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The role of norepinephrine in learning: Cerebellar motor learning in rats

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The Role Of Norepinephrine in Learning:

Cerebellar Motor Learning in Rats

by

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of the requirements for the degree of
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DEDICATION

This work is dedicated to my mother, father, sister and every person I have encountered in my journey.

To the memory of Vicente Gallardo Silva and Laszlo Szilasi, who taught me the meaning of seeking true reason. And to my role model as a scientist and human being, Dr. Luis Francisco Hernandez.

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ABSTRACT

Delay classical eyeblink conditioning is an important model of associative, cerebellar dependent learning. Norepinephrine (NE) plays a significant modulatory role in the acquisition of learning; other neurotransmitter systems are also at play. The goal of this dissertation was to determine whether NE, GABA and glutamate (Glu) release is observed in cerebellar cortex during delay eye blink conditioning, and whether such release was selectively associated with training and not due only to stimulatory sensory input. The data support the hypothesis of noradrenergic and GABAergic system involvement in motor learning with NE as a modulator of early responding and GABA as a mediator of the learned response. In addition to neurotransmitter levels, we found that the local administration into the cerebellum of Rp-cAMP and propranolol impair the consolidation of learning when administered post training on the eyeblink conditioning task indicating that the β-adrenergic receptor and the cAMP downstream signaling cascade are

essential for memory consolidation. These results support the hypothesis of NE acting as a neuromodulator in the cerebellum for the acquisition of motor learning. A similar experimental design was applied to aged animals and the neurochemical pattern of release was haracterized by a delay in the response to eyeblink conditioning and smaller amounts of the neurotransmitter evoked by the paired US-CS. It is hypothesized that the impairment in aging could be due to excitotoxicity caused by chronic inflammation. The present study also approached this issue by targeting the pro-inflammatory cytokine TNF-α and we found that suppression of TNF-α in aged animals improved learning.

CHAPTER 1

Introduction

In this dissertation it is hypothesized that the release of norepinephrine (NE) in the cerebellum is necessary to activate the β-adrenergic receptor and its signal transduction cascade which are critical for cerebellar motor learning consolidation. This thesis has focused on the behavioral paradigm of classical eyeblink conditioning which is a well characterized cerebellar-dependent motor learning task. Despite the accumulation of work showing that NE in the cerebellum modulates the rate of acquisition of cerebellar dependent learning, little is known about the noradrenergic downstream temporal events, specifically the role of cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA). Other neurotransmitters such as gamma-aminobutyric acid (GABA) and glutamate (Glu) are hypothesized to play an important role in cerebellar motor learning. We hypothesized that GABA acts as a mediator of the learned response in the cerebellar cortex during the training and the pattern of GABA release becomes strengthened and sharpened in response to the behavioral task following several days of training. Furthermore, we hypothesize that increases in extracellular glutamate levels are observed as a consequence of the somatosensory projections to the cerebellum during training. Interestingly, there is an age-related decline in memory function in which neurotransmitter release is compromised perhaps as a result of chronic inflammation. For this reason we hypothesized that TNF-α inactivation improves learning in aged rats whereas the

activation of TNF-α in young rats impairs learning since inflammation and more specifically TNF-α may be a critical factor that is involved in the decline in classical conditioning behavior during the aging process.

Evidence of memory formation shows that distinct molecular mechanisms contribute to the multiphasic process of memory formation, however, little is known about the biochemical pathways underlying the induction and maintenance of these different memory phases. It has been shown that NE in the cerebellum modulates the rate of acquisition for a variety of motor learning tasks among them delayed classical eyelid conditioning. Many studies in diverse animal models lead to a convergence in the temporal dynamics of signaling cascades and how these cascades contribute to the distinct aspects of learning. Norepinephrine has been shown to have a modulatory role in cerebellar dependent learning. Noradrenergic inputs from the locus coeruleous (LC) inhibit spontaneous discharge but appear to augment the signal-to noise ratio for both excitatory and inhibitory neurotransmission, however little is known regarding noradrenergic upstream and downstream temporal events including the signal transduction cascades with the implication of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) in both long and short-term memory formation in numerous parts of the brain and specially in cerebellum which is our major focus. Our lab has recently shown that discrete localized blockade of β-adrenergic receptors or PKA in the cerebellum caused a significant deficit in acquisition of eyelid classical conditioning. As a consequence this result has led

us to our current interest in studying this mechanism of cerebellar memory formation. This dissertation combined pharmacological and neurochemical approaches to uncover different aspects of memory formation and molecular mechanisms using delay eyeblink conditioning as the behavioral task. The delay form of eyelid conditioning is a well-characterized system in which the implication of the cerebellum is clear and localized effects of learning can be measured before, during and after acquisition of training in different discrete cerebellar areas such as lobule HVI and interpositus nucleus (see (Steinmetz, 2000)).

1.1. Eyeblink Conditioning

Classical conditioning of the eye blink is a type of Pavlovian conditioning which is one of the simplest forms of associative learning by which animals, including humans, learn relations among events in the world so that their future behaviors are better adapted to their environments. In this method a discrete conditioned stimulus (CS) is paired with a discrete unconditioned stimulus (US) with particular temporal relationships between the CS and US. The untrained animal exhibits the eyeblink response only to the US; this unlearned behavior to the US is referred to as the unconditioned response (UR). Over the course of training sessions, the animal develops a conditioned response (CR) to the CS that mimics the UR, precedes the US in onset time, and peaks at about the time of US onset. As only two stimuli are involved, the learning or association of CS and US has to occur at the brain sites where the two forms of information converge (Rescorla, 2003; Kim & Thompson, 1997). The majority of work in this model

system has been performed in rabbits using the nictitating membrane, however more recently work has been performed in rats and mice. In the present studies we examine delay eyeblink conditioning in rats. Figure 1.1 is an illustration of the headgear design where the rats move freely in a training chamber that sits inside a sound-attenuating box.

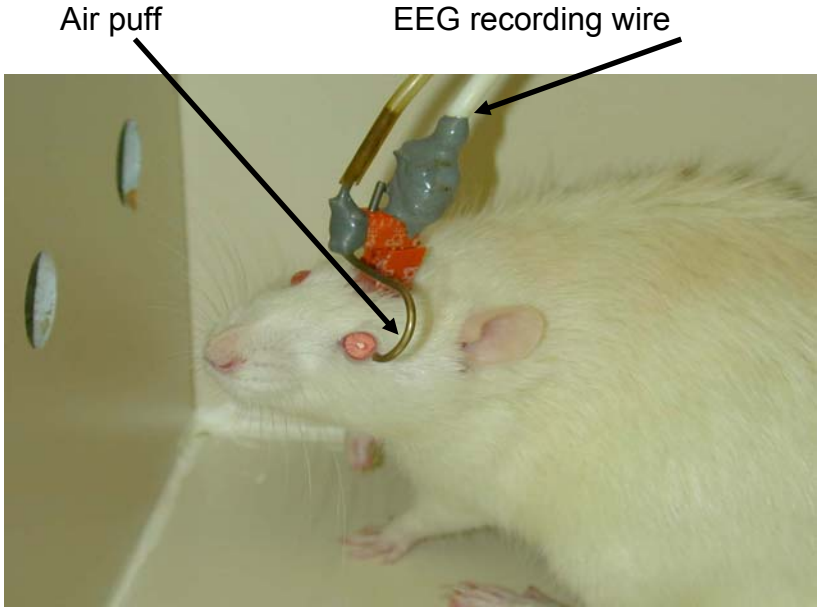


Figure 1.1. Rat in eye blink chamber with the head stage holding the EEG recording wire and the connection to the air puff tubing.

The discovery of a high proportion of cerebellar inhibitory synapses was the highlight of the cerebellar neuronal circuitry described in over 14 articles published between 1961 and 1966 by John C Eccles (Eccles et al., 1961; Eccles, 1967; Eccles, 1986). Eccles established basis to dissect out the neuronal circuitry of the cerebellum by taking advantage of recording and stimulation techniques with a high degree of temporal resolution (for review see (Ito, 2006).

David Marr published ‘A theory of cerebellar cortex’ in which he proposed two forms of input-output relations, one suitable for learning movements and the other for the maintenance of reflexes such as postures and balance (Marr, 1969). Marr’s theory predicted that synapses from parallel fibers to Purkinje cell (PC) are facilitated by the conjunction of presynaptic and climbing fiber activity. On the other hand, Eccles proposed that only two kinds of afferent fibers convey information to the cerebellum, the climbing fibers and mossy fibers with only one type of efferent fiber from the cerebellum, the PC which projects to the deep cerebellar nuclei (Eccles, 1967). Eccles’s established that the climbing fiber is uniquely distributed to a single PC on which it has a powerful excitatory action whereas the mossy fiber input is characterized by enormous divergence and it has excitatory as well as inhibitory actions on PC’s. Interestingly, Eccles assumed that in the cerebellar cortex the transfer of information from each small zone (beam) to another is not significant because the only association pathways would be by the Purkinje axon collateral and the basket cells which are weakly inhibitory. Later, evidence was established for noradrenergic central inhibition in the cerebellum when the pathway from LC to rat cerebellar PC are activated, this finding elucidated another important cerebellar input from the LC (Hoffer et al., 1973). Following this, several studies examined the effect of NE on the cerebellar PC. For instance, NE applied by microiontophoresis to rat cerebellar PC selectively depressed spontaneous neuronal discharges showing that NE increased the responsiveness of PC to afferent inputs, also it was found that NE exerted its action on PC through β-adrenergic receptors (Parfitt et al., 1990;

Parfitt et al., 1988; Freedman et al., 1976; Freedman et al., 1977; Moises et al., 1979; Woodward et al., 1991b).

Several decades after the anatomy and dynamics of the cerebellar circuitry were established there are still general disagreements in regard to whether learning occurs in the cerebellum. The plasticity for cerebellar learning is shared between the cortex and deep nuclei, but exactly how much each of these structures contribute to memory formation is still under debate. Extensive work has been done using very small and discrete interventions to impair either the cerebellar cortex or interpositus nucleus of the cerebellum. The issue is not whether the memory occurs in the cerebellum, but whether it happens in the cerebellar cortex or in the deep nuclei or may be in both. In order to address these questions several approaches have been used, such as electrolytic lesions using kainic acid and temporary inactivation (cooling, muscimol, anisomycin) to localize the learning process to the interpositus nucleus (Lavond, 2002) concluded that the interpositus nucleus is responsible and this is also supported by published works (Mojtahedian et al., 2007; Thompson et al., 1997).

However, other studies claim that memory formation in the cerebellum during eyeblink conditioning might be mediated mostly in the cerebellar cortex (Sanchez et al., 2002; Yeo, 2004; De Zeeuw & Yeo, 2005). In either case, lesions of the cerebellar cortex, interpositus nuclei or inferior olive prevent or diminish the expression of conditioned responses. Nevertheless, there is

agreement that all these structures are interconnected and rely on each other to perform the overall process of conditioned learning (Attwell et al., 2002b)

1.2. Norepinephrine and its involvement on motor learning

NE chemically known as 4-(2-Amino-1-hydroxyethyl)benzene-1,2-diol is both a hormone and neurotransmitter. In the peripheral nervous system NE is secreted by the adrenal medulla and the nerve endings of the sympathetic nervous system to cause vasoconstriction, an increase in heart rate, blood pressure, and blood glucose. The LC is the major brain area containing NE in the central nervous system (CNS) (Loughlin et al., 1986; Foote et al., 1983; Luppi et al., 1995; Aston-Jones et al., 1991). Projections from the LC innervate multiple brain areas which are involved with process such as learning, memory, attention and anxiety (Vavrejnova et al., 1990). Human clinical therapies in traumatic stress relate NE to enhanced encoding of memory for arousing stimuli as well as aversive events. Therapeutic approaches using drugs such as propranolol (β-adrenergic antagonist), MAO inhibitors and tricyclic antidepressants are currently and effectively being implemented (for review see(Southwick et al., 1999).

Multiple activities have been associated to the activation of adrenergic receptors in the CNS. There are ten different types of adrenergic receptors in the nervous system; α1A, α1B, α1C, α1D, α2A, α2B, α2C, β1, β2 and β3. Depending on the type of adrenergic receptor activated by NE, the spatial distribution, relative density of receptors subtypes, affinities and selectivity will determine when and

how the adrenergic receptors will be part of the memory formation process (Gibbs & Summers, 2002).

Cerebellar NE has been extensively studied in relation to associative motor learning in rats (Mason & Iversen, 1977; Bickford, 1993) and has been proposed to mediate cerebellar synapses (Bloom et al., 1972; Freedman et al., 1977). Also pharmacological evidence of noradrenergic central inhibition has been established by activating LC and recording in rat cerebellar PC (Hoffer et al., 1973). It has been shown that the local administration of propranolol injected prior to NE decreased cerebellar cyclic GMP (Haidamous et al., 1980). Noradrenergic receptors are known to be involved in cerebellar learning by increasing the "signal to noise" ratio of afferent inputs to cerebellar Purkinje neurons (Yeh & Woodward, 1983b; Woodward et al., 1991a). Most recently it has been suggested that cerebellar NE enhances GABA release by α_1-adrenoceptors, which are expressed in presynaptic terminals and somatodendritic domains, whereas NE suppresses the excitability of interneurons by α_2-adrenoceptors, which are expressed in presynaptic somatodendritic domains. This finding reveals the dual modulation of GABAergic inputs from interneurons to PCs as a possible mechanism for the fine-tuning of information flow in the cerebellar cortex (Hirono & Obata, 2006). On the other hand, studies using halothane-anesthetized rats have shown that within the medial septum, stimulation of the beta-adrenergic receptors by isoproterenol can mimic the

arousing effect of LC stimulation, while antagonist infusion blocks this effect (Berridge et al., 1996).

Motor skill learning requires repetitive training sessions, and there is a great amount of evidence that associative memory is a cerebellar dependent process (Jirenhed et al., 2007). Neuroimaging studies have reported clear cerebellar activation during the acquisition of a motor skill, followed by low activation after prolonged practice on a procedural learning or skill acquisition (Petersen et al., 1998; van Mier & Petersen, 2002). Using the vestibule-ocular reflex task (VOR), the modulatory role of NE has been shown. In these studies the administration of a β-adrenergic antagonist significantly reduced the adaptation of the VOR gain showing that the blockade of β-adrenergic receptors significantly decrease the ability to produce adaptative changes in VOR gain (Van et al., 1990; Heron et al., 1996). Brain areas other than the cerebellum are also involved in the acquisition and retention of skilled motor tasks (Sanes, 2000). For example, in the rotarod task mice show differential corticostriatal plasticity during fast and slow motor skill learning (Costa et al., 2004). These reports are consistent with the hypothesis that the β-adrenergic receptor is involved in the acquisition of novel motor tasks.

1.2.1. NE and memory

A role for norepinephrine in learning and memory has been elusive and controversial. A longstanding hypothesis states that the adrenergic nervous system mediates enhanced memory consolidation of emotional events and it this

hypothesis has been tested using several learning tasks in mutant mice conditionally lacking norepinephrine and epinephrine, as well as control mice and rats treated with adrenergic receptor agonists and antagonists. Murchison and colleagues found that adrenergic signaling is critical for the retrieval of intermediate-term contextual and spatial memories, but is not necessary for the retrieval or consolidation of emotional memories in general (Murchison et al., 2004). The role of NE in retrieval requires signaling through the β_1-adrenergic receptor in the hippocampus. Murchison demonstrated that mechanisms of memory retrieval can vary over time and can be different from those required for acquisition or consolidation. These findings may be relevant to symptoms in several neuropsychiatric disorders as well as the treatment of cardiac failure with β-blockers. Activation of the central noradrenergic system is associated with increased arousal, orienting to novel stimuli, selective attention, enhanced memory, and cardiovascular responses (Southwick et al., 2003). In rats, eliminating the central noradrenergic system leads to impaired fear conditioning (Neophytou et al., 2001), and in humans pharmacological suppression of noradrenergic responses with a β-adrenoceptor (AR) antagonist impairs memory of emotional events (van Stegeren et al., 1998). NE seems to play rather a role in the maintenance as well as for the induction of long term potentiation (LTP) in the dentate gyrus. Application of NE or β-adrenergic receptor agonists can induce LTP phenomena in the DG (Dahl & Sarvey, 1989; Stanton et al., 1989; Dahl & Li, 1994) and NE depletion or β-adrenergic receptor blockade have been shown to result in impaired DG-LTP in brain slices and anaesthetized animals

(Swanson-Park et al., 1999; Bliss et al., 1983; Stanton & Sarvey, 1987; Stanton & Sarvey, 1985; Bramham et al., 1997). It is clear that NE plays an important role in memory formation as well as arousal states.

1.2.2 The action of NE in the cerebellum

A number of modulatory signals are needed for powerful cerebellar learning and control in particular NE. The NE input to the cerebellum is the second largest modulatory input and distributes to all part of the cerebellar cortex with a patchy innervation pattern. The noradrenergic fibers project to all parts of the cerebellar cortex and originate from the dorsal and ventral parts of the LC. These fibers are found both around the glomeruli, making close contacts with granule cell dendrites, and around the PC dendrites (Kimoto et al., 1978). It is known that cultured cerebellar granule cells express adrenergic receptors (Dillon-Carter & Chuang, 1989) but due to the difficulty of recording from granule cells, there are not significant data showing the direct effect of NE upon granule cells. However, NE has been shown to increase cAMP in granule cells as well as astrocytes an important key messenger of neuronal plasticity (Morton & Bredt, 1998). Furthermore NE inhibits spontaneous PC discharge (Bickford et al., 1985a) possibly through both presynaptic adrenergic receptors on basket cells (Mitoma & Konishi, 1996) and enhancement of spontaneous spike firing of basket cells (Saitow & Konishi, 2000). However, unlike serotonin, and relative to the change in spontaneous activity, norepinephrine increases the responsiveness of the PC to its afferent excitatory inputs (Freedman et al., 1976). J.E. Cheun and H.H.

Yeh., showed that noradrenergic potentiation of cerebellar PC responses to GABA is mediated by cyclic AMP as intracellular intermediary, in other words NE exerts two types of long-term influences on PC, the activation of the beta-adrenergic receptors resulting in a raised intracellular levels of cyclic AMP, which leads to an increase of cyclic AMP-dependent protein kinase activity (Cheun & Yeh, 1996). This action of NE suggests that it can enhance rebound potentiation, which requires elevation of intracellular cAMP level (Kano et al., 1992b), and antagonize the suppression of rebound potentiation, which requires the suppression of cAMP level (Kano et al., 1992a). On the other hand, NE increases the expression of immediate-early genes, such as c-fos and Jun-B, in PC. Induction of immediate-early genes could then represent a mechanism by which sustained inputs are transformed into long-term biochemical changes that are required for the maintenance of cerebellar long-term plasticity, such as long term depression (LTD) (Pompeiano, 1998). NE receptors have also been found in the cerebellar nuclei, and NE modulates the GABAergic neurons inhibition of deep cerebellar neurons (Gould et al., 1997c).

1.2.3. Behavioral effects of NE

Naka et al, (2002) have had shown that an enriched environment specifically increases NE concentration in the parieto-temporo-occipital cortex, cerebellum and pons/medulla oblongata (by 17.0–37.5%) without changing the concentrations of NE, 5-HT or DA in other brain regions, including the frontal cortex and hippocampus. This finding agrees with our working hypothesis of NE

playing a important role on cerebellar motor learning, also NE has been shown to facilitate synaptic plasticity to regulate ocular dominance plasticity (Brocher et al., 1992) and to enhance learning and memory via the activation of beta-adrenergic receptors (Devauges & Sara, 1991). Accordingly, it is likely that the increased concentrations of extracellular NE will be observed following delay eyeblink conditioning which must be involved in synaptic plasticity and possible changes in leaning and memory. Several authors (Watson & McElligott, 1983; Watson & McElligott, 1984; McElligott & Keller, 1984); by using motor learning paradigms have established a modulatory role of NE in cerebellar dependent motor learning behaviors. On the other hand, either depletion of NE or blockade of β-adrenergic receptors impairs the ability of rats to improve performance on a runway task where the rats have to learn to walk on varying patterns of pegs that protrude from the runway walls (Watson & McElligott, 1983; Watson & McElligott, 1984; Bickford et al., 1992). Selective depletion of cerebellar NE produces a decline in acquisition of this task (Watson & McElligott, 1984) and furthermore the data from our lab shows that infusions of propranolol into the paramedian lobe of the cerebellum impair learning (Cartford et al., 2004b). Motor learning impairments in aged rats are correlated with the loss of cerebellar β-adrenergic receptor sensitivity (Bickford, 1993; Bickford, 1995). The behavioral impairment is noticed as a decrease in the rate of acquisition on the task, rather than a complete blockade of learning. Another motor learning task is the classical eyelid conditioning which has been linked to cerebellar modulation when the delayed paradigm is used. In this task the unconditioned stimulus (US) is either an air

puff or eye shock, the signal from which enters the cerebellum via the climbing fibers. The conditioned stimulus (CS) is usually a tone and is relayed via the granule cell-parallel fiber synapse. Subjects learn to link or associate the air puff with the tone and a new response is formed (a conditioned response) to the tone that anticipates the air puff. Conditioning related activity has been established for neurons within lobule HVI and the interpositus nucleus in rabbits as well as in rats (Berthier & Moore, 1986; Gould & Steinmetz, 1994; Rogers et al., 2001b). Lesions of the cerebellar lobule HVI (Yeo et al., 1985; Perrett et al., 1993; Nordholm et al., 1993); and interpositus nucleus (Clark et al., 1992; Krupa et al., 1993b); abolish the response of the classical eyelid conditioning. Mice containing PC mutant (pcd), which have a complete lack of cerebellar PCs, still acquire the eyelid response (Chen et al., 1996). Also jaundiced Gunn rats (a mutant with loss of PC) have normal to elevated levels of NE innervation and functional activity in cerebellar cortex and deep nuclei after degeneration of the PC layer (Onozuka et al., 1990; Kostrzewa & Harston, 1986; Ghetti et al., 1981; Clark et al., 1997; Clark & Lavond, 1996; Rogers et al., 2001a). By using electrophysiological techniques to examine the action of NE, NE seems to modulate the activity of other neurotransmitters in discrete cerebellar areas such as cortex and deep nuclei (Gould et al., 1997b). On the other hand, the fact of NE modulating this motor learning task is also supported by the capability of propranolol to delay the acquisition of the eyelid conditioning in rabbits (Gould, 1998) as well as in rats (Cartford et al, 2002). Early studies also showed that by doing electrolytic lesions of the LC a resistance to extinction in delay conditioning

in rabbits has been observed (McCormick & Thompson, 1982). Winsky and Harvey demonstrated that bilateral intraventricular administration of 6-hydroxydopamine causes retarded acquisition but not performance of conditioned eyeblink responses (Winsky & Harvey, 1992). Another cerebellar learning paradigm is the vestibulo-ocular reflex (VOR), has been demonstrated to be influence by cerebellar NE (McElligott & Freedman, 1988) and appears to be to mediated by β-adrenergic receptors since the β-adrenergic antagonist sotalol decreases the adaptation of the VOR gain when microinjected into the cerebellar flocullus of the rabbit (Pompeiano et al., 1991). As we can see there is an accumulation of evidence that clearly shows a significant role of NE in the acquisition phase of cerebellar dependent learning. It has been established that PCs receive excitatory inputs from parallel and climbing fibers, inhibitory GABAergic inputs from basket and stellate interneurons, and a noradrenergic input from the pontine nucleus LC (Eccles, 1967; Hoffer et al., 1973). Later on, NE was proposed as a "modulatory" input due to its capability to induce synaptic plasticity in PCs by selectively improving the "signal to noise" ratio of evoked versus spontaneous activity (Freedman et al., 1977). This enhances the sensitivity of cerebellar neurons to both excitatory and inhibitory afferent inputs by inhibiting spontaneous discharge, specifically with regard to action of NE on inhibitory neurotransmission within the cerebellum, when applied iontophoretically or via activation of the LC and potentiates GABA-induced inhibition of cerebellar Purkinje neurons (Parfitt & Bickford-Wimer, 1990; Woodward et al., 1979; Yeh & Woodward, 1983c; Cheun & Yeh, 1992). Based

upon the Marr-Albus theories of cerebellar motor learning and NE's modulatory action on neurotransmitter function Gilbert proposed that NE should have a role in the consolidation of memory within the cerebellum (Gilbert, 1975). The modulatory role of NE in the cerebellum is mediated through the β-adrenergic receptor (Yeh & Woodward, 1983d).

