, CA1 and DG [Two-way ANOVA of condition, $F(1, 30) = 96.218$, $p < 0.001$; $F(1, 30) = 11.486$, $p < 0.01$; and $F(1, 29) = 22.193$, $p < 0.001$, respectively]. Together, these data indicate that nectin-3 is essential for hippocampus-dependent learning and long-term, but not short-term, spatial memory.

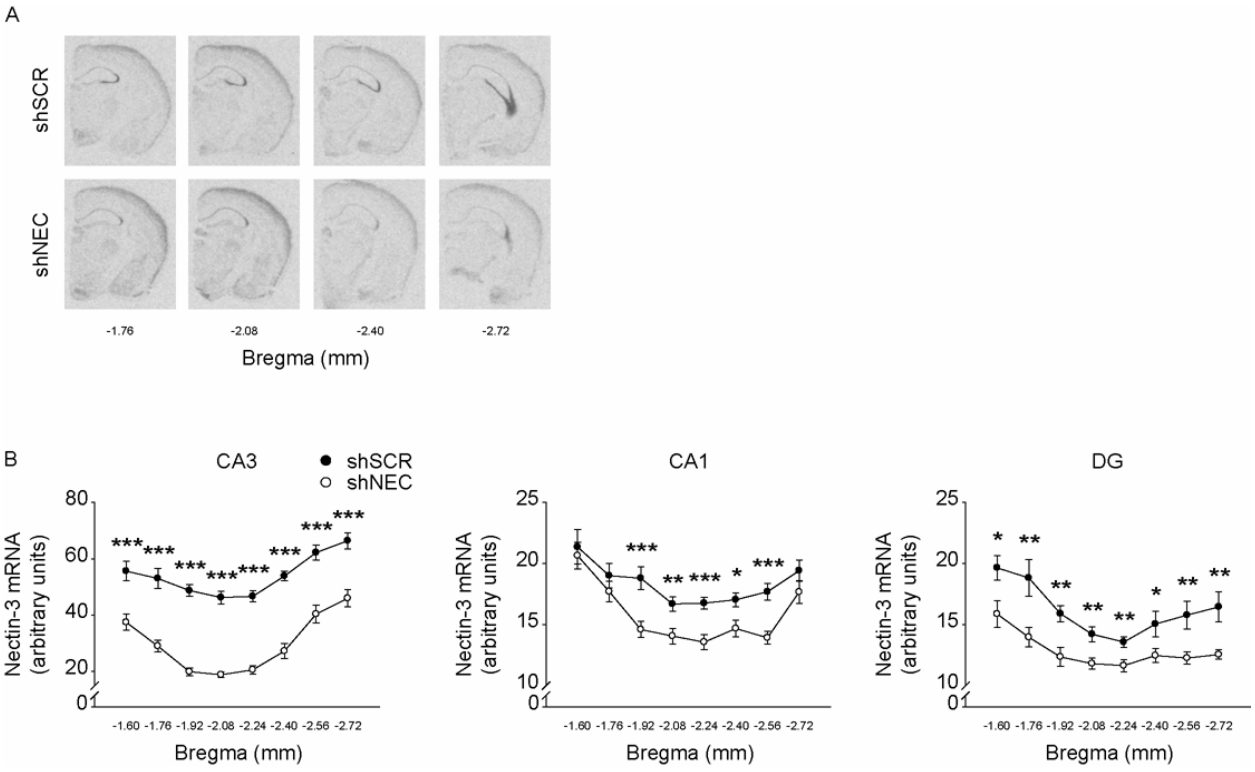

Figure 5. Verification of AAV-shNEC-induced hippocampal nectin-3 knockdown after behavioral testing. **(A)** Representative photomicrographs show the gene expression patterns of hippocampal nectin-3 in AAV-shSCR and AAV-shNEC mice. **(B)** Nectin-3 mRNA levels in CA3, CA1 and DG were significantly reduced by AAV-shNEC. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus AAV-shSCR group. n = 15-16 mice per group.

