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SEX HORMONE – DEPENDENT – RANK SIGNALING IN LEARNING AND MEMORY

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ABSTRACT

Sex hormones have multiple targets in the body and in the brain where they exert ubiquitous effects on mood and memory. Depression, anxiety disorders and cognitive deficits are one of the most common symptoms women in their 50s experience when they are entering menopause characterized by the decline of ovarian sex hormones. Since the world population is ageing significantly and women have a higher life expectancy than men they stay for more than one third of their life in an estrogen-deficient state, which makes them twice as likely to develop any kind of behavioral disorder compared to age-matched males.

Receptor-activator of NFκB ligand (RANKL), its tumor necrosis factor (TNF)-family receptor RANK and its decoy receptor osteoprotegerin (OPG) are key units in bone remodeling and are tightly regulated by sex hormones. Estrogen is known to upregulate OPG, whereas progesterone triggers RANKL expression. However the functional relevance of the RANK/RANKL/OPG axis in the brain especially in memory and mood known to be affected by estrogen and progesterone was entirely unknown. Here we report that RANK which is localized in neurons in the ventromedial hypothalamic nucleus (VMH) and RANKL have an important role in learning and memory but not in mood. Using tissue-specific Nestin-Cre and CamKII-Cre deleter mice it was shown that the depletion of RANK does not alter inhibitory avoidance (IA) learning and emotional behavior neither in female nor in male mice. However upon removal of the ovaries in Nestin-Cre $rank^{floxed}$ mice, mimicking postmenopause, the females exhibit severe learning deficits suggesting that RANK is necessary for memory formation in the absence of ovarian sex hormones. Mechanistically, the reduction of estrogen and progesterone triggers the upregulation of serum RANKL levels and downregulation of

OPG levels. Brain RANKL and OPG on protein and mRNA level are apparently not regulated by sex hormones, suggesting that the peripheral regulation of RANKL and OPG is affecting cognition.

Taken together, these data suggest that the RANK/RANKL/OPG system is an important player in learning and memory in female mice through peripheral regulation of RANKL and OPG.

ZUSAMMENFASSUNG

Geschlechtshormone spielen eine große Rolle in der Funktion des menschlichen Körpers, vor allem im Gehirn, wo sie ubiquitäre Einflüsse auf Stimmung und Gedächtnis haben. Dieser Einfluss zeigt sich vor allem durch den Rückgang von ovarialen Geschlechtshormonen in der Menopause, welche bei Frauen ab dem 50. Lebensjahr eintritt und häufig Symptome wie Depressionen, Angst- und Gedächtnisstörungen hervorruft. Durch die deutlich gestiegene Lebenserwartung, die bei Frauen allgemein höher ist als bei Männern, verbringen Frauen heutzutage länger als ein Drittel ihres Lebens nach der Menopause. Dies ist unter anderem eine der Hauptursachen warum Frauen ein doppelt so hohes Risiko für Verhaltensstörungen haben als Männer gleichen Alters.

Receptor-activator of NFκB ligand (RANKL), sein Rezeptor RANK und sein Decoy Rezeptor Osteoprotegerin (OPG) werden durch Geschlechtshormone reguliert und sind wesentlich am Knochenmetabolismus beteiligt. Östrogen stimuliert die Produktion von OPG, wobei Progesteron hingegen die Expression von RANKL anregt. Die funktionale Relevanz von der RANK/RANKL/OPG Achse im Gehirn besonders für das Gedächtnis und die Stimmung, das durch Östrogen und Progesteron beeinflusst wird, war jedoch bisher unbekannt.

In der vorliegenden Arbeit wird gezeigt, dass RANK, welcher in den Neuronen des ventromedialen Hypothalamus (VMH) lokalisiert ist und RANKL eine wichtige Rolle für Lernen und Gedächtnis, jedoch nicht für Stimmung tragen. Anhand von gewebs-spezifischen Nestin-Cre und CamKII-Cre deleter Mäusen wurde experimentell bewiesen, dass der Verlust von RANK das Lernverhalten im inhibitory avoidance (IA) Test und das emotionale Verhalten weder in weiblichen noch in männlichen Mäusen verändert. Interessanterweise bewirkte die

Entfernung von den Eierstöcken in Nestin-Cre *rank*^{floxed} Mäusen, welche die Postmenopause imitiert, schwere Lernverluste im IA Test. Dies deutet darauf hin, dass RANK bei einem Mangel an Östrogen für den Lernprozess unerlässlich ist. Mechanistisch gesehen wird durch den Verlust von Östrogen und Progesteron die Konzentration von RANKL im Serum erhöht und der OPG Level vermindert. RANKL und OPG mRNA und Protein werden im Gehirn offensichtlich nicht durch Geschlechtshormone reguliert, was darauf hinweist, dass die periphere Regulation von RANKL und OPG das Lernvermögen beeinflusst.

Zusammenfassend zeigen diese Resultate, dass das RANK/RANKL/OPG System eine essentielle Rolle im Lernen und Gedächtnis spielt.

1. INTRODUCTION

Populations around the globe are ageing rapidly. The percentage of people 60 years of age or older increased from 9.2 in 1990 to 11.7 in 2013. Projections indicate that this percentage will grow to 21.1 by 2050 and that the majority of the older population will be female. This disproportionality increases with age due to higher life expectancy of females, which is projected to be around 90 years of age in 2050 (United Nations, 2013).

Increasing life expectancy is associated with higher morbidity and prolonged life-time spent in dependency. The older population will mainly suffer from "the four giants of geriatrics", memory loss, depression, urinary incontinence and falls or immobility. Women are especially affected due to higher life expectancy and due to a loss of sex hormones that occurs at menopause. Thus, women will spend one third of their lives in menopause. In contrast, men are reproductively-capable well into their 80ties. Therefore, the process of ageing and the related diseases will gain more importance in biomedical research in order to improve the quality of life in particular the life of women (Lobo, 2014; United Nations, 2013).

1.1. Mood and Emotions

Emotions are defined as consistent responses to internal or external events, which means that emotions are not only created through external stimuli but they are also influenced by hormones and neurotransmitters such as dopamine, noradrenaline, serotonin, oxytocin, cortisol, or estrogen (Kandel et al., 2000c; Neumann, 2000). Emotional responses range from euphoria to elation, pleasure, surprise, anger, anxiety, disappointment, sadness, grief, despair, or depression. Emotions are closely connected to the arousal of the nervous system resulting in neural, physical and psychological changes that influence behavior and cognition. Thus emotions and cognition are interconnected. An emotional state lasting for weeks or more is defined as mood. Significant disturbances in a persistent emotional state or mood of humans and animals are characterized as mood disorders. Common forms of mood disorders are depression and anxiety (Kandel et al., 2000c; Purves et al., 2001). It has been shown that anxiety disorders have a lifetime prevalence of about 30 % and they contribute to developing depression (Kessler et al., 2005; Tye et al., 2011). However the symptoms of depression and anxiety disorders are extensive and heterogeneous and cover emotional, motivational, cognitive and physiological domains, which makes it hard to fully model certain kinds of mood disorders in rodents.

The limbic system is a complex of different brain structures located on both sides of the thalamus. It is primarily responsible for the emotional life and partly involved in the formation of memories (Catani et al., 2013; Rajmohan and Mohandas, 2007). It regulates endocrine and autonomic functions upon emotional stimuli such as altering the heart rate or the cutaneous blood flow. The limbic system includes the hippocampus, the amygdala, and parts of the neocortex which create the input and processing side, which means that the environmental information is received and subsequently processed and adjusted. The processed information is used to control effectors such as the olfactory bulb, some thalamic and septal nuclei together with the hypothalamus providing output of the limbic system. Most of the regions are interconnected with each other in order to allow information transfer (Catani et al., 2013; LeDoux, 2000; Mac, 1949; Rajmohan and Mohandas, 2007).

A lot of what we know about the anatomy of mood came from seminal studies in rodents, where many assays have been developed that allowed us to dissect relevant brain regions and molecular mechanisms underlying mood (Hrabé de Angelis et al., 2006). In the present study we took advantage of several such tests to study anxiety and depression. In brief, we examined anxiety levels in mice by using the elevated plus maze (EPM) test and assessed depression with the forced swim test (FST), tail suspension test (TST) and the sucrose preference test (SPT) detailed below.

1.1.1. Elevated plus maze (EPM)

The EPM is a commonly used and validated behavioral test for rodents to study and identify brain regions and mechanisms underlying anxiety-related behavior (Lister, 1987; Pellow et al., 1985). Moreover it is used to analyze or identify novel anxiolytic drugs, which decrease anxiety, and anxiogenic drugs, which increase anxiety. Briefly, in this task the rodent is placed on the central platform of the plus-shaped elevated apparatus consisting of two open and two enclosed arms. Duration or time spent exploring each arm are recorded. During this time the rodent is faced with a strong approach-avoidance conflict. Compared to other anxiety assays using noxious stimuli such as electric shock, loud noises and predator odor which generate a conditioned response, EPM reflects an unconditioned fear response to open spaces and heights and the rodent's preference for dark, enclosed safe spaces called thigmotaxis (Pellow et al., 1985). Depending on the kind of anxiety-test used different brain regions and neural circuits are activated (Duncan et al., 1996; Hale et al., 2006; Knapp et al., 1998; Sandner et al., 1993). It has been shown by Fos immunoreactivity, a marker for neuronal activity, that exposure to the EPM involves the medial prefrontal, cingulated and ventrolateral orbital cortices, nucleus accumbens (NAcc), the paraventricular nucleus of the hypothalamus (PVH), the dorsomedial hypothalamus (DMH), the amygdala and the lateral septum (LS) (Duncan et al., 1996). Moreover, the amygdala microcircuitry including the basolateral amygdala (BLA), the central amygdala (CeA), the centrolateral (CeL) and the centromedial (CeM) nuclei plays a central role in anxiety behavior in the EPM test (Tye et al., 2011). Likewise, in

humans, it has been reported that patients having generalized anxiety disorders exhibit abnormal activity in the BLA and CeM (Etkin et al., 2009).

1.1.2. Forced swim test (FST)

The FST is a widely used task to assay for depression (Porsolt et al., 1977b). Many human studies reported that the exposure to stress and adversity are critical factors that increase the risk for developing depression (Agid et al., 2000; Agid et al., 1999; Caspi et al., 2003). The FST can partly model this type of depression as it uses acute stress and examines how the rodent can deal with it. Placing a rodent in an inescapable and stressful situation for a short period of time creates acute stress. It is marked by an immediate burst of activity while the animal is struggling to escape, followed by complete immobility when the animal gives up escape attempts. In models of depression, animals which are depressed give up struggling significantly earlier and spend longer time immobile during the testing phase (Castagne et al., 2011). This behavior can be reversed with anti-depressants and has been shown to be independent of fatigue (Porsolt et al., 1979; Porsolt et al., 1977a). In this particular task the rodent gets placed in a cylinder filled with water for 10 minutes. The animal's activity is recorded, and immobility is automatically interpreted. The tail suspension test (TST) which is the "dry" version of the FST, is also based on the adoption of a passive response in a stress situation. However instead of exposing the rodent to water, it gets suspended by its tail for 6 minutes (Choi et al., 2013; Krishnan and Nestler, 2008).

Several studies showed by testing antidepressant drugs that the hypothalamic-pituitary-adrenal (HPA) axis is involved in the behavioral response in the FST and the TST (Wang et al., 2013; Yamada et al., 2013). The HPA axis includes PVH containing neuroendocrine neurons that regulate the pituitary gland and the adrenal cortex. Projections from the amygdala and the hippocampus lead to the hypothalamus and facilitate the activation of the HPA axis (Smith and Vale, 2006). Moreover it has been reported that the amygdala is a critical part in the behavioral response in the FST by assessing injection of antidepressant drugs directly into the amygdala (Duncan et al., 1986).

1.1.3. Sucrose preference test (SPT)

The SPT is based on reward and does not cause the animal stress, unlike the FST and TST (Moreau, 1997). Many humans suffering from depression have a reduced response to positive emotional stimuli like food, sex and social interaction. Likewise, healthy rodents have an innate preference for sweet food, however, those suffering from depression, do not. In the SPT the rodents is given a free choice between water and sucrose fluid over several consecutive days. Failure to develop a preference to sucrose is indicative for anhedonia and depression and can be reversed with anti-depressants (Russo and Nestler, 2013).

It has been shown that the reward-circuitry plays a crucial role in depression. The core of this circuit comprises dopaminergic neurons in the ventral tegmental area (VTA) belonging to the midbrain which project to GABAergic neurons in the nucleus accumbens (NAcc) which is part of the striatum. Moreover this core unit receives innervations from the prefrontal cortex (PFC), hippocampus, the amygdala and the lateral hypothalamus (LH) being also partly involved in depression-like behavior (Krishnan et al., 2007; Nestler and Carlezon, 2006; Russo and Nestler, 2013).

1.2. Learning and Memory

Knowledge is gained about the world through learning and memory is described as the mechanism for storing such learnt information. When the acquired knowledge is consciously or unconsciously retrieved, this is called recall (Sweatt, 2010a). Different anatomical structures and combinations of regions in the brain are used for different types of memories (Sweatt, 2010a). Memory can be either short-lived or can last for the lifetime of the organism. Depending on the behavior-altering stimulus, long lasting memory can require repetition or can be made by a single event. For example, in the fear-based learning paradigm for rodents, the inhibitory avoidance (IA) task, a single electrical shock administered to rodents leads to long-lasting memory (Whitlock et al., 2006). During long-term memory a period of consolidation

happens which underlies a set of cellular and molecular processes including synthesis of new RNA and proteins causing formation of new synapses changes in synaptic structures and circuits. Short-term memory relies more on persistent firing of neuronal action potentials which involves signaling events such as phosphorylation, activation of ion channels and calcium influx and changes in the release and function of neurotransmitters at particular synapses (Goelet et al., 1986; Kandel et al., 1986; Sweatt, 2010a).

Two different forms of memory exist: 1. Implicit or non-declarative memory and 2. Explicit or declarative memory (Sweatt, 2010a, b). Implicit memory is gained without conscious awareness, like learning to ride a bicycle or to walk, whereas explicit memory underlies the conscious recall of previously learnt facts and experiences (Sweatt, 2010a, b). Any kind of gained information is first processed in one of the cortical areas such as temporal, parietal, cingulated, olfactory and prefrontal cortex which react to visual, auditory and somatic signals (Figure 1.1). In order to reach long-term storage of declarative memory the information of the behavior-modifying stimulus from the cortical areas is transmitted to the parahippocampal cortex and further to the hippocampus. However, depending on the stimulus the hippocampal regions are communicating with other brain parts such as the amygdala and the hypothalamus which are mainly involved in emotional memory (Kandel et al., 2000a; Sweatt, 2010b).

Implicit memory including motor memory mostly involves the striatum, cerebellum, brainstem, parts of the amygdala and some specific sensory and motor systems (Sweatt, 2010a, b). Although these different brain regions work independently they can operate as parallel processors in order to increase the overall “memory throughput” of the CNS. In figure 1.1 the complex information storage and the communication between the different parts of the brain are indicated.

There are many different learning tests which are used to gain a better insight into learning and memory and the neurobiology of different disorders (Crawley, 2007). In the present study we used the prepulse inhibition (PPI) task to examine basic synaptic functions and the inhibitory avoidance (IA) test to analyze fear-based learning.

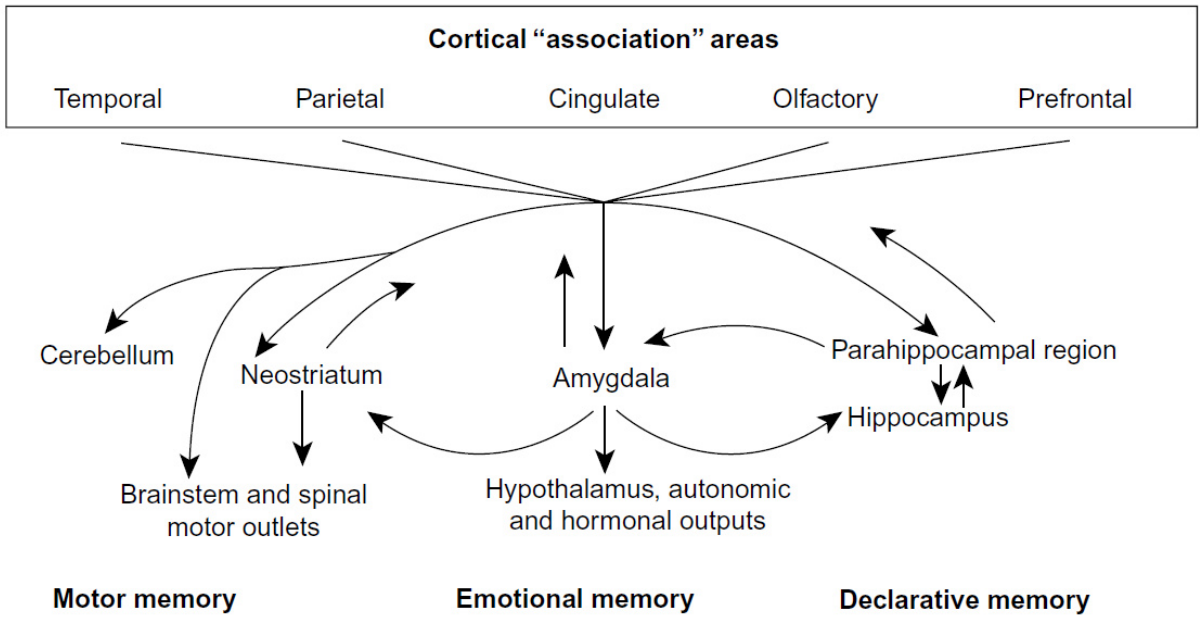


Figure 1.1: A current model of the memory system, how it works and which parts of the brain are involved.

All kinds of information such as visual, auditory and somatic signals are first processed in one of the cortical areas including temporal, parietal, cingulated, olfactory and prefrontal cortices. Depending on the information it gets then transmitted to different parts of the brain. The storage of declarative memory occurs mostly in the hippocampal regions whereby communication to the amygdala and hypothalamus is possible. The implicit memory including motor memory involves the cerebellum, striatum, brainstem and partly the amygdala. (adopted from Sweatt, 2010b)

1.2.1. Prepulse inhibition (PPI)

PPI is a test which analyzes basic reflexes and basic synaptic functions of the rodent (Basavaraj and Yan, 2012). It tests the simple ability of the organism to ignore non-harmful stimuli (Larrauri and Schmajuk, 2006; Li et al., 2009). The PPI involves an altered response to an auditory stimulus. The rodent is presented to an auditory startling stimulus, reaction to which is altered by fear, attention, habituation or sensitization etc. In the second part of the test, a weaker prestimulus, the prepulse, precedes the startling stimulus. A healthy rodent will react with an attenuated startle response. The underlying neural circuit in this task involves three parts: first the auditory system detects the acoustic signal and differentiates the signal. The information gets then transferred to the limbic system which is mainly responsible for

cognitive information processing and modulates the activity of the motor system through the caudal pontine reticular nucleus (Swerdlow et al., 2001). It has also been shown that parts of the hypothalamus are involved in PPI. For example Dashti et al reported that leptin which acts mainly in the VMH and the arcuate nucleus decreases the startle response (Dashti et al., 2013).

The PPI was originally used in human neuropsychiatric research to measure sensorimotor gating, or basic synaptic function. People with PPI deficits are often diagnosed with mental and neurodegenerative diseases like schizophrenia, Alzheimer's Diesease or Parkinson's disease. Nowadays PPI is a commonly used method for screening antipsychotic drugs. (Valsamis and Schmid, 2011)

1.2.2. Inhibitory avoidance (IA)

To test long-term memory a well-established form of fear conditioning, called the inhibitory avoidance task is used. IA is similar to fear conditioning a form of classical conditioning pioneered by Ivan Pavlov in the 1920s (Pavlov, 1927). It is based on the learning process that certain environmental stimuli forecast aversive events, which is of great importance in the wild as it is used in the animal's defensive behavior system (Fanselow, 1994). In the last 10 years fear conditioning and IA gained more and more attention as they are seated in the interface of memory and emotion.

The IA apparatus consists of two compartments, one is bright illuminated whereas the other is dark. Both are separated with a sliding door. During the training the mouse is place in the lit box. After a few seconds the door opens and the mouse has now the choice to enter the dark box containing an electrified floor grid. Rodents naturally tend to move to the darkness as they are nocturnal animals. Once the mouse enters the dark chamber the door shuts and the animal receives a mild foot shock. In the testing phase when the animals are placed back into the light box, the latency to crossover to the dark side is measured and is termed IA memory (Brioni et al., 1989). The IA task underlies several phases which are distinct on the molecular level. The acquisition of the task is characterized by a labile disruptable stage of memory.

Through a process of consolidation, accompanied with RNA and protein synthesis, the memory is converted into a long-lasting state. The recall of memory turns it back to a labile state which is referred to as reconsolidation. (Bryan et al., 2009; Curzon et al., 2009; Maren, 2001; Nader, 2003; Wilensky et al., 2000)

There is still very little known about the molecular events that underlying memory consolidation and reconsolidation. However it has been shown that protein synthesis (Nader et al., 2000) and the expression of the transcription factors CCAAT enhancer binding protein β (C/EBPβ) and nuclear factor-κB (NF- κB) are essential in different brain regions such as the amygdala and the hippocampus during consolidation and reconsolidation in the IA test (Freudenthal et al., 2005; Milekic et al., 2007; Taubenfeld et al., 2001a; Taubenfeld et al., 2001b). Furthermore, it has been reported that the activity of Src family tyrosine kinases is required in the hippocampus during formation and retrieval of IA memory (Bevilaqua et al., 2003; Pereira et al., 2007).

The neuronal circuits critical in the formation and retrieval of IA memories are described in figure 1.2. Several brain regions have been shown to participate in this fear circuit including the amygdala, hippocampus and hypothalamus (Gross and Canteras, 2012). The amygdala mediates fear responses and it is also able to associate the stimulus with emotional output, in this case with fear. The short-lasting and mild electric foot shock, animals receive in the IA task, reaches the amygdala via two pathways (LeDoux, 2003). One pathway is quickly processed through the sensory thalamus to the amygdala. Output projections from the amygdala lead to the hypothalamus which initiates immediate fear responses. A detailed description of fear processing in the hypothalamus will be given in following chapter. The indirect pathway involves the cortex and the hippocampus in order to further process the information of the stimulus and to create an association with the context in which the shock was received. In this case the brain will “look at all options” and “decide” whether or not to fear the stimulus (Gross and Canteras, 2012; LeDoux, 2000) .