1.2.4. The NE signal transduction cascade and learning in the cerebellum.

As mentioned above NE is known to be a neuromodulator and can increase the "signal to noise" ratio of afferent inputs to cerebellar Purkinje neurons. This effect is mediated through the β-adrenergic receptor (Yeh & Woodward, 1983e; Woodward et al., 1991a) involving the adrenergic signaling cascade which involves the familiar G protein coupled in which adenyl cyclase, cAMP, and PKA are activated and lead to such well studied phenomena as the downstream phosphorylation of cAMP responsive element binding protein (CREB) and subsequent expression of genetic material required for protein synthesis (see figure 1.2). The fact that CREB phosphorylation can also be mediated via Ca++ signaling mechanisms and protein kinase C (PKC) and that multiple signal cascades have been implicated to play a role in learning and memory (see (Selcher et al., 2002; Ma & Huang, 2002; Kornhauser & Greenberg, 1997) and paragraph below on LTD) it is likely that more than one molecular mechanism is necessary for learning and consolidation of memories in any given brain location.

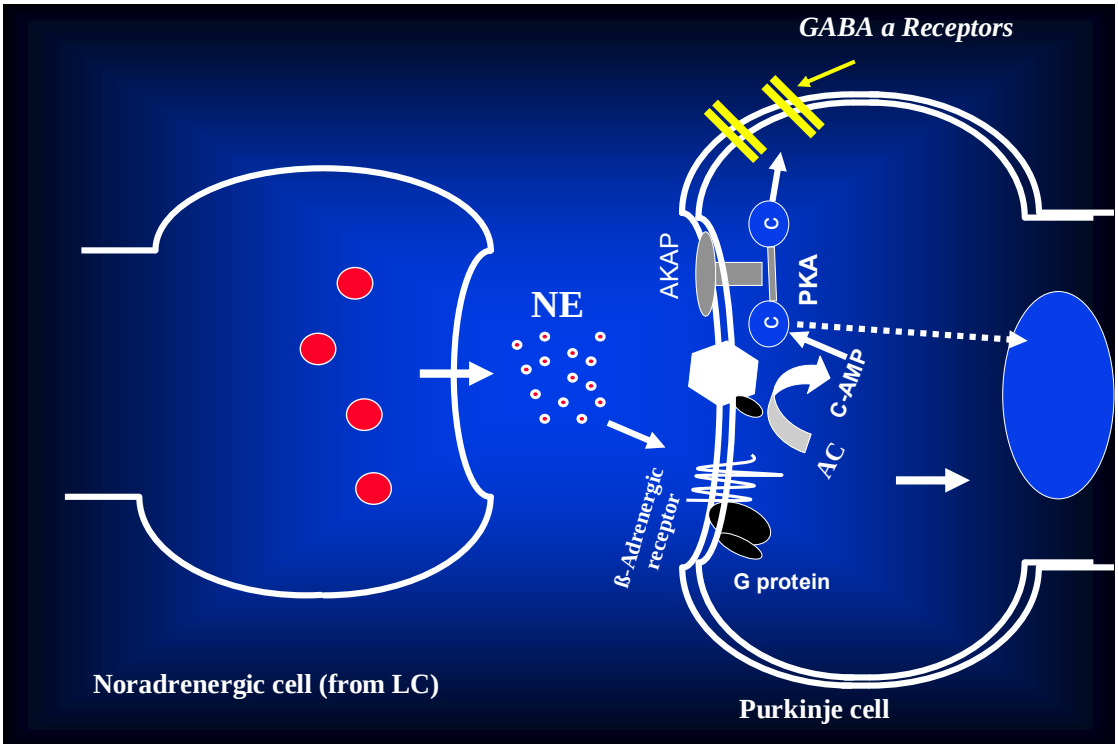


Figure 1.2. The hypothesis predicts that release of NE from presynaptic terminals acts upon β-adrenergic receptors to increase c-AMP and activation of PKA by releasing the catalytic subunit (c) from the regulatory subunits. There is a potential regulatory role of AKAP's in this process that is unknown at this time. One known effect of this cascade is a modulation of GABAa neurotransmission via PKA that is observed in Purkinje cells and also in the cells in the interpositus nucleus (Gould et al., 1997d). There is a short term increase in GABA neurotransmission (NE is also known to modulate mossy fiber (MF) and parallel fiber (PF) inputs). A second role for PKA activation relates to a potential phosphorylation of CREB and subsequent transcription of proteins that modulate neuronal activity or lead to new synapse formation.

In other brain areas (such as hippocampus), cAMP, PKA and phosphorylated CREB (pCREB) have been implicated as major players in the establishment of synaptic changes necessary for both short-term and long-term memory formation (Vianna et al., 2000; Baldwin et al., 2002; Taylor et al., 1999; Muller, 2000;

Shobe, 2002). Behavioral studies in long term potentiation (LTP) and long term depression (LTD) support this finding (Nayak et al., 1998; Rotenberg et al., 2000; Huang & Kandel, 1996a; Huang et al., 1994). It has been shown in eyelid conditioning that blockade of protein kinases impairs acquisition but not retention in rabbits (Chen & Steinmetz, 2000).

1.2.5. Cerebellar motor learning: long-term depression (LTD) and long term potentiation (LTP)

In the past decade there have been advances in understanding the cellular mechanims of LTD at parallel fiber-purkinje cell synapses. LTD in the cerebellum has been established as a mechanism the activation of voltage-gated Ca+2 channels, ionotropic (AMPA) and metabotropic (mGluR1) glutamate receptors as well as the stimulation of the PKC and nitric oxide (NO) formation. Thus, LTD is now well supported by recent experiments on transgenic mice (for review see (Daniel et al., 1998). A prominent, but still controversial hypothesis is that LTD of parallel fiber-PC synapses is at least one substrate for synaptic plasticity that underlies motor learning in the cerebellum. Most of the work done in this area has pointed to an accepted role for Ca++, PKC, MAP kinases, protein phosphatases, metabotropic glutamate receptors, nitric oxide, among other signaling pathways, yet the vast majority of studies have found no role for PKA in either short term or late-phase LTD (Hartell et al., 2001; Linden, 1994; Linden, 1996; Kawasaki et al., 1999). Late phase LTD is protein synthesis dependent and requires CaMKIV and CREB (Ahn et al., 1999). A very convincing argument

for the role of LTD and PKC in motor learning comes from a study of transgenic mice with PC specific inhibition of PKC. These mice do not demonstrate LTD and do not show modulation of gain in VOR (De Zeeuw et al., 1998), but yet, the adaptation mechanism to compensate the no natural condition implicit in the transgenic mice should still be considered. This is in contrast to an obvious role for NE and PKA in at least some forms of LTP in the hippocampus (Heginbotham & Dunwiddie, 1991; Huang & Kandel, 1996b; Frey et al., 1993; Roberson et al., 1996). PKA also plays a role in LTP in the cerebellum (Salin et al., 1996; Jacoby et al., 2001; Kimura et al., 1998) specifically, the action of PKA is proposed to be in cerebellar granule cells (Linden & Ahn, 1999) however, the regulation of PKA by NE has not been determined.

1.3. Intracerebral Microdialysis

Microdialysis is a technique for the *in vivo* assessment of cerebral neurotransmitters and their metabolites. The technique has been improved dramatically to reduce probe size in order to target smaller brain regions such as the anterior interpositus nucleus of the cerebellum, reduce the amount of gliosis which improves the diffusion barrier and hence the levels of neurotransmitters recovered and even extends to the use of multiple probes (Hernandez et al., 1986b). During microdialysis artificial cerebral spinal fluid (aCSF) is perfused through the internal tube, flows down into the microdialysis probe and back up the outer tube where it is collected for assay. Neurotransmitters and their metabolites diffuse from an area of high concentration to an area of low

concentration (through probe cellulose membrane) and flow with the perfusate up the outer tubing to the collection vial where it is immediately refrigerated. In order to correlate memory to neurotransmitter levels in vivo microdialysis was coupled to delay eye blink conditioning (see figure 1.3 for illustration of head gear and microdialysis setup).

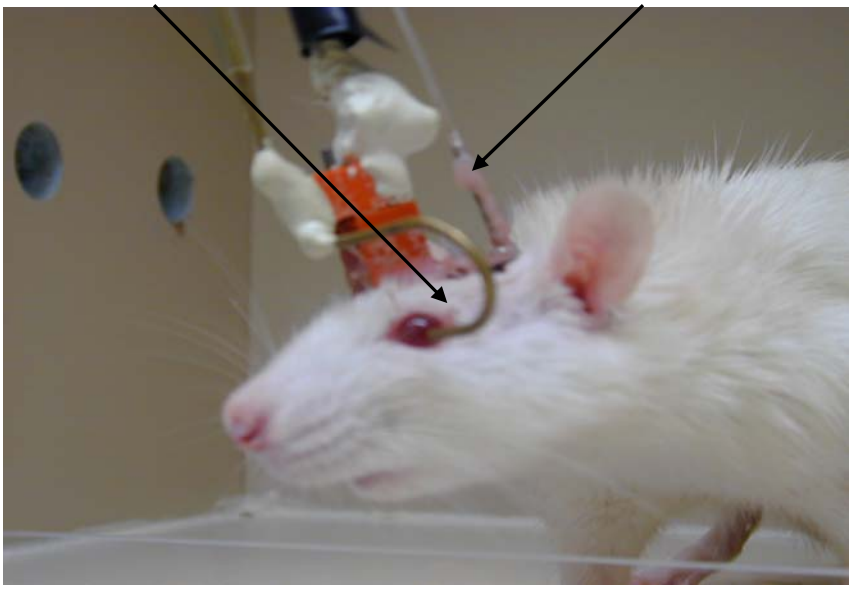


Figure 1.3. Rat in the eye-blink conditioning chamber with the head stage-air puff and the microdialysis probe targeting the cerebellum.

1.4. Other neurotransmitters

Other neurotransmitter systems are also implicated in delay eyeblink conditioning tasks. Administration of the glutamate AMPA receptor antagonist CNQX or the GABA-A receptor antagonist picrotoxin into the cerebellar cortex completely and reversibly impairs fully established conditioned response's (CR's), suggesting

that GABAergic and glutamatergic transmission are involved in CR performance (Attwell et al., 2002b). It has been suggested that the basal GABAergic output from the cortex onto the interpositus nucleus modulates CR expression, whereas timing of CR's is modulated by the stimulus activated inhibition (Bao et al., 2002). The expression of CR's can be interrupted by both GABA mediated inactivation of the interpositus neurons with muscimol as well as up-regulation of activity with picrotoxin (Aksenov et al., 2004).

1.4.1 Glutamate

Glutamate (GLU) is an excitatory neurotransmitter which is essential in the development of synaptic plasticity and is involved in cognitive functions like learning and memory. Glutamate is ubiquitious throughout the mammalian brain and is involved in cellular metabolism (Attwell, 2000; Petroff, 2002). GLU is formed by the precusors glutamine and α-ketoglutarate and subsequently packaged into vesicles for future release into the synaptic cleft (Tapiero et al., 2002). Glu is released from vesicles in presynaptic terminals by a Ca^{2+}-dependent mechanism that involves voltage-dependent calcium channels (Anderson & Swanson, 2000; Meldrum, 2000). The synaptic release of GLU is controlled by a wide range of presynaptic receptors (Anderson & Swanson, 2000). These include both Group II and Group III metabotropic GLU receptors and also cholinergic (nicotinic and muscarinic) receptors, adenosine (A1), κ-opioid, $GABA_B$, cholecystokinin and neuropeptide Y (Y2) receptors (Anderson & Swanson, 2000). Once released into the synaptic cleft, Glu is bound to either

pre- or post-synaptic receptors, reuptaken via the glutamate transporter and repackaged, diffuses away from the synaptic cleft, or is internalized by GLU transporters on glial cells (Anderson & Swanson, 2000; Attwell, 2000; Daikhin & Yudkoff, 2000). GLU is converted into the inhibitory GABA with the enzyme glutamic acid decarboxylase (GAD) which is highly abundant in the cerebellum (Sherif, 1994).

1.4.2 GABA

In 1965, GABA was established as a major inhibitory neurotransmitter in the CNS (Curtis & Watkins, 1965). GABA is synthesized by the enzyme GAD and taken up by glial-cells and neuronal mitochondria where it is transaminated to succinic semialdehyde and subsequently oxidized to succinic acid then enters the citric acid cycle in the glial cell (Sherif, 1994). Once GABA is released, it binds to GABA receptors which have been classified into three major subtypes ($GABA_A$, $GABA_B$ and $GABA_C$) on the basis of pharmacological and physiological data (Bowery et al., 1984; Mody et al., 1994). The major output pathway of the cerebellum is via the PC which are GABAergic. It has been suggested that the basal GABAergic output from the cerebellar cortex onto the interpositus nucleus modulates CR expression, whereas timing of CR's is modulated by the stimulus activated inhibition (Bao et al., 2002). The expression of CR's can be interrupted by both GABA mediated inactivation of the interpositus neurons with muscimol as well as up-regulation of activity with picrotoxin (Aksenov et al., 2004).

1.5. Aging as a process in which the modulatory effect of NE on PC is compromised

Aging-associated deficits on motor learning have been linked to dysfunction of the noradrenergic system (Cartford et al., 2004a; Bickford et al., 1985b; Bickford et al., 1986) which is thought to be caused by the loss of noradrenergic enhancement of the relative responsiveness of PCs to afferent inputs in aged animals.

Age-related pathologies are characterized by a pronounced imbalance in immune functions like glial hyperactivity with altered antigen expression of microglia in aged rodents (Perry et al., 2003a; Cunningham et al., 2007b). Chronic inflammation is known as one of the multiple age-related pathologies that involves the activity of several products, including cytokines (Murray et al., 1997b; Murray & Lynch, 1998a). Cytokines are proteins that mediate the response of the body's defense system to injury and mediate diverse inflammatory processes. The presence of altered levels of cytokines in the central nervous system has been implicated several aged-related and neurodegenerative diseases (Benveniste & Benos, 1995). Cytokines are secreted by activated microglia and can be either pro-inflammatory cytokines, among them tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL1-β), anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor beta 1 (Uccelli et al., 2005b). Pro-inflammatory cytokines are chronically increased in the aging brain (Godbout et al., 2005b) and more specifically, TNF-α

and TNF-β are significantly elevated in the cerebellum of aged rats (Gemma et al., 2002). In this study rats were fed an anti-oxidant enriched diet for six weeks which resulted in a significant reduction in both TNF-α and TNF-β levels. These diets have also been shown to improve classical eyeblink conditioning performance in aged rats and are directly correlated with a reduction in the expression of TNF-α and TNF-β levels in the cerebellum (Cartford et al., 2002b). The pro-inflammatory cytokine TNF-α binds TNF-α receptors that are expressed on both neurons and glial cells (Benveniste & Benos, 1995). TNF-α is synthesized and released by astrocytes, microglia and some neurons (Lieberman et al., 1989; Chung & Benveniste, 1990; Morganti-Kossman et al., 1997). TNF-α levels are usually increased in many CNS disorders, including ischemia (Liu et al., 1994), trauma (Goodman et al., 1990) and multiple sclerosis (Rieckmann et al., 1995). In these pathological conditions, the expression and release of TNF-α can occur as soon as one hour after an insult to the brain and long before neuronal death (Liu et al., 1994; Wang et al., 1994; Allan & Rothwell, 2001).

Given the evidence stated above for a role of inflammation in aging and specifically the role for an increase in TNF-α in the cerebellum with age, one goal of the studies outlined in this dissertation was to examine the impact of TNF-α on cerebellar dependent learning and the concomitant release of NE using microdialysis.

In summary, NE is implicated as an important factor in cerebellar plasticity that underlies motor learning behaviors. Specifically, it has been postulated that NE plays a role in consolidation of the performed behavior (Gilbert, 1975). One goal of this dissertation was to characterize the presynaptic release of NE during eyeblink conditioning in the rat and to investigate the timing of the influence of NE on acquisition of the learned response. Furthermore, there is evidence for declines in cerebellar dependent motor learning with age, as well as changes in NE signal transduction. One aspect of aging that may impact NE signaling and motor learning is an increase in inflammatory cytokines, specifically TNF-α. Thus, another goal of this dissertation was to examine the impact of TNF-α on cerebellar motor learning and NE signaling.

CHAPTER 2

Norepinephrine, GABA and Glutamate as a Substrate of Memory Formation in Cerebellar Eye Blink Conditioning

2.1 Abstract

Delay classical eyeblink conditioning (EBC) is an important model of associative, cerebellar dependent learning. Norepinephrine (NE) plays a significant modulatory role in the acquisition of learning; however, other neurotransmitters are also involved. The goal was to determine whether NE, Gamma-aminobutyric acid (GABA) and Glutamate (Glu) release are observed in cerebellar cortex during EBC, and whether such release was selectively associated with training. *In vivo* microdialysis coupled to EBC was performed. NE release was observed in EBC and peaked on day 1 then diminished with subsequent days of training. No changes in baseline NE release were observed in pseudo-conditioning indicating that NE release is directly related to the associative learning process. GABA release was also observed only during paired training but increased in magnitude over days of training. Glu release was observed during both paired and unpaired training. These data support the hypothesis that NE is a modulator of early responding and GABA is a mediator of the learned response. Age related deficits in eyeblink conditioning are linked to the loss of noradrenergic activity in the cerebellum. The present study used eyeblink conditioning coupled to microdialysis to demonstrate that, in contrast to young rats, NE, GABA and Glu

levels that occur during eyeblink conditioning show different patterns of release which appear to be correlated to the age-related impairment in the acquisition of learning.

2.2 Introduction

Delay eyelid conditioning is an excellent model of cerebellar motor learning. NE is known to play a modulatory role in cerebellar-dependent learning. NE induces synaptic plasticity in Purkinje neurons by selectively improving the signal to noise ratio of evoked versus spontaneous activity, enhancing the sensitivity of cerebellar neurons to both excitatory and inhibitory afferent inputs (Siegel & Freedman, 1988). Based upon the Marr-Albus theories of cerebellar learning Gilbert proposed that NE should have a role in the consolidation of memory within the cerebellum (Gilbert, 1975). Behavioral evidence which shows the involvement of NE in memory consolidation in the cerebellum is observed in several cerebellar dependent paradigms. For example in rod running motor learning, a cerebellar-dependent task, the ability of rats to learn is reduced following lesion of the locus coeruleus (LC) (Khachaturian et al., 1983). This effect is localized to cerebellar NE (Watson & McElligott, 1984) and is specific to the β-adrenergic receptor (Bickford et al., 1992). Adaptation of the vestibulo ocular reflex (VOR) is modulated by noradrenergic inputs (McElligott & Freedman, 1988) and appears to be mediated by the β-noradrenergic receptor (Pompeiano et al., 1991). Cerebellar delay classical conditioning has been shown to be modulated by NE. Electrolytic lesions of the LC induce resistance to

extinction in rabbits (McCormick & Thompson, 1982) and 6-hyroxydopamine (6-OHDA) has been shown to retard acquisition but not performance of conditioned eyelid responses in rabbits (Winsky & Harvey, 1992). Blockade of the β-adrenergic receptor retards the acquisition of learned responses in delay conditioning tasks in rats (Cartford et al., 2002a; Cartford et al., 2004b). NE is also implicated in non-cerebellar dependent tasks. LC neurons fire prior to a target cue in a vigilance task in monkeys and during reversal of task contingency, the LC response to the new stimuli precedes behavioral responding (Aston-Jones et al., 1997)

Other neurotransmitter systems are also implicated in delay eyeblink conditioning tasks. Administration of the Glu AMPA receptor antagonist CNQX or the GABA-A receptor antagonist picrotoxin into the cerebellar cortex completely and reversibly impairs fully established conditioned response's (CR's), suggesting that GABAergic and glutamatergic transmission are involved in CR performance (Attwell et al., 2002b). It has been suggested that the basal GABAergic output from the cortex onto the interpositus nucleus modulates CR expression, whereas timing of CR's is modulated by the stimulus activated inhibition (Bao et al., 2002). The expression of CR's can be interrupted by both GABA mediated inactivation of the interpositus neurons with muscimol as well as up-regulation of activity with picrotoxin (Aksenov et al., 2004). Aging-related deficits have been reported for the acquisition of CR in a variety of motor learning task. In particular, loss of cerebellar noradrenergic function has been directly correlated with the decreased

ability to learn the rod walking task (Bickford, 1995). Currently, the correlation between aged-related impairments in motor plasticity and specific neurophysiological deficits in the cerebellar PC is accepted (Bickford, 1993).