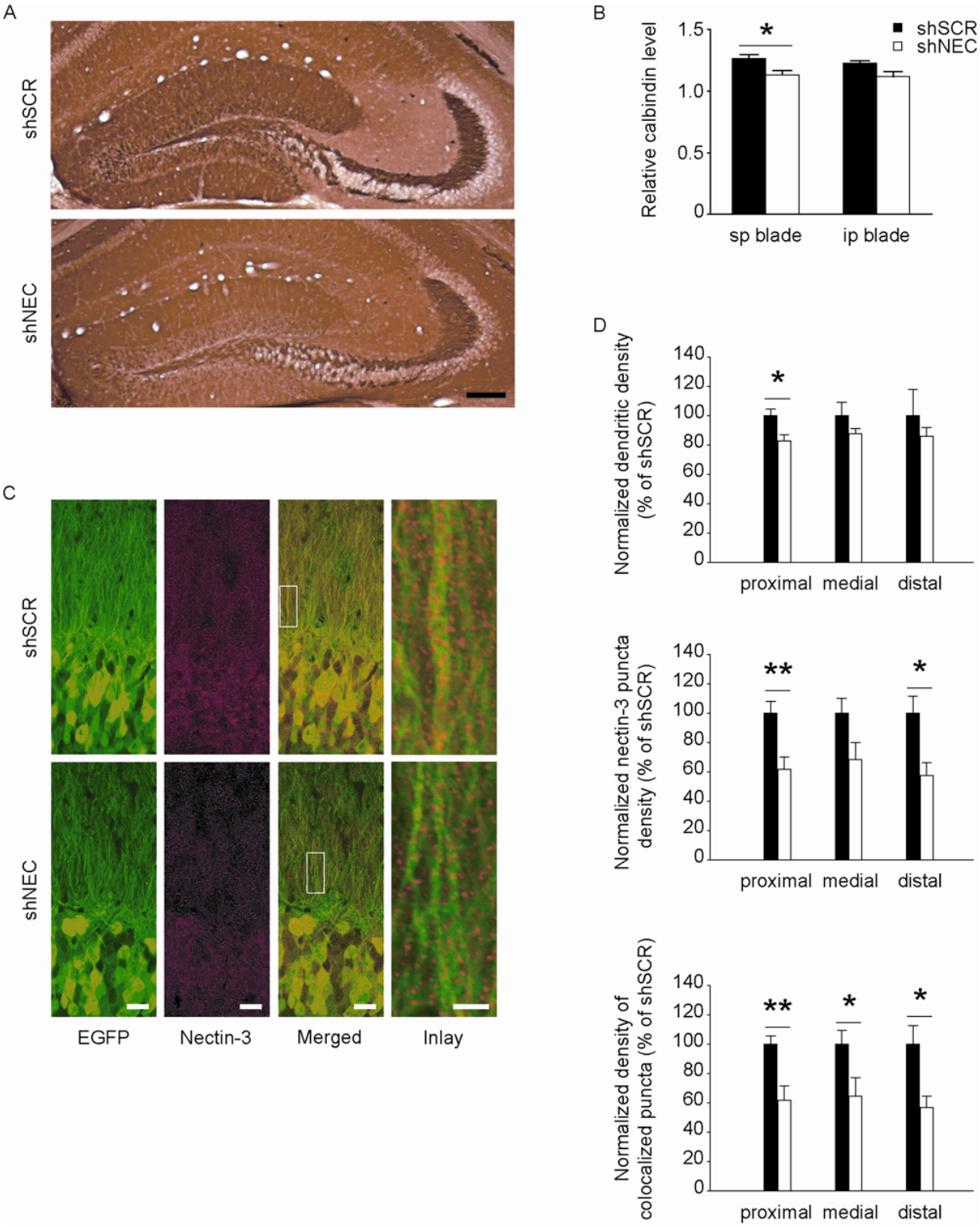

Figure 6. Effects of nectin-3 knockdown on apical dendritic complexity of dentate granule cells. **(A)** Representative immunohistochemical images show the expression patterns of calbindin in the hippocampus injected with AAV-shSCR or AAV-shNEC. Scale bar = 200 μm. **(B)** Calbindin immunoreactivity in the molecular layer of suprapyramidal (sp) but not infrapyramidal (ip) blade was significantly reduced by nectin-3 downregulation. **(C)** Representative confocal images taken from the suprapyramidal blade of DG showing EGFP and nectin-3 labeled granule cells and apical dendrites. Scale bars: left and middle two panels, 20 μm; right panels, 5 μm. **(D)** Nectin-3 knockdown reduced the density of proximal dendrites in granule cells and decreased the number of EGFP and nectin-3 colocalized synaptic puncta. * $p < 0.05$; ** $p < 0.01$ versus AAV-shSCR group. Mice: **(A, B)**, n = 3 per group; **(C, D)**, n = 6 per group.

Nectin-3 knockdown evokes dendritic regression and spine loss in DG

Nectins have been implicated in the formation, maintenance and remodeling of synapses in hippocampal neurons (Honda et al., 2006; Lim et al., 2008; Majima et al., 2009; Mizoguchi et al., 2002). However, whether nectin-3 modulates the remodeling of dendrites and spines has not been addressed. We first performed calbindin immunohistochemistry, which can be used to assess the morphology of apical dendrites (Yamashita et al., 2011), and found that AAV-shNEC significantly reduced calbindin immunoreactivity in the molecular layer of dentate suprapyramidal blade ($p < 0.05$, unpaired *t* test; Figure 6A and 6B). To further assess the long-term *in vivo* effects of nectin-3 knockdown on hippocampal morphology in adult mice, we measured the complexity of apical dendrites of dentate granule cells, the majority of which was infected by AAV (Colon-Cesario et al., 2006). We randomly selected ROIs in the medial and outer molecular layers of suprapyramidal blade, where the numbers of corresponding EGFP-expressing granule cells were comparable (Figure 6C). In line with calbindin immunostaining results, apical dendritic density was decreased in AAV-shNEC mice, most prominently in the proximal dendritic segments ($p < 0.05$, unpaired *t* test). Moreover, nectin-3 puncta density and EGFP and nectin-3 colocalized puncta density were significantly lower in AAV-shNEC mice compared to the controls (see Figure 6D for details).

Next, we quantified the number of large-headed mushroom-like spines and spines with small heads (thin and stubby spines) in the apical dendrites of granule cells (Figure 7). Compared to the controls, AAV-shNEC mice had less mushroom spines, whereas the number of thin and stubby spines was preferentially reduced at 10 μm and 20 μm from the IML, resulting in a decrease in overall spine density at 10-50 μm from IML (see Figure 7B for details). These data indicate that

silencing nectin-3 function mainly destabilizes mature, mushroom-like spines that harbor larger areas of PSD.

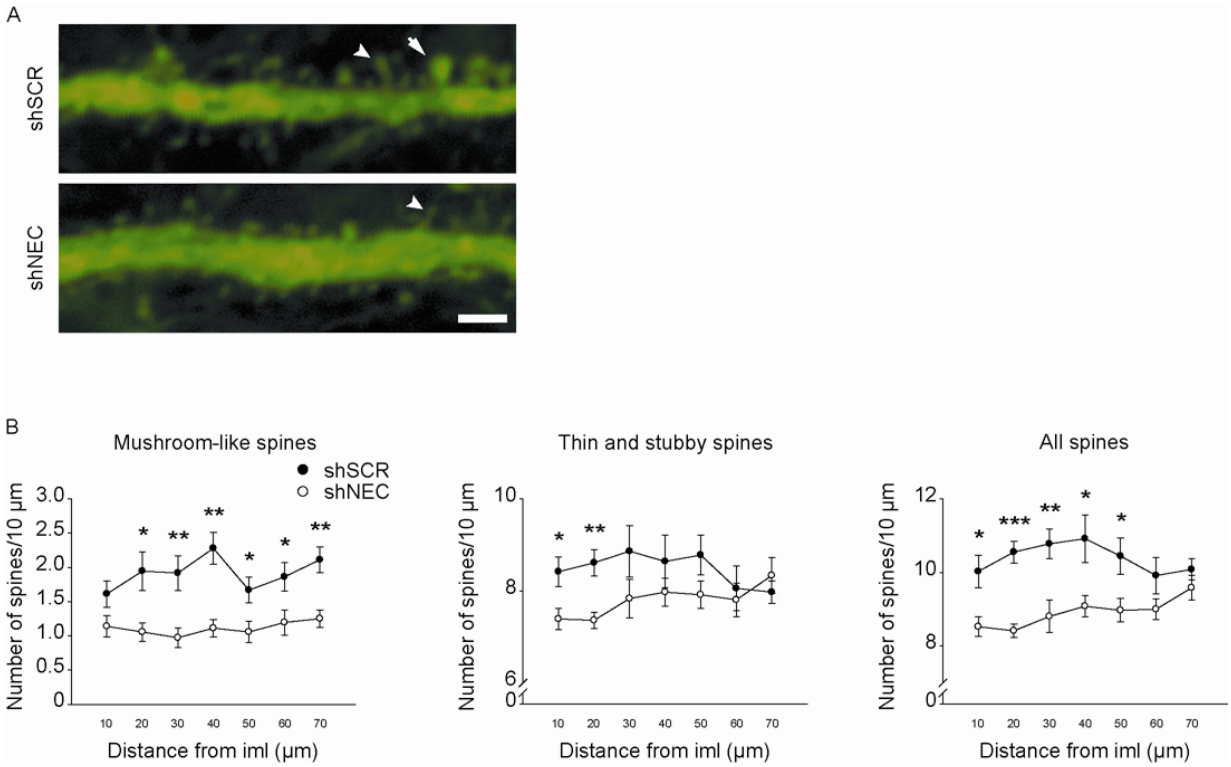

Figure 7. Effects of nectin-3 knockdown on apical dendritic spine density in dentate granule cells. **(A)** Representative photomicrographs showing EGFP-expressing apical dendrites and spines of granule cells. Arrow indicates a large-headed mushroom-like spine. Stealth arrows indicate small-headed thin spines. Scale bar = 2µm. **(B)** Hippocampal nectin-3 knockdown reduced the number of mushroom-like spines at all distances from the inner molecular layer (iml), and preferentially reduced the number of thin and stubby spines at 10 µm and 20 µm from iml, resulting in an decrease in overall spine density at 10-50 µm from iml. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus AAV-shSCR group. n = 6 mice per group.