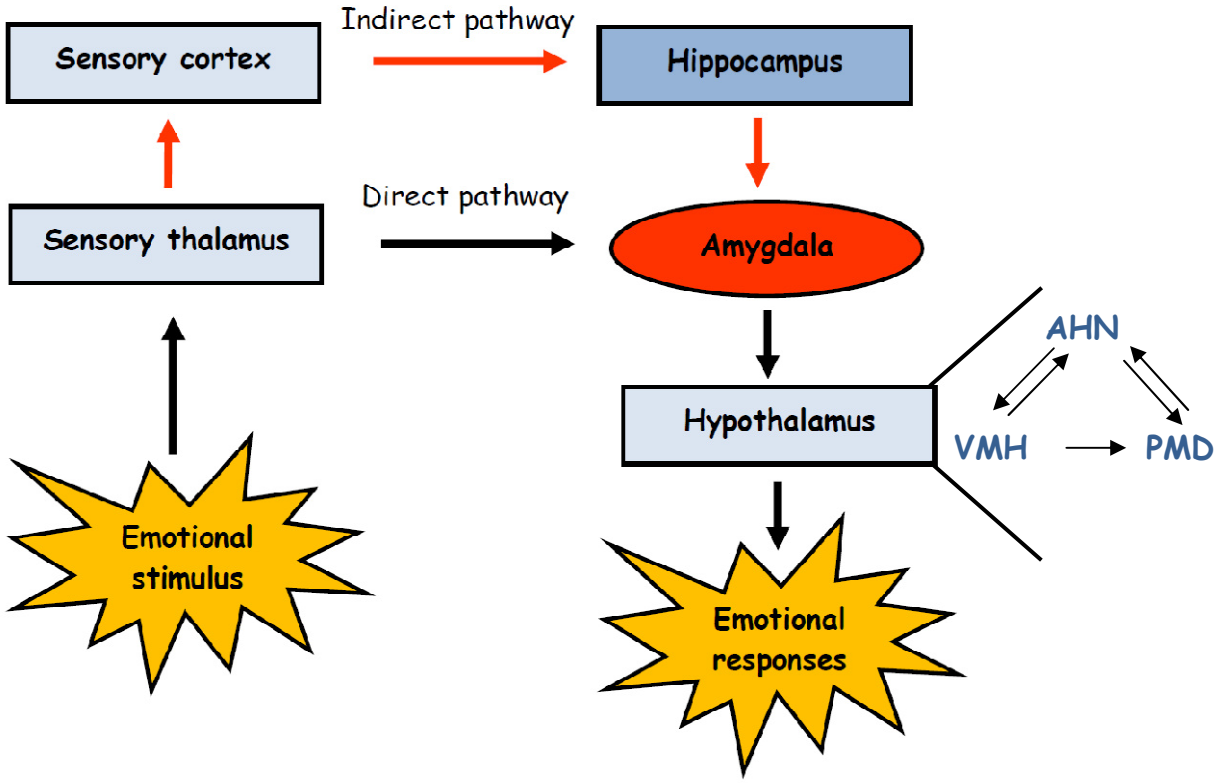


Figure 1.2: Fear circuit.

Two pathways exist in processing the information of an emotional stimulus. The direct pathway involves the sensory thalamus, amygdala and the hypothalamus which senses immediate fear responses. The indirect pathway takes longer as the information of the stimulus gets further processed and context is established by involving additionally the sensory cortex and the hippocampus. The triangle shows a more detailed description of the information flow in the hypothalamus which involves the ventromedial hypothalamus (VMH), the anterior hypothalamic nucleus (AHN), and the premammillary nucleus (PMD).

1.3. Hypothalamus

The hypothalamus is a portion of the brain located at both sides of the third ventricle right below the thalamus. It belongs to the limbic system which regulates emotional life and links the nervous system to the endocrine system via the pituitary gland. The synthesized releasing hormones in the hypothalamus activate or inhibit the secretion of the pituitary hormones (Guillemin, 2005). The main functions of the hypothalamus include the regulation of metabolic processes, the autonomic nervous system, emotional and sexual behavior (Kandel et al., 2000b). It does so via the integration of many different external and internal signals. Amygdala and hippocampus process emotional reactions and memory through their tight connection with the hypothalamus (Kandel et al., 2000b). As specific regions of the hypothalamus lack the blood brain barrier (BBB) it also serves as the brain's "window" to the homeostasis of the organism, and is sensitive to many cues from the body: gonadal steroids, autonomic inputs, stress, invading microorganisms and blood-borne stimuli such as leptin and insulin as well as large proteins and small cytokines such as tumor necrosis factors (TNFs) to which receptor activator of NFκB (RANKL) belongs (Rodriguez et al., 2010). These signals act on different nuclei of the hypothalamus depending on the specialized function of each. For example the preoptic area (POA) is responsible for thermoregulation (Boulant, 2000) whereas the dorsomedial hypothalamic nucleus (DMH) is involved in circadian activity, feeding, drinking and body-weight regulation (Bernardis and Bellinger, 1998; Mieda et al., 2006). The paraventricular hypothalamic nucleus (PVH) gets mainly activated by stress or physiological changes. Mammillaries body (MB) are important for memory function (Vann, 2010). The ventromedial hypothalamic nucleus (VMH) is responsible for feeding, fear, thermoregulation and sexual activity (McClellan et al., 2006). As already mentioned earlier, the VMH is an integral part of the fear circuit, which receives inputs from the amygdala. Information is than transmitted directly or through the anterior hypothalamic nucleus (AHN) to the dorsal premammillary nucleus (PMD) which leads to an emotional response (Figure 1.2) (Cezario et al., 2008; Gross and Canteras, 2012).

VMH lesion and Fos expression studies on rodents revealed that the VMH plays an important role in fear learning paradigms (Colpaert and Wiepkema, 1976; Trogrlic et al., 2011)

1.4. Amygdala

The amygdala is located within the temporal lobes of the brain and belongs to the limbic system. It is primarily involved in processing memory, emotional reactions and stress (Rasia-Filho et al., 2000). The amygdala consists of 13 nuclei and receives sensory information such as auditory, visual, olfactory, gustatory and visceral inputs from cortical and subsortical regions. The output projections lead to the cortical hypothalamic, hippocampal and brain stem regions (Sah et al., 2003). It has been shown that the amygdala is important in fear and fear-based learning such as fear conditioning and inhibitory avoidance which involves freezing behavior, potentiated startle, release of stress hormones and alterations in blood pressure and heart rate by activating the autonomous nervous system via the central nucleus of the amygdala (Sah et al., 2003). Depending on the types of stimuli distinct parts of the amygdala are activated. For instance, in fear conditioning and IA it has been shown that the lateral amygdala (LA), basolateral amygdala (BLA) and the central amygdala (CeA) are involved (Huff et al., 2013). In contrast, predator cues activate the LA, the basomedial amgydala (BMA) and the medial amygdala (MEA) (Gross and Canteras, 2012).

Studies in humans suffering from the rare Urbach-Wiethe disease characterized with focal bilateral amygdala lesions reported that these people fail to show any fear-related behavior (Feinstein et al., 2011; Kucuk et al., 2012). In addition, amygdala lesions in rodents exhibit partial retention of memory in IA (Liang et al., 1982; Parent et al., 1995). There is much evidence that changes in the synaptic density occur in the amygdala during fear learning but it is also well-known that the amygdala modulates plasticity in other brain regions that are substrates for memory storage (Cahill et al., 1999; McGaugh, 2002; McGaugh et al., 1996). Thus the issue whether the amygdala is the site of acquisition for fear learning or rather serves for modulating the strength of memory storage in other brain areas such as the hippocampus remains to be resolved (Sah et al., 2003).

1.5. Estrogen actions in the brain

Estrogens are essential hormones which are necessary from the very beginning when life begins at fertilization, throughout development till senescence of all vertebrates (Katzenellenbogen, 1996). The main sites of female sex hormone production are the ovaries. The release of estrogens is mainly controlled through the hypothalamus- pituitary-gonadal (HPG) axis. In the 1960s Jensen and Jacobson discovered that intracellular estrogen receptors (ERs) mediate the actions of estrogen (Jensen and Jacobson, 1962). ERs belong to the nuclear receptor superfamily and form a complex with estrogen which binds to specific DNA sequence called hormone response element (HRE) found in promoters of many genes, and thus regulates gene expression (Klinge, 2001). This is called the genomic mechanism of estrogen signaling. More recently, novel estrogen signaling via nonnuclear receptors which mediates rapid effects has emerged (Harrington et al., 2006). Receptors in close proximity to the plasma membrane of neurites, soma, dendritic spines and axon terminals mediate these nongenomic effects by activating distinct intracellular signaling cascades (Prossnitz and Maggiolini, 2009; Raz et al., 2008). There are a variety of estrogen's nongenomic effects which include activating cyclic adenosine monophosphate (AMP) and mitogen-activated protein kinase (MAP kinase) pathways; modulating G protein coupling and affecting calcium currents; influencing calcium channels and calcium entry; protecting neurons from damage by excitotoxins and free radicals; and acting on the excitability of neuronal and pituitary cells (Brinton, 2001; Kelly and Levin, 2001; Kelly and Wagner, 1999; Lee and McEwen, 2001).

Besides ER expression in classical estrogen target tissues such as uterus, mammary gland or bone they are also present in neurons and glia cells in the brain (McEwen, 2002). In the brain, ERs were first found in the hypothalamus and the pituitary gland as these regions are tightly related to reproduction and sexual behavior. Subsequently, it was discovered that ERs are expressed throughout the brain, regulating many different functions, including cognition (Pfaff, 1980). Two different types of ERs have been described: ER-α and ER-β which differ in their binding affinities for their ligand (Toran-Allerand, 2004). ER-α and ER-β were found

in different brain regions such as the cerebral cortex, basal forebrain, amygdala, thalamus, hypothalamus particularly in the VMH, PVH and POA, mesencephalon, pons, cerebellum and medulla oblongata. In the hippocampus, exclusively ER-α was detected. (Pérez et al., 2003; Pfaff, 1980)

Interestingly, it has been reported that estrogens play a very important role in the sexual differentiation of the brain during neonatal life. Testosterone is secreted first, then it either binds to androgen receptors or it gets aromatized to estrogen which is necessary in the masculinization of the brain (Kandel et al., 2000a; McEwen, 1981). In these studies structural and functional differences between male and female brain were not only found in the hypothalamus but also in other parts like the hippocampus, cerebral cortex, amygdala, midbrain, brainstem and spinal cord (McEwen, 2002; McEwen and Alves, 1999). Several studies showed sex differences in the size of rodents' hippocampi which might explain the different ways used to solve spatial navigation problems used by female and male rats (Gaulin and FitzGerald, 1986; Williams and Meck, 1991). In humans similar differences in spatial problem solving between women and men have been reported (Kimura, 1992).

Intriguingly, studies have also shown that estrogen stimulates the outgrowth of neurites in cultures of hypothalamus, amygdala and hippocampus and is required for the outgrowth of axons of estrogen-responsive neurons (Diaz et al., 1992; Kandel et al., 2000a; Lorenzo et al., 1992; von Schassen et al., 2006). Therefore, the brain is not only able to control the secretion of estrogen through the HPG axis but can also respond to estrogen through one of its specialized receptors (Morrison et al., 2006). Furthermore estrogens affect also several neurotransmitter systems such as cholinergic (Dumas et al., 2006), catecholaminergic (Leranth et al., 2000), serotonergic (Bethea et al., 2002)and GABAergic (Milner et al., 2001) neurons which are important in cognition, mood and locomotor activity. Taken together, the available data indicate that ER-α and ER-β are expressed in key brain regions involved in learning and memory as well as in mood and play an important role in regulating the anatomy and the connectivity of memory- and mood-relevant brain regions.

1.5.1. Estrogen in mood

It is very well known that mood fluctuates across the menstrual cycle of women (Alexander et al., 2007). Moreover women are more likely to develop various kinds of mood disorders compared to men, especially in menopause when circulating estrogen levels have dropped (Kessler et al., 1993). Depression and anxiety are the most prevalent psychological symptoms related with estrogen deficiency in postmenopausal women (Pisani et al., 1998). It has been reported that 8% to 47% women entering menopause suffer from depressive symptoms (Avis et al., 2001; Schmidt, 2005). Furthermore the prevalence of mood disorders in surgical induced menopause is also as high as in natural menopause transition (Paoletti et al., 2001; Taylor, 2001).

In vivo studies investigating the role of female sex hormones in mood-related behavior in female rodents also reported a strong correlation between emotional behavior and different phases of the estrous cycle. Female rodents with high endogenous estrogen levels in late proestrus when sexually receptive behavior is displayed exhibited a decreased anxiety-like behavior in several different tasks such as elevated plus maze compared to females in diestrus with low concentrations of estrogen, ovariectomized rodents and male rodents (Frye et al., 2000; Frye and Walf, 2002; Marcondes et al., 2001; Walf and Frye, 2007; Walf et al., 2009). Moreover high circulating estrogen levels induced also antidepressant-like effects and decreased the immobility time in the forced swim test (Frye and Walf, 2002). Additionally, studies on pregnant rats with persistently elevated estrogen levels exhibited also a reduced anxiety and depression behavior (Zuluaga et al., 2005). Furthermore the administration of exogenous estrogen in a physiological concentration either subcutaneously or directly into the amygdala reversed ovariectomy-induced depression and anxiety-like behavior (Bernardi et al., 1989; Estrada-Camarena et al., 2003; Frye and Walf, 2004; Frye and Wawrzycki, 2003; Walf and Frye, 2005).

Interestingly, testosterone was also shown to cause similar anxiolytic and anti-depressive effects like estrogen. Subcutaneous injection of testosterone in ovariectomized rats increased

the time spent in the open arm of the elevated plus maze indicating decreased anxiety-like behavior (Frye and Lacey, 2001). Frye et al reported that aged mice subcutaneously administered with testosterone or its metabolites decreased the time spent immobile in the forced swim test (Frye and Walf, 2009). The underlying molecular mechanism of estrogens effects on anxiety and depression are still not fully understood. However it was reported that estrogen is activating the HPA axis especially the hippocampus and the amygdala, important parts of the limbic system. Studies showed for the hippocampus as well as for the amygdala that estrogen injection induces expression of c-Fos, an immediate early gene (Insel, 1990; Rudick and Woolley, 2000) and the formation of new dendritic spines (Gould et al., 1990; Nishizuka and Arai, 1982).

1.5.2. Estrogen in learning

Beside mood disorders cognitive decline is also associated with the transition to menopause. It has been reported that women are twice as likely to develop cognitive disorders as age-matched men (Barrett, 1999; Lee et al., 2012). However due to the greater longevity of women it is still unclear if cognitive decline is only induced by the drop of physiological estrogen levels or if it is a normal ageing process which will be discussed in the next chapter. Nevertheless there are studies which revealed benefical effects of hormone replacement therapy in postmenopausal women, e.g. reviewed by (Sherwin, 2006). Intriguingly, young and elderly men treated with estrogen revealed better cognitive performance in spatial and verbal memory (Cherrier et al., 2001; Kampen and Sherwin, 1996).

In vivo studies with rats reported that the reduced physiological levels of estrogen in diestrus and ovariectomy caused learning deficits in the inhibitory avoidance task and fear conditioning (Milad et al., 2009; Walf and Frye, 2007). A similar result with orchiectomized male rats was provided by Frye and collegues. The depletion of testosterone induced IA learning deficits (Frye and Seliga, 2001). However in the ovx and aged females as well as in orchiectomized (orh) males cognitive function was improved by the administration of estrogen for the females and testosterone for the males (Frye et al., 2005; Frye and Seliga, 2001;

Walf and Frye, 2007). Furthermore it has been reported that the administration of estrogen either pre or post-training led to different outcomes. Pre-training treatment led to discrepant effects on the rodents' performance (Chesler and Juraska, 2000; Foster et al., 2003), whereas post-training had more consistent mnemonic effects (Rhodes and Frye, 2004). As estrogen administration influences activity and arousal (Morgan and Pfaff, 2001; Morgan et al., 2004; Ogawa et al., 2003) it has been suggested that estrogen during training might change activity and/or arousal and thus also the performance and the memory consolidation process (Walf and Frye, 2005).

Although it appears now that estrogen has mainly positive effects on cognition, various studies showed opposite results. For example, testing rodent behavior in estrus having high physiological estrogen levels revealed either worse or unchanged performance in different learning tasks (Berry et al., 1997; Frye, 1995; Galea et al., 1995; Stackman et al., 1997; Warren et al., 1995). Taken together, it appears that estrogen's variable effects on cognition are dependent on the timing of and on the nature of the cognitive task.

1.6. Estrogen in the ageing brain and Menopause

The female gonads are the key organs of the reproductive system where differentiation and release of mature oocytes occur. The number of ovarian follicles which contain a single oocyte is fixed even before birth. At the time of birth the ovaries hold about one to two million follicles. This number drops throughout the whole life-time of a female, by puberty it has already declined to less than half (McGee and Hsueh, 2000). The follicles also produce estradiol, testosterone and progesterone which are not only essential for the maturation of an oocyte but also for multiple processes in the entire body including the central nervous system (McGee and Hsueh, 2000). Estradiol and Progesterone are secreted in a cyclic manner throughout the menstrual cycle until the time-point has reached where the ovaries cease to release any female sex hormones. This natural phase of a woman's normal aging process is called menopause and starts around the age of 50 years (Faddy and Gosden, 1996; McGee and Hsueh, 2000).

At this time-point the follicle number has fell to an all-time low of about 1000 cells which are not able anymore to release a single fertile oocyte. The woman enters a new stage where the menstrual bleeding and thus the reproductive ability terminates and the body has to adapt to a nadir in circulating estradiol and progesterone (Faddy and Gosden, 1996). Due to this change to a prolonged hypoestrogenic state many women are faced with several health problems like hot flashes, vaginal dryness and infections, night sweats, depression, anxiety disorders, sleeping problems, mood swings, joint aches and pains but also with severe diseases like osteoporosis, coronary heart disease and neurodegenerative disorders including any kind of cognitive deficits. It has been reported that about two times more women suffer from cognitive disorders than age-matched men (Barrett, 1999; Lee et al., 2012).

Due to the higher life-expectancy of women they live now for more than a third beyond the cessation of their ovarian function. Thus hormone replacement therapies (HRTs) either with estrogen alone or a combination of estrogen and progesterone are very common to mitigate several menopausal symptoms. However whether or not estrogen and progesterone can protect the brain from cognitive disorders in humans is still controversial (LeBlanc et al., 2007; Prelevic et al., 2005; Resnick et al., 2004). The decline of estrogen levels over menopause is also accompanied by a decline in functional capacity of cognitive and other behavioral processes as they are influenced by estrogen. Therefore these processes are in principle subject to reversal by estrogen replacement therapy (McEwen and Alves, 1999).

Neurodegenerative disorders are characterized by the loss of neurons and synapses in certain parts of the brain such as hippocampus and prefrontal cortex, which correlates with deficits in learning and memory (DeKosky and Scheff, 1990; Selkoe, 2002; Terry et al., 1991). Studies showed that estrogen has neuroprotective and anti-apoptotic effects (Morrison et al., 2006). It can counteract excitotoxins and free radicals which cause cell damage and cell death through apoptosis. In vitro studies reported that the increase in mitochondrial reactive oxygen species by amyloid β-protein (Aβ) can be suppressed by the actions of estrogen through its stabilization effects on mitochondrial membrane potentials and prevention of adenosine triphosphate (ATP) depletion and thus prevents cell damage. (Mattson et al., 1997; Wang et al., 2001)

One would predict that HRT is beneficial in the prevention and treatment of cognitive aging and Alzheimer's disease (AD). Many human studies reported that HRT lowered the risk of developing cognitive disorders (Jacobs et al., 1998; Tang et al., 1996; Waring et al., 1999; Zandi et al., 2002). Similar positive effects of HRT were also shown in rodent studies. Ovx rodents mimicking postmenopause in women performed better in different learning paradigms involving different parts of the brain such as hippocampus, amygdala, hypothalamus and cortex when they underwent HRT. As mentioned previously, HRT induced additionally anti-depressive and anxiolytic behavior in ovx rodents, thus alleviating common postmenopausal symptoms women suffer from (Kalandakanond-Thongsong et al., 2012; Koss et al., 2004; Pandaranandaka et al., 2006, 2009). However there are many rodent studies which showed opposite results (Diaz-Veliz et al., 1997; Mora et al., 1996). And even the largest clinical trial of HRT the Women's Health Initiative Memory Study (WHIMS) which was randomized, double-blind and placebo-controlled, testing the effect of estrogen alone and in combination with progesterone did not find any significant effect on reducing the risk for AD. WHIMS concluded that the use of HRT for preventing dementia or cognitive decline in women 65 years of age or older is not recommended (Shumaker et al., 2004). These discrepant findings in human as well as rodent studies may be related to the type and dose of estrogen used, the timing of initiation of HRT and the age of the women. HRT that was started several years after menopause in women did not show any positive effects and was even harmful (Sherwin, 2007a, b). Thus it appears that estrogen has only beneficial effects on neurological function and cell survival in the brain if the neurons are still healthy, as soon as neurological health is compromised estrogen can even exacerbate the degeneration process.

Taken together, there is considerable evidence at the molecular as well as the cognitive level supporting the use of HRT during menopause for the prevention and treatment of cognitive and mood disorders. However, the efficacy of estrogen depends on the type of estrogen, the time of initiation and age (Genazzani et al., 2007).

1.7. RANK/RANKL/OPG and its functions

Bone remodeling and morphogenesis is a lifelong and physiologically controlled process that involves the formation of new bone by osteoblasts and the resorption of bone by osteoclasts in a coordinated and balanced manner (Felix et al., 1996; Roodman, 1996). These dynamic processes of osteoblast and osteoclast activity can be easily disturbed and imbalanced by any perturbation like hormonal, inflammatory or growth factor changes, which leads to severe skeletal abnormalities such as osteoporosis characterized by crippling bone damage and increased bone resorption or ostropetrosis which manifests in the increase of bone mass (Mostov and Werb, 1997; Reddi, 1997). Osteopetrosis is an extremely rare inherited disorder in contrast to osteoporosis which mostly affects post-menopausal women, rheumatoid arthritis or immobilized patients, and even astronauts (Boyle et al., 2003; Theill et al., 2002; Wronski and Morey, 1983). Osteoblasts are specialized, mononuclear and terminally differentiated cells that stem from the mesenchymal lineage (Caetano-Lopes et al., 2007; Pittenger et al., 1999), whereas osteoclasts are large multinucleated cells that develop from the monocyte/macrophage haematopoietic lineage (Boyle et al., 2003; Nijweide et al., 1986) (Figure 1.3 A).

With the discovery of the RANK signaling pathway in osteoclasts in the late 1990s by several groups a new insight into the mechanisms of osteoclatogenesis and bone remodeling was gained (Lacey et al., 1998; Nakagawa et al., 1998; Simonet et al., 1997; Yasuda et al., 1998b). This signaling pathway includes three key molecules, namely receptor activator of NFκB ligand (RANKL; also known as tumor necrosis ligand superfamily member 11 (TNSFSF11), TNF related activation induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), its receptor RANK (also known as TRANCE-R, TNFRSF11A, OFE, PDFR, TRANCE-R, ODAR, CD265) and the RANKL-decoy receptor osteoprotegerin (OPG or TNFRSF11B) belonging all to the tumor necrosis factor-tumor necrosis factor receptor (TNF-TNFR) superfamily. RANKL is a homotrimeric type II transmembrane protein. However it can be released from the cell surface after proteolytic cleavage

by many extracellular proteases such as a disintegrin and metalloproteinase (ADAM) (Hikita et al., 2006; Wada et al., 2006). RANKL was first found on the surface of osteoblasts and mediates osteoclastogenesis by binding to RANK which is a heterotrimeric transmembrane protein sitting on the surface of osteoclast progenitors (Anderson et al., 1997; Hsu et al., 1999; Nakagawa et al., 1998). Besides RANK, there is a second receptor for RANKL, the soluble receptor OPG which blocks the interaction of RANKL with RANK. Thus OPG is responsible for counterbalancing RANK/RANKL mediated osteoclastogenesis by acting as a neutral decoy receptor for RANKL ultimately leading to bone formation (Simonet et al., 1997; Yasuda et al., 1998a).