Eyeblink conditioning in particular has been shown to be a good paradigm to evaluate the effects of antioxidant-enriched diets on cognitive ability of aged rats (Cartford et al., 2002b; Gemma et al., 2002). In these studies the authors showed that anti-oxidant enriched diets can reverse the learning impairment while the levels of the cytokine TNF-α were reduced in aged rats. This suggests that the high levels of TNF-α found in aged rats might be one of the major reasons learning deficits are observed during aging. To date work examining the role of neurotransmitters in delay eyeblink conditioning has used either the application of agonists or antagonists to investigate the postsynaptic effects of these neurotransmitters (Bao et al., 2002; Krupa & Thompson, 1997; Mamounas et al., 1987; Schreurs & Alkon, 1993). However, questions remain regarding presynaptic release of neurotransmitters during performance of the delay conditioning task. The present study uses in vivo microdialysis to examine the temporal patterns of release of NE, GABA, and Glu during eyeblink conditioning training to examine critical presynaptic events and whether the aged-related deficits in learning are reflected in the neurochemical orchestration within the cerebellum during the eyeblink conditioning.

2.3 Methods

2.3.1 Animals and surgery

Male F344 rats four and twenty two months of age were used in this study. Room temperature was kept at 72 °F and the dark/light circle was 12-h (lights were on from 7:00 AM to 7:00 PM). Animal number was the minimum required for reliable statistical test results. Rats were anesthetized with pentobarbital (10 mg/kg, i.p.) and ketamine (25 mg/kg, i.p.) and placed in a stereotaxic instrument. Anesthesia level was monitored every 10 min and maintained in such a way that the withdrawal reflex to paw pinch was absent during surgery. If the experimenter noted any spontaneous movement or minute vocalization an additional charge of ketamine (10 mg/kg) and pentobarbital (5 mg/kg) was given. A 10 mm long guide shaft made of 21-gauge stainless-steel tubing (Plastics One) was inserted into the cerebellum. The guide shaft was attached to the skull by jeweler screws and cemented with dental acrylic. In young rats the coordinates to implant the guide cannulae for the microdialysis probe into the cerebellar lobule HVI (simplex, and interpositus nucleus) were AP-11.3, ML +2.5 and DV -2.5 mm in reference to bregma for young rats, while in aged rats were AP -11.8, ML +2.5 and DV -2.5 mm. In the same surgical session rats were prepared for eyelid training by fixing a small ITT/Cannon connector strip to their skull to hold gold pin connectors to EMG wires that were run under the left eyelid. This method has been previously published by our lab (Cartford et al., 2002b). Rats were allowed to recover for one week after the surgery procedure before starting the eyeblink conditioning training and microdialysis. Each animal was used for only one

experimental condition. All procedures were carried out in accordance with the institutional guidelines (IACUC) and with USA National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3.2 Microdialysis procedure

Microdialysis probes were lab-made with cellulose hollow fiber (MW 13,000) attached to stainless steel tubing, with a 45 cm length of fused silica capillary (internal diameter [I.D.] 76 μm; outside diameter [O.D.] 150 μm) inserted into the cellulose tube (Hernandez et al., 1986a). The effective length of the dialysis piece was 3 mm.

2.3.3 Training

The rats were habituated to the training chamber and headstage cable for three days. The training consisted of 50 trials each training trial consisted of a 250 ms baseline, a 400 ms CS period, and a 100 ms US period. The tone was 500 ms in duration and overlapped the airpuff for 100 ms. The training tone was 3 kHz and the airpuff 10 psi. Hardware and software used to train and analyze data were manufactured by J.Tracy, J.Green and Joe Steinmetz, (Bloomington, Indiana). Eyelid EMG data was collected, amplified, rectified, and integrated. Learned responses were determined using a 10 standard deviation criterion for eyelid amplitude elevated during the CS period when compared to the baseline. Alpha responses to the tone were excluded from learned response analysis by using a 70 ms discrimination/exclusion window. Learning was measured as the

percentage of learned (conditioned) responses (CR's) made in each training session.

2.3.4 Microdialysis and eye-blink conditioning procedure

The night prior to the experiment the probe was inserted into the cerebellum of rats via the guide cannula. The inlet of the probe was connected to a syringe pump filled with artificial cerebrospinal fluid (aCSF) solution (134.9 mM NaCl, 3.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM $NaHCO_3$ at pH 7.4) and the perfusion flow rate was set at 0.1 µl/min during 12 hours (overnight) to allow recovery from the probe insertion damage. The next morning the flow rate was set at 2 µl/min for 2 hours and the head stage (ITT/Cannon connector**)** was coupled to the rat head stage for the EEG recording and airpuff delivery, microdialysates were collected every 10 minutes. Microdialysis samples were collected for one hour prior to training baseline (B), during training (T)(18-20 min) and for two hrs post-training (PT).

2.3.5 Neurochemical analysis

Immediately after each sample collection, one microliter of sample was taken from the vial and placed into another vial for GLU and GABA analysis, the rest of the sample (nineteen microliters) was acidified with 2 µl of 0.1 M HCL and all the samples immediately frozen for High Performance Liquid Chromography (HPLC) and Capillary Zone electrophoresis (CZE) analysis.

2.3.6. HPLC: Norepinephrine

Catecholamine Analysis: The microdialysis samples that were frozen at -80 °C were unfrozen for the analysis in the HPLC. This method is published and routinely used by our laboratory (Hall et al., 1986; Bowenkamp et al., 1997). The detection of NE, 3,4-Dihydroxyphenylacetic acid (DOPAC), was performed using an isocratic HPLC system (Beckman, Inc., Fullerton, CA), at a flow rate of 1 ml/min. This system is coupled to a dual-channel electrochemical array detector (model 5100A, ESA, Inc., Chelmsford, MA), E1 = +0.35 mV and E2 = -0.25 mV, using an ESA model 5011 dual analytical cell. The compounds of interest were separated with reverse-phase chromatography, using a C18 column (4.6 mm x 100 mm, 3 µm particles, ODS Thermo Hypersil; Keystone Scientific, Bellefonte, PA) with a pH 4.1 citrate-acetate mobile phase, containing 10% methanol and 0.45 mM 1-octane-sulfonic acid. Data were quantified using Totalchrom software V6.2 (Perkin Elmer) based on peak area, in comparison with an external standard calibration curve.

2.3.7. Capillary electrophoresis: Glu and GABA

This method has been published (Rada et al., 2003) and used by our laboratory. A capillary electrophoresis system equipped with an argon laser tuned to 488 nm was used (Model R2D2, Meridialysis Co., Merida, Venezuela). A carbonate buffer (20 mM carbonate/bicarbonate) was the running buffer to transport the microdialysis sample through the capillary when detecting glutamate. Detection

of GABA from the samples required a different running buffer consisting of 23 mM borate with 120 mM sodium dodecyl sulfate and 1% methanol. The samples or standards were sucked into the anodic end by applying a negative pressure (19 psi or 1.34 kg/cm for 0.5 s) at the cathodic end of the capillary. Electrophoretic separation was achieved by applying a high voltage between the anode and the cathode for 12 min, 22 kV for glutamate and 26 kV for GABA.

2.3.8. Derivatization

Fluorescein isothiocyanate (FITC) was conjugated with glutamate and GABA as the fluorescent chromophore. Optimal concentrations of FITC and the calibration curves for both amino acids have been reported previously. Dialysates and standards were derivatized mixing 1 µl (sample or standard solution) with 1 µl of a solution containing FITC (1 mM/acetone) - carbonate buffer (20 mM) 1:1 mixture. A syringe loaded with FITC–carbonate mixture was placed in a precision pump, and 1 µl of the mixture was delivered into a tube containing 1 µl of microdialysis sample. The samples reacted overnight (14 hr) at room temperature in a water-saturated chamber that minimized evaporation. Homoglutamine (10–5 M) was used as an internal standard and was mixed in the carbonate buffer used to derivatize samples and standards.

2.3.9. Histology

After completing the experiment, the animals were overdosed with sodium pentobarbital and decapitated and the brains were dissected, placed in 10%

formalin for 24 h and then transferred to 15% sucrose with 10% formalin for 24 hrs. The brains were then frozen, sectioned (40 μm) on a freezing microtome and stained with cresyl violet for verification of the probe placement and extensions of the lesion. Only animals with probe placements verified through cerebellar cortex and lobule HVI (simplex, and interpositus nucleus) region were used for data analysis, also animals that presented high level of damage or hemorrhage due to the microdialysis probe insertion were discarded from the study.

2.3.10. Statistical analyses

Analyses were performed using conditioned responses (CR's) and area under the curve (AUC) of neurotransmitter concentration as the dependent measures. Data from experiments were subjected to a two-way analysis of variance of either conditioned responses or area under the curve (AUC) for the neurotransmitter, followed by subsequent post hot tests. Supernova was the statistical software utilized in these analyses. A p value of <0.05 was considered to be statistically significant.

2.4 Results

2.4.1 Young Rats

Microdialysis was performed in the cerebellar cortex on rats during training in the delay eyelid conditioning task. Rats learn this task over days and were significantly different from pseudo-conditioned performance and days 2 – 5 (Figure 2.1). Neurotransmitter release was examined on all five days of training.

However the microdialysis performed and plotted for neurotransmitter release on each day was done with independent groups.

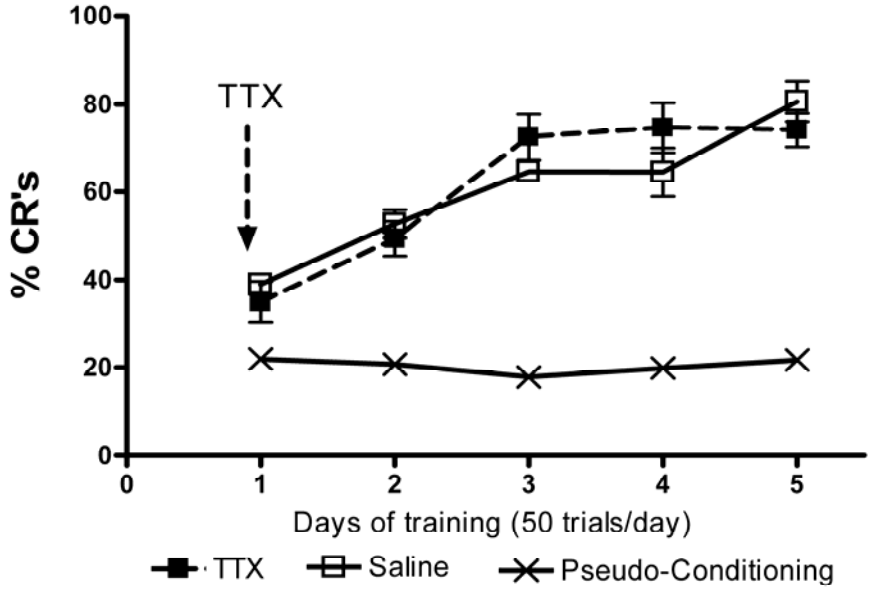


Figure 2.1. Effect of TTX on cerebellar dependant eyelid conditioning. Eyelid conditioning was performed over 5 days. Rats received either vehicle or TTX twenty minutes prior to eyelid conditioning on the first day of training. The Y-axis shows the percentage of conditioned response (% CR's), the x-axis represents daily training sessions of 50 trials. Both TTX and vehicle treated rats learned this task over days (represented by higher % CR) whereas rats who under went psuedoconditioning did not learn the task. Black squares represent TTX treated rats, open squares represent control rats and X represents pseudoconditioning. Control and TTX treated rats learned significantly more than psedoconditioning treated rats ($p<0.05$).

2.4.1.1 Norepinephrine

NE release was observed in the microdialysate on all 5 days of training, however the temporal pattern and magnitude of release changed over days of training (Figure 2.2A-E). On day one of training there was an increase in NE detected in the microdialysate that peaked at the end of the behavioral training and remained

significantly above baseline for 70 minutes after the training session. A 2 (Treatment: conditioning, pseudoconditioning) x 5 (Day: 1-5) between subjects ANOVA revealed a significant main effect of treatment, [F (1, 27) = 84.20, MSe = 91975.60], with conditioning resulting in higher AUC of NE than pseudoconditioning (Fig 2.2F). This indicated that the NE release was specific for the learning condition and was not the result of sensory stimulation alone. There was also a significant main effect of day, [F (4, 27) = 4.30], with AUC generally decreasing over days. The Fisher's LSD test revealed that day 1 resulted in significantly higher AUC than days 2, 4 and 5. As shown in Figure 2.2F, there was no significant interaction between day and treatment [F (4, 26) = 2.20]. NE release was significantly greater in the conditioning group on days 1-5 compared to the pseudoconditioning group. These findings can be observed in Figures 2.2A-E which illustrates the time window of NE release once the training started. On all days a clear increase in NE levels were observed with the onset of training in the conditioning group. Interestingly, over days NE levels returned to baseline levels faster, indicating a larger amount of release with a longer duration was observed on the beginning days of training. In contrast, the pseudoconditioning group did not show this pattern of transient NE release which indicates that the NE release observed (conditioning group) is directly related to the learning process in the eyeblink task.

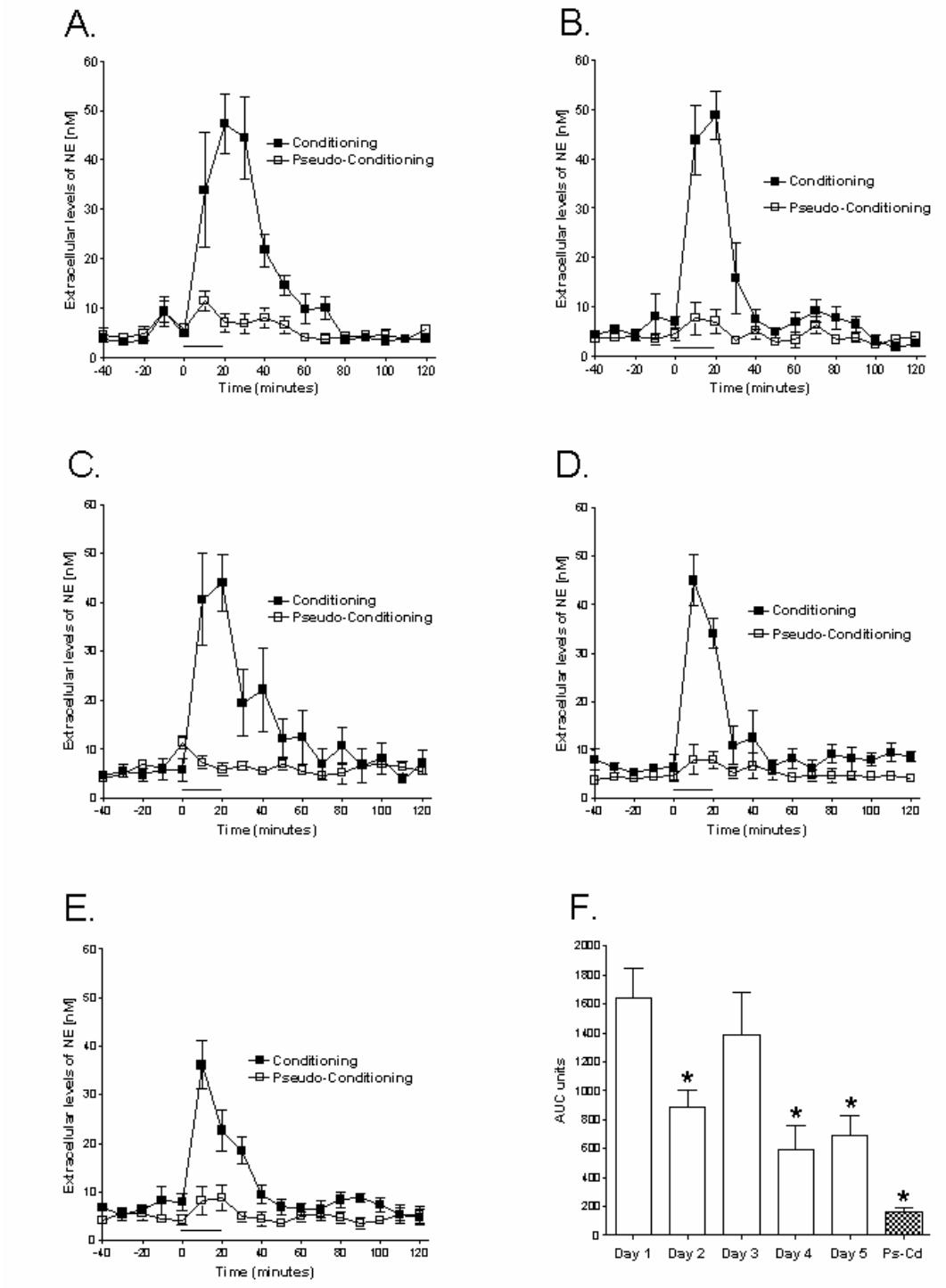


Figure 2.2. Temporal release of norepinephrine during eyelid conditioning. Microdialysis was performed in the cerebellar cortex on rats during training in the delay eyelid conditioning task. The time window of NE release once eyelid

conditioning starts can be observed on day 1 (A), day 2 (B), day 3 (C), day 4 (D) and day 5 (E). On day one of training (A) there was an increase in NE that peaked at the end of the behavioral training and remains significantly above baseline for 70 minutes after the training session. On all days (A-E) a clear increase in NE levels was observed with the onset of training in the conditioning group. Data are expressed as NE [nM] (y-axis) over time in 10 minute dialysate samples (x-axis). Black squares represent conditioning, open squares represent pseudoconditioning. F) Area under the curve (AUC units) representation of NE release for each day of training during Eyelid conditioning. Note that the pseudoconditioning group (Ps-Cd) did not show this pattern of transient NE release (indicates that the NE release observed (conditioning group) is directly related to the learning process in the eyeblink task.)

2.4.1.2 GABA

GABA release was also examined over days of training and it can be observed in Figure 2.3 that the amplitude of GABA release increased over days of training and the time course shortened. Data are expressed as percent of baseline and mean basal levels were 0.12 μM (± 0.1). A (treatment: conditioning, pseudoconditioning) x 5 (day: 1-5) between subjects ANOVA revealed a significant main effect of treatment, [F (1, 27) = 92.70, MSe = 33192.80], where conditioning resulted in significantly more GABA release (expressed as AUC units) than the pseudoconditioning (see figure 2.3F). There was also a significant interaction between treatment and day, [F (4, 27) = 4.70], which revealed that GABA release was significantly greater in the conditioning group on day 1 compared to days 2, 3 and 5 ($p<0.05$), one reason for this difference is observed when looking at the time course of the GABA release over days of training. The maximum amplitude of release was lower on Day 1, yet the release is spread over time, thus leading to an overall larger amount of GABA measured over baseline. Figures 2.3A-E illustrates the time window of GABA release once

the training starts. The peak magnitude of release increased over time and the time frame of the response sharpened so that GABA release is sharply timed with the performance of CR's by day 3 and sharpens further up to day 5. As observed with NE, the conditioning group resulted in significantly higher AUC on all days except day 5, when compared to the pseudoconditioning group ($p<0.05$), suggesting that the GABA release is associated with learning of the conditioned response.

