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Original Article

Role of TNFα Induced Inflammation in Delay Eyeblink Conditioning in Young and Aged Rats

Daniel Paredes[1, #], Sandra Acosta[1], Carmelina Gemma[1, 2], and Paula C. Bickford[1, 2*]

[1]Department of Neurosurgery and Brain Repair, USF Tampa FL, and [2] James A. Haley VAMC, Tampa FL, USA
[#]Current address: Laboratory of Molecular Biology, NINDS, Bethesda, MD, USA

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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 cytokine TNF-α and improve classical eyeblink conditioning performance. Therefore we tested the hypothesis that the proinflamatory cytokine TNF-α may be a critical factor that modulates classical conditioning behavior. If increased levels of endogenous cerebellar TNF-α negatively affect performance on the eyeblink conditioning task in aged rats, then exogenous administration of TNF-α in young rats should result in an impaired acquisition and/or retention of eyeblink conditioning memory. On the other hand, the reduction or blockage of the age-related increase in cerebellar TNF-α levels in aged rats should result in an improvement in memory. Young (3 month old) F344 rats were pretreated with an intracerebellar injection of recombinant rat (rr)TNF-α or denatured (rr)TNF-α prior to eyeblink conditioning coupled to microdialysis. The results showed that young rats treated with rrTNF-α have a decreased rate of learning compared to the control group. Norepinephrine which has been shown to play a critical role in cerebellar learning tasks presented a shift on training day one of young rats resembling that observed in aged rats. In a second experiment aged (22 month old) F344 rats were pretreated with intracerebellar microinjection of anti-rat TNF-α three times a week for 4 weeks prior to eyeblink conditioning training couple to microdialysis. Aged rats showed a better performance in the conditioned responses when compared to controls. The release of norepinephrine 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 learning and the pattern of NE release during eyeblink conditioning.

Key words: Learning; cytokines; memory; consolidation; aging; microglia

Norepinephrine (NE) is released from the locus coeruleus (LC) to the cerebellar cortex (among other brain areas) [1-3] and exerts a modulatory effect on the action of other neurotransmitters in the cortex and deep nuclei of the cerebellum [4]. 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 [5,6]. Procedural learning has been widely associated with noradrenergic innervation to the cerebellar cortex in rats [7-14]. Delay classical conditioning is a well established cerebellar-dependent learning paradigm and is modulated in part by NE [14,15]. When the β-adrenergic receptor antagonist, propranolol is administered systemically or locally (cerebellum), acquisition of the learned response on the delay classical conditioning in rats is impaired [9,16]. Furthermore, the administration of 6-hydroxydopamine depletes NE storage and prevents animals from regaining proficiency on a motor learning task [11,17]. Aging-associated deficits on motor learning have been linked to dysfunction of

***Correspondence should be addressed to:** Dr. Paula C. Bickford, Department of Neurosurgery and Brain Repair, USF Tampa FL, USA. *E-mail:* pbickfor@health.usf.edu
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the noradrenergic system 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 [10,18,19].

Age-related pathologies are characterized by a pronounced imbalance in immune functions like glial hyperactivity with altered antigen expression of microglia in aged rodents [20,21]. Chronic inflammation is known as one of the multiple age-related pathologies that involve the activity of several products, including cytokines [22,23]. 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 [24,25]. 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 (TGF-β1) [26,27]. Pro-inflammatory cytokines are chronically increased in the aging brain [28]. TNF-α and TNF-β are significantly elevated in the cerebellum of aged rats and diets rich in anti-oxidants reduced both TNF-α and TNF-β levels [29]. 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 [30].

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

MATERIALS AND METHODS

Animals and surgery

Male F344 rats 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 stereotactic 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 were allowed to recover for one week after the surgery procedure before the eyeblink conditioning training coupled to 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.

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). In a second experiment aged (20 month old) F344 rats were pretreated with intracerebellar microinjection of 2 uL of anti-rat TNF-α (30ng) three times a week for 4 weeks prior to eyeblink conditioning training with microdialysis.