The binding of soluble or membrane-bound RANKL to RANK leads to receptor oligomerization whereby tumor necrosis factor receptor associated factor 6 (TRAF6) gets recruited to specific sites on the cytoplasmatic tail of RANK (Armstrong et al., 2002) (Figure 1.3 B). TRAF6 is an essential molecule in the entire cascade as TRAF6 mutant mice exhibit severe osteopetrosis due to a partial block in the formation of mature osteoclasts (Lomaga et al., 1999). The binding of TRAF6 to RANK induces the activation of signaling pathways such as NFκB and MAPK among several other pathways (Yavropoulou and Yovos, 2008) (Figure 1.3 B). MAPK signaling is an evolutionarily conserved mechanism, which is essential in directing cellular responses to environmental stimuli and thus regulating cell survival, adaptation, gene expression, differentiation, mitosis and apoptosis (Pearson et al., 2001). The phosphorylation and activation of MAPKs lead to activation and nuclear translocation of c-Fos and c-Jun, which together with nuclear factor of activated T-cells c1 (NFATc1) induce transcription of the genetic program required for osteoclastogenesis, osteoclast differentiation and activation (David et al., 2002; Kenner et al., 2004; Wagner, 2002; Yavropoulou and Yovos, 2008). Mice deficient in c-fos (Grigoriadis et al., 1994) or NFATc1 exhibited osteopetrosis due to an osteoclast differentiation defect (Aliprantis et al., 2008). Moreover the activation of NFκB is a crucial step in osteoclast formation, which was demonstrated by the finding that mice lacking NFκB subunits p50 and p52 are osteopetrotic (Iotsova et al., 1997). The RANK/RANKL pathway is also coupled to calcium signaling via the phosphatidylinositol-related enzyme phospholipase Cγ (PLCγ). PLCγ mediated increase of intracellular Ca^{2+} is required for the

activation and translocation of NFATc1 (Takayanagi, 2005) and NFκB (Komarova et al., 2003).

Additionally to RANKL, macrophage colony-stimulating factor (M-CSF) is essential in blocking apoptosis progenitors (Arai et al., 1999; Lacey et al., 1998). Hence, Dai et al showed that M-CSF knockout (KO) mice exhibited osteopetrosis due to a lack of macrophages and osteoclasts (Dai et al., 2002). Interestingly, at the same time when the RANK/RANKL/OPG system was discovered in bone remodeling it was reported that RANKL is also expressed on T-cells and regulates adaptive immunity (Anderson et al., 1997; Wong et al., 1997) and is involved in lymph node organogenesis (Kong et al., 1999b). Moreover it was shown that osteoclastogenesis is directly regulated by RANKL produced by activated T-cells (Kong et al., 1999a). Genetic deletion of RANKL or RANK in mice resulted in severe osteopetrosis, defective tooth eruption, lack of lymph nodes and osteoclasts and defects in thymocyte and B-cell differentiation (Kong et al., 1999b). These findings provided a first cross-talk between skeletal and immune system, which gave birth to the new field “osteoimmunology” (Walsh et al., 2006).

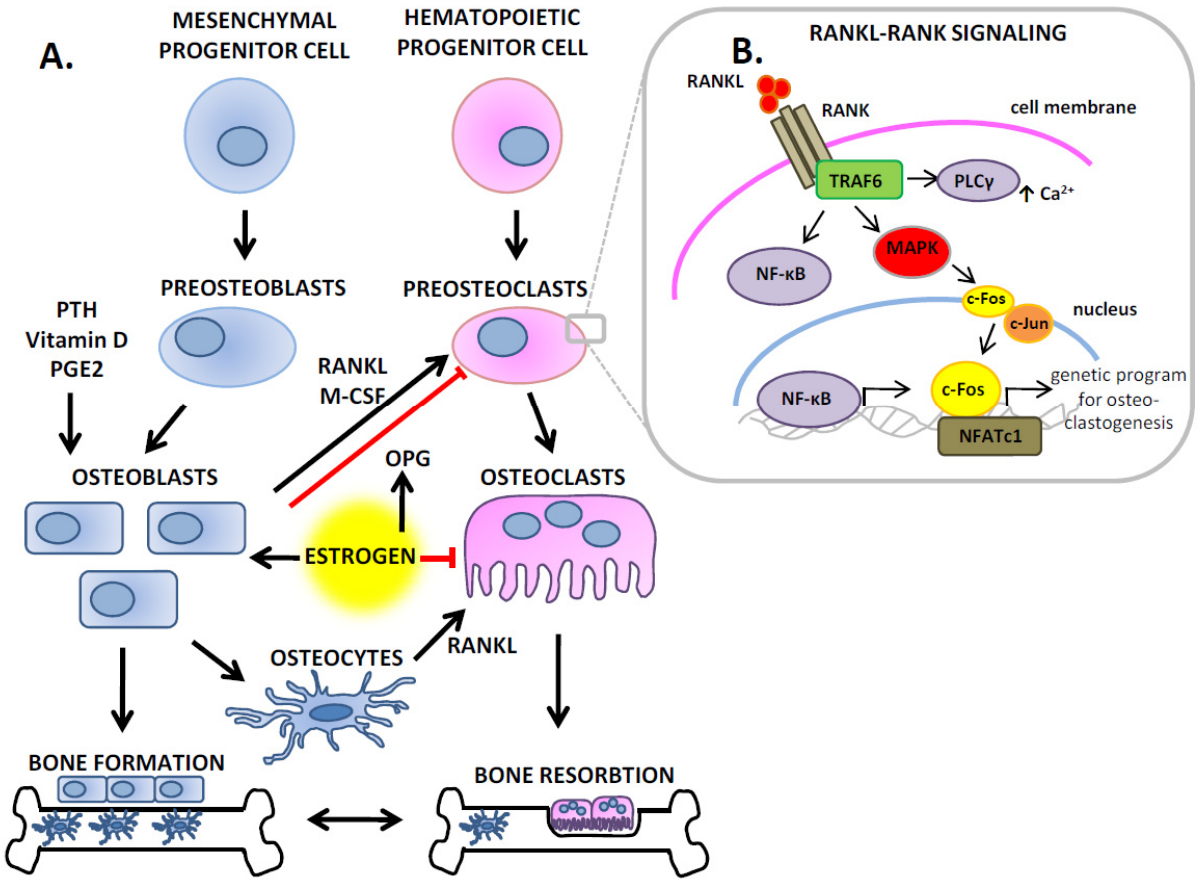


Figure 1.3: RANK signaling pathway in bones.

(A) Osteoblasts are single-nucleated and arise from a mesenchymal stem cell lineage whereas osteoclasts are multinucleated and arise from a hematopoietic stem cell lineage. Osteoblasts which can be induced by several factors like PTH, Vitamin D and PGE2 are necessary for bone formation. The formation of osteoclasts is induced by RANKL and M-CSF which leads in turn to bone resorption. OPG which is upregulated by estrogen acts as a decoy receptor for RANKL and blocks osteoclastogenesis thereby leading to bone formation. **(B)** Downstream signals of RANK/RANKL. Binding of RANKL to RANK leads to the recruitment of TRAF6 which in turn activates NFκB, MAPK, or PLCγ among other pathways. PLCγ mediates the increase of intracellular Ca^{2+} which is important for the activation and translocation of NFATc1 (modified from Nagy and Penninger).

1.8. Estrogen and RANK/RANKL/OPG

The expression of the triad RANKL, RANK and OPG is under strict regulation of several different hormones and cytokines including Vitamin D3, parathyroid hormone (PTH) and its related protein (PTHrP), prolactin, prostaglandin E2 (PGE2), tumor necrosis factor α (TNFα) or interleukins IL-6 and IL-1β which promote all osteoclastogenesis (Akatsu et al., 1991; Akatsu et al., 1989; Evely et al., 1991; Lam et al., 2000; Takahashi et al., 1988; Theill et al., 2002). It was already in the 1930s when the American physician and endocrinologist Fuller Albright set the first link between female sex hormones and bone metabolism. He realized that an increased number of his postmenopausal female patients suffer from osteoporosis. Also studies in which male and lactating female pigeons were compared showed osteoporotic conditions in males compared to females. However he could improve the bone matrix formation in his postmenopausal female patients and in the male pigeons by the administration of 17β-estradiol (E2). Thus, he hypothesized that osteoporosis is caused by an E2 deficiency and can be counteracted by the exogenous administration of E2 which stimulates the osteoblasts and in turn restores bone mass (Albright et al., 1940; Kyes and Potter, 1934; Pfeiffer and Gardner, 1938).

Later studies confirmed the positive effects of E2 in bone formation by applying HRT containing a combination of E2 and progesterone or E2 alone in postmenopausal women (Cauley et al., 1995; Ettinger et al., 1985; Prince et al., 1991). In the 1980s functional E2 receptors on bone cells were discovered (Eriksen et al., 1988), whereby the effects of E2 on the cell type is cell type specific, on osteoclasts it acts pro-apoptotic whereas on osteoblasts anti-apoptotic (Almeida et al., 2010; Hughes et al., 1996; Kousteni et al., 2007). Rapid loss of E2 in menopause causes a prolongation of the life span of osteoclasts and therefore enhanced remodelling of the skeleton enventually leading to osteoporosis. Further studies on osteoclastogenesis have reported that E2 can also block bone resorption by inhibiting proinflammatory cytokines such as IL-1β, IL-6, TNF-α, M-CSF, or PGE2 (Manolagas, 2000; Pacifici, 1996).

However with the discovery of the new triad of the TNF ligand and receptor family RANK, RANKL and OPG, a new E2 regulation of bone remodeling had been also identified. It was

reported that E2 upregulates OPG whereas it downregulates RANKL on the mRNA as well as the protein level (Bord et al., 2003; Hofbauer et al., 1999; Shevde et al., 2000). It does so either through a direct mechanism or indirectly via E2-responsive mediators. Hofbauer and Pacifici showed that IL-1 and TNF-α increase RANKL, OPG and M-CSF levels, whereas PGE2 increases RANKL but decreases OPG (Hofbauer et al., 2000; Pacifici, 1996)

Interestingly, mammary gland morphogenesis which is controlled by sex and pregnancy hormones including E2, progesterone, prolactin and PTHrP, involves also the signaling of RANK, RANKL and OPG. Loss of RANKL or RANK results in a complete block in the formation of a lactating mammary gland and, hence, all pups died because of a lack of milk production by their mothers (Fata et al., 2000). RANKL is not expressed in virgin mammary glands but it increases during pregnancy when the levels of progesterone, prolactin and PTHrP are elevated. In contrast RANK and OPG expression is not changing with hormone levels (Srivastava et al., 2003). Later studies showed that the RANK/RANKL system plays a central role in progestin-driven breast cancer which is mainly induced through HRT containing synthetic progesterone derivatives in postmenopausal women (Schramek et al., 2010). It was reported that progesterone expands the population of Lin-CD24+CD49fhi mammary epithelial stem cells which are found in mammary tumors (Asselin-Labat et al., 2010; Pece et al., 2010). In vivo studies showed that administering progesterone leads to a massive induction of RANKL (Schramek et al., 2010). These findings suggest that the RANK/RANKL/OPG axis plays a key role in many physiological processes especially in menopausal women and appears to play a key role in hormone driven breast cancer.

1.9. RANK/RANKL/OPG in the brain

There is still little known about RANK/RANKL/OPG functions in the brain including behavior, compared to bone metabolism, immunity, and mammary gland formation. Hanada et al. showed RANK and RANKL expression in the brain, in particular in specific areas of the hypothalamus such as the preoptic area (POA), the medial septal nucleus (MSn) and the lateral septal nucleus (LSn) (Hanada et al., 2009). This brain region is responsible for thermoregulation and thus fever (Morrison et al., 2008). In the past many studies identified mediators of fever like lipopolysaccaride (LPS), a bacterial cell wall component which acts as an exogenous pyrogen and, endogenous pyrogens such as the pro-inflammatory cytokines IL-1β, TNFα, or IL-6. Both exgenous and endogenous pyrogens trigger the release PGE2 mediated by phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2). PGE2 binds then to prostaglandin E receptor 3 (EP3) expressing neurons in hypothalamic specific regions and thereby induces an increase in body temperature (Bartfai and Conti, 2010; Elmquist et al., 1997; Luheshi, 1998; Morrison et al., 2008). Hanada et al. reported that RANKL injections directly into the brain induced a sever fever response and diminished circadian activities whereas peripheral intraperitoneal (i.p.) injections did not have any effects. No changes in body temperature were detected in CNS specific conditional knock-out RANK mice, which strongly indicates that RANK/RANKL is indeed involved in the febrile response. Importantly, children with a homozygous RANK mutation also showed an abolished febrile response while suffering from pneumonia. Moreover, CNS specific conditional knock-out RANK female mice had an elevated body temperature at the resting phase. This change in body temperature appears to depend on the presence of female sex hormones (Hanada et al., 2009).

This study provided the first evidence that the RANK/RANKL/OPG axis acts in the CNS where it plays a key role in the inflammatory fever response. Additionally, a connection between female sex hormones and female thermoregulation influenced by the RANK/RANKL/OPG system was found.

A recent study reported another involvement of the RANK/RANKL/OPG axis in the CNS specifically in ischemic stroke where an anti-inflammatory and neuroprotective effect was

found. Shimamura et al. used a model of transient ischemia called middle cerebral artery occlusion (MCAo) and showed elevated mRNA and protein levels of RANK, RANKL and OPG which were specifically expressed in activated microglia and macrophages (Shimamura et al., 2014). By applying MCAo on OPG KO mice they noted an increased RANK/RANKL signaling contributing to a reduced infarct volume and decreased cerebral edema. A similar outcome was also shown after intracerebroventricular injection of active RANKL into wild type (WT) mice with MCAo. An increase in infarct volume with a reduced RANK/RANKL signaling was noted after the administration of anti-RANKL neutralizing antibody. In vivo and in vitro studies further showed that the reduction of infarct volume correlated with RANKL mediated downregulation of microglial inflammatory cytokines IL-6 and TNFα and thus prevented neuronal death. However, Shimamura and colleagues did not analyze whether female sex hormones also regulate this specific RANK/RANKL/OPG axis in ischemic stroke, as it is known that stroke has a higher incidence in women. These discoveries revealed a role of the RANK/RANKL/OPG axis in the brain.

1.10. Preliminary results

Preliminary studies were performed with RANK/Nestin female mice which specifically delete RANK in neurons and astrocytes, by Vanja Nagy, a post-doctorate fellow in our lab. As RANK, RANKL and OPG are under strict regulation of sex hormones and estrogen plays a critical role in mood and anxiety, she tested those mice for anxiety and depression by using an elevated plus maze (EPM) test (Figure 1.4 A) and the tail suspension test (TST) (Figure 1.5 A). Sham-operated (intact) and ovariectomized (ovx) RANK/Nestin mice were used. However in the EPM no significant difference between control or RANK/Nestin knockout (KO) animals in the amount of time spent exploring the open arms of the maze was detected (Figure 1.4 B). Ovariectomy of control or RANK/Nestin KO animals had no effect on anxiety (Figure 1.4 C). Together, these data indicated that the RANK/RANKL/OPG axis is not involved in regulating anxiety, in the presence or absence of circulating female sex hormones. Although women with reduced estrogen levels often have elevated anxiety levels (Arpels, 1996; Terauchi et al., 2013), rodent studies have shown controversial results of estrogen's influence on anxiety level in the EPM test (Diaz-Veliz et al., 1997; Mora et al., 1996; Nomikos and Spyraki, 1988). Of note, we were unable to detect any influence on anxiety by ovariectomy, in contrast with other studies which reported increased anxiety (de Chaves et al., 2009; Lagunas et al., 2010).

Many studies showed that estrogen-deficiency induces depression in female mice (de Chaves et al., 2009; Heydarpour et al., 2013). The TST is a despair-based depression test. The mouse is put in an inescapable, stressful situation by suspending it by its tail whereby the time spent immobile is recorded (Figure 1.5 A). We were unable to detect depression as defined by increased time spent immobile in the ovx controls as compared to sham controls. Thus the absence of RANK had no apparent effect on depression in the TST (Figure 1.5 B). Several reasons could account for these discrepancies of estrogen's effect on anxiety and depression in our experimental setup, including age of ovariectomy, recovery time after surgery, and/or

mouse strain differences. Taken together, it appears that RANKL/RANK is not involved in despair-based depression and anxiety in mice.

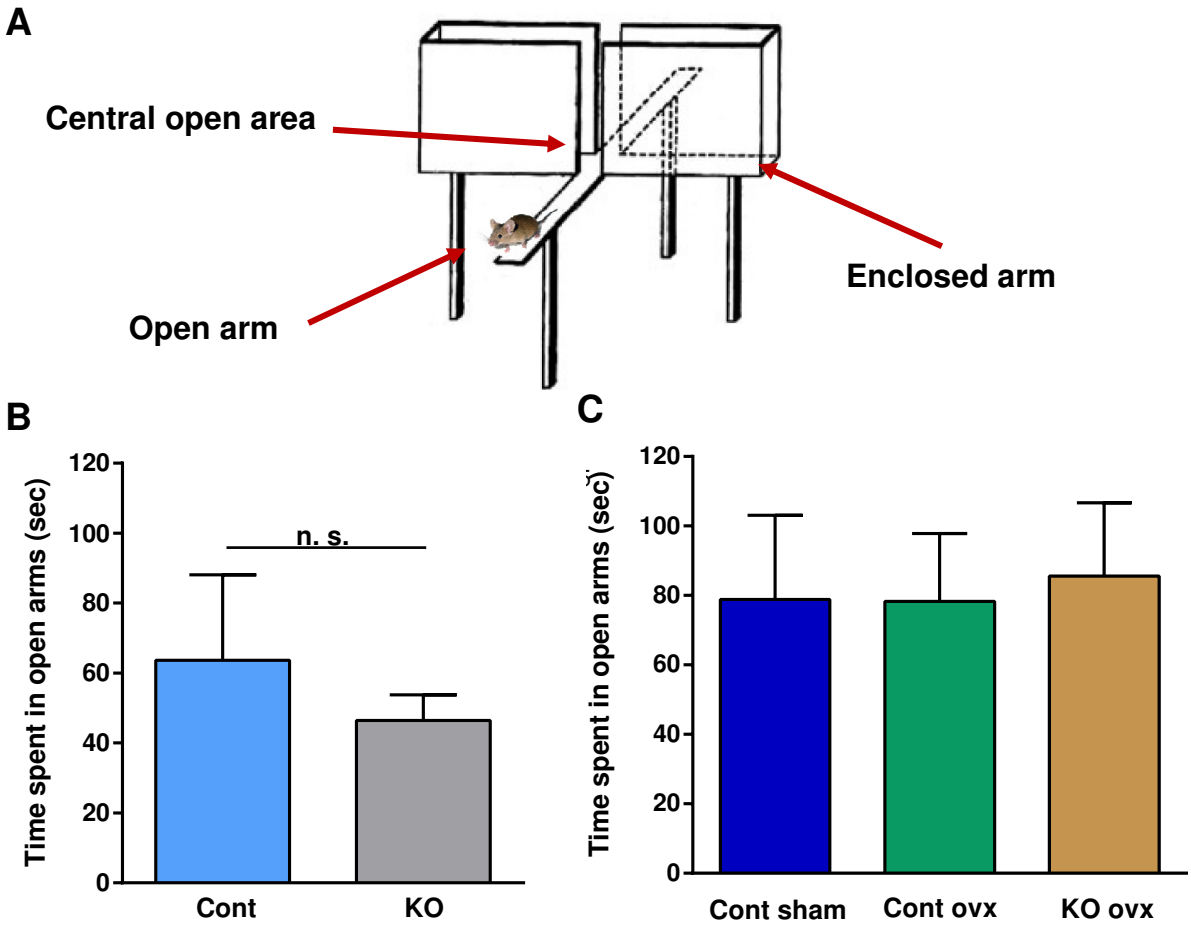


Figure 1.4: RANK deficiency in the brain does not influence anxiety levels in the elevated plus maze in the presence or absence of female sex hormones.

(A) Schematic drawing of the EPM test. The mouse is placed on the central open area facing open and enclosed arms and allowed to explore the maze for 5 minutes. The time spent in the open arms is recorded. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(B,C)** Anxiety levels measured as time spent in open arms of RANK/Nestin controls (Cont, n=15), RANK/Nestin KOs (KO, n=16), sham-operated RANK/Nestin controls (Cont sham, n=7), ovx RANK/Nestin controls (Cont ovx, n=10) and ovx RANK/Nestin KOs (KO ovx, n=14) in EPM. **(B)** RANK/Nestin controls and KOs did not show any significant difference (n.s.) in anxiety levels. **(C)** Ovariectomy in sham-operated RANK/Nestin controls and RANK/Nestin KOs do not influence anxiety compared to sham-operated RANK/Nestin controls. The y-axis in both diagrams show the time spent in open arms. Values are shown as means ± SEM (one-way ANOVA with Tukey's multiple comparisons test).

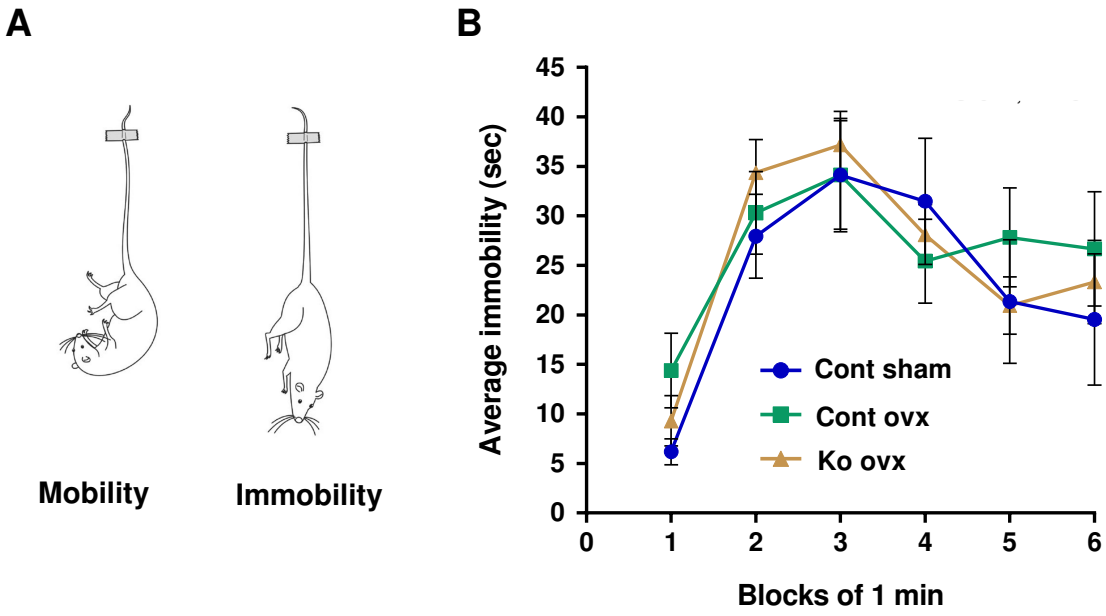


Figure 1.5: RANK deficiency in ovariectomized mice does not affect depression-like behavior in the test and tail suspension test.

(A) Schematic representation of mobile and immobile posture of the mouse in the TST. The mouse gets suspended by its tail and the time spent immobile every minute was recorded. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(B)** Despair-based depression tested in sham-operated RANK/Nestin control (n=6), ovx RANK/Nestin control (n=11) and ovx RANK/Nestin KO (n=13) mice. No difference between the different groups was detected indicating that RANK is not involved in depression-like behavior neither in the presence nor in the absence of sex hormones. Values are shown as means ± SEM (one-way ANOVA).

1.11. Objectives

There is a strong interaction between sex hormones and the brain. As estrogen declines with age the cognitive ability is also reduced. Thus, in menopause the risk of developing various neurological disorders including any type of cognition or mood disorders is quite high. Moreover, estrogen replacement studies in humans as well as rodents showed that estrogen has neuroprotective and neurotrophic effects and thus preserves mental capabilities.