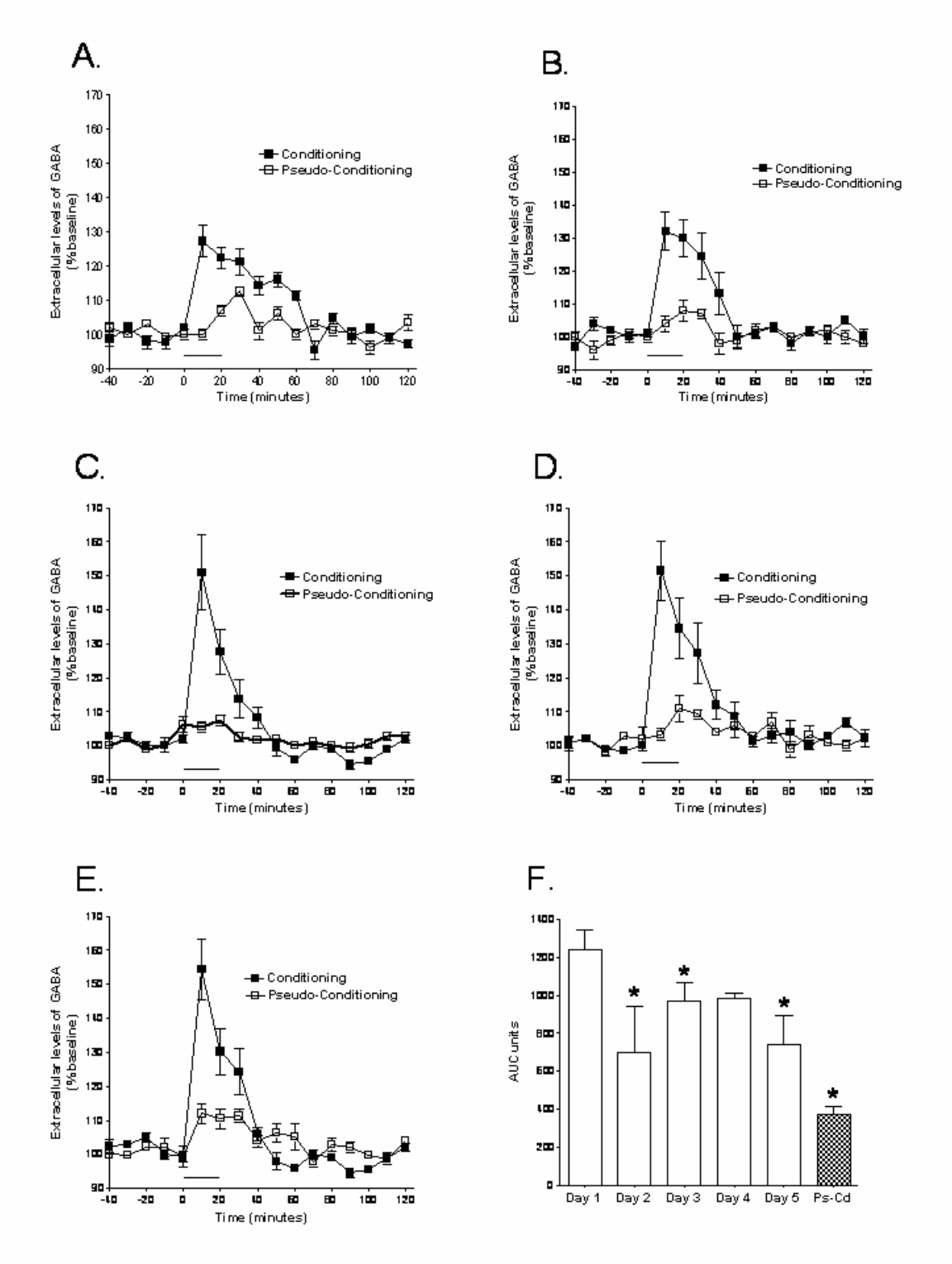


Figure 2.3. Temporal release of GABA during eyelid conditioning. Microdialysis was performed through the cerebellar cortex and interpositus nuclei on rats during training in the delay eyelid conditioning task. The time window of GABA

release once eyelid conditioning starts can be observed on day 1 (A), day 2 (B), day 3 (C), day 4 (D) and day 5 (E). GABA release was significantly greater in the conditioning group on day 1 compared to days 2, 3 and 5 ($p<0.05$). Peak magnitude of release increases over time and the time frame of the response sharpens so that GABA release is sharply timed with the performance of CR's by day 3 and sharpens further up to day 5. Data are expressed as percent of baseline (% baseline) (y-axis). Black squares represent conditioning, open squares represent pseudoconditioning. (F) Area under the curve representation of GABA release for each day of training during eyeblink conditioning. As was observed with NE, the conditioning group resulted in significantly higher AUC for GABA on all days except day 5, when compared to the pseudoconditioning group (Ps-Cd) ($p<0.05$), suggesting that the GABA release is associated with learning of the conditioned response.

2.4.1.3 Glutamate

As can be seen in figure 2.4F, a 2 (treatment: conditioning, pseudoconditioning) x 5 (day: 1-5) between subjects ANOVA of the AUC of Glu did not reveal main effects of day, [F (4,31) = 0.40, MSe = 2854.80] or treatment, [F(1,31) = 0.0036], or any interaction between treatment and day, [F(4,31) = 1.50]. Interestingly, figures 2.4A-E shows that comparable levels of Glu were released during training on the eyeblink task for both the conditioning and the pseudoconditioning groups.

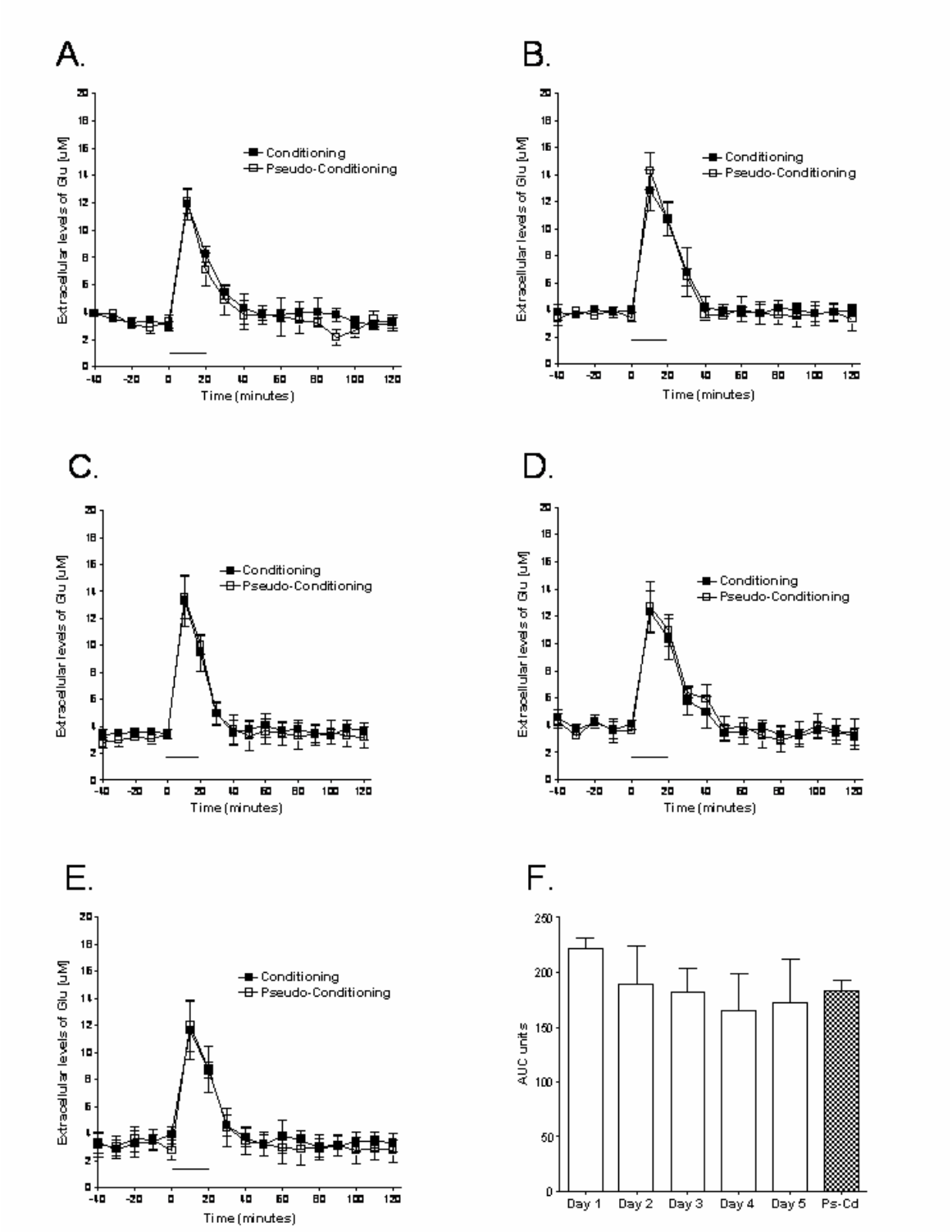


Figure 2.4. Temporal release of glutamate during eyelid conditioning. Microdialysis was performed in the cerebellar cortex on rats during training in the delay eyelid conditioning task. The release of Glu over time during eyelid

conditioning is shown on day 1 (A), day 2 (B), day 3 (C), day 4 (D) and day 5 (E). Comparable levels of glutamate were released during training on the eyeblink task for both the conditioning and the pseudoconditioning groups. Data are expressed as Glu [μM] (y-axis). Black squares represent conditioning, open squares represent pseudoconditioning. (F) Area under the curve representation of Glu release for each day of training during eyelid conditioning and pseudoconditioning (Ps-Cd, average of days). There were no differences in AUC of Glu.

2.4.1.4 Tetrodotoxin (TTX) effect on neurotransmitter release

In a subset of young rats TTX was administered twenty minutes prior to training on the first day of training and administration continued for the duration of the microdialysis collection. TTX was administered to test the impulse dependence of neurotransmitter release with microdialysis. The behavior of the rats was analyzed and there were no significant differences in learning between groups (figure 2.5). There was a decrease in amplitude of the unconditioned response on the TTX treatment group in two of the six rats, suggesting that there was a suppression of performance of the blink response for those two animals (data not shown). The microdialysates were analyzed for NE, GABA and Glu. The effect of TTX on NE release is observed in Figure 2.5A-B, where TTX reduced the AUC of NE compared with controls demonstrating an impulse dependent nature of NE release ($p<.05$). The effect of TTX on GABA release was also a reduction in the AUC for GABA (Figures 2.5D). A similar effect was observed with glutamate (Figures 2.5E-F). TTX was administered twenty minutes before the eyeblink conditioning training started, and it is notable that the extracellular levels for every neurotransmitter (NE, GABA and Glu) were depleted from their basal levels, indicating tonic release for NE, GABA and Glu. Despite the depletion in

basal extracellular levels of neurotransmitters, once the training sessions started each particular neurotransmitter was released following the same temporal pattern as the non-TTX groups. However the total amount of release was significantly lower compared to the control groups. It is interesting to see that there were not significant differences on percent CR's reached on day one of training. This behavioral outcome was unexpected, however it should be considered that the low concentration of TTX used might have not been enough to impair the circuitry flow of sensory inputs carrying the US and CS. Even with the reduction in tonic release (due to the presence of TTX) of NE, GABA and Glu, it is also possible that the signal to noise ratio on the PC was sufficient to relay essential information for memory formation as a greater percentage of CR's were reached on day two of training. The data show that TTX depleted baseline neurotransmitter levels and attenuated the neurochemical response to training. However, taking into consideration changes in evoked neurotransmitter levels relative to baseline levels it is apparent that neurotransmitter release for both TTX and non-TTX are similar. Therefore, despite the depletion on presynaptic activity through the cerebellar cortex and interpositus nuclei, if the signal to noise ratio remains the same throughout the training sessions, it might still be possible that memory encoding occurs. This would explain why in aged animals, which have attenuated levels of NE, GABA and Glu, if training persists the rats develop CR's. Therefore, it is not surprising that the TTX group show levels of CRs comparable to the non-TTX group. More experiments need to be conducted to

clarify this explanation, and if it is shown to be true it will support the concept of NE signaling facilitating the signal to noise ratio of the PC.

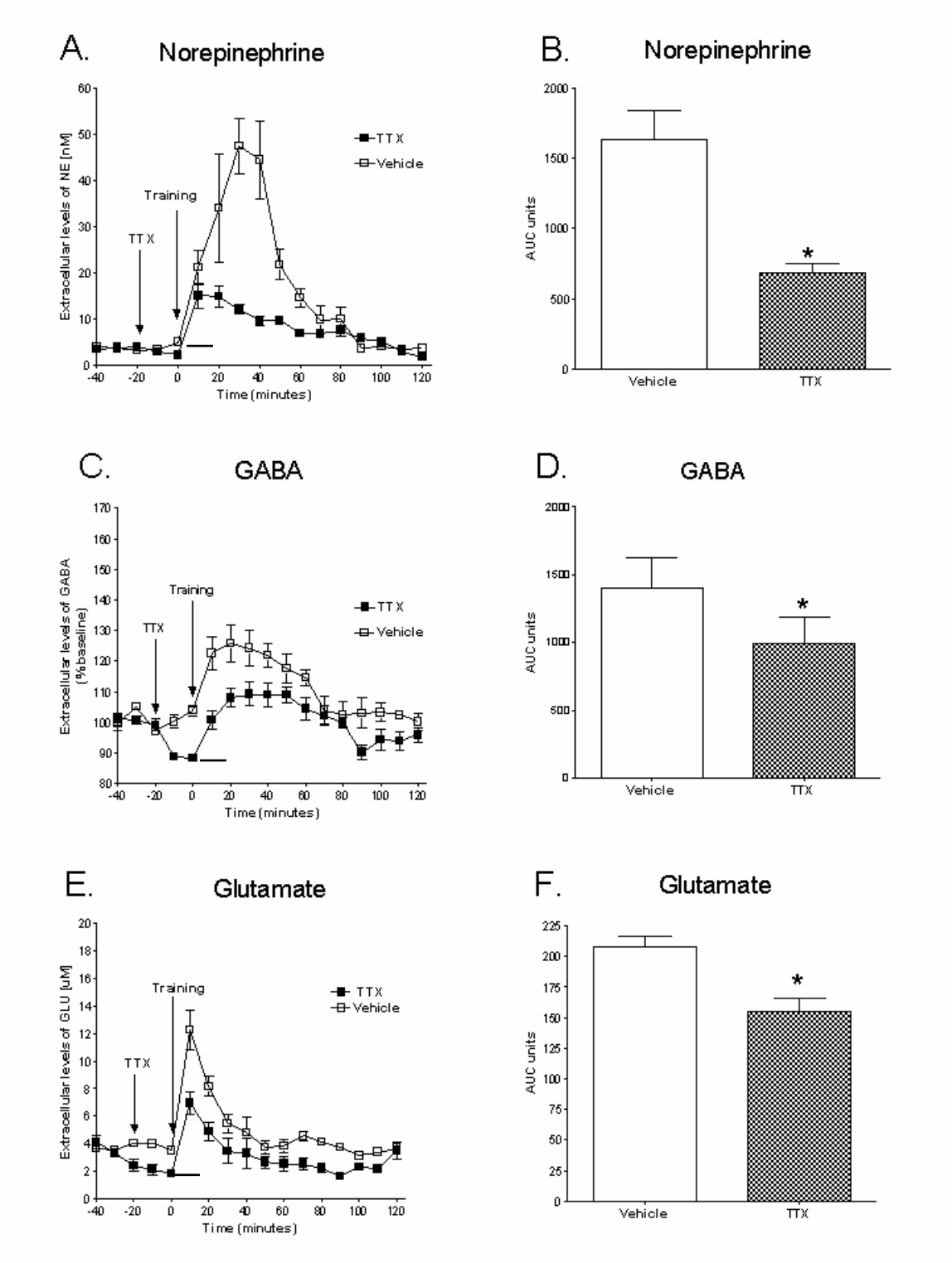


Figure 2.5. Tetrodotoxin (TTX) effect on neurotransmitter release during eyelid conditioning. TTX was administered 20 minutes prior to training on the first day of training (indicated by arrow) and administration continued for the duration of the microdialysis collection. Training began at time point 0 (indicated by arrow) and lasted approximately 20 minutes. (A) Shows extracellular NE levels [nM] over time (minutes) on day 1 of conditioning and (B) shows the area under the curve (AUC) of NE release during eyeblink conditioning on day 1. TTX reduced the amount of NE in the microdialysate when compared with control demonstrating an impulse dependent nature of NE release. (C) Extracellular GABA levels (% baseline) over time (minutes) on day 1 of conditioning. Note the reduction in basal levels after TTX administration compared to vehicle. (D) AUC of GABA release during eyeblink conditioning on day 1. There was a slight reduction in the GABA levels after TTX. (E) Extracellular Glu levels [μM] over time on day 1 of conditioning. (F) AUC of Glu release during eyeblink conditioning on day 1.

2.4.2 Aged Animals

Microdialysis was performed in the cerebellar cortex on rats during training in the delay eyelid conditioning task. As can be seen in figure 2.6 aging and young rats performed significantly better than pseudoconditioning, however young rats learned the task faster with more accuracy compared to aging rats ($p<0.05$). The pattern of release follows a different dynamic with might reflex why the rats takes longer (need more training sessions) to learn a CR comparable to young rats. These changes include a delay in the release in respond to the training conditioning sessions and also the magnitude for the release is significantly smaller compare to young rats. Long lasting release is also observed in aged rats, which could reflect impairments in the metabolism (clearance) of the neurotransmitters such as the re-uptake systems for Glu and NE.

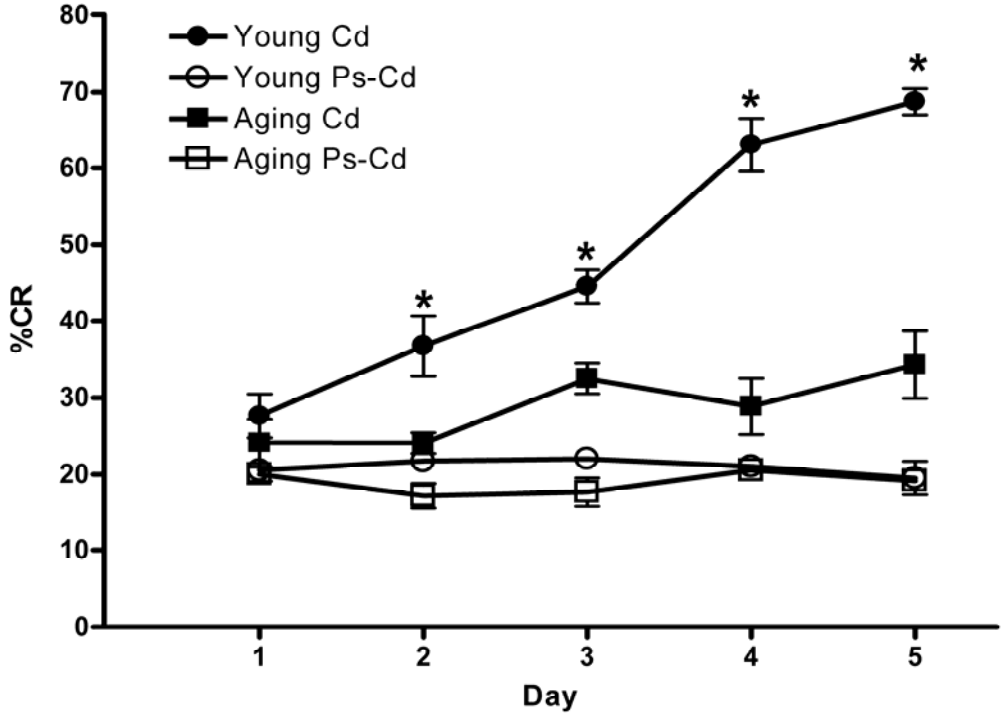


Figure 2.6. Cerebellar dependant eyelid conditioning is impaired in aging. Eyelid conditioning was performed over 5 days. The Y-axis shows the percentage of conditioned response (% CR's), the x-axis represents 5 daily training sessions of 50 trials. Black circles represent young conditioning, open circles are young pseudoconditioning, black squared represent aging conditioning, open squared are aging pseudoconditioning. Young rats learned this task over days (represented by higher % CR) whereas rats who under went did not learn the task. Both aging and young rats performed significantly better than pseudoconditioning, however young rats learned the task faster with more accuracy compared to aging rats (p<0.05). * indicates difference between young Cd and young Ps-Cd.

2.4.2.1 Norepinephrine

NE release was examined on days 1, 3 and 5 of training. NE release was observed in the microdialysate on all 3 days of training, however the temporal pattern and magnitude of release changed over days of training (Figure 2.7A-C). In contrast to what is observed in young rats, NE levels increased faster and

return to baseline sooner after days of training. A 2 (treatment: conditioning, pseudoconditioning) x 3 (day: 1,3,5) between subjects ANOVA revealed a significant main effect of learning, [$F(1,9) = 29$, $p<.05$], with conditioning resulting in higher AUC of NE than pseudoconditioning (Fig 2.7D). This indicates that NE release is specific for the learning condition and is not the result of sensory stimulation alone. There was also a significant main effect of day, [$F(2,9) = 7.2$], with AUC generally increasing over days in the aged animals. Figure 2.7D illustrates a significant interaction between day and treatment [$F(2,9) = 5.6$] with NE release being significantly greater in the conditioning group on days 3 and 5 compared to the pseudoconditioning group. These findings can be observed in Figures 2.7A-C which illustrate the time window of NE release once the training starts. On all days an increase in NE levels are observed in the conditioning group whereas, the pseudoconditioning group did not show this pattern of transient NE release which indicates that the NE release observed (conditioning group) is directly related to the learning process in the eyeblink task.

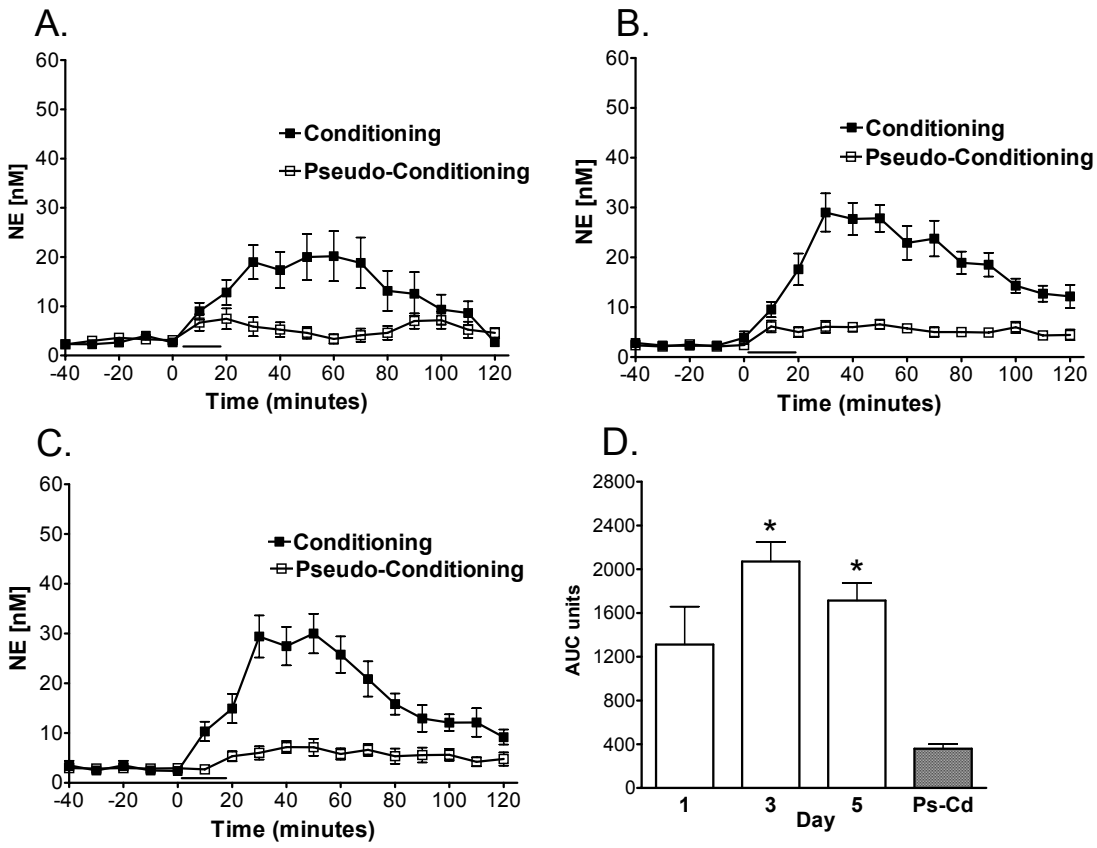


Figure 2.7. Temporal release of norepinephrine during eyelid conditioning in aged rats. Microdialysis was performed in the cerebellar cortex on aged rats during training in the delay eyelid conditioning task. The time window of NE release once eyelid conditioning starts can be observed on day 1 (A), day 3 (B) and day 5 (C). Data are expressed as NE [nM] (y-axis) over time in 10 minute dialysate samples (x-axis). Black squares represent conditioning, open squares represent pseudoconditioning. D) Area under the curve (AUC units) representation of NE release for each day of training during eyelid conditioning.