Training of behavior in a delay classical eyeblink conditioning task

The rats were placed in the behavioral chamber and hooked to the headstage cable for habituation purpose during 15 minutes 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, 80dB 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 80 ms discrimination/exclusion window. Learning was measured as the percentage of learned (conditioned) responses (CR's) made in each training session.

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.

RESULTS

Young Rats:

In figure 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 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.

Aged rats:

Figure 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 (30 ng 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 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 when comparing the total release over the first 50 minutes (see figure 2B). The insert to figure 2B shows the area under the curve (AUC) for NE release from time point 0 to 50 minutes from the beginning of training, this is significantly higher in the treated rats compared with control. A second aspect of comparison was a change in the time course of NE release. In a previous study [31] we demonstrated that in aged rats not only was there a decrease in total amplitude of NE release during learning, but there is a delay in the peak of NE release. Note that in the anti-TNF-α group NE release

begins to increase over baseline sooner and reaches maximum amplitude sooner than the IgG group.

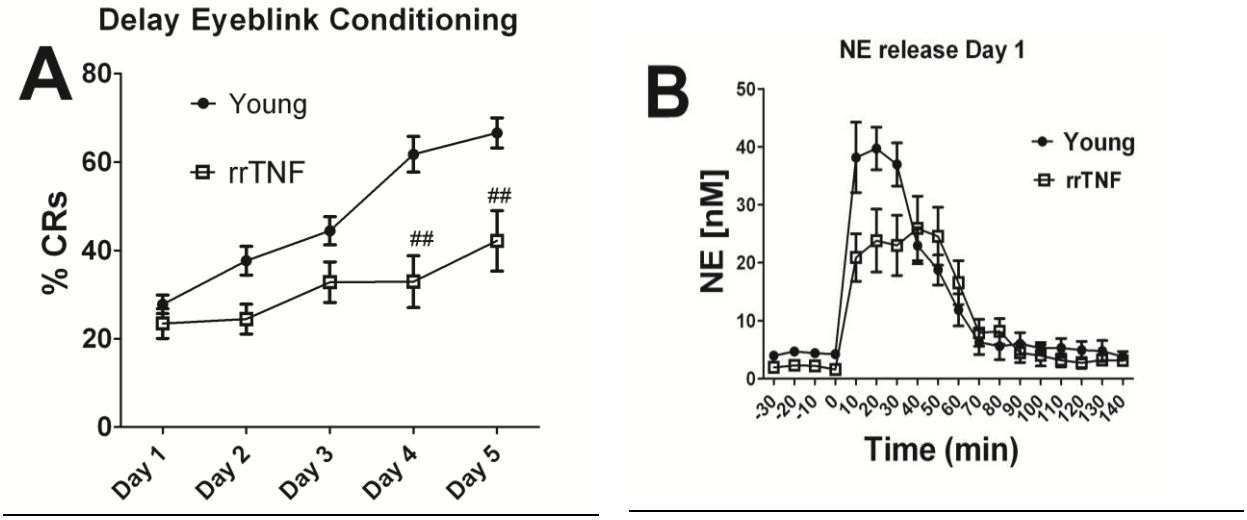

Figure 1 Effects of TNF-α in young rats. Performance on eyeblink conditioning task (A) and the time course of NE release (B) in young F344 rats. A) Rats which received control infusions (rrTNF-α heated) showed progressive learning (increases in %CR) over 5 days. Whereas, rats given rrTNF-α infusions did not show significant improvement in %CR over days, and were significantly different from control on days 4 and 5 (# indicates $p<0.001$ 2 Way ANOVA followed by Bonferroni posttests). (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.

DISCUSSION

The main goal of this study was to evaluate whether the proinflamatory cytokine TNF-α, which is found to be increased with aging, is 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 also affecting the pattern of NE release. 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. Thus, 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. Others work discuss 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 [32-36]. However we must take into consideration the fact that the pattern of NE release was shifted during learning indicating that increased

levels of TNF-α interfere with this signal which has been shown to be important for memory consolidation [31]. This may well alter the signal to noise ratio essential to trigger a meaningful signal on the cerebellar purkinje cells necessary to promote memory formation.