RANK, RANKL and OPG are under regulation of female sex hormones especially OPG which is shown to be upregulated by elevated estrogen levels and RANKL which responds to progesterone. It has been reported that RANK is expressed in the brain, specifically in the preoptic area of the hypothalamus, the preoptic area (POA) and the medial septal nucleus (MSn) of the hypothalamus. The hypothalamus plays a key role in fear circuits and in fear-based learning. Moreover is it a key unit in the hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis and is involved in the neurobiology of mood disorders such as stress disorders and depression. Considering the evidence that the hypothalamus is rich in estrogen receptors and mood and memory are influenced by sex hormones, we sought to investigate the role of RANK/RANKL/OPG in learning and memory as well as mood regulated by female sex hormones. Preliminary data showed that RANK did not have an effect on anxiety and depression neither in the absence nor in the presence of estrogen. Thus the aim of my study was to further explore RANK's influence on mood and to elucidate the role of RANK/RANKL/OPG axis in learning and memory. To address these questions, several brain-specific knockout mice were generated: Nestin-Cre $rank^{floxed}$ mice with specific deletions of RANK in neurons and astrocytes, CamKII-Cre $rank^{floxed}$ mice which delete RANK only in neurons and GFAP-Cre $rank^{floxed}$ mice which have lost RANK expression only in astrocytes. For simplification all the three tissue-specific RANK knockout mice are abbreviated as follows RANK/Nestin KO, RANK/CamKII KO and RANK/GFAP KO. All these mouse strains were tested in different behavior tests including different depression models and a fear-based learning paradigm the inhibitory avoidance task.

2. MATERIALS AND METHODS

2.1. Generation of brain-specific RANK knockout mice

All animal experiments conformed to the International Guiding Principles for Biomedical Research Involving Animals and were approved by the authorities. Mice were group-housed as 4-5 animals per cage and maintained on a 14-h light/10-h dark cycle. Food and water were available ad libitum throughout the entire the entire study. Mice carrying a *rank*^{floxed} allele have been generated by Hanada and colleagues (Hanada et al., 2009). In order to generate mice carrying a null allele of *Rank (rank*$^{\Delta}$ allele) *rank*^{floxed} mice were crossed to β actin-Cre ubiquitous deleter mice (Lewandoski and Martin, 1997). For brain-specific deletion of RANK mice carrying the *rank*^{floxed} or the *rank*$^{\Delta}$ allele were crossed to either Nestin-Cre (Dubois et al., 2006), GFAP-Cre (Hirrlinger et al., 2006) or CamKII-Cre (Dragatsis and Zeitlin, 2000) mice. *Rank*^{floxed}, *Rank*$^{\Delta}$ and Nestin-Cre, GFAP-Cre and CamKII-Cre mice were backcrossed to C57BL/6 mice before generating Nestin-Cre *rank*$^{floxed/\Delta}$ GFAP-Cre *rank*$^{floxed/\Delta}$ and CamKII-Cre *rank*$^{floxed/\Delta}$ mice. Nestin-Cre acts in neural precursors leading to gene deletion in neurons and astrocytes (Tronche et al., 1999; Zimmerman et al., 1994). GFAP-Cre specifically inactivates genes in astrocytes (Johnson et al., 1995; Marino et al., 2000). CamKII-Cre deletes in neurons specifically in memory-relevant brain regions, mainly in the hippocampus and amygdala and to a lesser extent in the hypothalamus depending on the specific CamKII Cre line (Casanova et al., 2001).

2.1. Genotyping

Genomic DNA was digested over night (o/n) at 55°C in genomic DNA lysis buffer (100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) with Proteinase K solution (10mg/ml). The next day the lysate was heated at 99°C for 10 minutes to inactivate the enzyme and diluted with 200 ul of water. The isolated genomic DNA was amplified by PCR using the following three primers for *Rank*:

Forward Primer: 5'-GGCAG AACTC GGATG CACAG ATTGG-3'

Reverse Primer: 5'-AGTGT GCCTG GCATG TGCAG ACCTT-3'

Mutant allele: 5'-CTGGT GGTTG TTCTC CTGGT GTCAT-3'

Nestin-Cre, GFAP-Cre and CamKII-Cre transgenes were detected using the following primers:

Forward Primer: 5'-CTGCCACGACCAAGTGACAGCAATG-3'

Reverse Primer: 5'-GCCTTCTCTACACCTGCGGTGCTAA-3'

PCR conditions: 95°C/3min, 95°C/30sec,60°C/30sec, 72°C/30sec, step 2-4 5 cycles, 95°C/15sec, 54°C/15sec,72°C/30s, step 6-8 33 cycles

PCR samples were analyzed on a 2% agarose gel. PCR products were 390 bp (RANK floxed allele), 256 bp (RANK wild-type allele) and 566 bp (RANK Δ allele).

The following subgroups for all tissue-specific RANK knockout mice were made: The KO group included $rank^{floxed/floxed}$, $rank^{\Delta/\Delta}$, $rank^{floxed/\Delta}$ mice carrying the Cre recombinase. All the other mice carrying either a wild type allele with or without the Cre recombinase were grouped as controls since the presence of Cre recombinase did not affect the phenotype.

2.2. Immunostaining

Mice were anesthesized with an intraperitoneal (i.p.) injection of Ketasol-Xylasol (Animedica) in 0.1 phosphate buffered saline (PBS) (1:1:8) and transcardially perfused with 10 ml of 0.1 M PBS (pH 7.4) followed by 10 ml of 4% paraformaldehyde in 0.1 M PBS (PFA). Brains were removed, post-fixed with the same fixative o/n at 4°C and kept for 24 hours in 0.1 M PBS containing 30% sucrose. Brains were then embedded in TissueTek (Sakura) and frozen on a dry ice block. 20 µm thick frozen serial sections were obtained by cryosectioning and stored at -80°C. Following immunohistochemical staining all brain sections were examined using an Axio Imager Z2 widefield fluorescence microscope with a CoolSnap HQ2 camera and further analyzed with ImageJ.

RANK immunostaining

For all RANK immunostainings including co-immunolabeling with anti-NeuN and anti-GFAP the frozen brain sections were rehydrated 3 times in 0.1 M PBS for 5 minutes. After blocking for 2 hours with 0.1% Triton-X 100/5% donkey serum/2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.1 M PBS at room temperature (RT), the sections were incubated o/n at 4°C with anti-RANK antibody (dilution 1:13, R&D systems #AF692) and anti-NeuN (dilution 1:200, Millipore #MAB377) or anti-GFAP (dilution 1:1000, Millipore #MAB3402) in staining buffer (0.05% Triton-X 100/2.5% donkey serum/1% BSA). Sections were washed three times in 0.1 M PBS for 10 minutes followed by incubation for 1 hour at RT with anti-goat donkey fluorescent antibody (1:400 for RANK, Alexa Flour 488, Invitrogen #A-11055) and anti-mouse goat antibody (1:400 for NeuN and GFAP, Alexa Fluor 568 #A-11031) in staining buffer. After 3 washing steps in 0.1 M PBS for 10 minutes the sections were mounted in mounting media with 4',6-diamidino-2-phenylindole (DAPI) to stain the cell nuclei (Vectashield, VECTOR Laboratories) and protected with coverslips.

C-Fos immunostaining

For all c-Fos immunostainings including co-labeling with NeuN and GFAP, brain sections were washed in 0.1% Triton X 100 in 0.1 M PBS (PBST) for 10 minutes.

After blocking for 30 minutes in 1% BSA in PBST the sections were incubated o/n at 4°C with anti-c-Fos antibody (1:400, Abcam #ab87655) and anti-NeuN (1:200, Millipore #MAB377) or anti-GFAP (1:1000, Millipore #MAB3402) in blocking solution. The next day sections were washed three times with PBST for 10 minutes followed by incubation for 2 hours at RT with anti-goat donkey fluorescent antibody (1:400 for c-Fos, Alexa Flour 488, Invitrogen #A-11055) and anti-mouse goat antibody (1:400 for NeuN and GFAP, Alexa Fluor 568 #A-11031). The sections were again washed three times in PBST for 10 minutes before they were mounted in mounting media with DAPI to stain the nuclei (Vectashield, VECTOR Laboratories) and protected with coverslips.

2.3. Ovariectomy/Orchiectomy

Female and male mice between the ages of 10-12 weeks were anesthesized with Ketasol-Xylasol (Animedica) in 0.1 M PBS (1:1:8). Bilateral ovariectomy (ovx) in females was performed through abdominal skin and muscle incision under sterile conditions. Ovaries were located and carefully pulled out of the abdominal cavity, a knot was made around the fallopian tubes just below the ovaries, which were then removed together with the adherent fat tissue. One stitch was made using non-absorbable suture (Ethicon) on the muscle wall, and two stiches were made to close the skin incisions. Bilateral orchiectomy (orh) in males involved a single incision on the ventral side of the scrotum. The testicle was carefully pulled out of the scrotum, and a knot was made around the vas deferens. The testicles were then removed, and the incisions were closed with one stitch using non-absorbable sutures as above. Sham operations for both females and males were performed using the same procedure as ovx or orh except that the organs were not excised (Idris, 2012). The mice were allowed to recover for at least 4 weeks before they were tested and analyzed. For simplification, the following subgroups of mice were used throughout the entire thesis: “Cont sham” for sham-operated controls, “Cont ovx” and “Cont orh” for ovx and orh controls and “KO ovx” and “KO orh” for ovx and orh RANK mutant mice.

2.4. Estrous cycle determination

All the C57BL/6 female mice used for the determination of the estrous cycle were 10 to 14 weeks of age. The murine estrous cycle lasts for about four to five days and its four stages are termed proestrus, estrus, metestrus and diestrus (Byers et al., 2012). The different stages of estrous were determined by the cytological evaluation of vaginal smears. Proestrus is characterized by the presence of mainly nucleated and some cornified epithelial cells whereas estrus shows only cornified epithelial cells. Metestrus is marked with the presence of cornified epithelial cells and polymorphonuclear leukocytes. In diestrus which is the longest stage lasting up to 48 hours vaginal smears show primarily polymorphonuclear leukocytes. Vaginal smears were collected by using a cotton tipped swab wetted in 0.1 M PBS and inserted into the vagina. The cells on the swab were transferred to a glass slide and stained with Giemsa solution for 2 minutes (Sigma-Aldrich). After washing the slides in water for 1 minute they were viewed under bright field illumination. (Byers et al., 2012) Vaginal swabs were obtained 3 times daily and only normal cycling mice were included in the study. Mice classified as being in diestrus or estrus were sacrificed and blood and tissue collected for analysis. In order to verify the stage of the estrous cycle, blood was collected for 17β-estradiol analysis and one uterine horn was removed for measuring its thickness. Both procedures are described below.

2.5. Uterus analysis

Ovx mice, or those in either estrus or diestrus stage of the cycle were sacrificed by cervical dislocation and one uterine horn was removed for further analysis. The uterine horn was fixed for 2 to 3 hours in 4% PFA and embedded in paraffin. The paraffin blocks were sectioned until the mid-sagittal plane was reached. Sections were dewaxed in two steps by using xylene for 5 minutes each followed by rehydration in ascending concentrations of ethanol (100, 95, 70, 30% EtOH) for 5 minutes each. After staining for 5 minutes with Hematoxylin (Sigma-Aldrich) the sections were rinsed with tap water and destained with 5% hydrochloric acid

(HCl) in 0.1 M PBS. The sections were again rinsed with tap water and treated with 30% and afterwards with 70% EtOH for 5 minutes each. Sections were then stained with eosin (Sigma-Aldrich) for 16 seconds followed by a treatment with 95% and 100% EtOH for 5 minutes each. Finally the sections were put two times for 5 minutes in xylene, mounted in Permount mounting medium (eBioscience), and sealed with a coverslip. All the slides were automatically scanned using a Mirax Scanner (Zeiss) and the thickness of the uterine tissue was measured by using Panoramic viewer (3DHISTECH).

2.6. ELISA and sample preparation

For the determination of RANKL and OPG protein concentrations in the sera and brains ELISA kits from R&D Systems (#MTR00, #MOP00) were used. RANK protein levels were analyzed with an ELISA kit from Abcam (#ab119606) and 17β-Estradiol (E2) levels with a kit from Calbiotech (#ES180S-100). All ELISAs were performed according to the manufacturer's instructions.

2.6.1. Serum samples

Blood was collected from mice in estrus, diestrus or from ovx and sham-operated control mice by using the submandibular bleeing method (Golde et al., 2005). Serum was prepared using BD microtrainer SST tubes (BD). Briefly, blood was allowed to clot for 30 minutes and then centrifuged for 1.5 minutes at 9000g. Serum samples were diluted in sample buffer: 1:2 for RANK and RANKL, 1:5 for OPG ELISA and undiluted for 17β-Estradiol measurements.

2.6.2. Brain samples

Ovx mice or mice in estrus or diestrus stages were sacrificed by cervical dislocation. Fore- and midbrain or the hypothalamus were dissected out and immediately snap-frozen. Before further analysis brain samples were homogenized in RIPA buffer (50 mMTris- HCl pH 7.6, 150 mMNaCl, 5 mM $CaCl_2$, 0.05% NaN_3 and 1% Triton X-100) either manually with a

Teflon homogenizer (fore-/midbrain) or automatically using the bead-beating Precellys® 24 homogenizer (hypothalamus, Bertin Technologies). Homogenates were centrifuged at 4°C for 10 min at 12000g. Pellets were discarded and supernatants used for ELISA. Before the samples were analyzed they were further diluted in RIPA buffer: 1:2 for RANK and RANKL and 1:10 for OPG measurements.

2.7. mRNA quantification

2.7.1. RNA isolation

Hippocampus and hypothalamus of ovx and sham-operated control mice or mice in estrus and diestrus stages were dissected out and total RNA was prepared using the RNeasy Mini Kit (Qiagen #74104) according to the manufacturer's protocol. The final RNA was eluted in 40 µlRNase-free water and the concentrations were measured using the NanoDrop 1000 Spectrophotometer (Peqlab).

2.7.2. cDNA synthesis

The iScriptcDNA synthesis kit (Bio-Rad #170-8890) was used for the synthesis of first-strand cDNA templates from the purified RNA. 700 ng of total RNA were transcribed using 5x iScript reaction mix and iScript reverse transcriptase. All steps were carried out according to the manufacturer's protocol.

2.7.3. Quantitative real-time PCR (qPCR)

iQ™SYBR®GreenSupermix (Bio-Rad) was used for qPCR using MyiQ and iQ5 Real Time PCR Detection Systems (Bio-Rad). The qPCR mix, primers used and the reaction protocol are stated below. Gene expression was normalized to the level of β-actin mRNA.

RANKL forward primer: 5'-CTGAGGCCCAGCCATTTG-3'

RANKL reverse primer: 5'-GTTGCTTAACGTCATGTTAGAGATCTTG-3

qPCR mix:12.5 µl iQ mix, 11.5 µl cDNA, 1 µl Primer mix (forward + reverse, 10 µM each)

PCR conditions: 95°C/1min, 95°C/10sec, 60°C/30sec, 72°C/30sec, cycle to step 2 for 40 more times, 65°C/5min.

2.8. Behavioral tests

2.8.1. Experimental design

To test the effects of RANKL/RANK on mood and learning in genetically modified animals several well-established behavioral assays were performed with mice between the ages of 10-14 weeks. Figure 2.1 shows the experimental flow of all behavior tests performed, starting with the least stressful assay. Anxiety and fear was tested by elevated plus maze (EPM), depression was examined by tail suspension test (TST), forced swim test (FST) and sucrose preference test (SPT). Basic reflex was analyzed by prepulse inhibition (PPI) and long-term memory was tested by inhibitory avoidance (IA). Ample recovery time was provided for the animals that underwent more than one test (at least 24 hour between tests).

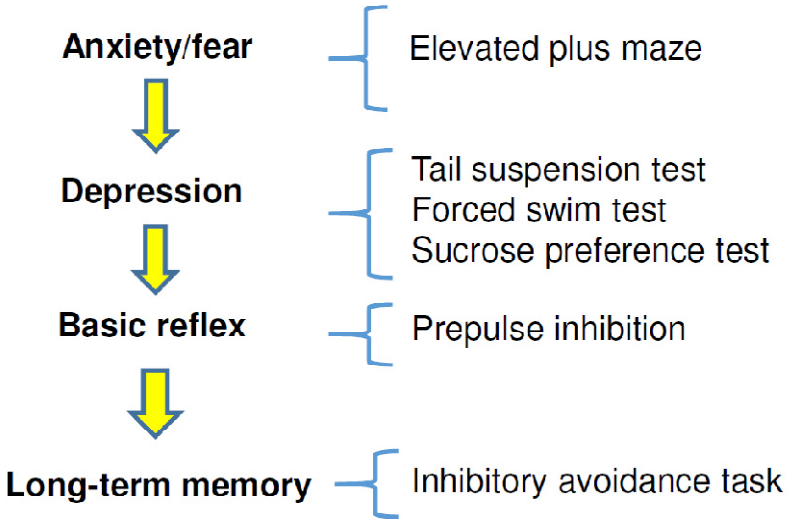


Figure 2.1: Experimental flow of all the behavior tests.
The experimental workflow of all the behavioral tests in chronological order.

2.8.2. Elevated plus maze (EPM) test

For all behavioral tests animals were habituated to get used to the testing environment. The elevated plus maze (EPM) is a commonly used test to evaluate the levels of anxiety in rodents. It was first described by Pellow and co-workers and reflects a physiological conflict between the animal's curiosity to explore the new surrounding and their natural drive to stay in a secure, closed, dark space, which is known as thigomotaxis (Pellow et al., 1985). The apparatus consists of a central platform and two open and two enclosed arms. It is elevated from the ground in order to prevent mice from jumping off. For analysis the mouse is placed in the center of the maze facing an open arm. The animal was allowed to explore the maze for the duration of 5 minutes while it was being recorded. The amount of time spent in each arm was automatically analyzed by ANY-maze software (Stoelting). Time spent in each arm was averaged within experimental groups. One-way ANOVA was used to evaluate significance levels. P-values smaller than or equal to 0.05 were considered significant.

2.8.3. Forced swim test (FST) and tail suspension test (TST)

The forced swim test (FST) and tail suspension test (TST) are two simple and commonly used models for the assessment of depression in rodents invented by Porsolt and colleagues in 1977 (Porsolt et al., 1977b). Both tasks are based on the principle that, as the mouse is exposed to an inescapable situation, it will react with vigorous activity as an attempt to escape. This will be followed by complete immobility which is interpreted as a state of despair and depression-like behavior as the mouse becomes aware that there is no escape from that stressful situation. The immobile stage can be reversed by anti-depressants (Chenu et al., 2006; Einat et al., 2001).

In brief, for the FST each mouse is placed in a clear Plexiglas cylinder (65 cm tall x 25 cm diameter) filled with tepid water. The mouse is kept in the water for 10 minutes, while its activity is being recorded by ANY-maze software (Stoelting). After initial attempts to escape from the water the mouse adopts immobile postures and makes only movements necessary to

keep its head above water. The mean total time spent immobile was recorded every 2 minutes. After testing, animals were dried with paper towels and the cages were put on a heating pad.
The TST is the "dry" version of the FST in which immobility is induced by suspending the mouse by its tail (Choi et al., 2013; Krishnan and Nestler, 2008). Adhesive tape was wrapped around the animal's tail and a suspension hook was put through the adhesive tape so that the mouse hangs in a straight line with its tail. Mice were observed for a total of 6 minutes and the time spent immobile was recorded manually with a stopwatch. The mean total time spent immobile was calculated for each group. One-way ANOVA was used to evaluate significance levels. P-values smaller than or equal to 0.05 were considered significant.

2.8.4. Sucrose preference test (SPT)

The sucrose preference test (SPT) is a non-stressful experimental model for testing depression in mice (Moreau, 1997). In comparison to FST and TST, which are despair-based depression models, the SPT is reward-based in which the natural preference of mice for sweet food is utilized. For 5 consecutive days single-housed mice were given a choice between two drinking bottles, one containing 2% sucrose solution and the other water. Both bottles contained a volume of excess of what the mice consumed during the five days. In order to prevent possible effects of side preference in drinking behavior the position of the bottles were switched daily. The consumption of sucrose solution and water was measured by weighing the bottles daily at the same time. The sucrose preference was calculated as a percentage of total volume of sucrose intake over the total volume of fluid intake (water and sucrose) and averaged over the 5 testing period. The mean of sucrose intake of each test group was calculated for each group. Reduced preference for sucrose is indicative of anhedonia, the inability to experience pleasure (Steckler et al., 2005; Strekalova et al., 2004). One-way ANOVA was used to evaluate significance. P-values smaller than or equal to 0.05 were considered significant.

2.8.5. Prepulse inhibition (PPI)

Prepulse inhibition (PPI) is a test which examines basic synaptic functions called synaptic gating (Basavaraj and Yan, 2012). It is based on the principle that the reaction to a stimulus will be reduced if preceded by another, non-harmful stimulus of lower intensity. In the case of PPI, auditory stimuli were used to test synaptic gating. The mouse is placed on a movement-sensitive platform in a small chamber to record the mouse startle response (SR-LAB Startle Response System, San Diego Instruments). The reaction to the sounds presented to the mice are part of a well-defined synaptic circuit which starts at the auditory cortex and terminates with a stereotyped front and hind leg muscular twitch which is detected and recorded by the platform (Larrauri and Schmajuk, 2006). At the start of the test, the mouse is presented by an intense acoustic startle stimulus (120 dB). In the second part of the test, the mouse is presented with a series of acoustic stimuli of lower intensity (80, 85, 90 and 95 dB) which precede the 120 dB startle pulse. Normally, the prepulse will result in attenuation of the startle response. Deficits in PPI are often indicative for abnormalities in sensorimotor gating associated with impaired cognitive function (Larrauri and Schmajuk, 2006; Li et al., 2009). The mean percentage of the startle reflex after PPI over the startle response after the loud tone was calculated for each trial cohort. Two-way ANOVA was used to evaluate significance levels. P-values smaller than or equal to 0.05 were considered significant.

2.8.6. Inhibitory avoidance (IA)

The inhibitory avoidance (IA) task is a fear-based learning paradigm (Brioni et al., 1989). The test apparatus consists of two compartements equally partitioned by a sliding door (Siemens). The safe compartment is brightly illuminated whereas the shock compartment is dark. Before the testing is initiated, mice are handled by the experimenter for a few minutes for at least 5 consecutive days. The tests last for three days. On day one, the mice are allowed to explore the testing chamber. Initially, they are placed in the safe compartment for 30 seconds, after which, the automatic sliding door opens and provide the mice with a choice to enter the dark compartment. As mice are nocturnal animals, they will enter the dark compartment and the

automatic door will close behind them. The mice are allowed to explore the dark compartment for 30 seconds after which they are placed back to their home cage. The next day, mice are subjected to the same procedure; however, once they enter the dark compartment, they will receive an electric foot shock (2s, 300μA) via the floor grid (Coulbourn precision animal shocker). 30 seconds later the mouse is returned to its home cage. IA memory is tested 24 hours post-training by placing the rodent back in the lit compartment. Retention is assessed by measuring latency to reenter the dark chamber. Mice are provided a total of 5 minutes in the light compartment. Two-way ANOVA was used to evaluate significance levels. P-values smaller than or equal to 0.05 were considered significant.