2.4.2.2 GABA

GABA release was also examined over days of training and it can be observed in Figure 2.8 that the amplitude of GABA release increased over days of training and the GABA levels remained elevated above baseline. A (treatment: conditioning, pseudoconditioning) x 3 (day: 1,3,5) between subjects ANOVA revealed a significant main effect of treatment, [$F(1,8) = 85.0$, $p<0.05$], where

conditioning resulted in significantly more GABA release (expressed as AUC units) than the pseudoconditioning (see figure 2.8D). There was also a significant interaction between treatment and day, [F (2,8) = 3.7, p<0.05], which revealed that GABA release was significantly greater in the conditioning group on day 1 compared to day 5 (p<0.05), one reason for this difference is observed when looking at the time course of the GABA release over days of training. The pattern of release changes across days so that by day 5 GABA remains elevated 2 hours after training. This can be observed in figures 2.8A-C which illustrates the time window of GABA release once training starts.

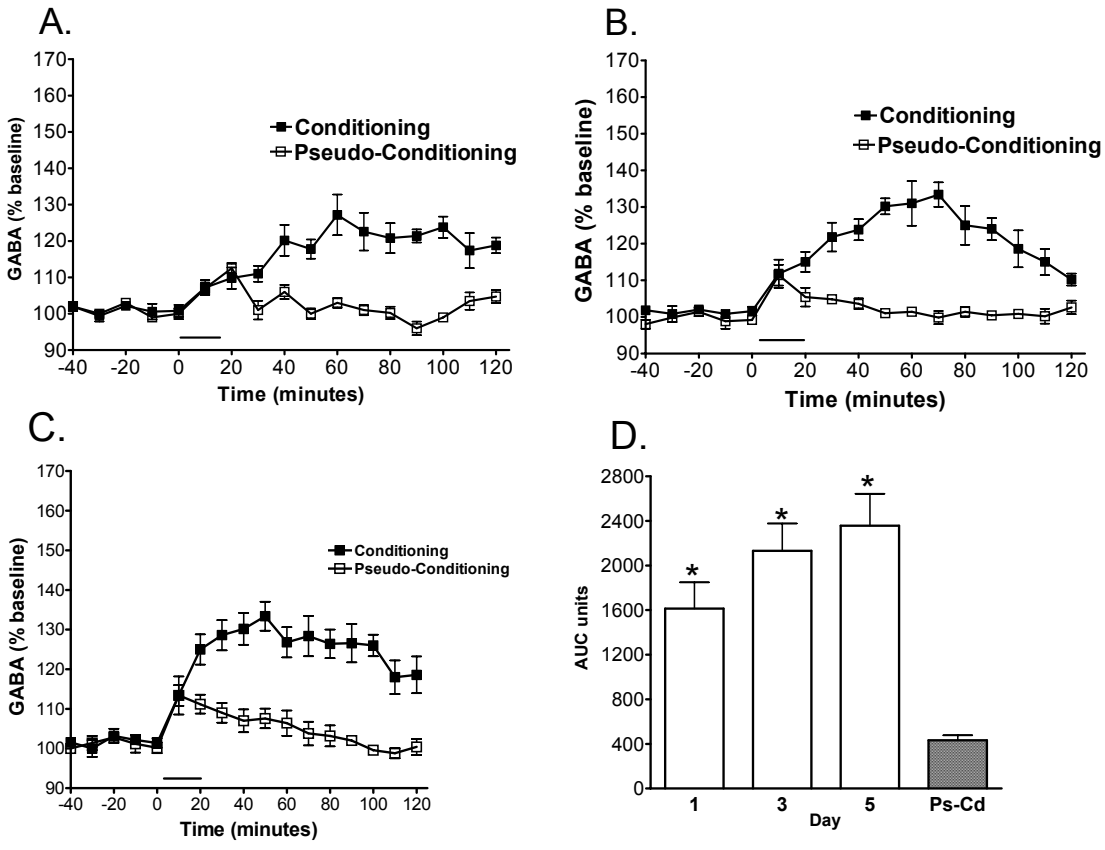


Figure 2.8. Temporal release of GABA during eyelid conditioning in aged rats. Microdialysis was performed in the cerebellar cortex on aged rats during training in the delay eyelid conditioning task. The time window of GABA release once

eyelid conditioning starts can be observed on day 1 (A), day 3 (B) and day 5 (C). GABA release was significantly greater in the conditioning group on day 1 compared to day 5 ($p<0.05$). The pattern of release in aged rats changes across days so that by day 5 GABA remains elevated 2 hours after training. Data are expressed as percent of baseline (% baseline) (y-axis). Black squares represent conditioning, open squares represent pseudoconditioning. (D) Area under the curve representation of GABA release for each day of training during eyeblink conditioning

2.4.2.3 Glutamate

As can be seen in figure 2.9D, a 2 (treatment: conditioning, pseudoconditioning) x 3 (day: 1,3,5) between subjects ANOVA of the AUC of Glu did not detect main effects of day, [F (2,6) = 2.2] or learning, [F(1,6) = 0.041], or any interaction between treatment and day, [F(2,6) = 0.66]. Interestingly, figures 2.9A-D shows that comparable levels of glutamate are released during training on the eyeblink task for both the conditioning and the pseudoconditioning groups.

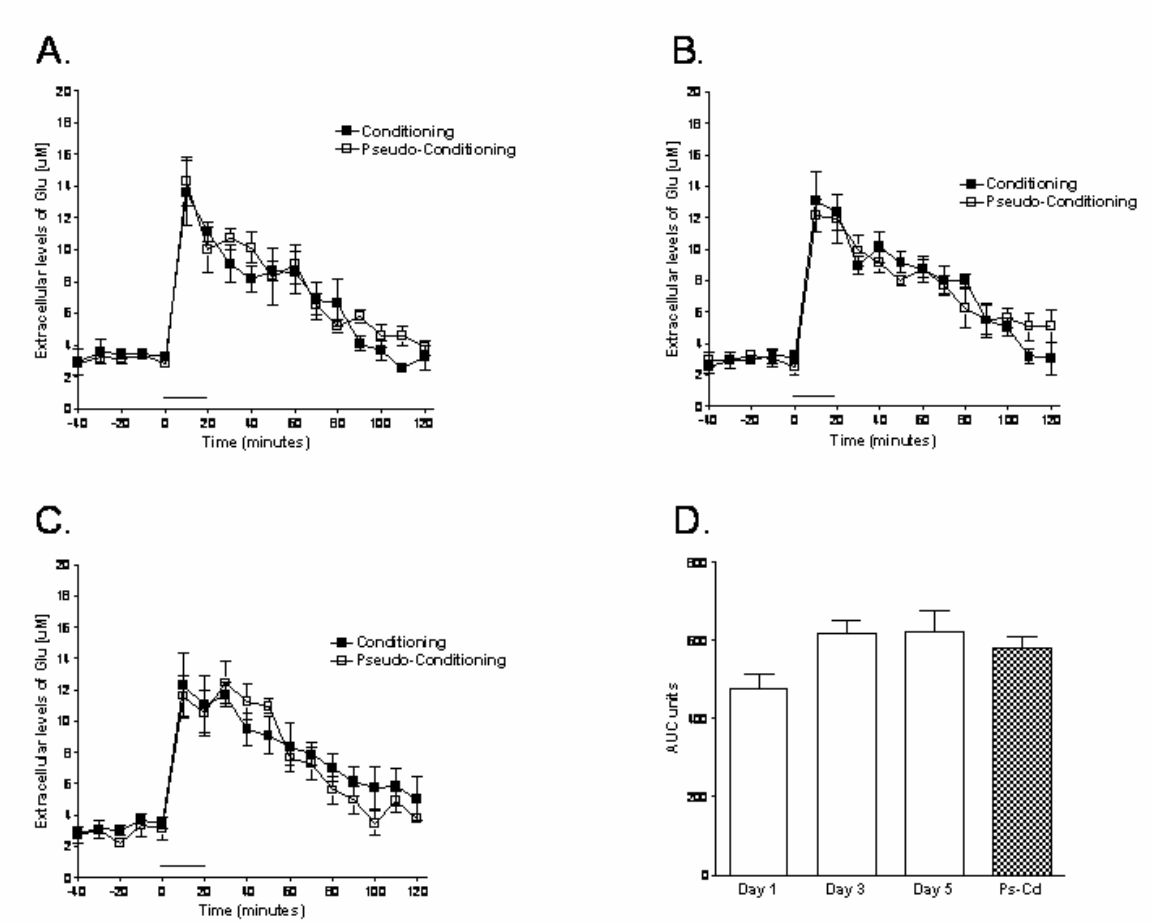


Figure 2.9. Temporal release of glutamate during eyelid conditioning in aged rats. Microdialysis was performed in the cerebellar cortex on aged rats during training in the delay eyelid conditioning task. The release of Glu over time during eyelid conditioning is shown on day 1 (A), day 3 (B) and day 5 (C). Comparable levels of glutamate are released during training on the eyeblink task for both the conditioning and the pseudoconditioning groups. Data are expressed as Glu [μM] (y-axis). Black squares represent conditioning, open squares represent pseudoconditioning. (D) Area under the curve representation of Glu release for each day of training during eyelid conditioning and pseudoconditioning (Ps-Cd, average of days). There were no differences in AUC of Glu.

2.5 Conclusions

The overall results show a significant role of the neurotransmitters NE, GABA and GLU occurring during the acquisition of the CR in the delay eyelid conditioning paradigm. The literature shows that these neurotransmitters participate in different ways and are essential for memory formation, consolidation and extinction (Attwell et al., 2002a; Yeo, 2004; De Zeeuw & Yeo, 2005; Weis et al., 2004; Farley & Alkon, 1985). To date, there is no direct evidence showing presynaptic release *in vivo* while the animals perform the learning task. In this study, the extracellular levels of neurotransmitters reported correspond to those captured by the active zone of the microdialysis probe from the lobus simplex and interpositus nucleus. We show the temporal pattern of release which occurs during training on the delay eyeblink conditioning task and the fact that there were no significant changes in NE and GABA release for the pseudo-conditioning groups shows the specificity of NE and GABA release related to the associative learning task and that it is not due to sensory stimulus activation by the CS and/or US, which was the case for GLU release. Our data also show that aged rats have a deficit in the neurotransmitters NE, GABA and Glu associated to the learning of the CR of eyeblink conditioning. This deficit seems to be directly associated with the impairments on the acquisition of CR's observed in aged rats.

2.5.1 Norepinephrine.

We demonstrate that extracellular levels of NE are increased in the cerebellum during training sessions of eyeblink conditioning from day 1 through day 5. On day 1, NE release remains increased over baseline for 60 minutes, whereas, as training progresses over days, the peak becomes smaller and NE remains elevated for a shorter time period so that by day 4 the NE signal returns to baseline within 30 minutes. In contrast, when pseudo-conditioning was examined there is no change in NE release during or after training on any day of training. This clearly indicates that the increase in NE which was observed during and after CS-US paired training is specifically linked to the combination of both the CS and US.

NE induces synaptic plasticity in purkinje neurons by selectively improving the signal to noise ratio of evoked versus spontaneous activity, enhancing the sensitivity of cerebellar neurons to both excitatory and inhibitory afferent inputs (Moises et al., 1979). The importance of NE during acquisition of motor learning tasks is supported by reports that 6-hydroxydopamine induced lesions of the LC disrupt cerebellar motor learning on a runway task (Watson & McElligott, 1983; Bickford, 1995) and classical eyeblink conditioning (Winsky & Harvey, 1992). Furthermore, blockade of postsynaptic β-adrenergic receptors with systemic administration of propranolol disrupts acquisition of the delay eyeblink conditioning in both the rabbit (Gould & Steinmetz, 1996) and rat (Cartford et al., 2002a). Both propranolol and Rp-cAMPS (activate cAMP-dependent PKA)

directly administered into the cerebellum impair acquisition of CRs (Cartford et al., 2004b). Interestingly, once the CR has been established, neither propranolol nor Rp-cAMPS block performance of acquired CRs. Overall, the amount of NE released decreases across days of training consistent with a role of NE early in acquisition and possibly in consolidation (Gilbert, 1974). This is consistent with work showing the firing of LC neurons demonstrates good discrimination within the first 500 trials of reversal of contingency in a visual discrimination task in monkeys (Kubiak et al., 1998) (monkeys are still showing a high number of errors), again suggesting that NE is important during the acquisition phase of learning of this task. Deficits in the NE re-uptake system of aged rats have been reported and could be associated to the delay in NE release observed and also with the long lasting overflow. We did not observe that NE returned to basal levels at least during the period of time that we collected the microdialysis samples. Our data agree with previous reported data showing deficits in the noradrenergic system in aged animals which been suggested to be directly related with the deficit in learning.

2.5.2. GABA.

As was observed with NE, GABA was released in a learning dependent manner and was specifically associated with paired conditioning and not pseudo-conditioning. As GABA is the predominant neurotransmitter in the cerebellum it was expected that if the cerebellum and interpositus nucleus are involved in the learned response, GABA release would be observed during the time

corresponding to the training period. Interestingly, during the first days of training, the increase above baseline in extracellular levels of GABA extends beyond the training period for over 60 minutes. This pattern changes across days of training where the time frame of GABA release shortens and at a time where the behavioral response is nearly maximal the GABA release is now primarily only observed during the behavioral testing.

Much of the work examining GABAergic transmission using either GABA agonists or antagonists has been directed at examining the functional role of the cerebellar cortex and cerebellar nuclei in regard to the memory formation and timing. Discrete infusions of GABA agonists and antagonists into the cerebellar cortex and interpositus nucleus support the hypothesis that both areas are involved in plasticity (Bao et al., 2002; Krupa & Thompson, 1997; Mamounas et al., 1987; Schreurs & Alkon, 1993). For example, the expression of CRs is mediated by LTD at the granule to purkinje synapses and by LTP at the mossy fiber synapses in the cerebellar nuclei (Mauk et al., 1998). GABAergic circuitry in the cerebellar cortex has also been postulated to play a role in post training memory consolidation (Cooke et al., 2004). Authors demonstrate that delayed infusions of muscimol into the cerebellar cortex at 5 or 45 minutes produced significant impairments of consolidation, suggesting that about 1 hour after training there is a period of cortical activity that is necessary for consolidation of the learned response. This time window is similar to that observed for GABA release (and NE release) observed in this report on the first few days of training.

GABA release likely reflects the activity of the cerebellar circuitry following training. During the later phase of training (days 4 and 5) there is a more discrete release of GABA which shows a higher peak while the timing is confined to the training session. At this time the behavioral response is well trained and the GABA release pattern likely reflects the concomitant activity of the cerebellar circuitry that is associated with the CR. In contrast, GABA release (in aged rats) was significantly delayed compared to the young rats and this delay might indicate that not just the noradrenergic system is impaired but also the activity of the PCs. This could be an indication of over activity of the incoming signaling carried out by the climbing and mossy fibers, however this does not rule out the possibility that other factors such as impairments in postsynaptic currents on the PC are contributing to produce this delay in GABA release.

2.5.3. Glutamate.

The GLU release measured in this report likely reflects the inputs to the cerebellar cortex and interpositus which is activated in both paired and unpaired conditions and explains why we observe Glu release in both conditions. A different scenario is observed for the NE and GABA as described above. Changes in the dynamics of NE release over time suggest that NE plays a major role in the first days of training. The change in timing of the GABA response correlates with the learned response in the rat and possibly reflects the activity of purkinje neurons and interpositus nucleus neurons that show entrained firing that develops over days of training. Glutamate has been shown to have significant

post-synaptic effects during training. For example, infusion of the NMDA antagonist AP5 disrupts eyeblink conditioning (Chen & Steinmetz, 2000). However, our results only reflect the pre-synaptic release of glutamate from afferents to the cerebellar cortex and support the idea that learning of the conditioned response does not take place before the signals reach the cerebellum as there is no change in afferent activity across days of training. It is interesting that Glu release happens in both, paired and unpaired trials of the conditioning sessions and the time period for the release is concomitant with the time period in which the sensory input to the cerebellum is happening. However in aged rats it is observed that even after the training finished extracellular Glu remains above the basal levels. This finding suggests a possible deficit in the Glu re-uptake system, which could be the cause of the prolonged release observed for NE and GABA once the training starts. Specifically, Glu levels in aged rats could be the result of over activity of microglia which would lead to a deficit in the Glu re-uptake system. This possibility still remains as one possible cause until more experiments are done to clarify the causes for this pattern of release of Glu in aged rats.

2.5.4. Effect of TTX on neurotransmitter release.

In a subset of young rats TTX was administered (through reverse dialysis) twenty minutes prior to training on the first day of training and administration continued for the duration of the microdialysis collection. TTX was administered to test the impulse dependence of neurotransmitter release with microdialysis. The behavior

of the rats was analyzed and there were no significant differences in learning between groups (figure 2.1). There was a decrease in amplitude of the unconditioned response in the TTX treatment group in two of the six rats, suggesting that there was a suppression of performance of the blink response for those two animals (data not shown). The microdialysates were analyzed for NE, GABA and Glu. The effect of TTX on NE release is observed in figure 2.5A-B, where TTX reduced the amount of NE in the microdialysate when compared with control demonstrating an impulse dependent nature of NE release. The effect of TTX on GABA release was also a reduction in the AUC for GABA (Figures 2.5C-D). A similar effect is observed with glutamate (Figures 2.5E-F).

TTX depleted NE, GABA and Glu basal levels, indicating tonic release for the measured neurotransmitters. Despite the depletion in basal extracellular levels of neurotransmitters, once the training sessions started each particular neurotransmitter was released with similar temporal patterns as the non-TTX groups. However the total amount of release was significantly lower compared to baseline levels of the control groups. It is interesting to see that there were not significant differences in the CR's levels reached on day one of training. This behavioral outcome was unexpected, however we should consider that the low concentration of TTX used might not have been enough to impair the sensory inputs carrying information about the US and CS. Even with the reduction in tonic release (due to the presence of TTX) of NE, GABA and Glu, it is also possible that the signal to noise ratio on the PC was sufficient to relay essential

information for memory formation as a greater percentage of CR's were reached on day two of training. The data show that TTX depleted baseline neurotransmitter levels and attenuated the neurochemical response to training. However, taking into consideration changes in evoked neurotransmitter levels relative to baseline levels it is apparent that neurotransmitter release for both TTX and non-TTX are similar. Therefore, despite the depletion on presynaptic activity through the cerebellar cortex and interpositus nuclei, if the signal to noise ratio remains the same throughout the training sessions, it might still be possible that memory encoding occurs. This would explain why in aged animals, which have attenuated levels of NE, GABA and Glu, if training persists the rats develop CR's. Therefore, it is not surprising that the TTX group show levels of CRs comparable to the non-TTX group. More experiments need to be conducted to clarify this explanation, and if it is shown to be true it will support the concept of NE signaling facilitating the signal to noise ratio of the PC.

2.5.5 Conclusions.

In conclusion, this study monitored the dynamics of noradrenergic, gabaergic and glutamatergic release as a consequence of delay classical eyeblink conditioning in the cerebellum. These data show that NE and GABA release vary over time and are specific to the paired training while Glu release is unaltered over days of training and is also observed with unpaired training. The timing of the NE response is consistent with previous studies demonstrating a role for NE to facilitate acquisition. The timing of the GABA response is consistent with a

period of about 1 hour post training during which the cerebellar circuitry is active which may reflect a period of consolidation. The fact that NE is also elevated after training is consistent with a possible role for NE in the consolidation process. Furthermore, the data show an alteration in the pattern of release for NE, GABA and Glu in aged rats, strongly suggesting that the deficit observed in the acquisition of the CR's in aged rats is due to a lost in the synchronization of neurochemical patterns during the acquisition of CR on the eyeblink conditioning. Also, we have to consider that the changes in the neurochemical pattern observed in the aged animals could be a consequence of processes underlying aging, such as oxidative stress for which the radical theory of aging have become more accepted (Orr & Sohal, 1994; Orr et al., 1992). A decline in the capacity of normal antioxidant defense mechanism has been postulated as a causative factor in aging related decline in the normal activity of different physiological systems (HARMAN, 1956a; Ames et al., 1993a). Damage to proteins, DNA and membranes has also been reported in association with aging (Ames et al., 1993b; Bickford, 1993; Davies & Goldberg, 1987; HARMAN, 1956b; Gutteridge & Stocks, 1976). Chronic inflammation leads to an over activation of microglia and high levels of pro-inflammatory cytokines and has been shown to deplete learning in the eyeblink conditioning task (Cartford et al., 2002b). The present report agrees with previous data which has demonstrated that deficits in noradrenergic signaling seen in aging are directly associated to the decline in learning capabilities (Bickford et al., 1999). More experiments are required to address the different aspects associated with cognitive deficits observed during

aging. We have shown with microdialysis that extracellular levels of NE increase at the beginning of training and remain elevated above basal levels for up to 80 minutes after the training session has finished. If we consider the release of NE directly into the cerebellar cortex during the critical time for consolidation, it is reasonable to suggest that NE might also have a critical role providing a source of energy when it is required for encoding memory in the cerebellum. Therefore, we propose that in addition to improving the signal to noise ratio, NE also activates glycogenolysis, a source of energy, while the consolidation of the new memory is taking place. It will be necessary to do specific experiments to elucidate in more details how NE acts on astrocytes to provide the energy necessary for the novo protein synthesis as well as calcium mobilization within the cells which must be also critical during the consolidation of the memory process. The findings from these experiments could likely help to understand an important aspect of aged-related memory deficits in which there is a deficit in NE signaling.