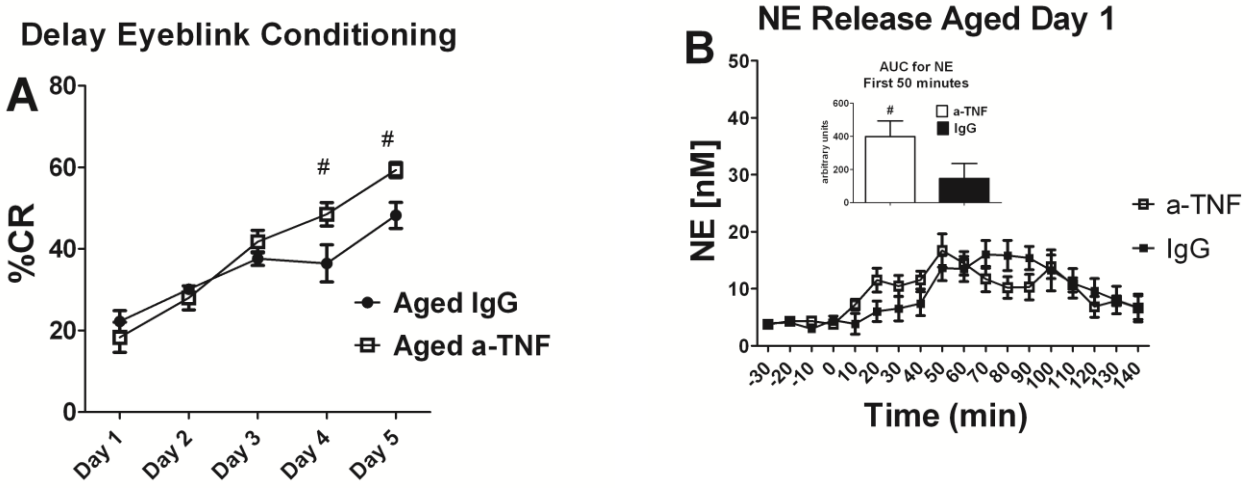

Figure 2 Effects of the blockade of TNF-α in aged rats. Performance on eyeblink conditioning task (A) and the time course of NE release (B) in 22 month old F344 rats. (A) Progressive learning over days (shown as increases in % CR) was shown in both IgG and anti-TNF-α injected aged rats. The anti-TNFα treated aged rats performed significantly higher %CR on days 4 and 5 (# indicates $p<0.05$ 2 way ANOVA followed by Bonferroni post-tests). (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. Insert shows the quantification of NE release during the first 50 minutes of release by measuring the AUC for NE release from time points 0-50 minutes and this is significantly higher in the treated rats versus IgG controls (# indicates $p<0.01$ students t-test). Note that in the anti-rat TNF-α group NE release appeared to rise above baseline sooner and reach a peak earlier than the IgG group indicating that the timing of NE release was more similar to the young rats in the treated group.

TNF-α in young rats

In our experimental design where young animals were pre-treated 3 hours 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 [37]. 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 [38]. 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 [39]. In such case, glutamatergic neurotoxicity due possible 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. One further caveat to consider with the administration of any pro-inflammatory cytokine is the possible effect of sickness behavior as this has in some cases been associated with cytokines crossing the blood brain barrier and disrupting learning [40-42]. This is especially true when LPS or some other toxin is given peripherally to the whole animal. However, there is evidence that effects of IL1β to disrupt learning are independent of peripheral effects on cortisol and other markers [43] suggesting that there are direct effects of cytokines on memory consolidation. As this study administered the cytokines directly into the cerebellum it is likely that the effects are limited to the cerebellum.

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 [29], 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 idea is supported by our previous finding in which aged rats during eyeblink conditioning training showed long lasting increases in extracellular glutamate compared to young rats [31]. 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 [38]. 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 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 learning which we have demonstrated in this research.

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