Furthermore, hippocampal l-afadin protein levels were reduced in AAV-shNEC mice (Figure 8). In short, these findings provide evidence that nectin-3 modulates structural remodeling in adult hippocampal neurons, probably through the actin filament (F-actin)-binding protein l-afadin.

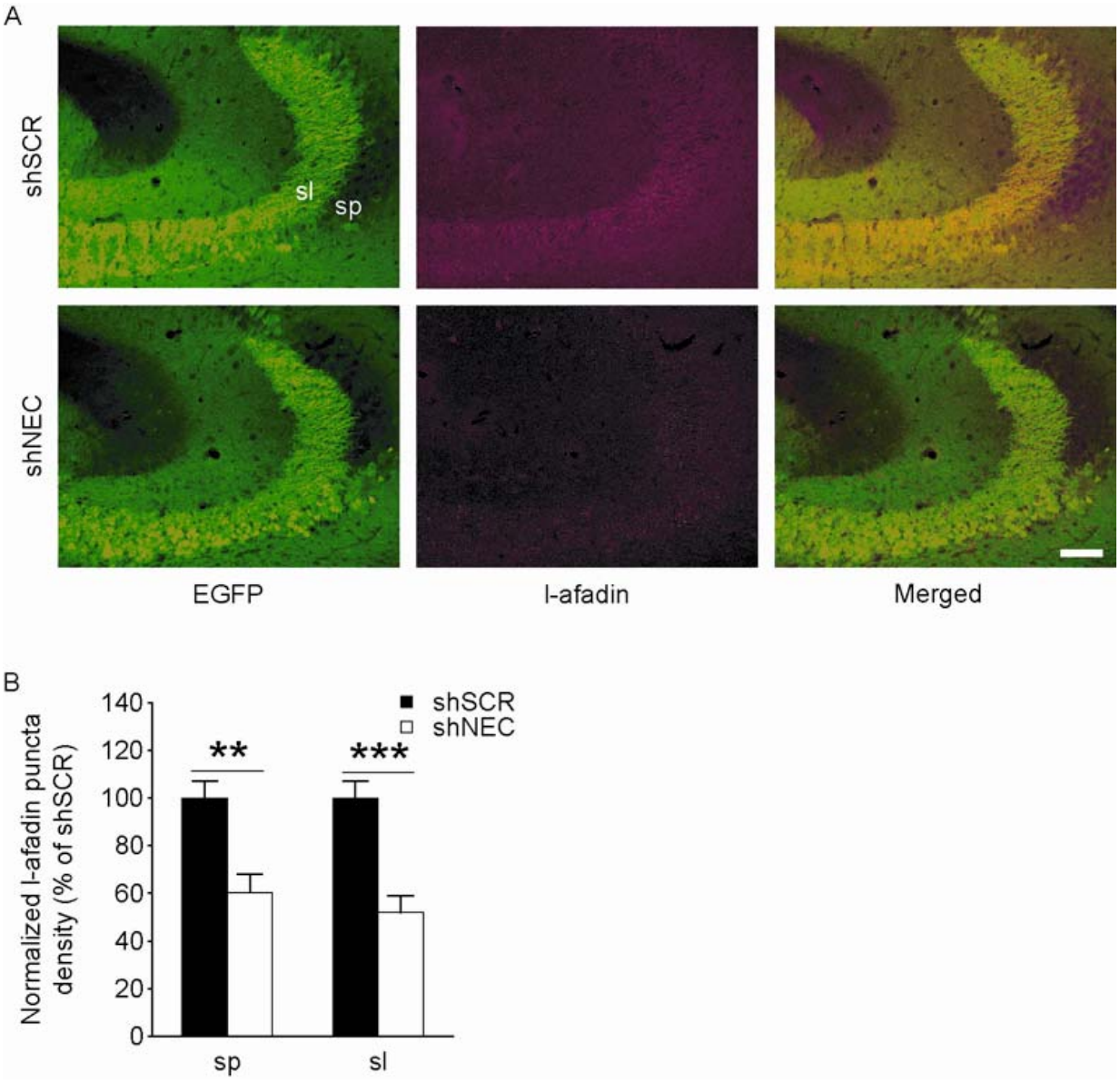

Figure 8. Effects of hippocampal nectin-3 knockdown on l-afadin protein expression levels. **(A)** Representative confocal images show EGFP and l-afadin immunoreactivity in area CA3. Scale bar = 100 µm. **(B)** L-afadin immunoreactivity was significantly reduced in the stratum pyramidale (sp) and stratum lucidum (sl) of area CA3 in AAV-shNEC mice. ** $p < 0.01$; *** $p < 0.001$ versus AAV-shSCR group. n = 6 mice per group.

Discussion

Postnatal stress impairs hippocampus-dependent learning and memory, and induces a reduction in CA3 nectin-3 levels via CRH-CRHR1 signaling. Site-specific suppression of nectin-3 in the adult hippocampus reproduces the effects of early-life stress and hampers spatial learning and memory, possibly through the destabilization of apical dendrites and spines. Together, these results

highlight the crucial roles of nectin-3 in mediating stress-evoked cognitive impairments and structural rearrangements, and provide a novel molecular link from CRH-CRHR1 signaling and dendritic (spine) remodeling.

Recurrent stress exposure, hippocampal CRH-CRHR1 signaling and nectin-3

By inactivating CRHR1 and overexpressing CRH in forebrain neurons, we previously found that impaired spatial learning and memory by early-life stress was dependent on hippocampal CRH-CRHR1 signaling, extending previous observations (Brunson et al., 2005; Ivy et al., 2010). Here, we showed that the expression levels of nectin-3, which is abundant in hippocampal area CA3, were altered by early-life stress in a CRHR1-dependent manner: early-life stress and postnatal forebrain CRH overexpression reduced CA3 nectin-3 levels in adult mice, while forebrain CRHR1 inactivation normalized nectin-3 expression in stressed mice. Interestingly, a similar effect could be observed in animals that experienced chronic stress in adulthood (Wang et al., 2011). These results suggest that the release of CRH upon stress challenge and the resultant activation of CRHR1 may inhibit the expression and even function of nectin-3. Indeed, the colocalization of dendritic spine-enriched CRHR1 with adherens junction-abundant nectin-3 in various neurons points to their potential functional interactions, and indicates that such interactions may be indirect considering their spatial distribution patterns.