3. RESULTS

3.1. RANK is expressed in the ventromedial hypothalamic nucleus

In 2009 Hanada and colleagues localized RANK to neurons and astrocytes of the preoptic area (POA), the medial septal nucleus (MSn) and the lateral septal nucleus (LSn) in the hypothalamus which all play a central role in thermoregulation (Hanada et al., 2009). Shimamura et al extended these studies by showing that RANK is also expressed in activated macrophages of the cortex after an ischemic episode (Shimamura et al., 2014).
To extend these previous studies, I performed immunofluorescence stainings of C57BL/6 female mouse mouse brains. These stainings showed that RANK is also expressed in the ventromedial hypothalamic nucleus (VMH) (Figure 3.1 A, B). RANK full body KO ($rank^{\Delta/\Delta}$) did not show any expression of RANK (Figure 3.1 C), thus confirming specificity of the anti-RANK antibody. Co-immunolabeling with either a neuronal (Anti-Neuronal Nuclei, Anti-NeuN) or astrocyte (Anti-Glial Fibrillary Acidic Protein) specific marker revealed that in the VMH RANK is specifically expressed in neurons (Figure 3.2 A) and not in astrocytes (Figure 3.2 B). Careful analysis of other brain regions including the cortex, hippocampus, striatum and amygdala did not show any detectable RANK staining (not shown). Of note, the VMH is not only involved in thermoregulation, feeding and sexual activity, but importantly also in processing fear and fear-based learning paradigms (Gross and Canteras, 2012; Kurrasch et al., 2007; Trogrlic et al., 2011). Trogrlic et al. identified a specific population of neurons in the VMH and the amygdala being involved in context fear conditioning, a fear-based learning

paradigm (Trogrlic et al., 2011). Thus, RANK is highly expressed in a key brain region involved in fear conditioning and learning.

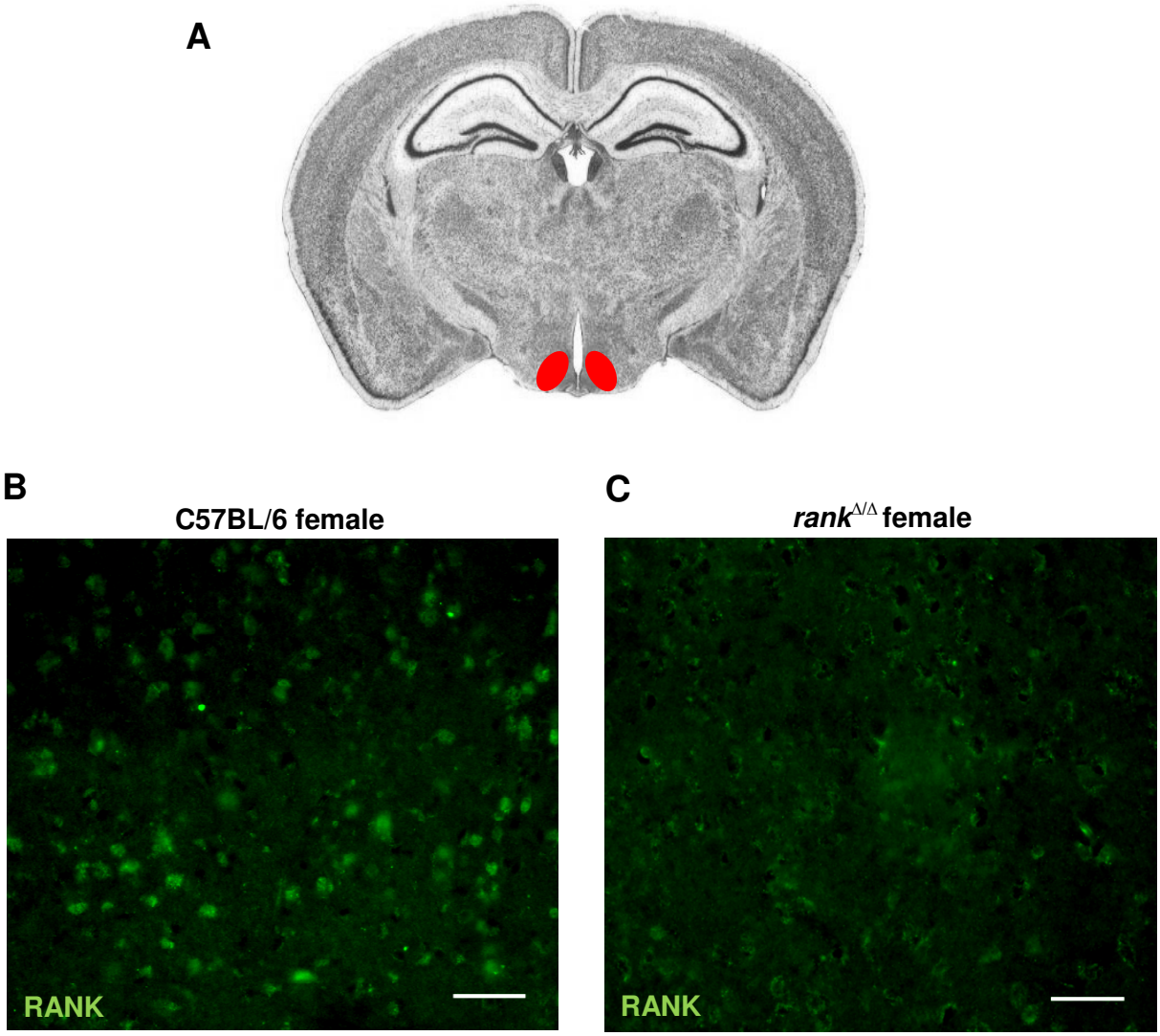


Figure 3.1: RANK is expressed in the VMH brain region.
(A) Coronal section of the mouse brain, the red circle marks the VMH region.**(B, C)** Immunofluorescence staining for RANK protein (green) in the brain of a **(B)** wild type C57BL/6 female mouse 12 weeks of age and of a **(C)** RANK$^{\Delta/\Delta}$female mouse. Mice were perfused with 4% PFA and the brain post-fixed and treated with 30% sucrose. Coronal cryosections were then stained with anti-RANK antibody. Images show the VMH brain region. Magnifications 20x. Bars =50µm.

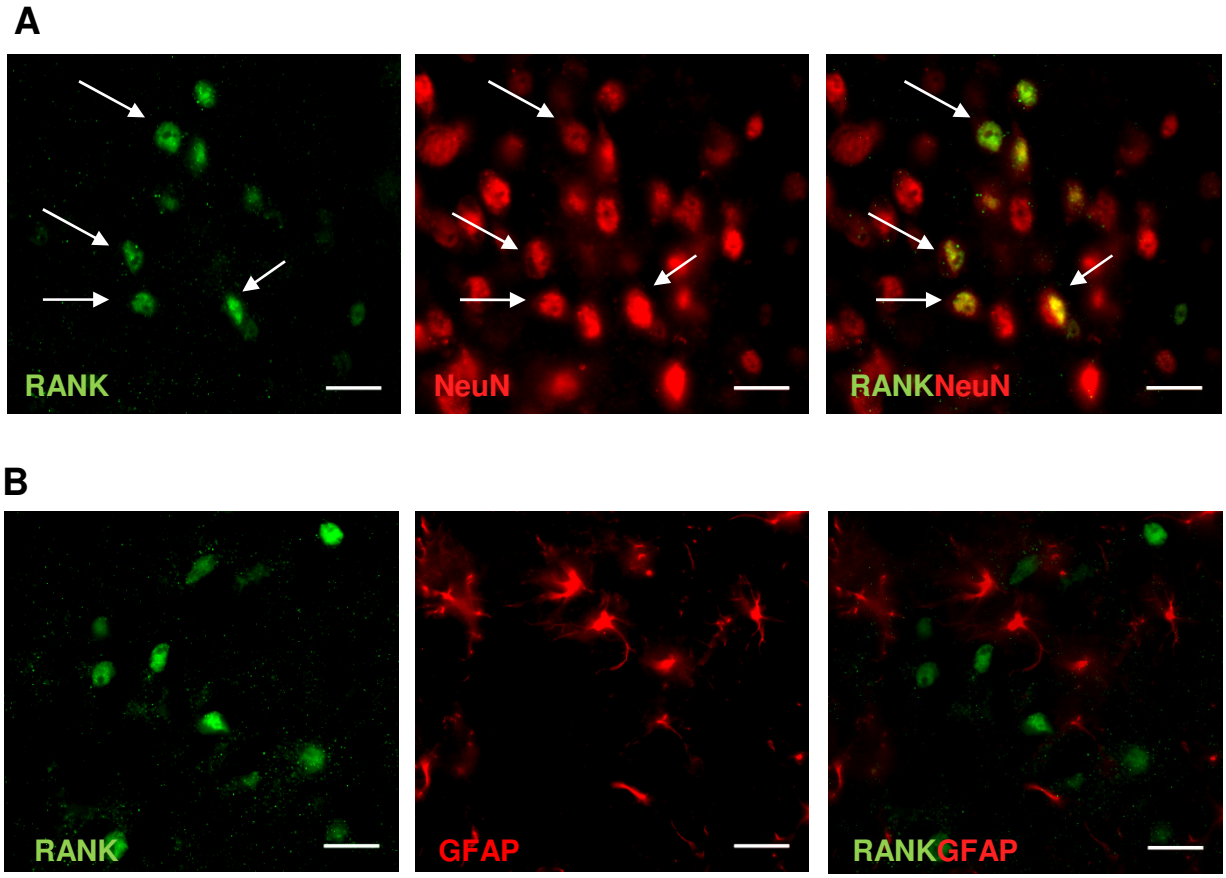


Figure 3.2: RANK is expressed on neurons but not astrocytes in the VMH brain region.
Immunofluorescence staining for **(A)** RANK protein (green) and NeuN (red) to label neurons and **(B)** RANK protein (green) and GFAP (red) to label astrocytes. Individual images and merged images show the VMH brain region of a wild type C57/6J female 12 weeks of age. The co-localization of RANK and NeuN was determined by the overlay of RANK and NeuN or RANK and GFAP (yellow, arrow). Magnifications 40x. Bars = 20µm

3.2. Female sex hormones regulate RANKL and OPG in the periphery but not in the brain

RANKL and OPG are under strict regulation of female sex hormones. It was reported that estrogen upregulates OPG and downregulates RANKL mRNA and protein levels in osteoblasts (Bord et al., 2003; Hofbauer et al., 1999; Khosla et al., 2002; Shevde et al., 2000; Szulc et al., 2001; Varsavsky et al., 2012). Considering evidence that estrogen influences mood and learning, we sought to investigate whether the RANK/RANKL/OPG axis was downstream of estrogen-regulated effects on the brain. Considering ER receptors are distributed throughout the brain (see introduction section 1.5) and that RANK is expressed in specific regions of the hypothalamus, we asked whether female sex hormones can also influence RANK, RANKL and OPG levels in the brain. In order to answer this question, we examined female C57BL/6 mice of various stages of the estrous cycle, as well as ovx females. First vaginal smears were collected from C57BL/6 female mice in order to determine the stage of the estrus cycle. The stages were then confirmed by measuring the thickness of the uterine horn as well as measuring serum 17β-estradiol levels. Figure 3.3 A shows paraffin sections of uterine horns stained with H&E. The uterine width positively correlated with the serum 17β-estradiol levels whereby the uterine thickness was at its peak in estrus when 17β-estradiol levels were the highest (Figure 3.3 B, C).

For further analysis we focused on three conditions in the female mouse: females in estrus which have high estrogen and low progesterone levels; those in diestrus with low estrogen and high progesterone levels; and estrogen and progesterone-deficiency in ovx mice. The hippocampus, hypothalamus (Figure 3.4), entire fore-/midbrain (Figure 3.5) and serum (Figure 3.6) were collected in order to measure mRNA and protein levels of RANK, RANKL and OPG. QPCR analysis of mRNA levels of RANK, RANKL and OPG revealed no change in expression of any of the three mRNAs evaluated in these brain regions (Figure 3.4). Furthermore, ELISA assays which were used to measure protein levels also showed no change of either RANK, RANKL or OPG in the brain (figure 3.5). In contrast, RANKL and OPG levels

were significantly altered in the serum (Figure 3.6). Serum OPG levels positively correlated with 17β-estradiol levels and peaked in estrus (Figure 3.6 B) whereas serum RANKL levels negatively correlated with 17β-estradiol and peaked in ovx mice (Figure 3.6 A), which was consistent with previous publications (Rogers et al., 2002; Szulc et al., 2001). Taken together, these data demonstrate that while sex hormone levels can modulate expression of RANKL and OPG in serum, there was no apparent influence of estrogen on RANK, RANKL and OPG levels in the brain.

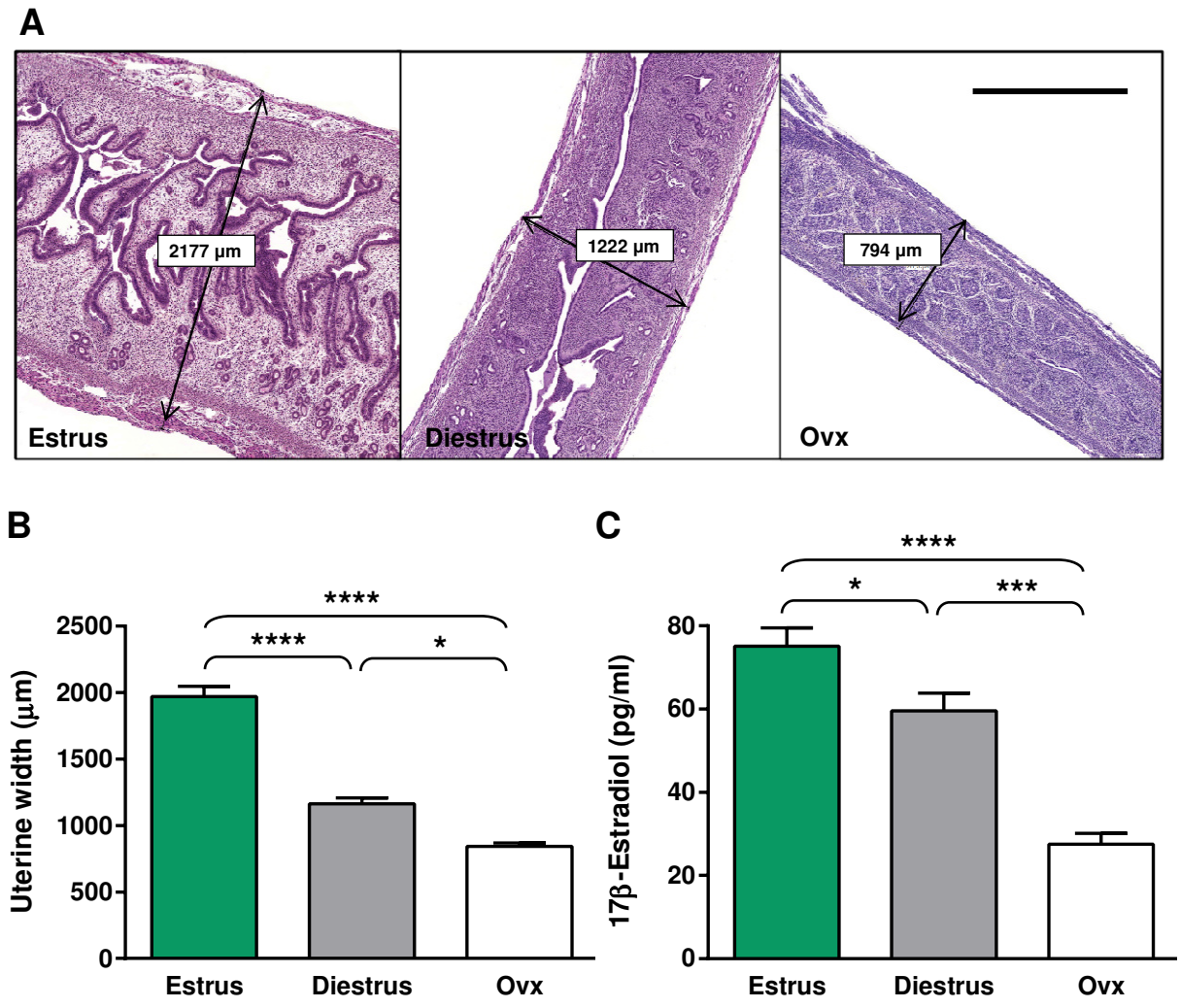


Figure 3.3: Uterine width positively correlates with 17β-estradiol levels.

(A) Histological evaluation of mouse uterine sagittal tissue sections stained with H&E. Two stages of the estrous cycle estrus and diestrus, and **t**he uterus of an ovx mouse are shown. Uterine thickness is indicated. Bar = 1 mm **(B)** Uterine width positively correlated with 17β-estradiol level. Estrus uteri (n=7) were wider than at diestrus (n=9) or ovx mice (n=3) (**P < 0.05; ****P<0.0001*). **(C)** Serum 17β-estradiol levels as a function of the estrous cycle. 17β-estradiol was highest in estrus (n=4) compared to diestrus (n=4) and ovx (n=5) (**P < 0.05; ***P<0.001; ****P<0.0001).* Values are shown as means ± SEM (one-way ANOVA with Tukey's multiple comparisons test).

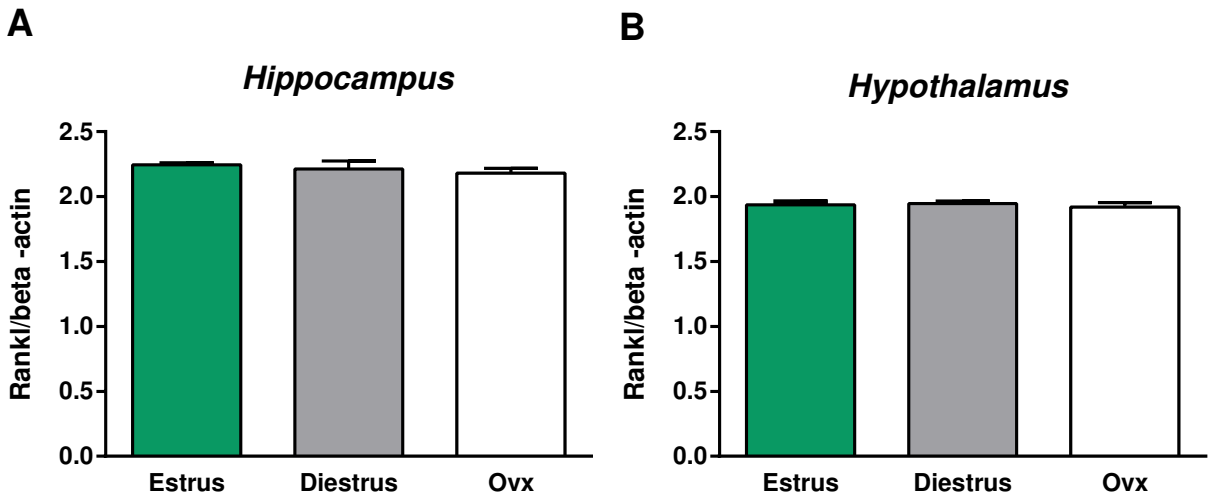


Figure 3.4: RANKL mRNA levels in the hippocampus and the hypothalamus.

(A) Hippocampal mRNA levels of *Rankl* in estrus (*n*=4), diestrus (n=3) and ovx (n=3). **(B)** Hypothalamic mRNA levels of *Rankl* in estrus (n=4), diestrus (n=3) and ovx (n=3). QPCR reactions were done in triplicates. Relative expression of *Rankl* to β-actin are shown as means ± SEM (one-way ANOVA with Tukey's multiple comparisons test).

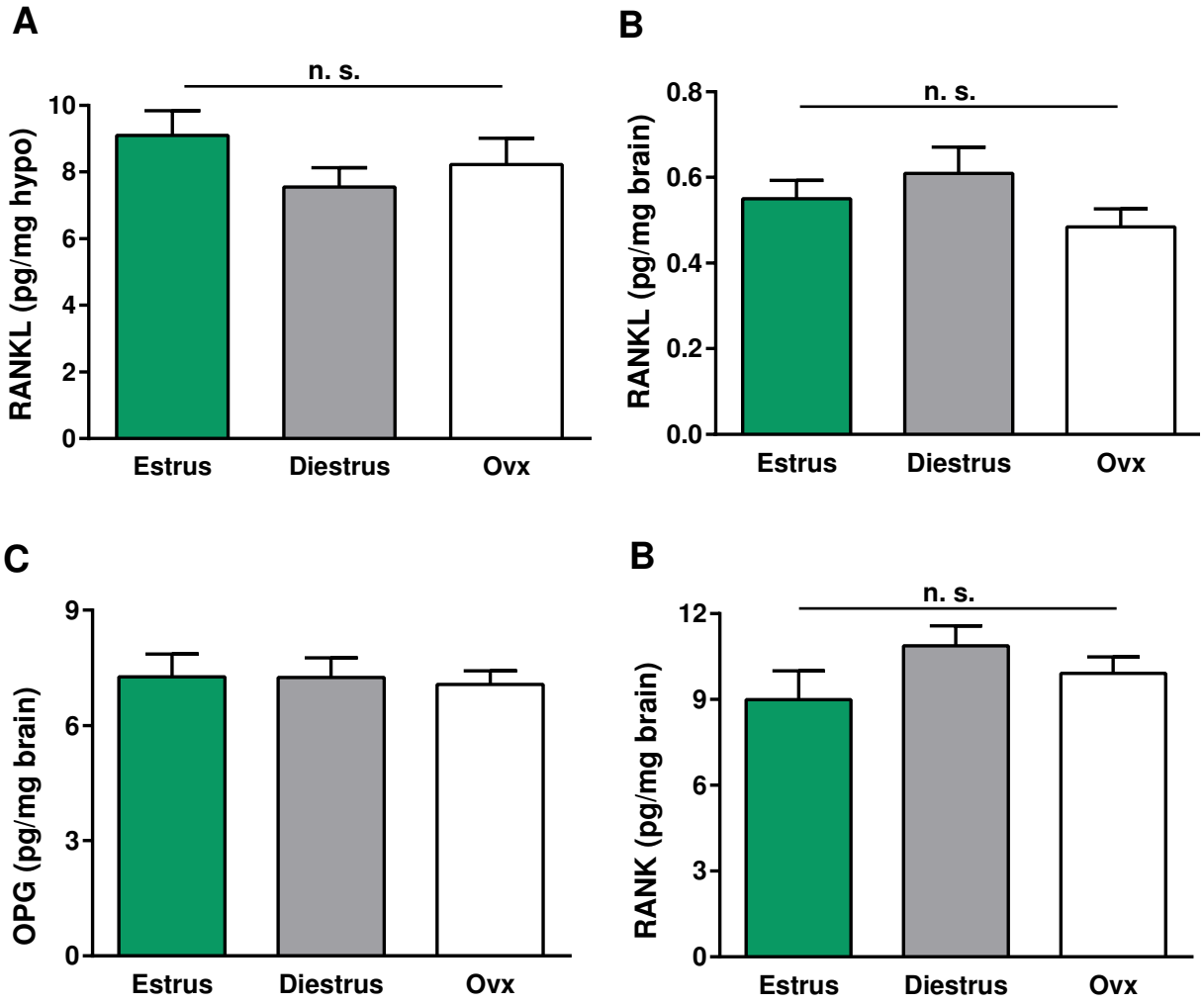


Figure 3.5: ANKL, OPG and RANK protein levels in the brain.

(A) Hypothalamic RANKL protein levels in estrus, diestrus and ovx (each n=3). **(B)** RANKL protein levels in the fore-/midbrain in estrus, diestrus and ovx (each n=9). **(C)** OPG protein levels in the fore-/midbrain in estrus (n=5), diestrus (n=4) and ovx (n=7). **(D)** RANK protein levels in the fore-/midbrain in estrus, diestrus and ovx (each n=4). RANKL, OPG and RANK protein levels in the hypothalamus and the fore-/midbrain in estrus, diestrus and ovx mice were determined by ELISA. The indicated brain regions were isolated, frozen, homogenized and then assayed. No significant (n. s.) regulation of RANKL, OPG and RANK in the brain was detected. Values are shown as means ± SEM of pg RANKL, OPG or RANK /mg tissue (one-way ANOVA with Tukey's multiple comparisons test).