CHAPTER 3

Beta-Noradrenergic Receptors in the Cerebellum Are Involved in Acquisition of Delay Classical Conditioning in Rats: Timing of Disruption

3.1 Abstract

The delay classical conditioning task in rats is a paradigm that depends primarily on the cerebellum. Several neuronal pathways are involved in the memory formation process for this task, including the noradrenergic projections from the locus coeruleus to the cerebellum. Previous evidence from our lab has shown that blocking beta-noradrenergic receptors with propranolol prior to training sessions significantly impairs acquisition in the eye blink task in rats. This was observed with both intraperitoneal injections and infusion directly into the cerebellar interpositus and lobule HVI. In a recent microdialysis study we have shown that there is a significant elevation in the extracellular levels of norepinephrine (NE) which occurs during the training sessions. Interestingly, the increased overflow of NE remains elevated for about 60 minutes after the training session has finished on day one, whereas this long lasting pattern of NE release decreases over days of training. Based upon these results we hypothesized that NE overflow that outlasts the training session might be involved in the consolidation of the learned behavior. In order to test this hypothesis, 1 uL of propranolol (100 uM) or vehicle (aCSF) was ipsilaterally infused into the cerebellar lobule HVI 5, 60 or 120 minutes after each training session. The rats which received a local infusion of propranolol showed impaired learning

compared to the control group (aCSF). In addition, activation of cAMP-dependent PKA was blocked with the local administration of Rp-cAMPS assessed at the same time points (5, 60 and 120 minutes post training). The results show a significant decrease in the acquisition of CR's. Indicating that both, β-adrenergic receptors and PKA signaling are essential during memory consolidation of delay eyeblink conditioning. These data also show that NE overflow, which remains elevated after the training session may be playing an important role in the process of memory consolidation by prolonging β-adrenergic activation beyond the training session period.

3.2 Introduction

Delay eyelid conditioning is a cerebellar learning task in which norepinephrine (NE) is known to play a modulatory role. NE induces synaptic plasticity in Purkinje neurons by selectively improving the signal to noise ratio of evoked versus spontaneous activity, enhancing the sensitivity of cerebellar neurons to both excitatory and inhibitory afferent inputs (Freedman et al., 1977; Moises et al., 1980). Based upon the Marr-Albus theories of cerebellar learning Gilbert proposed that NE should have a role in the consolidation of memory within the cerebellum (Gilbert, 1975). The involvement of NE in memory consolidation in the cerebellum is observed in several cerebellar dependent paradigms. For example, in a rod running motor learning task the ability of rats to improve performance is reduced following lesion of the locus coeruleus (LC) (Watson & McElligott, 1983). This effect is localized to cerebellar NE (Watson & McElligott,

1984) and is specific to the β-adrenergic receptor (Heron et al., 1996). Adaptation of the vestibulo ocular reflex (VOR) is also modulated by noradrenergic inputs (McElligott & Freedman, 1988) and appears to be mediated by the β-noradrenergic receptor (Pompeiano et al., 1991). Cerebellar delay classical conditioning has also been shown to be modulated by NE. Electrolytic lesions of the LC induce resistance to extinction in delay conditioning in rabbits (McCormick & Thompson, 1982) and 6-hyroxydopamine (6-OHDA) has been shown to retard acquisition but not performance of conditioned eyelid responses in rabbits (Winsky and Harvey 1992). This effect is also mediated by the β-adrenergic receptor as propranolol (administered either i.p. or locally into the cerebellum) retards the acquisition of learned responses in a delay conditioning task in rats (Cartford et al., 2002a; Cartford et al., 2004b). NE is also implicated in non-cerebellar dependent tasks. LC neurons fire prior to a target cue in a vigilance task in monkeys (Aston-Jones et al., 1994) and during reversal of task contingency, the LC response to the new stimuli precedes behavioral responding (Aston-Jones et al., 1997). The activation of adenylyl cyclase-cAMP-protein kinase A (PKA) intracellular signaling cascade has been shown to be essential for long-term memory consolidation in diverse brain areas including the hippocampal formation and prefrontal cortex (Squire & Zola-Morgan, 1991; Goldman-Rakic, 1987; Arnsten & Goldman-Rakic, 1987). However, the role of PKA cascade in the consolidation process is still under debate. In this study we manipulated the activity of the β-adrenergic receptors and PKA activity by administering microinjections of propranolol and Rp-cAMP directly into the

cerebellar cortex at specific post training periods within the time period that NE overflow remains elevated. The time period post training (in which we see elevated levels of NE) is though to be critical for memory consolidation (De Zeeuw & Yeo, 2005; Yeo, 2004; Krupa et al., 1993a; Bao et al., 2002; Nolan et al., 2002; Rogers et al., 2001c; Gould & Steinmetz, 1996)

Other neurotransmitter systems are also implicated in delay eyeblink conditioning tasks. Administration of the glutamate AMPA receptor antagonist CNQX or the GABA-A receptor antagonist picrotoxin into the cerebellar cortex completely and reversibly impairs fully established CR's, suggesting that GABAergic and glutamatergic transmission are equal, and essential for the CR performance (Attwell et al., 2002b). It has been suggested that the basal GABAergic output from the cortex onto the interpositus nucleus modulates conditioned response (CR) expression, whereas timing of CR's is modulated by the stimulus activated inhibition (Bao et al., 2002). The expression of CR's can be interrupted by both GABA mediated inactivation of the interpositus neurons with muscimol (GABA-A agonist) as well as up-regulation of activity with picrotoxin (GABA-A antagonist) (Aksenov et al., 2004).

To date much of the work examining the role of neurotransmitters in delay eyeblink conditioning have used either the application of agonists or antagonists to investigate the postsynaptic effects of these neurotransmitters. We have also recently demonstrated that NE is released in the cerebellum during training on

the delay eyeblink task and that the level of NE remains above background for up to 80 minutes post training. An important question that remains unanswered concerns the timing in which presynaptic release of neurotransmitters in the delay conditioning task are critical for consolidation of the new learned experience. This study uses delay eyeblink conditioning to examine critical post-synaptic events relating to the NE signal transduction cascade at different time points post training. It is hypothesized that the release of NE in the cerebellum during and post training activates β-adrenergic receptors and the subsequent PKA signal transduction cascade, which is critical for the consolidation of cerebellar motor learning.

3.3 Methods

3.3.1 Animals and surgery

Male F344 rats weighing 270–320 g were used in this study. Room temperature was kept at 72 °F and the dark/light circle was 12-h (light was on from 7:00 AM to 7:00 PM). Animal number was the minimum required for reliable statistical test results. Rats were anesthetized with Isoflurane and placed in a stereotaxic instrument. A 10 mm long guide shaft made of 21-gauge stainless-steel tubing was inserted into the cerebellum. The guide shaft was attached to the skull by jeweler screws and cemented with dental acrylic. The coordinates AP-11.4, ML +2.4 and DV -1.7 in reference to bregma was used to implant the guide cannulae for the microinjection into the cerebellar lobule HVI (simplex, and interpositus nucleus). In the same surgical session rats were prepared for eyelid training by

fixing a small ITT/Cannon connector strip to their skull to hold gold pin connectors to EMG wires that are run under the left eyelid. This method has been previously published by our lab (Cartford et al., 2002a). Rats are allowed to recover for one week after the surgery procedure before start the eyeblink conditioning training and microdialysis began. Each animal was used for only one experimental condition. All procedures were carried out in accordance with the institutional guidelines (IACUC) and with USA National Institute of Health Guide for the Care and Use of Laboratory Animals.

3.3.2 Training

The rats were habituated to the training chamber and headstage cable for three days. The training consisted of 50 trials each training trial consisted of a 250 ms baseline, a 400 ms CS period, and a 100 ms US period. The tone was 500 ms in duration and overlapped the airpuff for 100 ms. The training tone was 3kHz and the airpuff 10 psi. Hardware and software used to train and analyze data were manufactured by J.Tracy, J.Green and Joe Steinmetz, (Bloomington, Indiana). Eyelid EMG data was collected, amplified, rectified, and integrated. Learned responses were determined using a 10 standard deviation criterion for eyelid amplitude elevated during the CS period when compared to the baseline. Alpha responses to the tone are excluded from learned response analysis by using a 70 ms discrimination/exclusion window. Learning was measured as the percentage of learned (conditioned) responses (CR's) made in each training session.

On every experimental session 1 µL of propranolol (100 µM) or Rp-cAMP (80 nM) was administered over five minutes through the guide cannula with an infusion pump at intervals of 5, 60 or 120 minutes after the training. The control group received artificial cerebral spinal fluid at pH: 7.4.

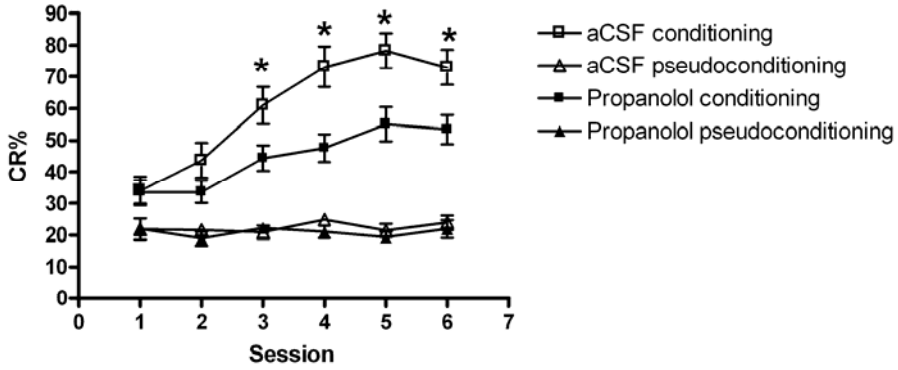
3.3.3 Design and Analysis

To analyze behavior for the eyeblink conditioning task, separate mixed model analyses of variance were used to analyze Drug and Session effects at each of the drug administration time points. Data from drug administration 5 minutes after training was analyzed using a three-way mixed model analyses of variance. For the analyses of the other time points 60 and 120 minutes data were analyzed using a two-way mixed model analyses of variance {[Drug (2): (Propanolol, aCSF)] x [Session (6): Day 1-3 AM and PM]}. Data were analyzed using a three-way mixed model analyses of variance {[Drug (2): (aCSF, Rp-cAMP)] x [Time of drug delivery (3): (5, 60, and 120 minutes)] x [Session (6): 1, 2, 3, 4, 5, 6]}. Post Hoc Analyses (Dunnett's) were used to test for Drug and Time effects. Comparisons were determined significant at the 0.05 alpha level. Percent Conditioned Response (CR %) and Amplitude of response (Conditioned and unconditioned) were the dependent measures.

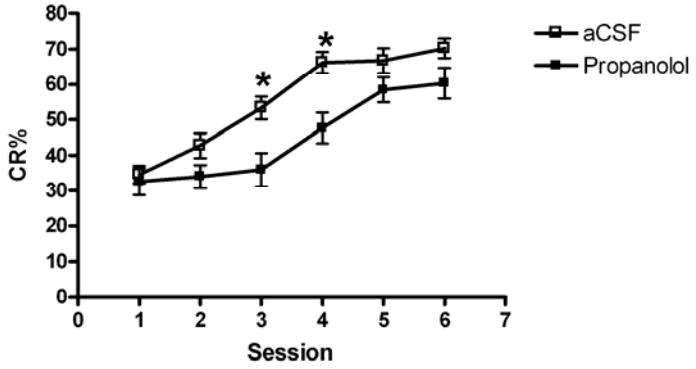
3.4 Results

Propranolol when administered 5, 60 or 120 minutes following training significantly disrupts learning of CR's. Figure 3.1A illustrates the attenuation of learning (shown as % CR) when propranolol is administered 5 minutes post training {significant drug x session interaction [$F(5,80) = 2.72$, $p<0.05$]}. The effect of propranolol is most pronounced at 5 minutes post training with rats learning significantly less than aCSF on sessions 3, 4, 5 and 6. A similar pattern is observed in rats treated with propranolol 60 minutes post training, however the magnitude of the difference is reduced. As can been seen in Figure 3.1B administration of propranolol 60 minutes after eyeblink conditioning reduced learning in sessions 3 and 4 {significant drug x session interaction [$F(5,40) = 2.58$, $p<0.05$]}. However, when propranolol was administered 120 minutes post training there was no difference with aCSF in learning acquisition interaction (see figure 1C) [$F(7,35) = 0.81$, $p = 0.812$]. These data show robust learning in aCSF treated rats and partial learning in propranolol treated rats when administered 5 or 60 minutes post training. The effect of pseudoconditioning on CR's did not differ over sessions or as a result of drug administration.

A.


CR%
Session
aCSF conditioning
aCSF pseudoconditioning
Propanolol conditioning
Propanolol pseudoconditioning

B.


CR%
Session
aCSF
Propanolol

C.

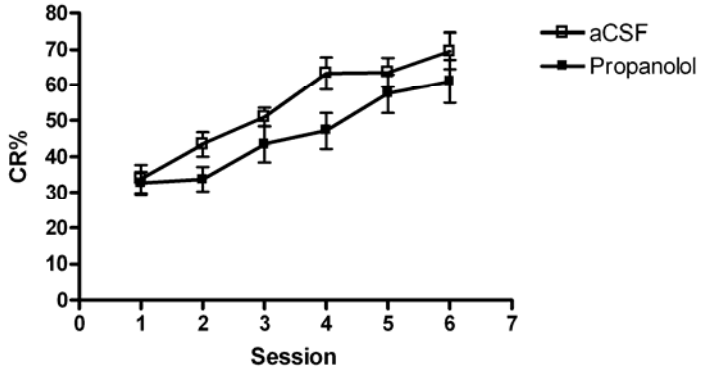

CR%
Session
aCSF
Propanolol

Figure 3.1. Propranolol administered 5, 60 or 120 minutes following training significantly disrupts learning of CR's. The X-axis shows training sessions over time, with session 1 being Day 1 AM, 2 Day 1 PM, 3 Day 2 AM, 4 Day 2 PM, 5 Day 3 AM, and 6 Day 3 PM. A) illustrates the attenuation of learning (shown as % CR) when propanolol is administered 5 minutes post training, with rats learning significantly less than aCSF on sessions 3, 4, 5 and 6 (indicated by *). Pseudoconditioning did not differ over sessions or as a result of drug administration. B) Propanolol administration 60 minutes after eyeblink conditioning reduced CRs relative to aCSF in sessions 3 and 4 (indicated by *). C) When propanolol was administered 120 minutes post training there was no difference with aCSF in learning acquisition. These data show robust learning in aCSF treated rats and partial learning in propranolol treated rats when administered 5 or 60 minutes post training.

Figure 3.2 shows the amplitude of the conditioned response (A, C, E) and the unconditioned response (B, D, F). Analyses of amplitude of both CR and UR for drug treatments 5, 60 and 120 minutes post training failed to detect any significant difference between aCSF and propranolol. The timing of the CR was also not affected by drug treatment. Neither CR onset or CR peak amplitude times were significantly different in any of the drug treatment groups (data not shown).

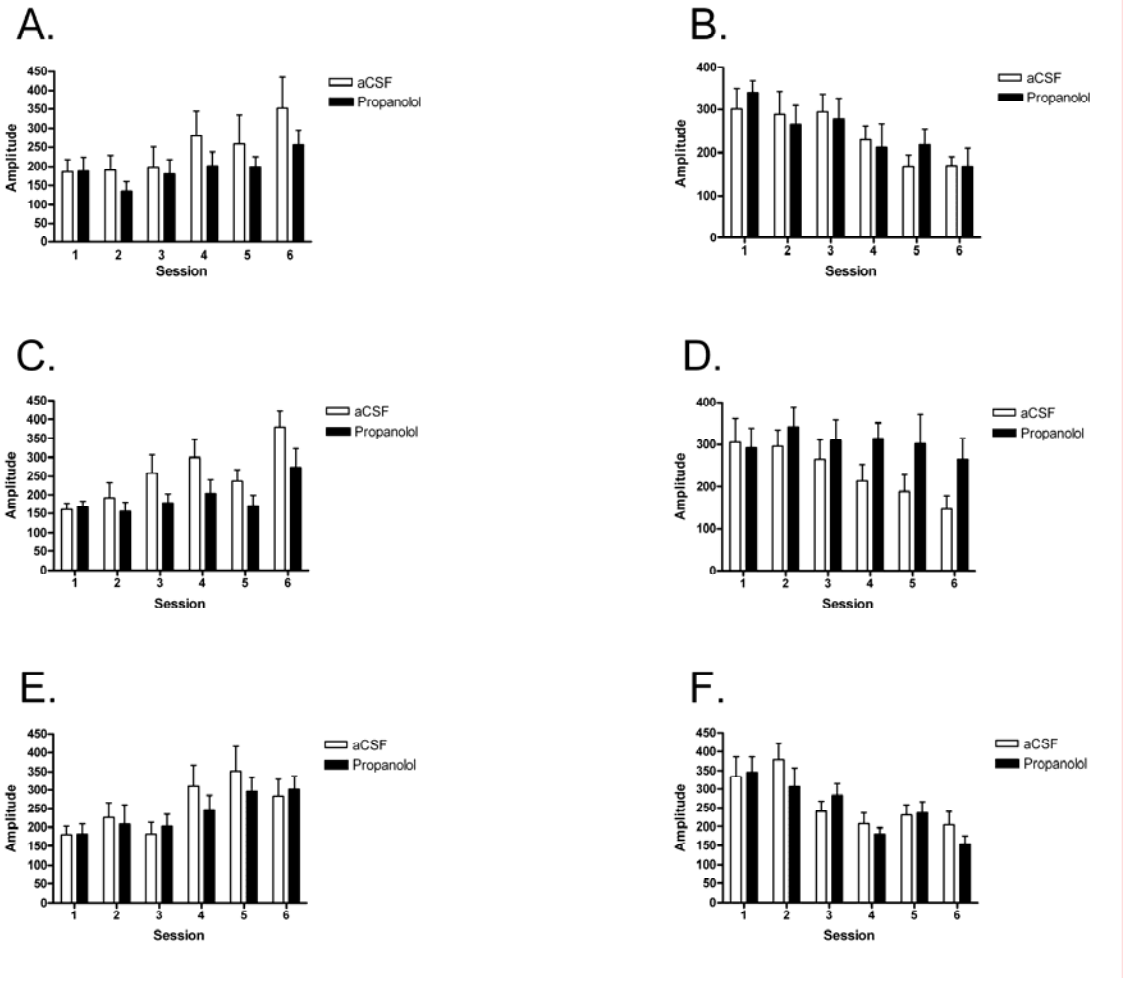


Figure 3.2. The amplitude of the conditioned response (A,C,E) and the unconditioned response (B, D, F) during eyeblink conditioning. The X-axis shows training sessions over time, with session 1 being Day 1 AM, 2 Day 1 PM, 3 Day 2 AM, 4 Day 2 PM, 5 Day 3 AM, and 6 Day 3 PM. Analyses of amplitude of both CR and UR for drug treatments 5, 60 and 120 minutes post training failed to detect any significant difference between aCSF and propanolol. A) CR 5 minutes post training. B) UR 5 minutes post training. C) CR 60 minutes post training. D) UR 60 minutes post training. E) CR 120 minutes post training. F) UR 120 minutes post training.

Rp-cAMP when administered 5, 60 or 120 minutes following training significantly disrupts learning of CR's. Animals which received infusions of ACSF improved from session 1 (mean = 29%) to session 6 (mean = 71%), demonstrating

learning (shown as % CR) over time, independent of time of drug delivery. Figure 3.3 illustrates the attenuation of learning when Rp-cAMP was administered 5, 60 or 120 minutes post training {significant drug x session x time of drug interaction [$F(10,120) = 2.20$, $p<0.05$]}. The effect of Rp-cAMP was most pronounced when administered 5 or 120 minutes post training with rats learning significantly less than aCSF on sessions 3, 4, 5 and 6. A similar pattern was observed in rats treated with Rp-cAMP 60 minutes post training, however the magnitude of the difference was reduced. These data show robust learning in aCSF treated rats and partial learning in Rp-cAMP treated rats when administered after training.

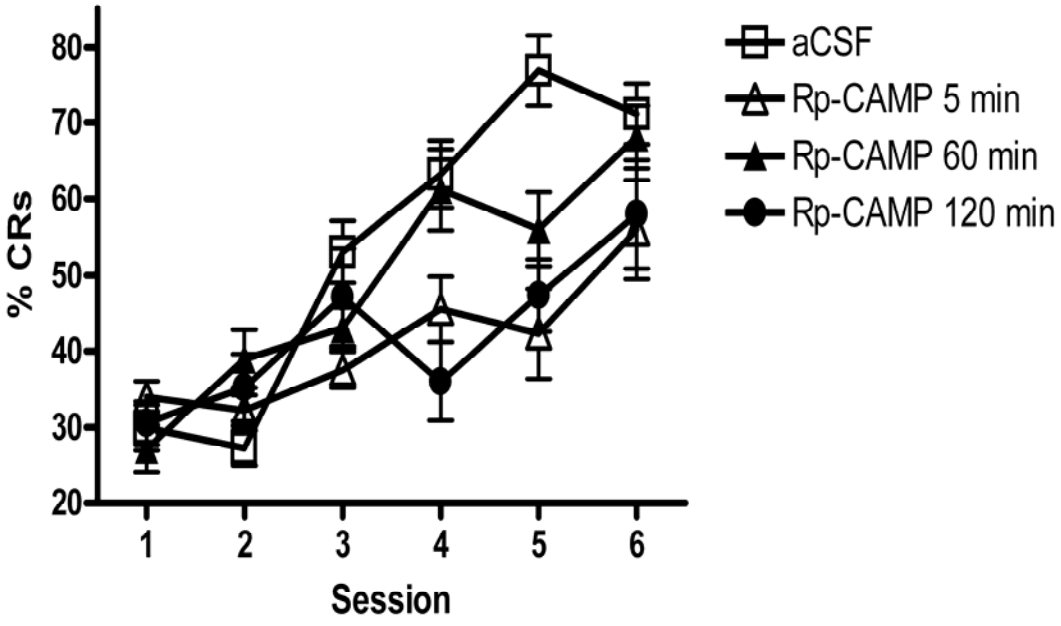


Figure 3.3. The Effect of Rp-cAMP on Eye Blink Conditioning. Rp-cAMP or aCSF was administered via local infusion into the interpositus nucleus of the cerebellum 5, 60 or 120 minutes post training on the eye blink conditioning task. Rats which received aCSF (open square) significantly improved learning over

sessions (p<0.05). Partial learning in Rp-cAMP treated rats was observed when administered 5, 60 or 120 minutes after training (p<0.05). Y axis shows percent conditioned responses (%CR). n=5 per condition.