Intriguingly, proximal dendritic branches of hippocampal pyramidal neurons and granule cells are increased in neonatal CRHR1-deficient mice (Chen et al., 2004a), whereas CRH application exerts the opposite effects (Chen et al., 2008). Similarly, we found that suppression of nectin-3 reduced the complexity of proximal dendrites in dentate granule cells. The overlapped effects on dendritic complexity provide further evidence for the functional interactions between CRHR1 and nectin-3. Nonetheless, the machinery through which CRHR1 and nectin-3 interacts merits further study.

Involvement of hippocampal nectin-3 in spatial learning and memory

Synaptic CAMs such as neuroligin-1 and neural cell adhesion molecule (NCAM) have been implicated in hippocampus-dependent cognition (Blundell et al., 2010; Dahlhaus et al., 2010), and are associated with chronic stress-induced cognitive decline (Bisaz et al., 2011; Borcel et al., 2008; Sandi, 2004). Our findings suggest nectin-3 as a novel CAM modulated by both early-life stress

and chronic stress in adulthood, yet little is known about its involvement in learning and memory. Because conventional nectin-3 knockout mice show microphthalmia (Honda et al., 2006), it has been difficult to evaluate cognitive performance in this transgenic mouse line. Using site-specific *in vivo* knockdown through AAV-mediated RNA interference, we showed that hippocampal nectin-3 was important for spatial learning and long-term, but not short-term, memory in adult mice. A direct link between reduced nectin-3 expression and stress-induced cognitive impairments was therefore revealed.

The nectin-afadin and cadherin-catenin complexes in adherens junctions

Synapse and adherens junction are specialized intercellular contacts in the nervous system. Synapses are the sites of neurotransmission, while adherens junctions maintain physical association between neurons. Adherens junctions localize alongside synapses (for example, on nearly 1/3 of dendritic spines in CA1 pyramidal neurons), and are implicated in modulating synaptic function and plasticity (Spacek and Harris, 1998).

The major molecular components of the adherens junction are the cadherin-catenin complex and nectin-afadin complex (Meng and Takeichi, 2009; Niessen and Gottardi, 2008). Cadherins mediate calcium-dependent homophilic adhesion, whereas post-synaptic nectin-3 and presynaptic nectin-1 mediate faster and more stable calcium-independent heterophilic binding, which is important for the preferential interactions between axonal terminals and dendritic spines (Beaudoin, 2006). Nectins binds to the F-actin-binding protein afadin, which bridges nectins to actin cytoskeleton and can interact with α-catenin (Takai et al., 2008a). The nectin-afadin complex colocalizes and cooperates with the cadherin-catenin complex to shape adherens junctions: nectin-3 recruits and promotes cadherin-mediated adhesion, whereas cadherins are required for nectin-3 function (Honda et al., 2006; Majima et al., 2009; Mizoguchi et al., 2002; Togashi et al., 2006).

Consistent with findings in nectin-3 knockout mice (Honda et al., 2006), nectin-1 remained unchanged in hippocampal lysates, but the puncta density of afadin was remarkably reduced in AAV-shNEC mice. Interestingly, necin-3, N-cadherin and β-catenin are all down-regulated in conditional afadin knockout mice (Majima et al., 2009), whereas N-cadherins are increased in nectin-3 overexpressing neurons (Togashi et al., 2006). Therefore, it is not surprising that l-afadin, and likely N-cadherin and catenins, were reduced in AAV-shNEC mice. These data indicate that

the expression of the nectin-afadin and the cadherin-catenin complexes is closely coupled, and their functional convergence is important for the regulation of adherens junctions and synaptic function.

Nectin-3 mediated axodendritic adhesion and structural plasticity

Cognitive deficits are correlated with the depletion of calbindin-D28k, a calcium-binding protein, in DG (Palop et al., 2003). In AAV-shNEC mice, impaired hippocampus-dependent learning and memory were associated with significantly reduced calbindin immunoreactivity in the molecular layer of DG, supporting the role of calbindin in cognition. As calbindin is enriched in dendrites of dentate granule cells (Celio, 1990), reduced calbindin levels indicate potential alterations in dendritic complexity (Yamashita et al., 2011). Accordingly, a reduction in dendritic arborization was noticed in the proximal segments of apical dendrites in DG. Furthermore, we observed that mature, large-headed mushroom-like spines were mainly decreased by nectin-3 suppression in the adult hippocampus. The elimination of proximal dendrites and destabilization of mushroom spines in AAV-shNEC mice were probably a result of impaired axodendritic adhesion.

It should be noted that, although the formation of adherens junctions is markedly impaired, the number of synapses remains unaltered in nectin-3 knockout mice (Honda et al., 2006). In comparison, we found that the number of spines is significantly reduced by suppressing hippocampal nectin-3 expression. The discrepancies between these findings are unclear. As the functional redundancy among various CAMs has been suggested (Giagtzoglou et al., 2009), one possibility is that other CAMs may compensate the effects of nectin-3 loss on synaptic density in conventional nectin-3 knockout mice. Another plausible explanation could be the regions investigated, that is, the stratum lucidum of area CA3 and the molecular layer of DG, respectively.

In addition, because cadherin-catenin complex has been shown to be essential for activity-dependent dendritic growth (Tan et al., 2010) and stabilization of dendritic spines (Abe et al., 2004), we could not rule out the possibility that the effects of nectin-3 on dendrite and spine morphology are partially mediated through the cadherin-catenin complex. Together, our results suggest that reduced nectin-3 levels result in instable axodendritic adhesion and consequently destabilize synaptic contacts.

In summary, based on our findings, we propose that repeated stress exposure persistently augments hippocampal CRH release and downregulates nectin-3 expression through CRHR1.

Disrupted function of the nectin-afadin complex leads to dendritic atrophy and spine modifications, which in turn impair hippocampus-dependent learning and memory.

Chapter 6

General discussion

- The programming effects of early-life experience on cognition: neural mechanisms and beyond
- Mechanisms and implications of cognitive impairments and structural alterations induced by chronic stress in adulthood: glucocorticoids or CRH?
- The nectin-afadin complex: a novel molecular machinery linking CRH-CRHR1 signaling to structural plasticity
- Summary

The studies presented in this thesis address the essential roles of the forebrain CRH-CRHR1 system in cognitive impairments and structural abnormalities caused by early-life stress or chronic stress in adulthood. Two synaptic adhesion systems, the neurexin-neuroligin complex and the nectin-afadin complex, are revealed to be associated with CRH-CRHR1 signaling and involved in stress-induced memory loss. Furthermore, novel evidence that nectin-3 may modulate spatial learning and memory and structural remodeling is provided.