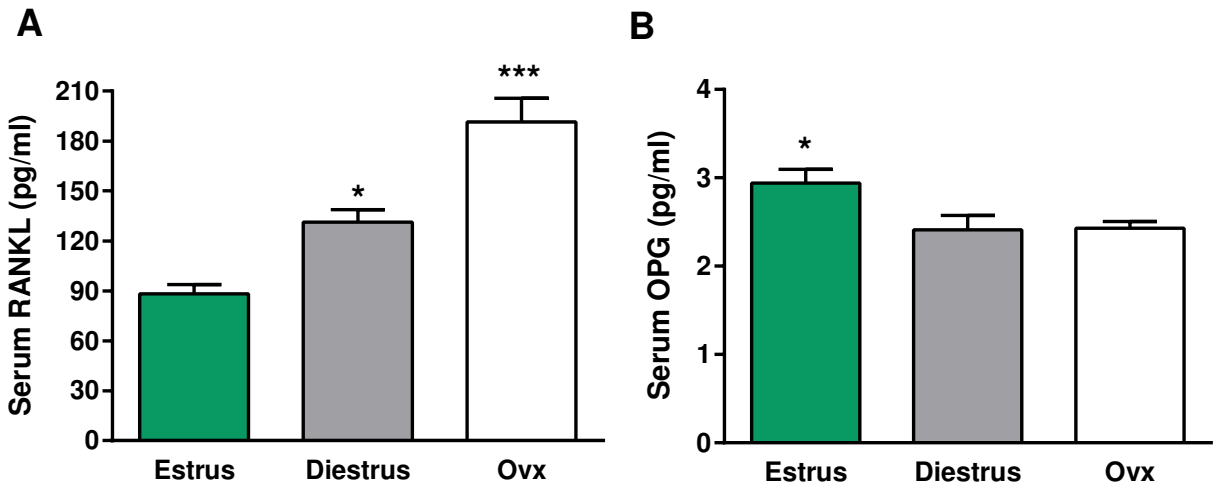


Figure 3.6: Female sex hormones regulate RANKL and OPG in the periphery.

(A) Serum RANKL was significantly elevated in ovx (n=7) (***$P<0.001$) and diestrus (n=9) (*$P < 0.05$) compared to estrus (n=5). **(B)** Serum OPG peaked in estrus (n=5) (*$P < 0.05$) as compared to diestrus (n=5) and ovx (n=8). Serum RANKL and OPG levels in estrus, diestrus and ovx mice were determined by ELISA. Values are shown as means ± SEM of pg RANKL, OPG or RANK/ml serum (one-way ANOVA with Tukey's multiple comparisons test).

3.3. I.v. RANKL injections induce c-Fos expression in the VMH

Considering that female sex hormones regulate RANKL and OPG in the periphery and apparently not in the brain, we next asked whether circulating RANKL can actually activate memory-relevant brain regions and thus affect learning. In order to answer this question we decided to inject RANKL in the periphery of the mouse and test its effects on learning. It has already been shown that i.p. injection of RANKL did not influence change in body temperature, and thus it has been suggested that circulating RANKL is unable to penetrate the blood brain barrier (Hanada et al., 2009). We therefore decided to inject RANKL into the tail vein of the mouse (i.v. injection) to test whether RANKL will be able to reach the brain. Increase in body temperature will have a detrimental effect on learning and locomotion so we therefore had to rule out any "side-effects" of exogenous RANKL that might influence the learning process of mice. In order to do this we first examined activity and body temperature after i.v. injection of 1µg RANKL. Figure 3.7 C and 3.8 C show the circadian activity one day before and one day after i.v. RANKL injection (red arrow) into female and male C57BL/6 male mice. As a control RANKL was boiled for 10 minutes to inactivate the protein and i.v. injected into female and male C57BL/6 mice. Measurements for both sexes revealed a clear nocturnal rhythm of spontaneous locomotor activity which is high in the dark period and low in the light period. No differences were observed in either light or dark phase of activity in the male or female mice following i.v. injection of active RANKL.

In addition body temperature was monitored to ensure i.v. injection did not trigger a febrile response. Again, active and inactive RANKL was injected i.v. into female (Figure 3.7 A, B) and male C57BL/6 mice (Figure 3.8 A, B). Rectal temperature was determined 0.5, 3, 6, 24 and 48 hours after injection. Both active as well as inactive RANKL led to a significant increase in body temperature of both sexes 30 minutes after injection which returned to base line 3 hours after injection (Figure 3.7 B, 3.8 B). The brief febrile response detected is commonly observed in tail vein injections and occurs because of stress induced by the handling of

the animals and the injection itself (Hanada et al., 2009). This brief increase in body temperature after i.v. injection did not alter activity of the animals (Figure 3.7 C, 3.8 C). Thus, i.v. injections of RANKL have no apparent effect on body temperature or the circadian clock.

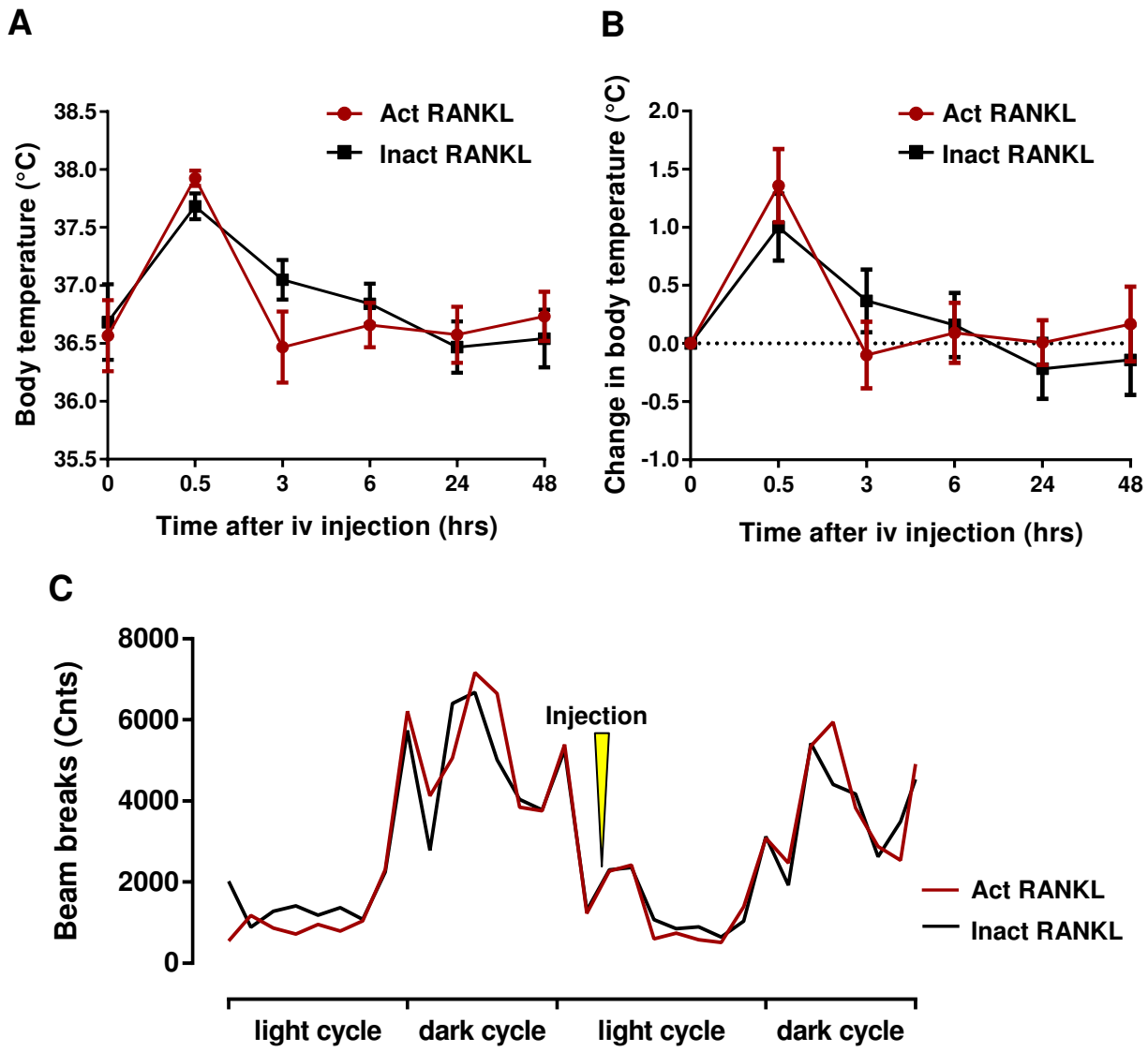


Figure 3.7: I.v. RANKL injections in C57BL/6 female mice do not alter body temperature and circadian activities.

(A) Body temperature and **(B)** change in body temperature in C57BL/6 female mice between the ages of 10 – 12 weeks after i.v. injection of 1 µg active RANKL (n=12) or inactive RANKL (n=12). Temperature was determined 0.5, 3, 6, 24 and 48 hours after injection using a rectal thermometer. No difference between active and inactive RANKL treatment were detected. **(C)** Circadian activity of C57BL/6 females i.v. injected either with 1 µg active (n=8) or inactive RANKL (n=7) was recorded for one week under a 14-h light/12-h dark cycle by using a three-dimensional (x,y,z) infrared light-beam. Data from one day before and one day after injection (yellow arrow) are shown. The beam breaks of the x-dimension were used for calculations. No differences in locomotor activity during the light and dark phase were detected comparing active with inactive RANKL treatment. Inactive RANKL was prepared by boiling RANKL for 10 minutes. Values are shown as means ± SEM (one-way ANOVA).

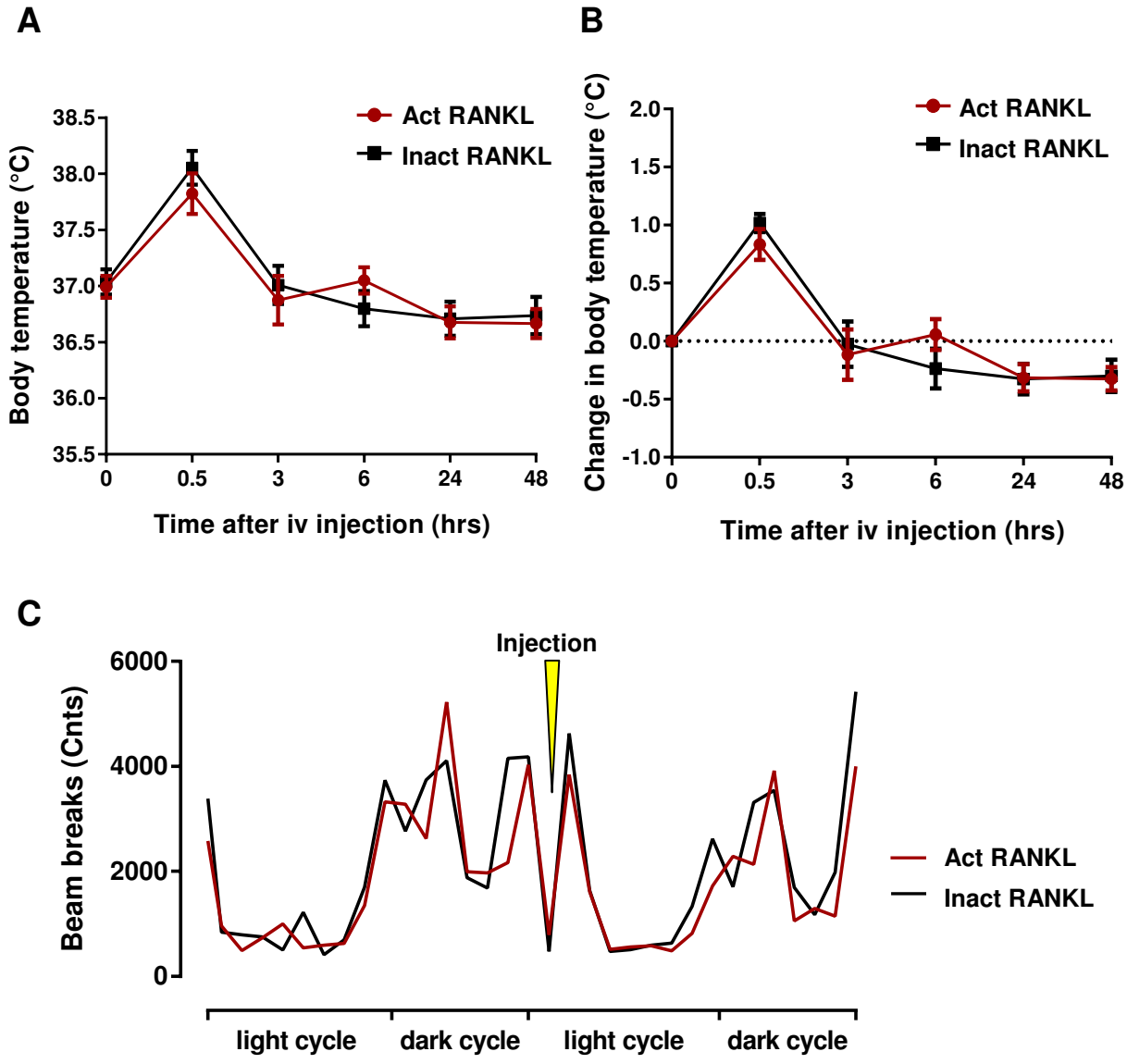


Figure 3.8: I.v. RANKL injections in C57BL/6 male mice do not alter their body temperature and circadian activities.

(A) Body temperature and **(B)** change in body temperature in C57BL/6 male mice between the ages of 10 – 12 weeks after i.v. injection of 1 µg active RANKL (n=12) or inactive RANKL (n=12). Temperature was determined 0.5, 3, 6, 24 and 48 hours after injection using a rectal thermometer. No difference between active and inactive RANKL treatment were detected. **(C)** Circadian activity of C57BL/6 males i.v. injected either with 1 µg active (n=8) or inactive RANKL (n=8) was recorded for one week under a 14-h light/12-h dark cycle. Data from one day before and one day after injection (yellow arrow) are shown. The beam breaks of the x-dimension were used for calculations. No differences in locomotor activity during the light and dark phase were detected comparing active with inactive RANKL treatment. Values are shown as means ± SEM (one-way ANOVA).

C-Fos is a critical downstream target of RANK signaling (Grigoriadis et al., 1994; Takayanagi et al., 2002). It is also an indirect marker for neuronal activity (Herrera and Robertson, 1996). To test whether peripheral RANKL could activate defined regions of the brain, we examined c-Fos levels in various brain areas following i.v. injections of RANKL. As a control, heat-inactivated RANKL was administered i.v. to male C57BL/6 mice. Mice were sacrificed 30 minutes after injection, perfused as described earlier, brains harvested and cryosectioned. Several sections of the brain were stained for c-Fos, NeuN and GFAP.

Careful analysis of the whole brain revealed that active RANKL triggered activation of c-Fos specifically in the VMH compared to mice treated with inactive RANKL (Figure 3.9). Co-localization studies of c-Fos with NeuN and GFAP indicated that c-Fos is specifically expressed in neurons (Figure 3.10 A-C), but not in astrocytes (Figure 3.10 D-F). To confirm this finding, we examined c-Fos activation in RANK/GFAP KO and RANK/Nestin KO males after i.v. injection of active RANKL. We reasoned that if we removed RANK expression from neurons we would no longer detect c-Fos activation in response to peripheral RANKL injection. Indeed, c-Fos expression was induced in the VMH of RANK/GFAP KO mice (Figure 3.10 G, J), but not in RANK/Nestin KOs (Figure 3.10 M). Again, co-immunolabeling with NeuN and GFAP revealed that c-Fos is only expressed within neurons (Figure 3.10 G-I) and not within astrocytes in these animals (Figure 3.10 J-L). Taken together, these results demonstrate that peripheral RANKL can activate the VMH as shown by c-Fos expression. Furthermore, peripheral RANKL specifically activates neurons in the VMH and not astrocytes.

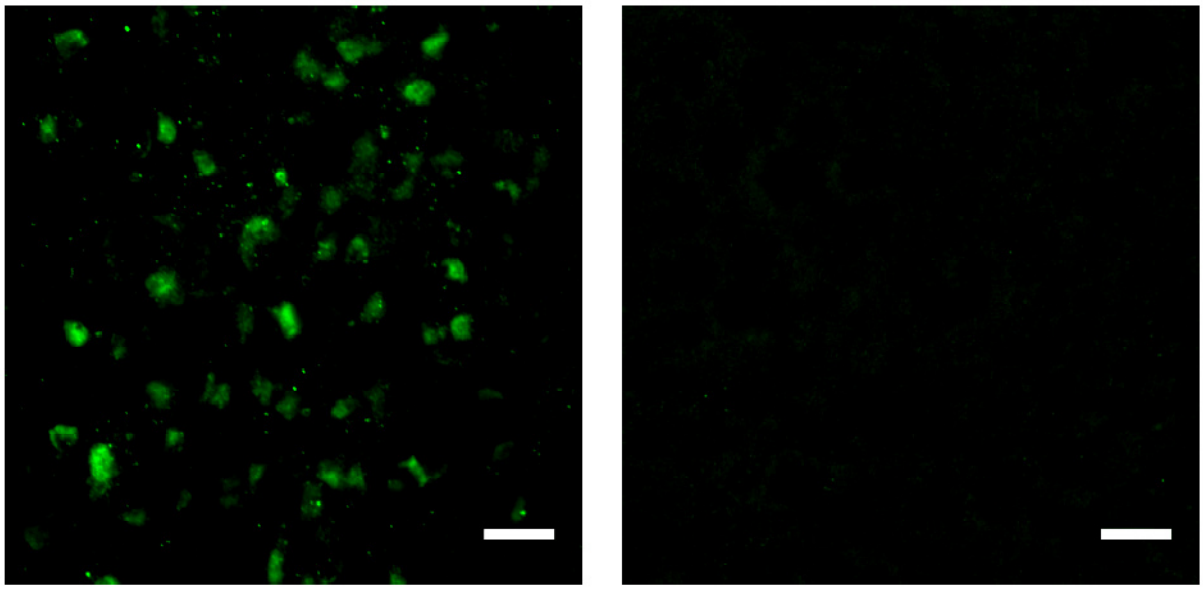
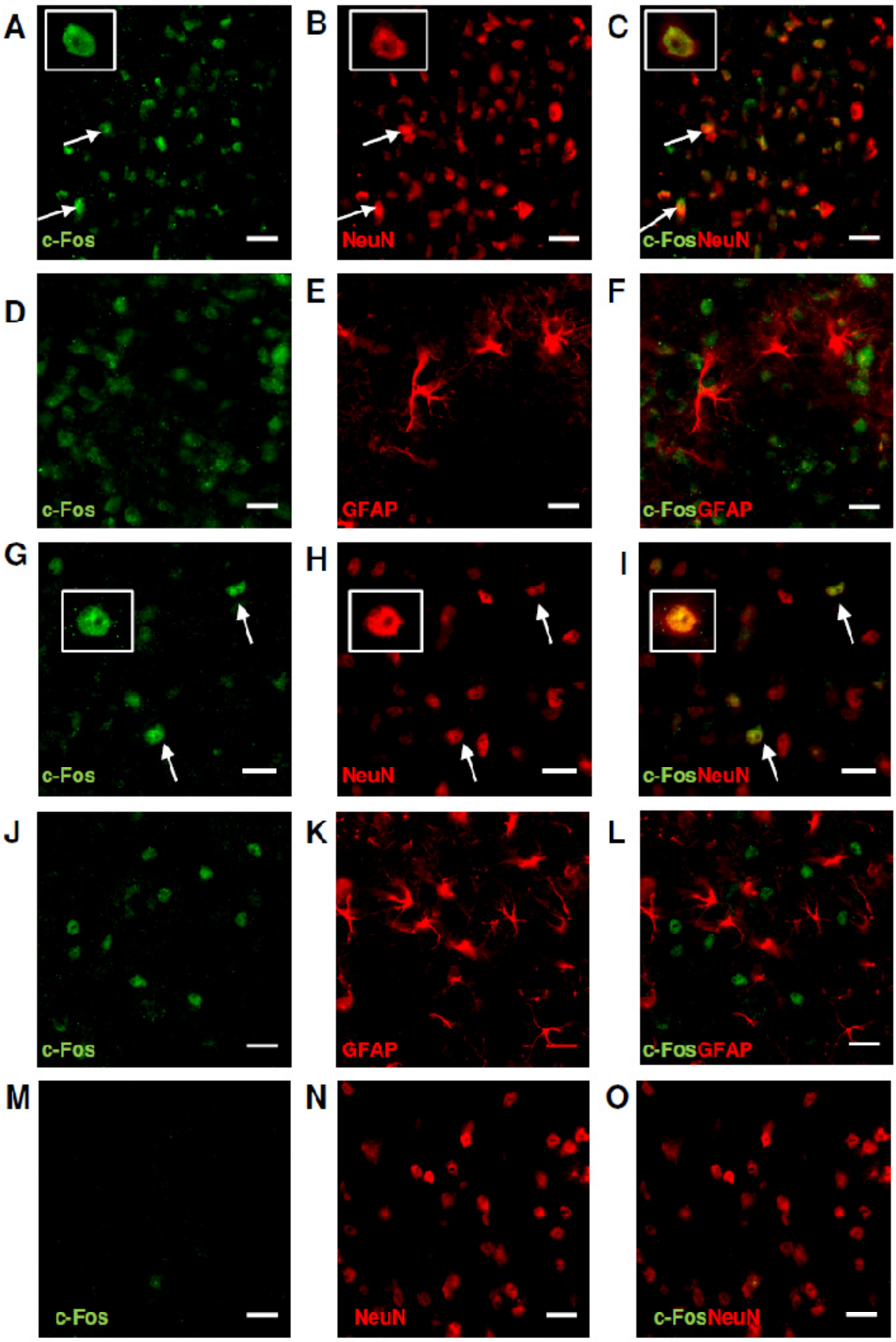


Figure 3.9: I.v. RANKL injection activates c-Fos in the VMH.

Immunofluorescence staining for c-Fos. I.v. injection of active RANKL in C57BL/6 male mice between the ages of 10 - 12 weeks induces the expression of c-Fos in the VMH 30 minutes after. I.v. injection of inactive RANKL in C57BL/6 males does not activate c-Fos at all. Inactive RANKL was prepared by boiling active RANKL for 10 minutes. Bars = 20 μm.


A
c-Fos
B
NeuN
C
c-FosNeuN
D
c-Fos
E
GFAP
F
c-FosGFAP
G
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H
NeuN
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J
c-Fos
K
GFAP
L
c-FosGFAP
M
c-Fos
N
NeuN
O
c-FosNeuN

Figure 3.10: I.v. RANKL injections activate c-Fos specifically within neurons in the VMH.

Immunofluorescence staining for c-Fos, neurons (NeuN, red) and astrocytes (GFAP, red) after i.v. injection of 1 µg RANKL into **(A-F)** C57BL/6, **(G-L)** RANK/GFAP KO and **(M-O)** RANK/Nestin KO male mice between the ages of 10 – 12 weeks. Merged images are shown to visualize co-localization (yellow, arrows and inset). RANKL activates c-Fos in the VMH of **(A, D)** of C57BL/6 and **(G, J)** RANK/GFAP KO mice, but not in **(M-O)** RANK/Nestin KO mice. C-Fos is only expressed within **(C, I)** neurons and not **(F, L)** astrocytes. All images show the VMH region of the brain. Magnification 20x, 40x (inset) Bar = 20 µm.