3.5 Conclusions

For several decades there have been investigations into the role of the cerebellum in the process of memory consolidation in the classical eyeblink conditioning task (Thompson et al., 1997). Despite the findings which have shown that the cerebellar cortex, cerebellar nuclei and inferior olive are all required in the processing of conditioned learning, less work has examined how noradrenergic inputs from the LC contribute to such processes. What is clear to date is that the noradrenergic inputs to the cerebellum from the LC excerpt both stimulatory and inhibitory effects when NE binds to receptors in the cerebellar cortex and the literature also supports a role of NE in arousal, emotion and learning (Cooke et al., 2004). The alpha and beta adrenoceptor have been found to be present in PC of the cerebellum (Rusakov et al., 2005), in which NE selectively inhibits the spontaneous activity of PC at the same time climbing fibers evoked activity remains either unaffected or augmented with NE action, thus increasing the signal to noise ratio of the evoked activity onto the PC (Luft et al., 2004). A vast body of data supports that aged-related decreases in catecholamines may contribute to impairments in learning acquisition on motor learning tasks (Luft & Buitrago, 2005; O'Dowd et al., 1994). It is well known that NE has profound effects in the cerebellum while a significant sensory input is

activated such as CS and US (through climbing and mossy fibers) during eyeblink conditioning which has led to a great interest in dissecting out the precise or discrete areas of the cerebellum in which memory formation takes place.

In this study we showed how the disruption of beta-adrenergic signaling in the cerebellar cortex diminished learning of conditioned responses (CR's) when propranolol was locally administered into the cerebellar cortex at 5 and 60 minutes post-training. The data show that noradrenergic signaling inputs to the cerebellar cortex are necessary for the consolidation process by which CR's are formed. Previous studies from our lab have shown that local administration of propranolol into the cerebellum prior to the training sessions disrupts the normal rate of learning of CR's (Cartford et al., 2002a). However in this study we showed that there is still significant activity of NE receptors even after the training session has finished indicating the possibility that noradrenergic activity is part of the overall process of memory consolidation. Our findings concur with previous data indicating that the depletion of NE signaling in the cerebellum of aged animals might be the primary cause of the learning deficit but not of the performance of CR's.

The local administration of propranolol at 5 and 60 minutes post training had the largest impact depleting the CR's acquisition while propranolol administered 120 min post training session did not cause a significant impairment in the acquisition

of CR's. It is important to note that when propranolol was administered 5 and 60 minutes post-training despite the depletion of CR's the rats still learned (see figures 3.1 and 3.2), indicating that even when the beta-noradrenergic signaling is blocked there must be other neurotransmitters or factors driving the consolidation of memory. It also has been shown that the blockade of non-NMDA receptors by CNQX infusion into the cerebellar cortex is also capable of disrupting learning (Attwell et al., 2001). In addition, the activation of GABA-A receptors in the cerebellar nuclei using muscimol can disrupt the acquisition of CR's (Hardiman et al., 1996; Krupa et al., 1993c; Ramnani & Yeo, 1996; Yeo et al., 1997). Most recently, Cooke and colleagues published an experiment where they demonstrated that cortical infusions of the GABA-A receptor agonist muscimol delayed by 5 or 45 minutes after a conditioning session disrupted learning, suggesting a role of the cerebellar cortex in consolidation (Cooke et al., 2004). Our data concur with this report providing evidence that there is critical post-training memory consolidation period which is mediated by the cerebellar cortex. Together these finding suggest that in addition to various neurotransmitters playing a role in the acquisition of CR's there are critical temporal effects which have been elucidated for GABA and NE which are critical for the acquisition of CR's.

The data presented in this study show that when beta-adrenergic receptors are blocked in the cerebellar cortex 5 and 60 minutes post-training, the acquisition of CR's are impaired, however, the rats still learn the task. Propranolol may

interfere with the consolidation process by the following mechanisms: First, it is known that NE is a neuromodulator in that it selectively improves the signal to noise ratio of evoked versus spontaneous activity which enhances the sensitivity of the cerebellar neurons to excitatory and inhibitory afferent inputs (Eccles, 1967; Hoffer et al., 1973). It is possible that the inhibition of the beta-noradrenergic signaling makes it difficult to enhance the required sensitivity of the cerebellar neurons to the sensory input carrying the US and CS signal through the climbing fibers and mossy fibers. In other words, the lack of the noradrenergic signal (elicited by propranolol administration) impairs the improvement of the signal to noise ratio of evoked versus spontaneous activity which may explain why the rats would require more training sessions in order to reach a significant conditioning response. Even when the rats received only six conditioning training sessions, it is plausible to predict that after a few more training sessions the rats could show better CR's. In figure 3.1 there was a significant difference in the groups that received aCSF and propranolol which started in session 3, however a trend towards learning remained for the rest of the sessions. When propranolol was administered 60 minutes post-training, a significant impairment was observed (compared to aCSF – see figure 3.1) on sessions 3 and 4 but sessions 5 and 6 did not show significant differences. These results demonstrate that when propranolol is administered into the cerebellar cortex, the rats might require more training sessions in order to develop similar levels of CR's as observed in the control group.

Another explanation for the depletion of CR's after blocking the beta-adrenergic receptor at different time points post-training could be associated with the activity that NE exerts by activating the beta-adrenergic receptors which leads to the activation of cAMP/PKA signaling cascade. Activation of the cAMP/PKA signaling pathway has been shown to enhance intracellular calcium and promote the release of vesicles containing GABA (Saitow et al., 2005). Furthermore, increases in intracellular calcium in GABAergic synapses are also modulated by AMPA receptors (Rusakov et al., 2005; Satake et al., 2004). Interestingly, Luft and colleagues demonstrated that motor skill learning depends on the novo synthesis of proteins in motor cortex after training. Based on the authors work they hypothesize that consolidation (of motor learning) requires modifications in neuronal circuitry or plastic changes in neuronal structure and established that the disruption of protein synthesis in inter trials can diminish consolidation (Luft et al., 2004; Luft & Buitrago, 2005).

In this case, protein synthesis may therefore require a pool of energy in the form of ATP in order to assemble and relocate the new synthesized proteins. Thus, it is essential that a reliable source of energy during the critical period of protein synthesis is available for intracellular signaling to occur and induce synaptic plastic changes. In this regard, NE has been shown to enhance glycogenolysis through astrocytes therefore providing the energy necessary to undergo memory consolidation (O'Dowd et al., 1994). It also has been shown that beta-adrenoceptors are highly localized in astrocytes (Stone & Ariano, 1989b; Aoki &

Pickel, 1992; McCarthy, 1983). It has been suggested that the noradrenergic action on glial cells might be the source of production and release of glucose for neural energy production (Stone & Ariano, 1989a). We have shown that extracellular levels of NE increased at the beginning of training and remained elevated above basal levels for up to 80 minutes after the training session (see chapter 2). If we consider the release of NE directly into the cerebellar cortex during the critical time for consolidation, it is reasonable to suggest that NE might also have a critical role providing a source of energy when it is required for encoding memory in the cerebellum. Therefore, we propose that in addition to improving the signal to noise ratio, NE also activates glycogenolysis, a source of energy, while the consolidation of the new memory is taking place. NE also activates glycogenolysis, a source of energy, while the consolidation of the new memory is taking place. NE exerts its facilitatory effect through β-adrenergic receptors and the activation of cAMP/PKA signaling cascade. Our results clearly demonstrate that when cAMP/PKA is blocked at different time points post training, the acquisition of CR is depleted indicating that the cAMP/PKA signaling cascade is an essential pathway in memory consolidation of eyeblink conditioning. It is important to consider that both PKC and PKA mediate histamine receptors which are dependent on their respective downstream pathways, Ca^{2+}-PKC and cAMP-PKA (Sakhalkar et al., 2005a). Rp-cAMP has been used as a tyrosine kinase inhibitor to study the downstream signaling cascade of histamine receptors (Sakhalkar et al., 2005b). In addition, histamine receptors have been shown to play a role in performance on both the rota-rod

and balance beam tasks through Histamine-2 receptors in the cerebellar interpositus nucleus. Therefore, it is possible that they are also involved in memory consolidation of eyeblink conditioning (Song et al., 2006). Futhermore, functional regulation of Ca^{2+} channels relies on phosphorylation processes. Protein kinase A and C and the Rp-cAMP could have affected Ca^{2+} channels that depend of the activity of PKA (Christ et al., 2004). It is necessary in the future to conduct experiments that utilize more specific antagonists to target the β-noradrenergic signaling cascade.

It will be necessary to do specific experiments to elucidate in more details how NE acts on astrocytes to provide the energy necessary for the novo protein synthesis as well as calcium mobilization within the cells which must be also critical during the consolidation of memory process. The findings from the proposed experiments could likely help to understand an important aspect of aged-related memory deficits in which there is a deficit in NE signaling.

CHAPTER 4

TNF-α inactivation improves learning in aged rats whereas the activation of TNF-α in young rats depletes learning

4.1 Abstract

Tumor necrosis factor alpha (TNF-α) is a multifunctional proinflammatory cytokine, which is a critical inflammatory mediator involved in aging and neurodegenerative diseases of aging. Previous work has shown that diets enriched with antioxidants reduce levels of the proinflammatory cytokine TNF-α in the central nervous system of aged rats. These diets have also been shown to improve classical eyeblink conditioning performance in aged rats. Therefore we tested the hypothesis that inflammation and more specifically TNF-α may be a critical factor that modulates classical conditioning behavior. If TNF-α increased cerebellar levels negatively affect performance on the eyeblink conditioning task, then exogenous administration of TNF-α in young rats should result in an impairment in the acquisition and/or retention of eyeblink conditioning memory. On the other hand, the reduction of the age-related increase in cerebellar TNF-α levels in aged rats should result in an improvement in memory. To address this question in experiment one young (3 month old) F344 rats were pretreated (via infusions into the cerebellar cortex lobus simplex) with 2 μL of recombinant rat (rr)TNF-α (50 ng) one day prior to training and then daily 3 h prior to eyeblink conditioning coupled to microdialysis for 5 consecutive training sessions with one session per day. The control group received the same dose of denatured rrTNF-α

(heated at 90 ° C for 15 minutes). The results showed that young rats treated with rrTNF-α have a decreased rate of learning compared to the control group. The neurochemical profile of neurotransmitter release measured with microdialysis on day 1 of training from young rats resembled that observed in aged rats. A significant sustained release of norepinephrine was observed after the training session of eyeblink conditioning. In a second experiment aged (22 month old) F344 rats were pretreated with intracerebellar microinjection a 2 uL of anti-rat TNF-α 0.3 pg/ml three times a week for 4 weeks prior to eyeblink conditioning training with microdialysis. Aged rats showed a better performance in the conditioned responses when compared to controls. The release of NE in this group reached basal levels sooner than the control group but not as early as the young rats. The results of these experiments demonstrate a critical correlation between TNF-α and the rate of conditioned learning acquisition.

4.2 Introduction

NE is released from the LC to the cerebellar cortex (among other brain areas) (Foote et al., 1983; Aston-Jones et al., 1986) and exerts a modulatory effect on the action of other neurotransmitters in the cortex and deep nuclei of the cerebellum (Gould et al., 1997a). This leads to amplification of the afferent inputs to the cerebellar purkinje cells (PC) and is thought to occur through the action on β-noradrenergic receptors (Yeh & Woodward, 1983a; Woodward et al., 1991b). Motor learning has been widely associated with noradrenergic innervation to the cerebellar cortex in rats (Bickford et al., 1992; Heron et al.,

1996; Cartford et al., 2004b; Bickford et al., 1986; Watson & McElligott, 1983; Bickford, 1995; Pompeiano et al., 1991; McCormick & Thompson, 1982). Delay classical conditioning is a well established cerebellar-dependent learning paradigm and is modulated in part by NE (McCormick & Thompson, 1982; Harvey et al., 1993). When the β-adrenergic receptor antagonist, propranolol is administered systemic or locally (cerebellum), the acquisition of learned response on the delay classical conditioning in rats is impaired (Cartford et al., 2002a; Cartford et al., 2004b). Furthermore, the administration of 6-hydroxydopamine depletes NE storage and prevents animals from regaining proficiency on a motor learning task (Watson & McElligott, 1983; Watson & McElligott, 1984). Aging-associated deficits on motor learning have been linked to dysfunction of the noradrenergic system (Cartford et al., 2004a; Bickford et al., 1985b; Bickford et al., 1986) which is thought to be caused by the loss of noradrenergic enhancement of the relative responsiveness of purkinje neurons to afferent inputs in aged animals.

Age-related pathologies are characterized by a pronounced imbalance in immune functions like glial hyperactivity with altered antigen expression of microglia in aged rodents (Perry et al., 2003b; Cunningham et al., 2007a). Chronic inflammation is known as one of the multiple age-related pathologies that involves the activity of several products, including cytokines (Murray et al., 1997a; Murray & Lynch, 1998b). Cytokines are proteins that mediate the response of the body's defense system to injury and mediate diverse

inflammatory processes. The presence of altered levels of cytokines in the central nervous system has been implicated in several aged-related and neurodegenerative diseases (Benveniste & Benos, 1995). Cytokines are secreted by activated microglia and can be either pro-inflammatory cytokines, among them tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL1-β), and anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor beta 1 (Uccelli et al., 2005a). Pro-inflammatory cytokines are chronically increased in the aging brain (Godbout et al., 2005a). TNF-α and TNF-β are significantly elevated in the cerebellum of aged rats (Gemma et al., 2002) and diet rich in anti-oxidant reduced both TNF-α and TNF-β levels. In addition, feeding aged F344 rats a diet enriched in spinach improves cerebellar β-adrenergic receptor function and improves motor learning that was associated with a decrease in oxidized gluthathione and the pro-inflammatory cytokine TNF-α (Cartford et al., 2002b).

The present study extended on previous findings to determine whether inflammation and more specifically TNF-α may be a critical factor that modulates classical conditioning behavior during the aging process. In this experiment TNFα was administered to young rats and blocked in aged rats young rats prior to eyeblink conditioning coupled microdialysis. The results of these experiments demonstrate a critical correlation between TNF-α, aging and learning.

4.3 Materials and Methods

4.3.1 Animals and surgery

Male F344 rats weighing 3 and 20 months old were used in this study. Room temperature was kept at 72 °F and the dark/light circle was 12-h (light was on from 7:00 AM to 7:00 PM). Animal number was the minimum required for reliable statistical test results. Rats were anesthetized with isoflurane and placed in a stereotaxic instrument. A double 10 mm long guide shaft made of 21 and 26 gauge stainless-steel tubing (separated by 1mm) were inserted into the cerebellum. The guide shaft was attached to the skull by jeweler screws and cemented with dental acrylic. The coordinates AP-11.4, ML +2.4 and DV -1.7 in reference to bregma was used to implant the guide cannulae for the microinjection into the cerebellar lobule HVI (simplex, and interpositus nucleus). In the same surgical session rats were prepared for eyelid training by fixing a small ITT/Cannon connector strip to their skull to hold gold pin connectors to EMG wires that are run under the left eyelid. This method has been previously published by our lab (Cartford et al. 2002). Rats are allowed to recover for one week after the surgery procedure before start the eyeblink conditioning training and microdialysis began. Each animal was used for only one experimental condition. All procedures were carried out in accordance with the institutional guidelines (IACUC) and with USA National Institute of Health Guide for the Care and Use of Laboratory Animals.

4.3.2. Treatment with rrTNF-α and anti-rat TNF-α

On completion of surgery young and aged rats were randomly assigned to different treatment groups. Young (3 month old) F344 rats were pretreated (via infusions into the cerebellar cortex lobus simplex) with 2 uL (50ng) of rrTNF-α one day prior to training and then daily 3 h prior to eyeblink conditioning coupled to microdialysis for 5 consecutive training sessions with one session per day. The control group received the same dose of denatured rrTNF-α (heated at 90 ° C for 15 minutes). The results show that young rats treated with rrTNF-α have a decreased rate of learning compared to the control group. The neurochemical profile of neurotransmitter release measured with microdialysis on day 1 of training from young rats resembles that observed in aged rats. A significant sustained release of norepinephrine is observed after the training session of eyeblink conditioning. In a second experiment aged (22 month old) F344 rats were pretreated with intracerebellar microinjection a 2 uL of anti-rat TNF-α 0.3 pg/ml three times a week for 4 weeks prior to eyeblink conditioning training with microdialysis.

4.3.3 Training of behavior in a delay classical eyeblink conditioning task

The rats were habituated to the training chamber and headstage cable for three days. The training consisted of 50 trials each training trial consisted of a 250 ms baseline, a 400 ms CS period, and a 100 ms US period. The tone was 500 ms in duration and overlapped the airpuff for 100 ms the training tone was 3 kHz and the airpuff 10 psi. Hardware and software used to train and analyze data were

manufactured by J. Tracy, J. Green and J. Steinmetz, (Bloomington, Indiana). Eyelid EMG data was collected, amplified, rectified, and integrated. Learned responses were determined using a 10 standard deviation criterion for eyelid amplitude elevated during the CS period when compared to the baseline. Alpha responses to the tone are excluded from learned response analysis by using a 70 ms discrimination/exclusion window. Learning was measured as the percentage of learned (conditioned) responses (CR's) made in each training session.

4.3.4 Design and Analysis

To analyze behavior for the eyeblink conditioning task, separate two-way mixed model analyses of variance were used to analyze Drug and Day effects {[Drug (2): (YOUNG: Control, rrTNF-α) or (AGED: IgG, Anti-TNF-α)] × [Day (5): 1-5]}. For the analyses of NE release separate two-way mixed model analyses of variance were used to analyze Drug and Time effects {[Drug (2): (YOUNG: Control, rrTNF-α) or (AGED: IgG, Anti-TNF-α)] × [Time (18): -30 to 140 minutes]}. Post Hoc Analyses (Dunnett's) were used to test for Drug and Time effects. Comparisons were determined significant at the 0.05 alpha level. Percent Conditioned Response (CR %) was used as the dependent measure.

4.4 Results

4.4.1 Young

In figure 4.1A it is shown that infusions of rrTNF-α (50ng) into the interpositus nucleus 24 and 3 hours before each day of training blocked learning on the classical eyeblink conditioning task (shown as percentage of conditioned response (% CR)) in young rats {significant drug x day interaction [$F(4,40) = 5.8$, $p<0.05$]}. Rats which received control infusions (denatured rrTNF-α) showed progressive learning (increases in % CR) over 5 days. On day 1 % CR was significantly less than days 3, 4 and 5. Also on day 3 % CR was significantly less than days 4 and 5. Rats given rrTNF-α infusions did not show a significant improvement in % CR over 5 days. These data suggest that rrTNF-α injected into the cerebellum of young rats significantly impairs the rats ability to learn the eyeblink conditioning task.

The time course of NE release in young rats during classical eyeblink conditioning is shown in figure 4.1B {significant drug x time interaction [$F(17,153) = 3.0$, $p<0.05$]}. Microdialysis was performed on day 1 of eyeblink conditioning and started 30 minutes before training and continued for 140 minutes once training started. Samples were collected every 10 minutes. NE release significantly increased above baseline levels for 60 minutes after training began in both control and rrTNF-α, suggesting that NE plays a critical role during the acquisition of learning. NE release reached baseline levels 70 minutes after the beginning of training. Infusion of rrTNF-α into the interpostius nucleus

significantly decrease the release of NE during the 20 minutes of training and 10 minutes following training ($p<.05$). These data show that TNF-α attenuates NE release in the cerebellum of young rats.