The programming effects of early-life experience on cognition: neural mechanisms and beyond

Early environmental factors shape brain development and cognitive function. In rodents, exposure to an enriched environment or mild stress in the first weeks of life, such as novelty exposure (Tang, 2001; Tang et al., 2006) or handling (Fenoglio et al., 2005; Meaney et al., 1988), predicts better adaptation and cognitive performance in adulthood. Conversely, early adversities such as an impoverished environment disrupt maternal behavior and the mother-pup interaction and impair hippocampal integrity, which may be manifested by attenuated or progressively deteriorated cognitive performance in the adult offspring (Brunson et al., 2005; Ivy et al., 2010; Rice et al., 2008). In humans, childhood traumatic events may lead to cognitive deficits during adult life, including deficits in attention, executive function, learning and memory (Hedges and Woon, 2011).

The neural mechanisms underlying the persistent effects of early experience on cognition have not been fully described. In the early life stages, there is a critical period when the central nervous system displays a heightened sensitivity to certain environmental factors (for instance, recurrent exposure to severe stressors). These risk factors may alter the trajectories of brain development and irreversibly influence the function of specific neural circuits (Andersen, 2003; Holtmaat and Svoboda, 2009). Dendritic spines, one of the key structural elements critical for synaptic function and plasticity, are highly dynamic and plastic (Segal, 2005). Interestingly, spines in the rat neocortex form and retract rapidly during the second postnatal week (Lendvai et al., 2000). The number of spines in the mouse neocortex also increases during the second and third postnatal weeks, but the turnover of spines decreases as the brain matures (Holtmaat et al., 2005). In accordance, synaptic density increases quickly during a short postnatal period, followed by a period of net synapse elimination (Holtmaat and Svoboda, 2009). Thus, a disruption of structural

and synaptic plasticity in key brain structures (for example, the hippocampus) during this critical period may permanently change the architecture of neural circuits, which subsequently contributes to functional impairments in adulthood (for example, impaired hippocampus-dependent declarative or spatial memory) (Fenoglio et al., 2006; Lupien et al., 2009). Consistent with previous findings, the data shown in **Chapter 3** revealed that early adversities induced spine loss in CA3 pyramidal neurons in adult wild-type mice, associated with the attenuation of LTP and a reduction in excitatory synapses in area CA3. Together, these data provide a detailed view on the structural substrates of early-life stress-induced cognitive deficits, and pinpoint the importance of environmental influences in the development of the brain and the programming of cognitive function.

Although early adversities are associated with defects in cognitive development and impaired stress responsiveness, a large proportion of individuals are resilient to early-life stress (Bradley et al., 2008; Feder et al., 2009). Recently, the CRH-CRHR1 system has been implicated in early-life stress-related psychopathologies (Binder and Nemeroff, 2010; Bradley et al., 2008; Smoller et al., 2005; Tyrka et al., 2009). In preclinical studies, evidence is accumulating on the involvement of the hippocampal CRH-CRHR1 system in modulating the programming effects of early-life experience on cognition (Brunson et al., 2001; Fenoglio et al., 2005; Heinrichs et al., 1996; Ivy et al., 2010). In line with these findings, we observed that the cognitive impairments in adult mice with early adverse experience were abolished by conditional inactivation of CRHR1 in forebrain neurons and mimicked by postnatal forebrain CRH overexpression. These findings delineate the importance of the CRH-CRHR1 system in mediating the effects of early-life stress as well as the neuroanatomical sites of action, and strengthen the notion that the complex and dynamic interactions between environmental risk factors and genetic predispositions may promote the onset of cognitive diseases (Lupien et al., 2009).

Early-life stress-induced cognitive alterations in adulthood used to be considered as maladaptive or detrimental. However, recent evidence demonstrates that the cognitive outcome of early adverse experience may have an adaptive value under specific conditions, that is, conditions reminiscent of the stressful environment early in life. Adult offspring receiving poor maternal care presented attenuated hippocampal LTP and spatial learning under non-stress conditions. Strikingly, in a highly stressful context, these animals showed enhanced LTP and learned better than the offspring of high maternal care mothers (Bagot et al., 2009; Champagne et al., 2008;

Oomen et al., 2010). Based on the match/mismatch concept proposed in recent years (Gluckman et al., 2009; Oitzl et al., 2010; Schmidt, 2011), neuropsychiatric disorders including cognitive diseases may not result from early-life adversity or susceptible genetic predisposition (except for rare mutations) *per se*, but instead from the interactions of both factors in combination with a mismatch of the early programmed and the later encountered environment.

Mechanisms and implications of cognitive impairments and structural alterations induced by chronic stress in adulthood: glucocorticoids or CRH?

The potential effects of chronic stress on hippocampal-dependent learning and memory have attracted much attention since the original reports that prolonged exposure to severe stress or excessive glucocorticoids in adult male rats compromised hippocampal neurons (Sapolsky et al., 1985) and their apical dendrites (Watanabe et al., 1992; Woolley et al., 1990), and reduced hippocampal volume (McEwen, 2000). A variety of spatial tasks has been applied in rodents and the results show that chronic psychological stress impairs spatial learning and memory (Conrad et al., 1996; Conrad, 2010; Luine et al., 1994; Venero et al., 2002), and may exacerbate cognitive deficits in animal models for cognitive diseases (Jeong et al., 2006; Tran et al., 2011). Consistent with these preclinical findings, chronic stress in humans may accelerate cognitive decline in older adults with mild cognitive impairments (Peavy et al., 2009). Moreover, clinical studies on stress-related disorders indicate that PTSD and depression patients have an impaired declarative memory (Bremner et al., 2004; Burt et al., 1995; Gilbertson et al., 2001) and reduced hippocampal volume (Bremner et al., 1995; Campbell et al., 2004). The close association between the impairments of hippocampus-dependent learning and memory and structural abnormalities in the hippocampus, such as atrophy of apical dendrites in CA3 pyramidal neurons (Magariños and McEwen, 1995; Watanabe et al., 1992), loss of excitatory glutamatergic synapses (Sandi et al., 2003; Sousa et al., 2000) and reorganization of the mossy fiber-CA3 synapses (Magariños et al., 1997; Stewart et al., 2005), underscores the importance of hippocampal integrity in the onset and development of cognitive deficits induced by chronic stress. Note that the loss of glutamatergic synapses and changes in the mossy fiber-CA3 synapses may reflect dendritic shrinkage and spine loss.