3.4. Depression is not induced by the loss of RANK in ovx mice

Preliminary studies of RANK/Nestin mice on mood suggested that RANK is not involved in emotional behavior such as anxiety and depression neither in the presence nor in the absence of female sex hormones. In order to confirm these results we employed two well established depression assays, the forced swim test (FST), a despair-based task (Porsolt et al., 1977b), and the sucrose preference test (SPT), a reward-based task (Moreau, 1997). Intact, sham operated and ovx RANK/Nestin controls and ovx RANK/Nestin KO mice were analyzed.

Since the FST requires the animal to struggle, there is a possibility that the surgical procedure to remove the ovaries interferes with the test. For this reason we used sham operated animals as a control. Like the tail suspension test (Figure 1.5) the FST involves scoring of immobile posture of mice forced to swim in an inescapable cylinder filled with water (Figure 3.11 A) (Can et al., 2012). Immobility in this task was recorded and scored using the ANY-maze software. As reported by others, ovariectomy of control mice induced a depressed state in female mice, shown by the increased time spent immobile as compared to sham controls (Figure 3.11 B) (de Chaves et al., 2009; Heydarpour et al., 2013). Ovx RANK/Nestin KO females also spent more time immobile as compared to sham controls, suggesting that RANK signaling did not reverse absence of estrogen-induced depression.

A second depression task, the reward-based SPT, was employed in order to rule out the possibility that the surgical procedure somehow interfered with the tasks in the FST. The SPT involves the mouse's innate preference for sweet food (Figure 3.12 A). A reduced amount of sucrose relatively to water consumed is indicative for anhedonia or depression (Russo and Nestler, 2013). Similarly to the TST (Figure 1.5), we did not observe changes in sucrose preference in ovx control animals as compared to sham control littermates (Figure 3.12 B). However, as in the FST and TST, we again could not detect a difference between ovx controls and ovx RANK/Nestin KO mice (Figure 3.12 B). As ovx RANK/Nestin KO and ovx

RANK/Nestin control mice had no significant difference in any of the tests employed these data indicate that neuronal RANK is not involved in depression.

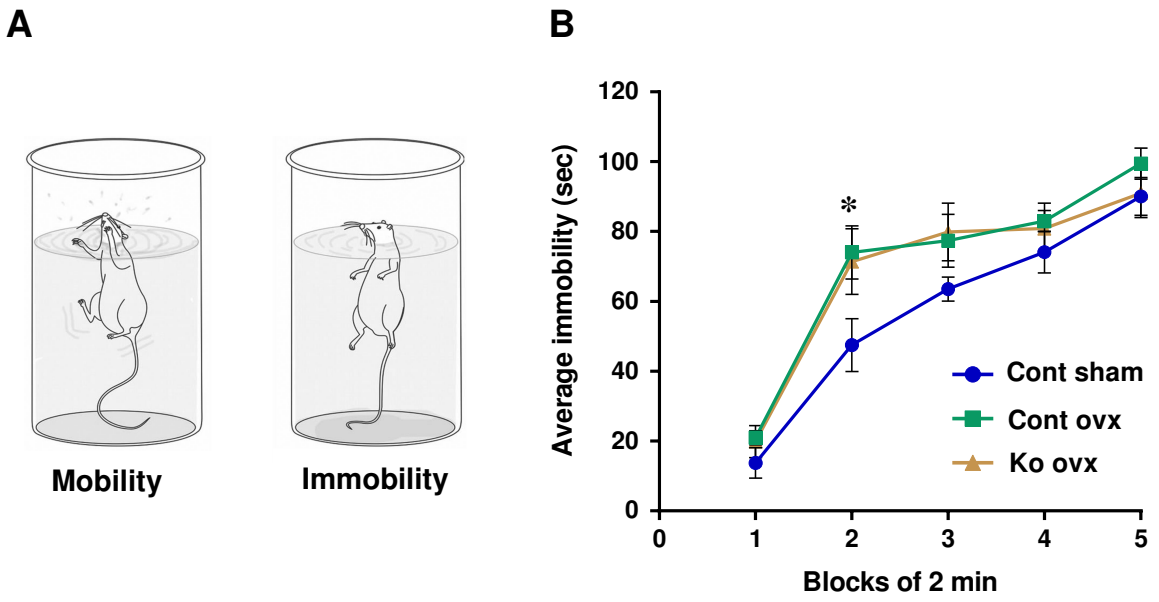

Figure 3.11: RANK deficiency in ovariectomized mice does not affect depression-like behavior in the forced swim test.

(A) Schematic representation of mobile and immobile postures as observed in the FST. The mouse is placed in a cylinder filled with water and forced to swim. The time spent immobile is recorded in an interval of 2 minutes. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(B)** Despair-based depression tested in sham-operated RANK/Nestin control (n=7), ovx RANK/Nestin control (n=4) and ovx RANK/Nestin KO female mice (n=11). Ovx RANK/Nestin controls and KOs spent significantly longer immobile after a time lapse of 4 min than sham-operated RANK/Nestin controls (*P<0.05). There was no difference between ovx RANK/Nestin controls and ovx RANK/Nestin KOs. Values are shown as means ± SEM (one-way ANOVA).

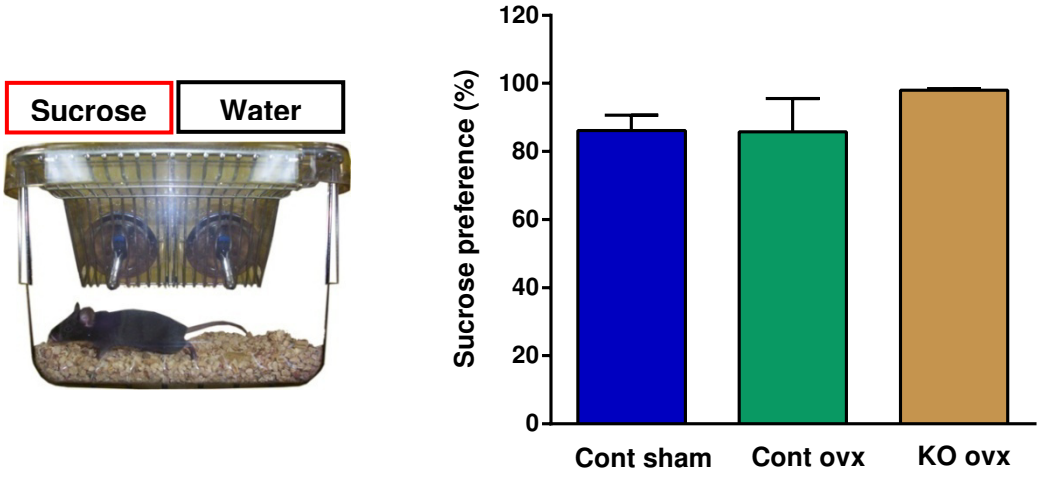

Figure 3.12: The loss of RANK in the brain and female sex hormones does not lead to depression-like behavior in the sucrose preference test.

(A) Schematic representation of the SPT in which the mouse has a free choice between sucrose solution and water. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(B)** All three groups, i.e. sham-operated RANK/Nestin control (n=7), ovx RANK/Nestin control (n=6) and ovx RANK/Nestin KO (n=2) female mice consumed more sucrose solution than water which is indicative for no depression. Values are shown as means ± SEM (one-way ANOVA with Tukey's multiple comparisons test).

3.5. RANK deficiency in the brain of ovariectomized mice leads to defects in sensorimotor gating

To assess basic synaptic functions, the PPI task was performed with sham-operated RANK/Nestin controls, ovx RANK/Nestin controls and ovx RANK/Nestin KO mice. Sensory filtering is tested in the PPI and thus impairments in the performance are indicative for defects in basic synaptic function (Basavaraj and Yan, 2012). Figure 3.13 A shows the set-up of the test. Briefly, in the first round the rodent is presented an intense acoustic stimulus (120 dB) to which it reacts with a startle response. This is followed by a second round where a weaker prepulse (80, 85, 90 and 95 dB) precedes the 120 dB startling stimulus and thus diminishes the startle response of the rodent. The final startle response was recorded relatively to the startle response after the pulse (Valsamis and Schmid, 2011). Figure 3.13 B shows that ovx RANK/Nestin KO mice exhibited a significantly stronger startle stimulus compared to ovx RANK/Nestin control and sham-operated RANK/Nestin control mice in the experimental set-up when the difference between the prepulse and the pulse was the largest (80 dB - 120 dB). There was no difference between animals that were either Cre positive or Cre negative, confirming that the effects are not simply a result of the presence of Cre recombinase (not shown). These data indicated that RANK might play an important role in sensorimotor gating.

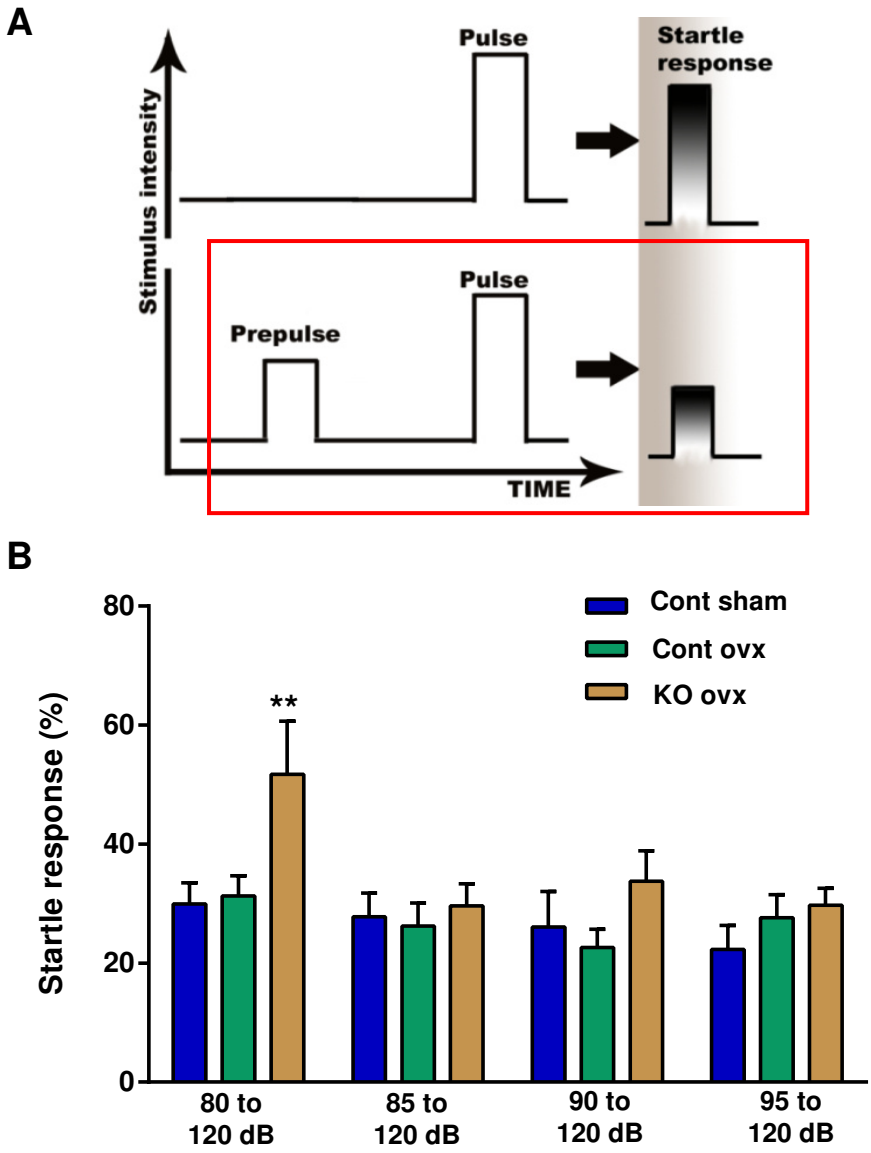

Figure 3.13: RANK deficiency in ovariectomized mice causes impairments in the prepulse inhibition task.

(A) Schematic representation of the PPI task. First, the startle response to a loud auditory stimulus (Pulse) is measured. In a second round a weaker prepulse precedes the pulse and attenuates the startle response creating the actual prepulse inhibition, which is marked in red. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(B)** PPI was tested in sham-operated RANK/Nestin control (n=14), ovx RANK/Nestin control (n=17) and ovx RANK/Nestin KO mice (n=19). Ovx RANK/Nestin KO exhibited a significant stronger startle response compared to the other two groups (**P<0.01) indicating dysfunction of senosorimotor gating. Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparsions

3.6. RANK is required for learning and memory in ovx female mice

In order to further elucidate possible defects in basic synaptic function of ovx RANK/Nestin KO mice, mutant and control animals were examined in a fear-based learning paradigm, the inhibitory avoidance (IA) task involving the amygdala, hypothalamus and hippocampus (Brioni et al., 1989). Importantly, a specific population of neurons in the VMH region, where RANK is expressed, is activated in fear-based learning (Trogrlic et al., 2011). Sham-operated and orh RANK/Nestin control and KO male mice which specifically delete RANK in neurons and astrocytes and RANK/CamKII KOs which have a loss of RANK only in neurons were analyzed in the IA task (Figure 3.14).

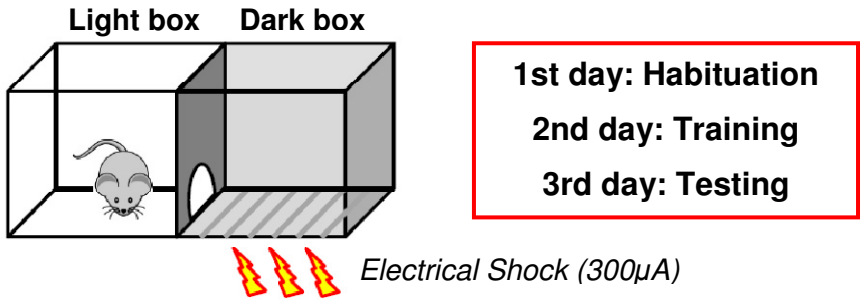

Figure 3.14: Schematic representation of the inhibitory avoidance learning paradigm.
The IA apparatus consists of a light and a dark box. On the first day the mouse gets habituated to the entire apparatus by placing it in the light compartment and allowing it to explore the apparatus for a maximum of 5 minutes. After the mouse enters the dark box, the sliding door will close and the mouse gets removed after staying there for 30 seconds. On the second day the mouse is placed back in the light box. As soon as the mouse enters the dark box it receives a footshock of 300 µA. Memory of the shock in the dark box is examined in the retention test 24 hours after. Latency to crossover to the dark side is recorded in the training and testing phase and compared.

Sham-operated RANK/Nestin KO females (Figure 3.15 A) and males (Figure 3.16 A) did not exhibit any learning deficits as they had a similar latency to enter the shock chamber compared to their controls. Ovariectomy in females did not alter the cognitive ability (Figure 3.15 B). However the deletion of RANK in the brain of ovx mice led to severe memory deficits in the IA task (Figure 3.15 B). Lack of motivation could be excluded as it was shown previously that neither the depletion of estrogen nor the loss of RANK altered anxiety levels or depression-like behavior examined in several tests (Figure 1.4, 1.5, 3.12). This data indicates that in ovx females RANK signaling is needed in fear-based learning and memory.

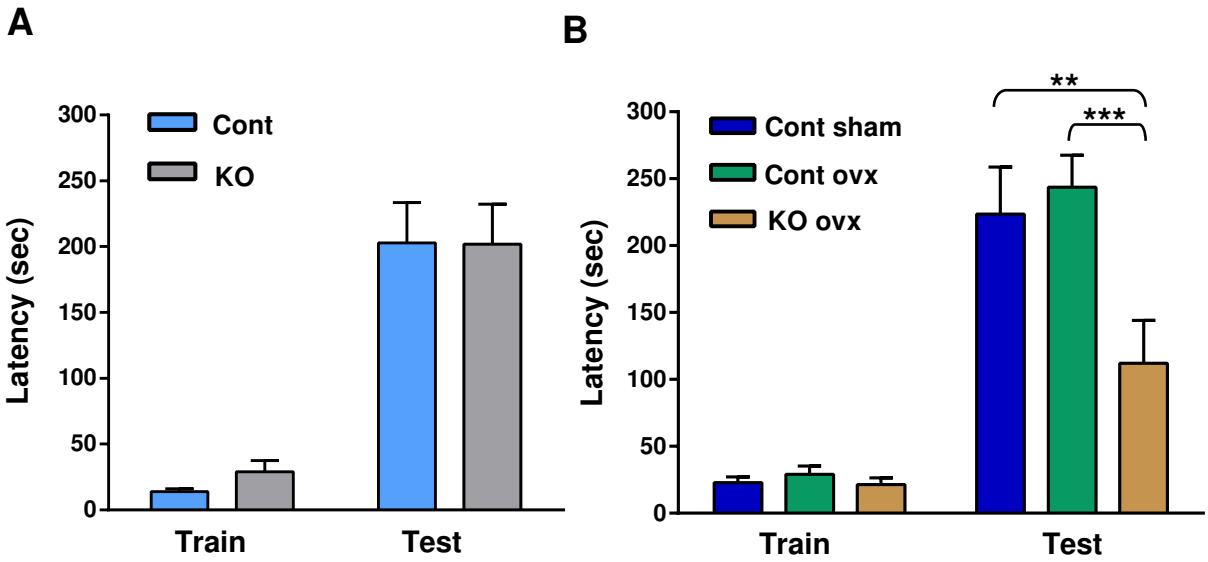

Figure 3.15: RANK deficiency in ovx female mice results in learning in the inhibitory avoidance task.

Fear-based learning of RANK/Nestin females was tested in the IA whereby the latency in the training phase and 24 hours later in the testing phase was measured. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(A)** RANK/Nestin KOs (n=16) did not show impaired memory compared to RANK/Nestin controls (n=17). **(B)** Ovx RANK/Nestin controls (n=10) did not alter cognition compared to sham-operated RANK/Nestin controls (n=6). However the deletion of RANK in the brain of ovx RANK/Nestin KO mice (n=13) resulted in a significant learning deficit (**$P < 0.01$; ***$P < 0.001$). Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparisons test).

In contrast to ovx females the loss of testosterone in orh males resulted in significantly reduced cognitive ability (Figure 3.16 B) confirming that testosterone is necessary for certain types of learning (Sandstrom et al., 2006; Spritzer et al., 2011; Spritzer et al., 2008). However orh RANK/Nestin KOs compared to orh RANK/Nestin controls exhibited similar latencies. Thus, the presence or absence of RANK does not influence learning in sham-operated or orh males.

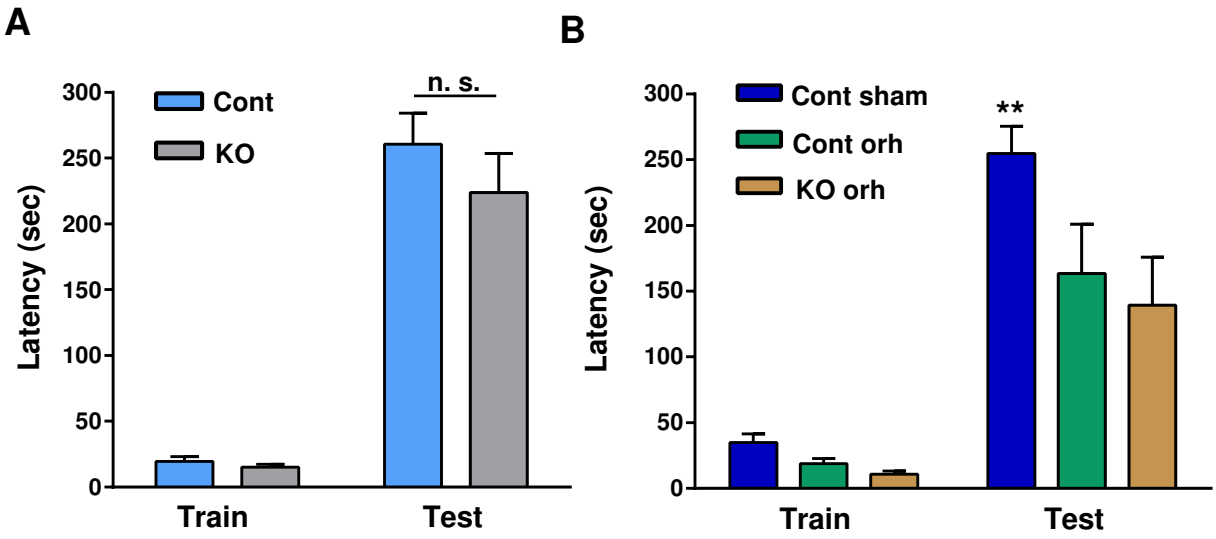

Figure 3.16: Loss of testosterone impairs inhibitory avoidance performance of male mice.
Fear-based learning of RANK/Nestin males was tested in the IA whereby the latency in the training phase and 24 hours later in the testing phase was measured. Mice between the ages of 10-14 weeks were used. Mice which underwent surgery recovered for at least 4 weeks. **(A)** No difference in learning between RANK/Nestin controls (n=16) and RANK/Nestin KOs (n=15) was detected. **(B)** Orh RANK/Nestin controls (n=12) and orh RANK/Nestin KOs (n=10) exhibited severe learning deficits (**$P < 0.01$) compared to sham-operated RANK/Nestin controls (n=14). Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparisons test).

To confirm the RANK/Nestin KO results, another neuronal Cre line was used, CamKII-Cre which specifically deletes in neurons located in memory-relevant brain regions (Casanova et al., 2001). Analysis of intact RANK/CamKII KO males and females using the IA test revealed similar results as RANK/Nestin KO males and females; RANK/CamKII KOs were not deficient in IA learning (Figure 3.17). Thus, the data in both RANK/CamKII KO mice and RANK/Nestin KO mice indicate that RANK-deficiency in intact animals does not cause

impaired learning and memory. In the future, we plan to test ovx and orh CamKII-Cre animals to further explore the role of RANK in memory deficits induced by loss of sex hormones.

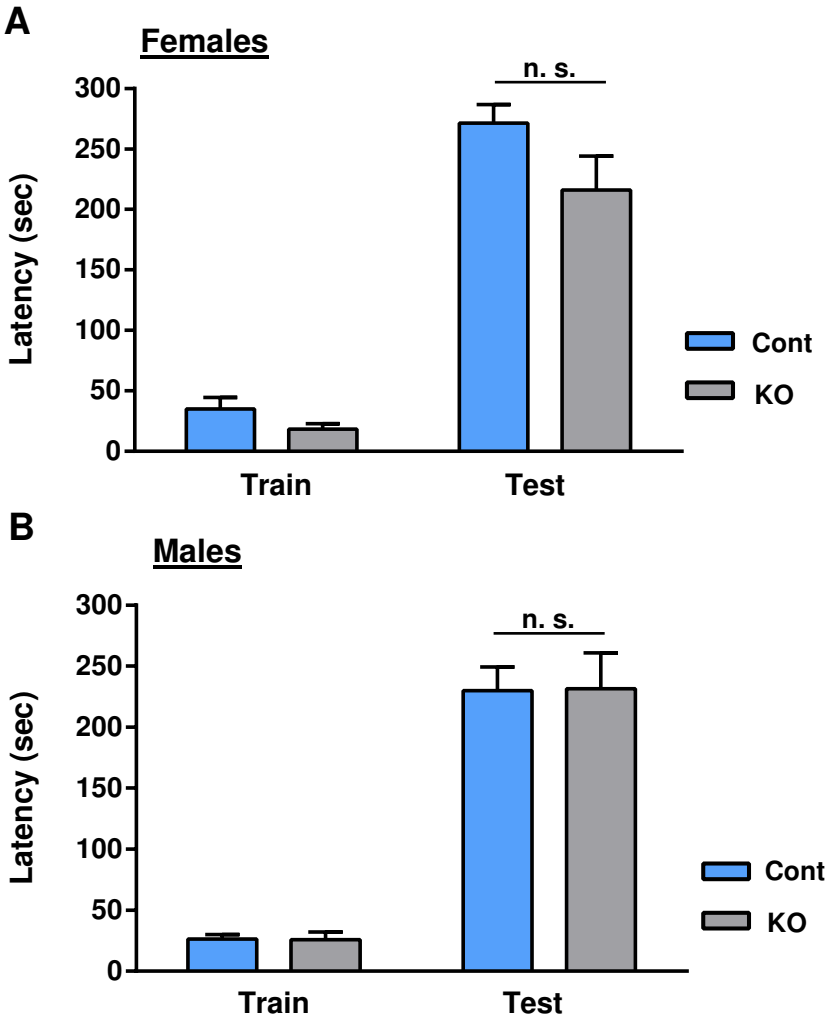

Figure 3.17: RANK/CamKII KO mice have intact memory function in the inhibitory avoidance task.