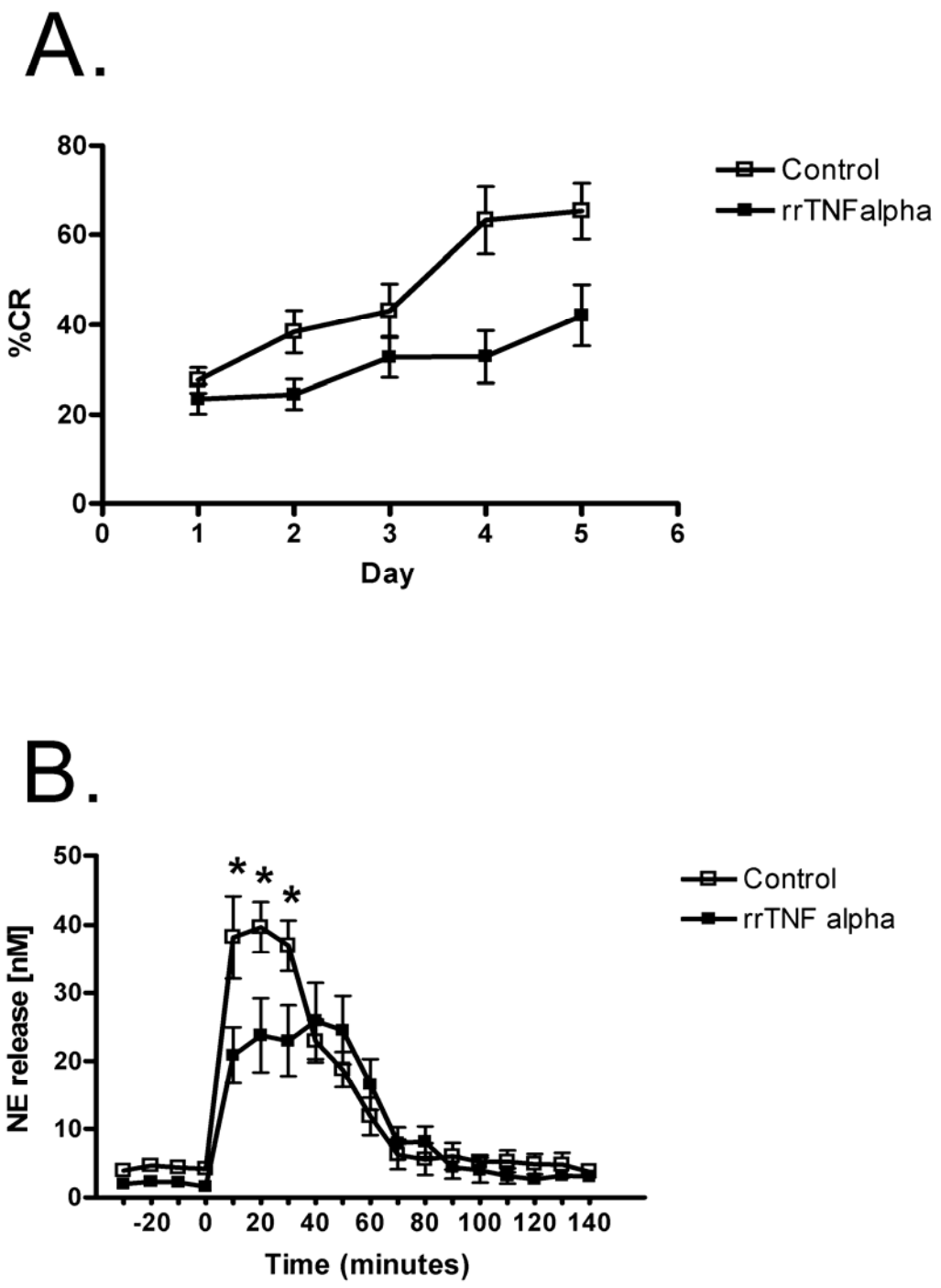


Figure 4.1 Effects of TNF-α in young rats. Performance on eyeblink conditioning task (A) and the time course of NE release (B) in young F344 rats. A) Rats which received control infusions (rrTNF-α heated) showed progressive learning

(increases in %CR) over 5 days. Whereas, rats given rrTNF-α infusions did not show significant improvement in %CR over days, suggesting that rrTNF-α significantly impairs the young rats ability to learn the eyeblink conditioning task. B) Microdialysis was performed in young rats and the time course of NE was recorded during eyeblink conditioning. To obtain basal level of NE, microdialysates were collected for 30 minutes before training (baseline) and continued for 140 minutes from the beginning of training (time points 0-20). NE release significantly increased above baseline levels during training in both control and rrTNF-α, reaching baseline levels 70 minutes after training began. However, infusions of rrTNF-α into the interpositus nucleus significantly lowered NE levels at time points 10-30 minutes (indicated by *) compared to controls, demonstrating that TNF-α attenuates NE release in the cerebellum of young rats.

4.4.2 Aging

Figure 4.2A shows learning (shown as increases in % CR over days) in aged rats which received infusions of IgG (control) or Anti-TNF-α into the interpositus nucleus {significant drug x day interaction [$F(4,40) = 3.6$, $p<0.05$]}. Anti-rat TNF-α was infused (0.3pg/ml in 2µl, during 5 minutes) three times a week for 4 weeks prior to eyeblink conditioning. Both groups demonstrate progressive learning (increases in %CR) over days. The percentage of conditioned responses was significantly lower on day 1 than days 2 - 5. Rats injected with anti-TNF-α performed significantly better (higher %CR) on days 4 and 5 compared to controls. These data suggest that the blockage of TNF-α in aged rats can improve learning on the eyeblink conditioning task.

The time course of NE release in aged rats which received infusions of IgG (control) or anti-TNF-α into the interpositus nucleus is shown in Figure 4.2B {significant drug x time interaction [$F(17,136) = 1.8$, $p<0.05$]}. NE release was significantly elevated in both conditions compared to baseline NE levels. Chronic

anti-rat TNF-α infusion resulted in significant increases above baseline NE levels (see figure 4.2B). The insert to figure 4.2B shows the AUC for NE release from time point 0 to 50 minutes from the beginning of training. Note that in the anti-TNF-α group NE release appeared to reach a higher peak of NE release sooner than the IgG group.

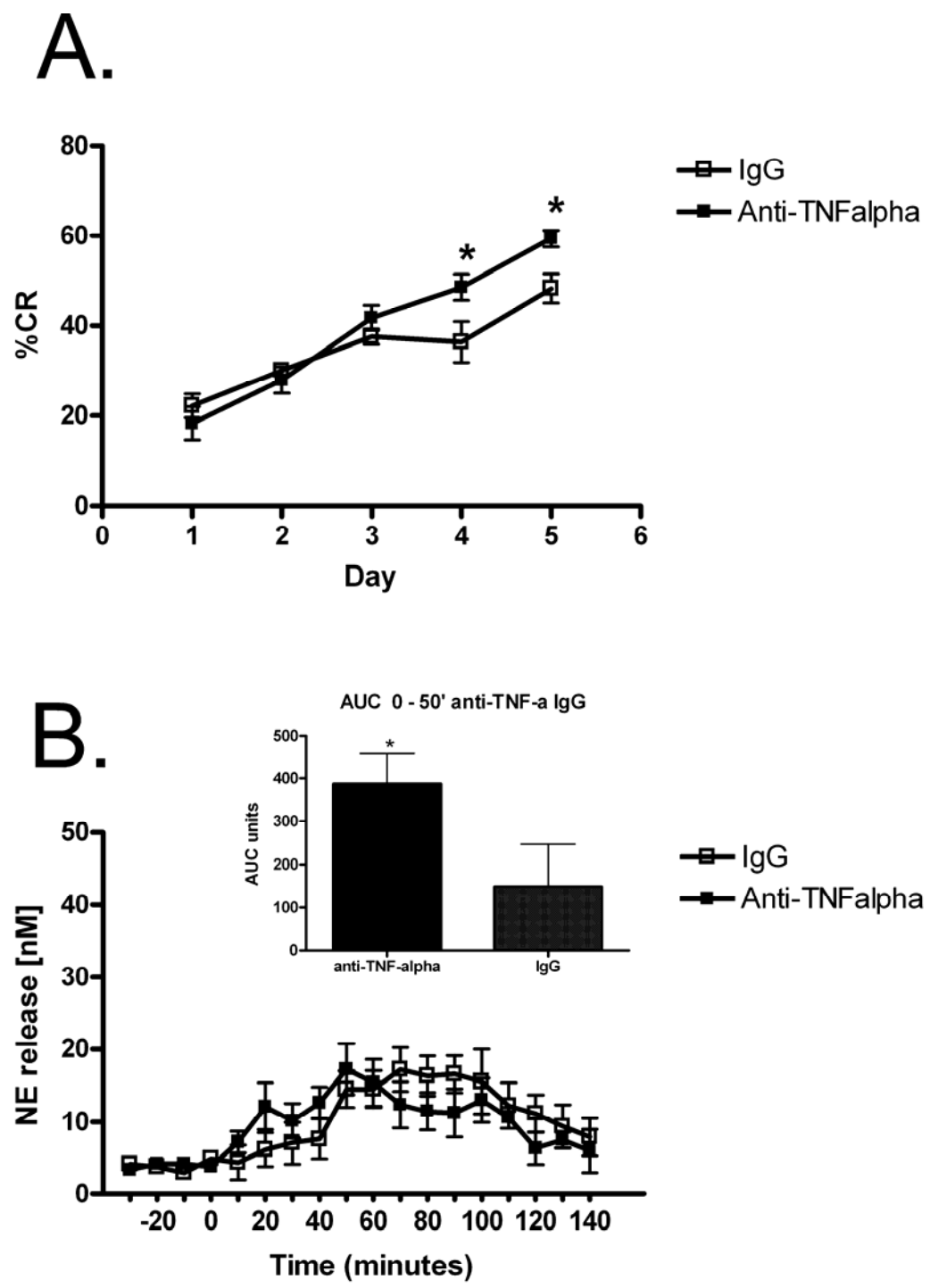


Figure 4.2 Effects of the blockade of TNF-α in aged rats. Performance on eyeblink conditioning task (A) and the time course of NE release (B) in 22 month

old F344 rats. A) Progressive learning over days (shown as increases in % CR) was shown in both IgG and anti-TNF-α injected aged rats. Interestingly, aged rats injected with anti-TNF-α performed significantly better (higher % CR) on days 4 and 5 compared to IgG controls (indicated by *), suggesting that the blockade of TNF-α in aged rats can improve learning on the eyeblink conditioning task. B) Shows the time course of NE release in aged rats which received infusions of IgG (control) or Anti-TNF-α into the interpositus nucleus. NE release was significantly elevated in both conditions compared to baseline. Chronic anti-rat TNF-α infusion resulted in a significant increase above baseline NE levels. Insert shows the AUC for NE release from time points 0-50 minutes. Note that in the anti-rat TNF-α group NE release appeared to reach a higher peak sooner than the IgG group.

4.5 Discussion

The main goal of this study was evaluate whether increases in TNF-α levels which are part of the aging process are to some degree responsible for the decline on memory formation capabilities.

In order to evaluate this hypothesis we did unilateral infusions of rrTNF-*α* through the deep nuclei and cortex in rat cerebellum and have found that the administration of rrTNF-α in young rats, prior to the training sessions of delay eyeblink conditioning significantly interferes with acquisition of CR's. On the other hand, when aged rats were treated with anti-TNF-α improvements in the acquisition of CR's where observed demonstrating that they were capable of learning faster than controls. The results show that pharmacological intervention targeting high levels of TNF-α present in the cerebellum of aged animals leads to an improvement in learning capabilities. Suggesting that the activity of TNF-α in some ways affects the processing of information to the cerebellum and hence interferes with the acquisition of CR's.

These results support the theory that cerebellar physiology is to some degree vulnerable to the presence of high levels of TNF-*α* which is evident since the local administration of recombinant TNF-*α* in young rats disrupts normal acquisition of CR's. The experimental design used with the young rats parallels an acute insult occurring just 5 minutes before the animals undergo eyeblink conditioning training. Chapter 2 discussed how there is a post-training timeline which is a process by which memory consolidation happens and pharmacological or molecular interventions during the consolidation process can interfere with the dynamics of memory consolidation (Mintz et al., 1994; Lavond et al., 1985; De Zeeuw & Yeo, 2005; Cooke et al., 2004; Attwell et al., 2002a).

TNF-α in young rats

In our experimental design where young animals were pre-treated 5 minutes before training sessions it is possible that the presence of TNF-α impacts the cerebellar region through the action on its receptors. TNF-α has two subtypes of receptors which have a broad spectrum of effects and have been reported to exist in areas such as cortex, brainstem, cerebellum and basal ganglia among other brain areas (Kinouchi et al., 1991). Cytokines, specifically recombinant human TNF-α has been reported to induce concentration-dependent and reversible alterations in the electrophysiological properties of axons in mammalian spinal cord (Davies et al., 2006a). This study provides evidence that elevated concentrations of TNF-*α* induce reversible depolarization of the

compound membrane potential (CAP) and reduction in CAP amplitude, sometimes to the point of extinction of the CAP, suggestive of impaired axonal conduction. Based on this report, it is plausible that local administration of TNF-α into the cerebellum might have impaired axonal conduction in a critical time in which the rats were receiving the eyeblink conditioning training and even for a critical period of time (post training session) for memory consolidation, in which case the depletion on the CR's acquisition occurred. Proinflammatory cytokines, specifically TNF-α have been reported to induce, through the classical I kappa B degradation pathway, a repression in excitatory amino acid transporter two (EAAT2) on astrocytes and increases the expression of AMPA receptors on synapses, which leads to elevated extracellular glutamate concentrations and in consequence facilitates the risk of glutamatergic neuronal toxicity (Sitcheran et al., 2005). In this case, glutamatergic neurotoxicity due to excessive glutamate activation might be involved in the depletion of CR's acquisition observed when the rats received a direct injection of TNF-α directly into the cerebellum. Given the previous facts it is very likely that the effect observed with TNF-α in young rats is due to an impairment on memory consolidation, in this case more experiments would have to be conducted to test this hypothesis, which can be assessed by administering the TNF-α at critical times after the training sessions of eyeblink conditioning. Another possible cause could be over saturation of signal input through the climbing and mossy fibers due to the glutamatergic overdrive and perhaps affecting the appropriate signal to noise ratio required to trigger a significant signal on the PC necessary to lead memory formation.

Anti-TNF-α in aged rats

All the possible mechanisms stated to explain the effect of TNF-α in young rats also apply to aged rats. For aged rats we must consider that chronic exposure to high levels of TNF-α are reported in aged rats (Gemma et al., 2002), which changes the scenario compared to young rats. Interestingly we observed a behavioral improvement regarding the CR's acquisition in aged rats by training day four and five showing that pretreatment with the antibody anti-TNF-*α* has reversed the cognitive impairment normally seemed in aged rats. This to a certain degree rules out neurotoxicity (due to high levels of Glu and apoptosis normally triggered by TNF-α) as a major cause of the CR impairment observed in aged animals (Sitcheran et al., 2005). This idea is supported by the fact that microdialysis performed in aged rats during eyeblink conditioning training showed (see chapter 1 of this dissertation) long lasting increases in extracellular glutamate compared to young rats. This could be partially due to the effect of TNF-α in the glutamate transporter system leading to a prolonged time for clearance of glutamate from the extracellular space.

Another possible mechanism by which treatment with anti-TNF-α is acting could due to an improvement on the impaired axonal conduction, since it has been reported that TNF-α alter the electrophysiological properties of axons in mammalian spinal cord (Davies et al., 2006b). Based on our results we can appreciate that NE release during training on eyeblink conditioning shifts (to an

earlier release pattern) as a result of anti-TNF-α treatment. This pattern of NE release peaks earlier and returns to baseline sooner, showing an improvement compared to the control group (see fig 4.2B). In addition, CR's improve on days 4 and 5 after treatment with anti-TNF-α supporting the idea of reversing the age related impairment, which could be due to an improvement in the axonal conduction as well as better re-uptake for extracellular glutamate by the glutamate transporter system. In addition to the finding presents in the current report there is still more to be done to understand the mechanisms by which treatment with anti- TNF-α is improving the capability of learning which we have demonstrated in this research.

CHAPTER 5

Conclusions

Using eyeblink conditioning coupled to microdialysis we have been able to monitor the activity of NE, GABA and Glu while the animals undergo classical delay eyeblink conditioning. Experiments were conducted in both young and aged rats, which gave us the opportunity to understand basic concepts about memory formation that have remained unclear until now. Delay eyeblink conditioning is a paradigm claimed to be mediated by the interplay of NE, GABA and Glu within the cerebellar network. The experimental design used in this research allowed the study of the complex neuronal circuitry in the cerebellum. Given the knowledge of the anatomy and physiology of the cerebellum, it is an excellent structure to explore in details the different mechanisms involved in neuronal plasticity underlying memory formation. The hypotheses tested in this dissertation utilized several decades of research and used behavioral pharmacology combined with neurochemical analytical techniques enabling us to answer exciting basic questions regarding the dynamics of memory formation.

We showed that the temporal pattern of NE and GABA release that occurs during training on the delay eyeblink conditioning task is linked to the associative learning task and that it is not due to sensory stimulus activation by the CS and/or US, which is the case for Glu release. Our results agree with the literature which shows that these neurotransmitters participate in different ways and are essential for memory formation, consolidation and extinction (Attwell et al.,

2002a; Yeo, 2004; De Zeeuw & Yeo, 2005; Weis et al., 2004; Farley & Alkon, 1985). It has been postulated that NE which is released in the cerebellum from the LC induces synaptic plasticity in purkinje neurons by selectively improving the signal to noise ratio of evoked versus spontaneous activity, enhancing the sensitivity of cerebellar neurons to both excitatory and inhibitory afferent inputs (Moises et al., 1979). Our data show that in fact NE is actively released in an impulse dependent manner into the lobus simplex and interpositus nucleus. This is the first direct evidence showing presynaptic release *in vivo* while the animals perform the learning task.

An interesting aspect of our findings are that the amount of NE released decreased across days of training which is consistent with a role of NE early in acquisition and possibly in consolidation (Gilbert, 1974). In addition, our findings agree with published work which showed that firing of LC neurons demonstrates good discrimination within the first 500 trials of a visual discrimination task in monkeys suggesting that NE is important during the acquisition or early phase of learning of this task (Kubiak et al., 1998). According to the pattern of release for NE during training sessions across days that we found, it can be inferred that the cerebellar circuit requires higher extracellular levels of NE in the early phase of training. On the other hand, NE as well as GABA were released in a learning dependent manner and were specifically associated with paired conditioning and not pseudo-conditioning which strengthens the idea that both neurotransmitters in play a role in the process of CR acquisition.

Much of the work examining GABAergic transmission has been done using a pharmacological approach by either administering GABA agonists or antagonists in order uncover the functional role of the cerebellar cortex and cerebellar nuclei and how it is involved in memory formation and timing (Bao et al., 2002; Krupa & Thompson, 1997; Mamounas et al., 1987; Schreurs & Alkon, 1993). In this dissertation we directly measured extracellular GABA levels by microdialysis. Interestingly, during the first days of training, the increase above baseline in extracellular levels of GABA extended beyond the training period for over 60 minutes. This pattern changed across days of training where the time frame of GABA release shortens and the amplitude of release increase after few days of training when the rats reached the maximal performance in CR's, at that time the release of GABA showed a higher peak while the timing was confined to the training session, at this time the GABA release pattern might reflect the concomitant activity of the cerebellar circuitry that is associated with the CR. Our data agrees with other's works in which has suggested that GABA plays a role in post training memory consolidation (Cooke et al., 2004).

According to our results Glu release reflects the inputs to the cerebellar cortex and interpositus which are activated in both paired and unpaired conditions on the eyeblink training the pre-synaptic release of glutamate from afferents to the cerebellar cortex and supports the idea that learning of the conditioned response does not take place before the signals reach the cerebellum as there are no changes in the pattern of release across days of training. Our results agree with

those indicating that Glu has significant post-synaptic effects during training (Chen & Steinmetz, 2000). The administration of TTX through reverse dialysis decreased the amount of neurotransmitter observed in the microdialysate for all three neurotransmitters indicating impulse dependent release for all three neurotransmitters. In another set of similar experiments but on aged rats, it was found that there was decreased capability to acquire CR's n eyeblink conditioning which is paralleled by a distorted pattern of NE, GABA and Glu release. When this pattern is compared to young animals it occurs with a significant delay in relation to the moment in which training sessions start. Also, the magnitude of release is depleted about 40% compared to young animals. The time-dependent change that occurs for NE and GABA in young rats doesn't happen in aged rats. These findings suggest an impairment of the orchestration carried by the neurotransmitters NE, GABA and Glu during memory formation and consolidation. The data show that the neurochemical network in the cerebellum becomes compromised with aging. More experiments need to be done in order to elucidate the causes of the neurochemical imbalance found in aged animals.

Furthermore, blockade of postsynaptic β-adrenergic receptors with discrete local administration of propranolol into the cerebellum disrupts acquisition of the CR when it is administered after the animal has been on training. This result clearly indicates the participation of β-adrenergic receptors during the consolidation process of the new memories. More experiments need to be performed to clarify the exact mechanism by which the β-adrenergic receptors are participating

during the memory consolidation process. Similar work has been done by administering propranolol before the training, this way it is clear that the activity of β-adrenergic receptors are critical during eyeblink conditioning (Gould & Steinmetz, 1996; Cartford et al., 2002a). Our findings show that noradrenergic inputs to the cerebellar cortex are necessary for the consolidation process by which CR's are formed.

The local administration of propranolol at 5 and 60 minutes post training had a large impact in depleting the CR's acquisition while propranolol administered 120 min post training session did not cause a significant impairment in the acquisition of CR's. It is known that NE is a neuromodulator in that it selectively improves the signal to noise ratio of evoked versus spontaneous activity which enhances the sensitivity of the cerebellar neurons to excitatory and inhibitory afferent inputs (Eccles et al 1967; Hoffer et al 1973). The effect of propranolol could be due to the lack of the noradrenergic signal (elicited by propranolol administration) impairs the improvement of the signal to noise ratio of evoked versus spontaneous activity which may explain why the rats would require more training sessions in order to reach a significant conditioning response. Another possible explanation for the depletion of CR's after blocking the beta-adrenergic receptor post-training could be associated with the activity that NE exerts by activating the beta-adrenergic receptors which leads to the activation of cAMP/PKA signaling cascade which has been shown to enhance intracellular calcium and promote the release of vesicles containing GABA (Saitow et al., 2005). Given the fact that NE

activates glycogenolysis, we are proposing that the blockade of β-adrenergic receptors might be interfering with the pool of energy in the form of ATP required to assemble and relocate the new synthesized proteins during the memory consolidation. This idea needs to be addressed with specific experiments to elucidate whether NE acts on astrocytes to provide the energy necessary for the novo protein synthesis as well as calcium mobilization within the cells as a essential part of the memory consolidation and how it correlate with aged-related memory deficits in which there is a deficit in NE signaling.

It has been postulated that in aged-related memory deficits an increase in by-products of oxidative stress is responsible for some of the damages found in the central nervous system in mammals (Harman, 1956; Leibovitz and Siegel, 1980), and perhaps triggering the release of cytokines. TNF-α has been found to be elevated in aged rats, and previous work has demonstrated that the elevated expression of TNF-α in aged rats can be reversed by diets rich in anti-oxidants and result in a reduction of TNF-α levels. Also, anti-oxidant diets improve the acquisition of CR's using eyeblink conditioning. We addressed some experiments to determine whether the activity of this cytokine can directly interfere with the acquisition of CR's. The findings showed that elevated levels of TNF-α in young rats depletes the acquisition of CR and the neurochemical pattern of release for the neurotransmitter NE become compromised. These results indicate that chronic neuroinflamation can be a leading factor by which aged animals present impaired capabilities of learning. Furthermore, we also

showed that intervention with antibodies anti-TNF-α can lead to an improvement in the ability of aged rats to acquire CR's. We also showed that with this pharmacological intervention the neurochemical pattern of release for NE can be slightly modified and look more like the patter release of young rats. This finding has tremendous impact in understanding which factors during aging can lead to depletion on cognitive abilities.

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