The causal relationship among glucocorticoid excess, dendritic retraction and cognitive impairments under chronic stress exposure remains to be determined. Sustained elevations of

glucocorticoids have been implicated in mediating the effects of chronic stress on dendritic morphology (Woolley et al., 1990) and spatial memory (Coburn-Litvak et al., 2003; Roozendaal et al., 2001; Wright et al., 2006), but the direct involvement of glucocorticoids in spatial memory deficits is absent in other studies (Conrad et al., 2007; Conrad et al., 1999; McLaughlin et al., 2007). As an alternative, it is hypothesized that chronic stress evokes CA3 dendritic shrinkage that disrupts HPA axis activity, and that both dendritic regression and the resultant glucocorticoid excess contribute to impaired hippocampus-dependent learning and memory (Conrad, 2006). However, hippocampal damage may be insufficient to disinhibit HPA axis activity and increase the levels of circulating glucocorticoids (Tuvnes et al., 2003). In addition, this hypothesis does not consider physiological alterations and structural changes in other hippocampal subregions such as CA1 dendritic complexity (Sousa et al., 2000) and adult neurogenesis in DG (Mirescu and Gould, 2006; Pham et al., 2003). Therefore, although it should be acknowledged that glucocorticoids partly mediate the deleterious effects of chronic stress on hippocampal morphology and cognition, the involvement of additional factors has to be explored.

As shown in **Chapter 4**, forebrain CRHR1 deficiency prevented object recognition memory impairments and attenuated spatial recognition memory deficits induced by chronic social defeat stress. Notably, postnatal CRHR1 inactivation did not influence spatial memory under basal conditions, which fits the idea that neuropeptides are crucial when the brain is challenged by stress (Hökfelt et al., 2003). At the cellular level, chronic social defeat stress reduced dendritic arborization in a CRHR1-dependent manner. These data demonstrate that CRH, acting through CRHR1, is another key stress mediator that modulates the cognitive and structural effects of chronic stress. Importantly, no apparent dysfunction of the HPA axis activity was found in stressed animals: hippocampal MR mRNA levels, basal plasma corticosterone levels and corticosterone response to acute stress were generally comparable among groups. Though enlarged adrenals and decreased hippocampal GR mRNA levels in stressed wild-type mice suggest a mildly disrupted HPA axis activity, it is unlikely that glucocorticoids fully account for the cognitive and morphological abnormalities in these animals.

The findings in **Chapter 4** underscore the importance of forebrain CRH and CRHR1 in mediating the impact of chronic stress on the brain and cognition. However, several issues remain to be addressed in future studies. First, it would be interesting to test the causal relationship among augmented hippocampal CRHR1 signaling, dendritic remodeling and stress-evoked

memory loss. Second, more studies are needed to characterize the potential interactions between CRH and glucocorticoids (and other stress mediators) in modulating the above-mentioned effects. Another interesting question yet to be answered is the spatiotemporal dynamics of CRH and CRHR1 during and after chronic stress exposure. Fourth, the mechanisms underlying the relatively rapid recovery of chronic stress-induced alterations (Conrad et al., 1999; Luine et al., 1994; McEwen, 2001) need to be investigated, although chronic stress-induced long-lasting changes have also been reported (Elizalde et al., 2008). Additionally, the differential effects of several factors, such as the intensity and controllability of the chronic stress (chronic predictable stress may facilitate spatial memory; see Parihar et al., 2011) and gender/sex hormones (McLaughlin et al., 2005) that may influence the cognitive performance under stress, should be considered.

In addition, we found that chronic stress acted via CRHR1 to regulate the expression of nectin-3, a synaptic CAM involved in synaptic formation and remodeling, in the mouse CA3 region. Interestingly, the sites of nectin-3 downregulation overlapped with the sites of dendritic shrinkage in CA3 pyramidal neurons in stressed wild-type mice. These findings encouraged us to examine also the effects of early-life stress on hippocampal nectin-3 expression and further, the potential role of nectin-3 in cognition and structural plasticity, which is discussed in the next section.

The nectin-afadin complex: a novel molecular machinery linking CRH-CRHR1 signaling to structural plasticity

The involvement of two synaptic adhesion systems, the neurexin-neuroligin complex and the nectin-afadin complex, in stress-induced cognitive decline and their potential interactions with the CRH-CRHR1 system is discussed in **Chapter 3-5**. The nectin-afadin complex emerges as a promising molecular candidate that may modulate stress- and augmented CRHR1 signaling-induced dendritic retraction, spine loss and cognitive impairments.

The nectin-afadin system consists of nectins (nectin 1-4; the function of nectin-2 and nectin-4 in the central nervous system has not been well described) and the F-actin binding protein l-afadin (the other isoform of afadin, s-afadin, lacks the actin filament binding domain) (Beaudoin, 2006; Takai et al., 2008a). Nectin-1 is preferentially localized at axon terminals while nectin-3 is present in both axons and dendrites. The differential distribution patterns of the two molecules allow for a strong, Ca^{2+}-independent heterophilic adhesion between presynaptic nectin-1 and

postsynaptic nectin-3, which is crucial for the establishment of synaptic contacts (Parrish et al., 2007). Indeed, overexpressing nectin-1 in dendrites causes aberrant and unstable dendrodendritic contacts that rarely result in productive adhesions (Togashi et al., 2006). An interesting aspect of the nectin-afadin system is that it colocalizes and cooperates with the cadherin-catenin complex, which mediates Ca^{2+}-dependent homophilic adhesion, to modulate the formation, stabilization and remodeling of synapses during development (Abe et al., 2004; Majima et al., 2009; Mizoguchi et al., 2002; Tan et al., 2010; Togashi et al., 2006; Yu and Malenka, 2003).

One of the main findings presented in **Chapter 5** is that nectin-3 may regulate structural remodeling in both dendrites and spines in the adult mouse hippocampus, highlighting the essential roles of nectin-mediated synaptic adhesion in structural and, possibly, functional plasticity in the mature brain. The evidence in **Chapter 4** and **Chapter 5** further reveals an association of cognitive deficits induced by either early-life stress or chronic stress in adulthood with decreased levels of nectin-3 in the hippocampal CA3 region. Notably, suppression of nectin-3 function in the hippocampus could impair spatial learning and long-term memory, reproducing the effects of repeated stress exposure on cognition. Therefore, our studies provide evidence that the novel synaptic CAM nectin-3 connects structural remodeling to cognitive function.

Moreover, we found that the modulation of nectin-3 expression by stress was dependent on CRHR1 signaling, and that CRHR1 and nectin-3 partially colocalized in neurons in the central nervous system. Based on these observations**,** the nectin-afadin complex appears to be a promising molecular candidate linking CRH-CRHR1 signaling to structural/synaptic plasticity. Intriguingly, a small proportion of nectin-3 has been found at postsynaptic sites (examined by immunogold staining and electron microscopy; personal communication by Dr. Igor Kraev), raising the possibility that CRHR1 and nectin-3 may interact directly. Nonetheless, as the majority of nectin-3 protein resides at adherens junctions, an interesting question to be answered is the molecular machineries bridging CRHR1 and nectin-3. Several studies suggest cofilin and Rho GTPases (including Rac and CDC42) as candidate targets shared by the CRH-CRHR1 system and the nectin-afadin complex (Chen et al., 2008; Takai et al., 2008a; Takai et al., 2008b). Screening of such molecules used by different synaptic adhesion systems (the nectin-afadin complex, the cadherin-catenin complex and the neurexin-neuroligin complex) and the CRH-CRHR1 system may provide insight into the synaptic mechanisms underlying the consequences

of stress on learning and memory. Additionally, the potential interplays between nectin-3 and other stress mediators merit future investigations.