Female and male RANK/CamKII KO and their respective control mice were tested in IA for their learning ability. Latency in the training and testing phase was measured in seconds. Mice between the ages of 10-14 weeks were used. **(A)** No significant (n.s.) difference between female RANK/CamKII KOs (n=14) and controls (n=18) was detected. **(B)** Male RANK/CamKII KOs (n=11) exhibited the same cognitive ability than their controls (n=20). Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparisons test)

3.7. A two-time i.v. RANKL injection impairs cognitive ability

Intact RANK KO females did not show IA memory deficits (Figure 3.15 A), however, ovx RANK/Nestin KOs had significantly reduced latency times as compared to ovx RANK/Nestin controls (Figure 3.15 B). Presumably, these effects are the result of increased circulating RANKL levels induced by the absence of ovarian sex hormones (Figure 3.6 A). In addition orh control and orh RANK/Nestin KO male mice also had significant IA memory deficits (Figure 3.16 B). Thus, the effects in the male mice could be also attributed to the increase in circulating RANKL levels as a result of androgen absence.

To test this hypothesis we injected RANKL into the tail vein of male and female control and RANK/Nestin KO mice in order to mimic the increase in RANKL induced by the loss of sex hormones and tested these animals in the IA memory task. RANK/Nestin KO and RANK/Nestin control male and female mice were either injected with 1 μg active or inactive RANKL 3 hours before training (Figure 3.18 A, B). We chose to inject 3 hours before training based on the findings that the brief body temperature increase in response to injections returns to basal levels at 3 hours (see Figures 3.7 and 3.8). In order to assay if exogenous RANKL can be actually detected in the brain 3 hours after i.v. injection, RANKL protein concentrations were measured in the hypothalamus by ELISA and compared to controls injected with inactive RANKL. The hypothalamus was chosen as it is an important part in fear-based learning paradigms and it is partly open to the blood-brain-barrier (Rodriguez et al., 2010). Indeed, protein RANKL levels were significantly elevated in the serum and hypothalamus 3 hours after i.v. injection of RANKL (Figure 3.18 A, B).

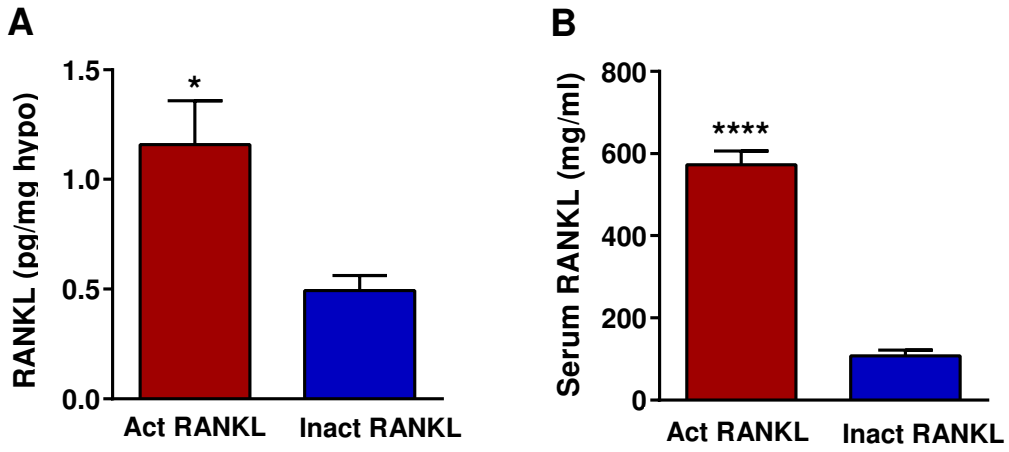

Figure 3.18: RANKL levels in the serum and hypothalamus after i.v. injection of RANKL.
(A) RANKL concentration in the hypothalamus (pg/mg hypo) and **(B)** in the serum (mg/ml) 3 hours afer i.v. injection of 1 µg active RANKL (n=5) or inactive RANKL (n=4) into C57BL/6 males. RANKL concentrations were determined by ELISA. Values are shown as means ± SEM (one-way ANOVA with Tukey's multiple comparisons test, **P<0.05; ****P<0.0001*).

Although we detected an increase in RANKL in the hypothalamus we did not detect any change in IA learning in any of the groups (Figure 3.19). We reasoned that one time injection of RANKL was not long enough to elicit the same effects as a prolonged sex hormone absence. For this reason, we injected RANKL twice: Male animals received the first injection of 1µg of RANKL 3 hours before training and the second 3 hours before testing. Analysis of these mice revealed that the active RANKL treatment induced severe learning deficits in both RANK/Nestin controls and RANK/Nestin KOs (Figure 3.20 A) as predicted. The experiments with female animals are ongoing. The results with male animals suggested that increase in peripheral RANKL levels causes severe impairment in IA learning. The results obtained with exogenously injected RANKL confirm the results showing that orchiectomy of RANK/Nestin control or KO animals induces impairment in IA learning. Data in the previous chapter shows that RANK is only expressed in the neurons in the VMH (Figure 3.2). In addition, we show that exogenous RANKL can activate only neuronal c-Fos expression in the VMH region of the hypothalamus (Figure 3.10 A-F). However, here we report that exogenous RANKL injection has an effect on IA learning in the control as well as in the RANK/Nestin KO. That RANKL is influencing learning in the absence of its neuronal receptor cannot yet be ex-

plained by the data we provide here and remains an open question and will be discussed in the later chapter.

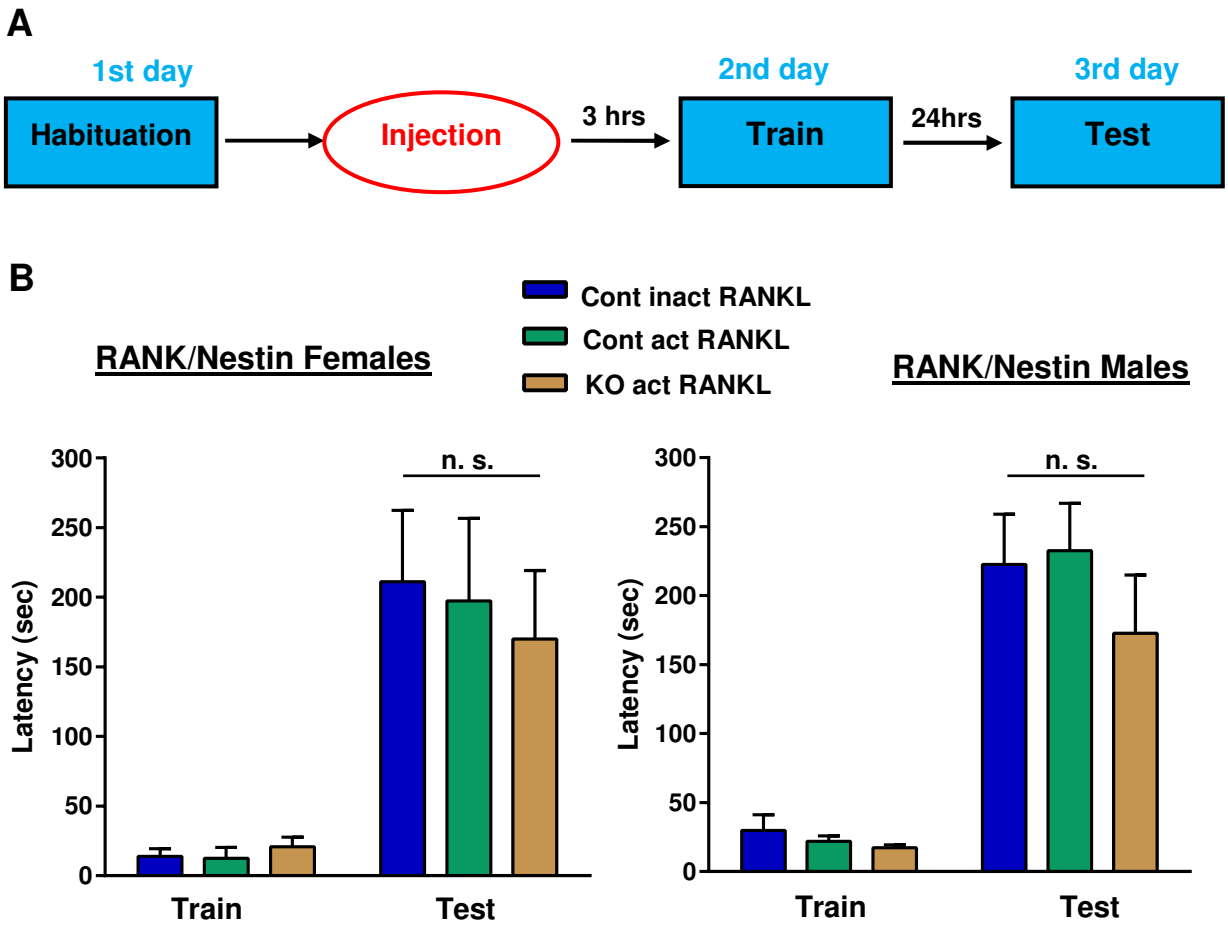

Figure 3.19: A one-time i.v. RANKL injection before training does not influence learning and memory.

IA performance of female and male RANK/Nestin controls and KOs which were injected with either 1µg active RANKL or inactive RANKL 3 hours before training. Retention testing was carried out 24 hours after training whereby the latency was measured. Inactive RANKL was prepared by boiling RANKL for 10 minutes. Mice between the ages of 10-14 weeks were used. **(A)** IA and injection scheme. **(B)** The three treatment groups within the RANK/Nestin females "Cont inact RANKL" (n=4), "Cont act RANKL" (n=4) and "KO act RANKL" (n=7) did not show any difference. **(C)** Also the three cohorts within the males did not differ in their cognitive ability (each n=9). Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparisons test).

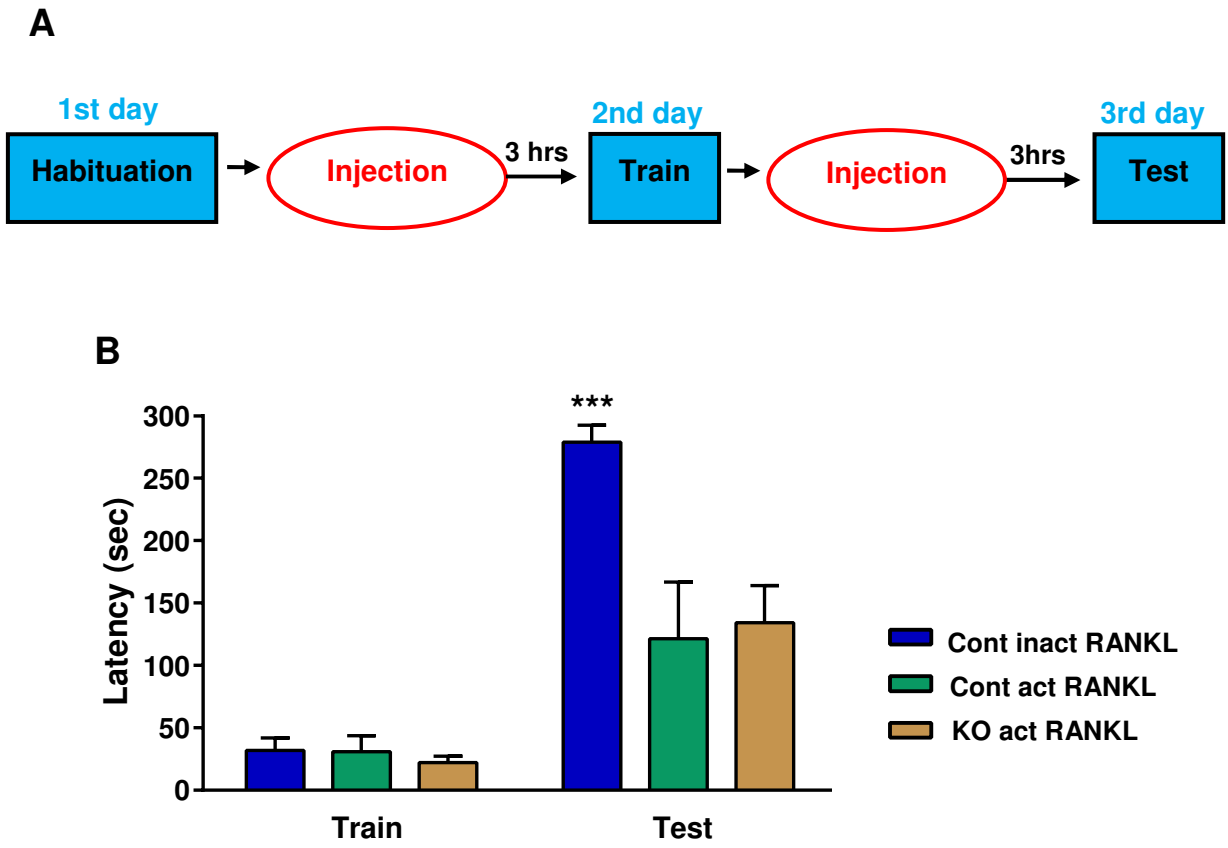

Figure 3.20: A two-time i.v. injection of RANKL in RANK/Nestin males causes impairments in learning.

IA performance of RANK/Nestin control (each n=6) and KO males (n=9) 10 – 14 weeks of age injected either with 1 µg active RANKL or inactive RANKL. Mice received a double injection, one 3 hours before training and one 3 hours before testing. Latency of the response was measured. **(A)** IA and injection scheme. **(B)** Active RANKL induced severe learning deficits in RANK/Nestin KOs as well as in RANK/Nestin controls (****P<0.001*). Values are shown as means ± SEM (two-way ANOVA with Tukey's multiple comparisons test)

4. DISCUSSION

Since the discovery of RANK, RANKL and OPG more than a decade ago the essential molecular mechanisms and regulation of bone remodeling have been elucidated (Kong et al., 1999b; Lacey et al., 1998; Nakagawa et al., 1998; Simonet et al., 1997; Yasuda et al., 1998a). However the RANK/RANKL/OPG axis is not only important in bone remodeling but also in the pathologic condition of bone turnover (Bucay et al., 1998; Leibbrandt and Penninger, 2008; Mizuno et al., 1998). Osteoporosis is a disease induced by an unbalance in the RANK/RANKL/OPG system. Especially women in their postmenopause when the production of estrogen ceased are highly vulnerable to develop osteoporosis, which indicates that there is a strong link between female sex hormones and the RANK/RANKL/OPG system (Leibbrandt and Penninger, 2008, 2009). RANK and RANKL were also found to play a crucial role in the formation of lactating mammary glands during pregnancy (Fata et al., 2000) and in the development of progestin-driven breast cancer (Gonzalez-Suarez et al., 2010; Schramek et al., 2010). Intriguingly, the hormone-regulated RANK/RANKL/OPG axis turned out to be also essential in the CNS for physiological thermoregulation in females, which might explain hot flashes experienced by postmenopausal women (Hanada et al., 2009). Taken together, it appears that the key units of bone metabolism are involved in several diverse sex hormone-regulated processes in the body including possible processes that have not been linked yet to RANKL/RANK.

It is well known that sex hormones impact the brain and on its functions including cognition and mood (Alexander et al., 2007; Barrett, 1999; Lee et al., 2012). Women experience more depressive periods and mood fluctuations during their lifetime than men do (Kessler et al.,

1993). Especially at menopausal transition women are often faced with depression, anxiety disorders, mood swings and cognitive disorders. However beside female sex hormones, testosterone was also reported to influence cognition. For instance rodent studies showed that the removal of testis caused learning deficits in males (Frye and Seliga, 2001). Thus the involvement of sex hormones in mood and cognition makes it attractive to study the RANK/RANKL/OPG axis in this field.

Intriguingly, we could identify RANK and RANKL as a critical mediator for hormone-dependent cognitive deficits. Previous experiments revealed that RANK is expressed in the hypothalamic POA/MSn region (Hanada et al., 2009). I now show that RANK is also expressed in the VMH. Whereas Hanada and colleagues found RANK expression in neurons as well as astrocytes in the POA/MSn RANK expression in the VMH region was restricted to neurons. The VMH is an integral part of the fear circuit and thus plays an important role in fear processing and in fear-based learning (Gross and Canteras, 2012). Furthermore, ERα and ERβ are expressed in the VMH which is thus receptive to estrogen and can in turn influence behavior.

RANK, RANKL and OPG are known to be tightly regulated by sex hormones. For instances, several studies revealed that estrogen upregulates OPG and downregulates RANKL mRNA and protein levels (Bord et al., 2003; Hofbauer et al., 1999; Shevde et al., 2000). Thus, the RANK/RANKL/OPG axis might act downstream of estrogen-regulated effects on the brain. Analyzing protein and mRNA levels of RANK, RANKL and OPG in different brain parts across the estrous cycle of mice, we observed in the brain that the triad is apparently not regulated neither on mRNA nor on protein level by female sex hormones. As expected, RANKL and OPG were strongly regulated in the periphery by female sex hormones. OPG levels positively and RANKL levels negatively correlated with physiological 17β-estradiol levels. This regulation of OPG in the blood was consistent with previous data (Szulc et al., 2001). Assuming that RANK/RANKL signaling is downstream of estrogen's effects on mood and learning, our data suggest that these effects rise from the peripheral regulation of RANKL and OPG and not from a direct control of RANKL expression in the brain. Interestingly, i.v. injection of exogenous RANKL in C57BL/6 male mice activate c-Fos, a critical downstream

target of RANK (Grigoriadis et al., 1994; Leibbrandt and Penninger, 2008; Takayanagi et al., 2002) and an indirect marker for neuronal activity (Herrera and Robertson, 1996) without changing body temperature and locomotor activities of the challenged mice. in contrast to Hanada et al., who showed that i.c.v. injection of RANKL, but not peripheral RANKL, induced hyperthermia (Hanada et al., 2009). This activation of c-Fos in the VMH indicated that peripheral RANKL might be able to influence behavior. C-Fos was specifically activated in neurons and not astrocytes in C57BL/6 mice. This finding is in line with our data that within the VMH RANK is only expressed in neurons. The deletion of RANK in neurons in RANK/Nestin KOs abrogated c-Fos induction whereas RANKL induced c-Fos expression was still observed in RANK/GFAP KOs.

Taken together, my data now shows on molecular level that the RANK/RANKL/OPG axis is involved in certain types of behavior in particular in females. Considering that the hypothalamus is an important part of the HPA axis which belongs to the neuroendocrine system and is involved in mood disorders such as stress disorders and depression (Wang et al., 2013; Yamada et al., 2013), we postulated that female sex hormones could regulate depression by modifying RANK/RANKL/OPG. Sham-operated RANK/Nestin and ovariectomized RANK/Nestin females mimicking postmenopause were examined in three experimental depression paradigms, namely, the forced swim test, tail suspension test and sucrose preference task, and one anxiety test, the elevated plus maze. However, none of these tests revealed any effects of RANK on depression and anxiety neither in the presence nor in the absence of female sex hormones. Furthermore, we tested RANK/Nestin mice in prepulse inhibition (PPI). Intriguingly, RANK deficiency in the absence of female sex hormones induced severe deficits in this task indicating that these mice have defects in sensorimotor gating. Since PPI deficits are often predictive for cognitive deficits RANK/Nestin mice were further tested using the inhibitory learning task. Importantly, in this test the loss of RANK in ovx females also caused severe impairments in their performance compared to ovx control mice and sham-operated RANK/Nestin mice. Sham-operated RANK/Nestin KO females exhibited normal memory formation. RANK/Nestin CamKII females reiterated the IA results from RANK/Nestin KO females, suggesting that the loss of RANK in the presence of ovarian sex

hormones does not lead to cognitive impairments. However the phenotype of ovx RANK/Nestin KO females has not been confirmed yet in RANK/CamKII females, a key experiment planned in the near future. Taken together it seems that in ovx female mice RANK is necessery for the formation of fear-based memory.

Our data further suggest that these effects on learning and memory are induced by the elevation of circulating RANKL levels. Similar to females, the loss of RANK in RANK/Nestin KO and RANK/CamKII KO male mice did not affect IA learning. However upon removal of the testes in RANK/Nestin KOs and controls the mice exhibited poor performances in the IA. Thus males need testosterone for intact memory formation, which is consistent with previously published data (Frye and Seliga, 2001). Previous studies showed that serum OPG levels positively correlate with testosterone levels (Szulc et al., 2001; Varsavsky et al., 2012). Thus the loss of testosterone leads to a decline in OPG levels and an increase in the serum RANKL/OPG ratio which could serve as an underlying mechanism for the learning deficits in orchiectomized RANK/Nestin males. Analysis of RANKL levels in orchiectomized mice needs to be done in future experiments.

In order to test our hypotheses stating that the learning deficits in orh RANK/Nestin KO and control males and ovx RANK/Nestin KO females arise from elevated peripheral RANKL levels we administered exogenous RANKL i.v. into RANK/Nestin males and females and checked their IA performance. However, in both sexes RANKL did not alter their cognitive ability although an increase in RANKL levels could be detected in the hypothalamus. A plausible reason could be that a one-time injection of RANKL was not sufficient to generate the same effects as a prolonged sex hormone depletion in ovx mice. Interestingly, twice injection of RANKL, one before training and one before testing, induced significant memory loss in male RANK/Nestin control and KO mice. Thus the two-time injection of RANKL confirmed our hypothesis that an elevated RANKL level impairs learning and memory. However the influences of exogenously administered RANKL in RANK/Nestin males on the brain still bear some discrepancies. RANK is expressed in neurons of the VMH. Moreover i.v. injection of RANKL in males was able to induce neuronal c-Fos expression in the VMH of control mice but not in RANK/Nestin KOs. However RANKL i.v. injection lead to memory deficits

in both RANK/Nestin controls as well as RANK/Nestin KOs. Thus, how exogenous RANKL is influencing learning in the absence of its receptor RANK remains to be resolved in future experiments? Though very unlikely, a possible reason could be that another receptor for RANKL besides RANK might exists in the brain. In order to confirm the phenotype of ovx RANK/Nestin KO females having severe learning deficits presumably due to elevated RANKL levels, IA learning needs to be examined in females after dual injections of RANKL in future experiments. Since it has been reported that estrogen treatment can improve learning and memory (Frye et al., 2007; Frye et al., 2005; Rhodes and Frye, 2004), it would be interesting to investigate if the administration of estrogen and/or progesterone can reverse the phenotype of ovx RANK/Nestin KO females.

Taken together, my results suggest that the RANK/RANKL/OPG axis is involved in learning and memory but not in emotional behavior. However, depending on the gender RANK, RANKL and OPG appear to affect memory differently. Especially, in the postmenopause of females it seems that RANK carries an important role in cognition. Nevertheless in order to further elucidate the underlying mechanism of RANK, RANKL and OPG's impact on learning and memory more experiments need to be performed. Moreover, the molecular downstream mechanisms need to be explored to get a fundamental molecular description how sex hormones can affect learning and memory via the RANKL/RANK system.

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