Summary

Based on the multiple levels of evidence provided in the previous chapters, a more detailed view on the mechanisms underlying early-life stress/chronic stress-induced cognitive deficits emerges, which can be summarized as follows (partly illustrated in Figure 1):

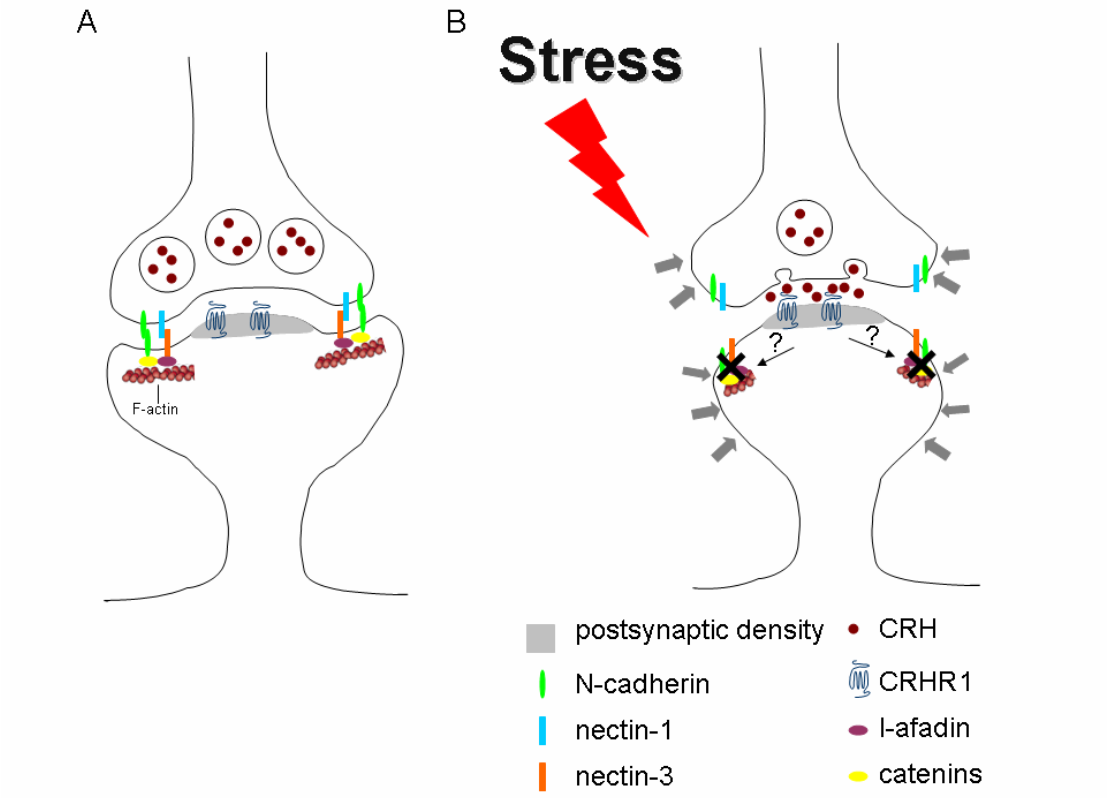

Figure 1. Molecular mechanisms of stress-induced spine disintegration. **(A)** Under normal conditions, spine morphology is maintained by stable axodendritic adhesion mediated via the nectin-afadin complex and cadherin-catenin complex. **(B)** Upon exposure to a severe stressor, CRH is rapidly released from presynaptic terminals and binds to CRHR1 anchored to the postsynaptic density. The activation of CRHR1 dephosphorylates cofilin (an actin-regulating protein; not shown in the figure) (Chen et al., 2008) and dysregulates the nectin-afadin and cadherin-catenin complexes through unidentified machineries, leading to impaired axodendritic adhesion and spine destabilization.

1. The CRH-CRHR1 system may interact with the nectin-afadin complex and the cadherin-catenin complex through specific scaffold/adaptor proteins or intracellular signaling

molecules.

2. Upon severe stress challenge, CRH is released from the presynaptic terminals of hippocampal interneurons and binds to CRHR1 that aggregates at the postsynaptic density of the dendritic spines of hippocampal pyramidal neurons.
3. The activation of CRHR1 inhibits the expression and function of nectin-3 in hippocampal pyramidal neurons, disrupts adherens junction function and synaptic adhesion, and weakens synaptic strength.
4. Reduced axodendritic adhesion impairs synaptic transmission, which may disrupt cognitive function.
5. Recurrent stress exposure either early in life or in adulthood leads to long-term impairments in axodendritic adhesion, resulting in spine destabilization and dendritic elimination, and finally spatial learning and memory deficits.

In conclusion, the findings described in this thesis broaden the CRH-CRHR1 hypothesis of repeated stress-induced cognitive deficits and provide further evidence on its underlying molecular mechanisms. Synaptic CAMs, especially nectin-3, are promising molecular targets to manipulate cognition and treat cognitive impairments.

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List of abbreviations

5-HTT	serotonin transporter
A	adrenaline
AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AFR	animal facility rearing
ANOVA	analysis of variance
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BGH	bovine growth hormone
CA1	cornu ammonis region 1
CA3	cornu ammonis region 3
CAM	cell adhesion molecule
CKO	conditional knockout
CN	communal nest
COE	conditional overexpression
CORT	corticosterone
CRH	corticotrophin-releasing hormone
CRH-BP	corticotrophin-releasing hormone binding protein
CRHR1	corticotrophin-releasing hormone receptor 1
DG	dentate gyrus
DNA	deoxyribonucleic acid
ED	early deprivation
EGFP	enhanced green fluorescent protein
EH	early handling
fEPSP	field excitatory postsynaptic potential
FKBP5	FK506 binding protein 5
FST	forced swim test
GCs	glucocorticoids
GR	glucocorticoid receptor
HFS	high-frequency stimulation

HPA axis	hypothalamic-pituitary-adrenal axis
IML	inner molecular layer
ITI	intertrial interval
KO	knockout
LC	locus coeruleus
LPS	lipopolysaccharide
LTP	long-term potentiation
MAP2	microtubule-associated protein 2
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
MS	maternal separation
NA	noradrenaline
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NH	non-handled
OE	overexpression
P	postnatal day (also pnd)
PB	phosphate buffer
PKR	protein kinase RNA-activated
PSD	postsynaptic density
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
RNA	ribonucleic acid
ROI	region of interest
RSG	retrosplenial granular cortex
Rt	reticular nucleus
RT	room temperature
SCCP	Schaffer collateral-commissural pathway
SEM	standard error of the mean
shRNA	short hairpin RNA
SHRP	stress hyporesponsive period

SN	standard laboratory conditions
SNRI	selective serotonin and noradrenaline reuptake inhibitor
SNS	sympathetic nervous system
SSRI	selective serotonin reuptake inhibitor
UTP	uridine triphosphate
VGAT	vesicular gamma-aminobutyric acid transporter
VGLUT1	vesicular glutamate transporter 1
VPL	ventral posterolateral nucleus
WPRE	Woodchuck post-transcriptional regulatory element
WT	wild-type
YFP	yellow fluorescent protein

Curriculum vitae

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Poster presentations

1. **Wang XD**, Rammes G, Wolf M, Scharf SH, Liebl C, Baram TZ, Deussing JM, Müller MB, Schmidt MV. Dissection of hippocampal CRH-CRHR1 signaling in early life stress-induced learning and memory deficits. **ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe**, Nice, France, March 2010.

2. Wolf M*, **Wang XD***, Scharf SH, Liebl C, Deussing JM, Baram TZ, Müller MB, Schmidt MV. Involvement of novel synaptic cell adhesion molecules in different mouse models of

cognitive impairment. **The 38th Annual Meeting of the Society for Neuroscience**, Washington, DC, USA, November 2008 (* shared authorship).

3. **Wang XD**, Wolf M, Liebl C, Scharf SH, Rice CJ, Baram TZ, Deussing JM, Müller MB, Schmidt MV. Effects of chronic stress in conditional corticotropin-releasing hormone receptor 1 knockout mice. **The 26th Collegium Internationale Neuro-Psychopharmacologicum Congress**, Munich, Germany, July 2008.

Oral presentations

1. Dissection of hippocampal CRH-CRHR1 signaling in early life stress-induced learning and memory deficits. **ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe**, Nice, France, March 2010.

2. Synaptic cell adhesion molecules, stress, and memory loss. **The 2nd Summer School of the International Research and Training Group**, Leiden, the Netherlands, June 2008.

List of publications

1. **Wang XD**, Wagner KV, Su YA, Hartmann J, Liebl C, Wolf M, Wurst W, Holsboer F, Deussing JM, Müller MB, Schmidt MV. Nectin-3 mediates stress-induced cognitive decline and structural remodeling. Manuscript in preparation.

2. **Wang XD**, Rammes G, Kraev I, Wolf M, Liebl C, Scharf SH, Rice CJ, Wurst W, Holsboer F, Deussing JM, Baram TZ, Stewart MG, Müller MB, Schmidt MV (2011). Forebrain corticotropin-releasing hormone receptor 1 is required for early life stress-programmed cognitive deficits. Submitted.

3. **Wang XD**, Chen Y, Wolf M, Wagner KV, Liebl C, Scharf SH, Harbich D, Mayer B, Wurst W, Holsboer F, Deussing JM, Baram TZ, Müller MB, Schmidt MV (2011). Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. **Neurobiology of Disease**, in press.

4. Su YA, **Wang XD**, Li JT, Guo CM, Feng Y, Yang Y, Huang RH, Si TM (2011). Age-specific effects of early MK-801 treatment on working memory in female rats. **NeuroReport**, in press.

5. Schmidt MV, **Wang XD**, Meijer OC (2011). Early life stress paradigms in rodents: potential animal models of depression? **Psychopharmacology (Berl)** 214, 131-140.

6. Wagner KV, **Wang XD**, Liebl C, Scharf SH, Müller MB, Schmidt MV (2011). Pituitary glucocorticoid receptor deletion reduces vulnerability to chronic stress. **Psychoneuroendocrinology** 36, 579-87.

7. Feng Y, **Wang XD**, Guo CM, Yang Y, Li JT, Su YA, Si TM (2010). Expressions of neuregulin 1β and ErbB4 in prefrontal cortex and hippocampus of a rat schizophrenia model induced by chronic MK-801 administration. **Journal of Biomedicine and Biotechnology** 2010, 859516.

8. **Wang XD**, Su YA, Guo CM, Yang Y, Si TM (2008). Chronic antipsychotic drug

administration alters the expression of neuregulin 1β, ErbB2, ErbB3, and ErbB4 in rat prefrontal cortex and hippocampus. **The International Journal of Neuropsychopharmacology** 11, 553-561.

9. Su YA, Si TM, Zhou DF, Guo CM, **Wang XD**, Yang Y, Shu L, Liang JH (2007). Risperidone attenuates MK-801-induced hyperlocomotion in mice via the blockade of serotonin 5-HT $_{2A/2C}$ receptors. **European Journal of Pharmacology** 564, 123-130.

Acknowledgements

The work appeared in this dissertation is the result of fruitful cooperation among excellent scientists from Germany and abroad. First and foremost, I am grateful to Dr. Mathias Schmidt, my mentor and friend from whom I learned the art of scientific research, for supervising my doctoral studies, revising the thesis and offering consistent encouragement and support. I also benefit greatly from advice and help given by Dr. Marianne Müller.

I wish to acknowledge the following collaborators for contributing valuable data and ideas and allowing them to be used in this thesis: Dr. Gerhard Rammes prepared and analyzed the electrophysiological data in Chapter 3; Dr. Igor Kraev prepared the dendritic spine data in Chapter 3; Dr. Jan Deussing and colleagues generated all the transgenic mouse lines used in this thesis; Dr. Yuncai Chen provided the dendritic morphology data in Chapter 4 and commented on the morphological analysis in Chapter 5. My special thanks go to Prof. Tallie Baram, who helped us set up the early-life stress paradigm and gave us critical suggestions and comments in Chapter 3 and Chapter 4.

I have the great fortune to work with my colleagues: Bianca Mayer, Christiana Labermaier, Christine Kohl, Dr. Claudia Liebl, Daniela Harbich, Jakob Hartmann, James Haiyang Xu, Klaus Wagner, Miriam Wolf, Sebastian Scharf, Dr. Yuji Kitaichi, and Dr. Yun-Ai Su, whose input has significantly enhanced this thesis and is highly appreciated!

I would like to thank Dr. Carsten Wotjak, Dr. Christoph Thöringer, Dr. Francesco Roselli, and Dr. Matthias Eder for marvelous technical guidance. I also warmly thank Claudia Kühne, Marcel Schieven, Robert Menz, and Stefanie Unkmeir for their technical assistance.

I would like to thank my parents and my wife for their understanding, encouragement, and love, which make my scientific endeavor in a country thousands of miles away not so lonely and unbearable.

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 31.03.2011