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Title: Brain-derived tumor necrosis factor-alpha and its involvement in noradrenergic neuron functioning involved in the mechanism of action of an antidepressant

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Running title: The role of TNF in the mechanism of action of desipramine

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Abbreviations: TNF, tumor necrosis factor-α; NE, norepinephrine; 5-HT, serotonin; FST, forced swim test; i.p., intraperitoneal; icv, intracerebroventricular

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Abstract

The present study documents a role for brain-derived tumor necrosis factor-α (TNF) in the mechanism of action of the antidepressant drug desmethylimipramine (desipramine). To establish this role, field-stimulation and superfusion of rat hippocampal slices was employed to investigate the regulation of norepinephrine (NE) release by TNF. Chronic desipramine administration transforms TNF-mediated inhibition of NE release to facilitation, dependent upon α_2-adrenergic receptor activation. Chronic intracerebroventricular (icv) microinfusion of polyclonal TNF antibody (pTNF-Ab) similarly transforms TNF inhibition of NE release to facilitation. To determine whether this transformation is due to desipramine-induced inhibition of TNF bioactivity in the brain, rats were icv microinfused with recombinant rat TNF (rrTNF) for 14 days, either alone, or with simultaneous i.p. desipramine administration. TNF-regulation of NE release in hippocampal slices isolated from these rats was compared to slices isolated from rats chronically administered desipramine alone. While simultaneous microinfusion of rrTNF with chronic desipramine administration prevents the transformation induced by desipramine, microinfusion of rrTNF enhances TNF-inhibition of NE release. These cellular events correspond to changes in immobility, analyzed by the forced swim test (FST). ICV microinfusion of rrTNF increases the duration of immobility of rats in the FST, compared to rats microinfused with aCSF. Desipramine administered chronically decreases immobility duration, which is mimicked by icv microinfusion of pTNF-Ab and prevented by simultaneous icv microinfusion of rrTNF. Thus, icv microinfusion of rrTNF with concomitant desipramine administration opposes decreases in neuron-associated TNF levels, required to transform presynaptic sensitivity to TNF, which is necessary for the drug to be efficacious.

The etiology of depression as well as the mechanism of action of antidepressant drugs has been investigated for over 40 years, yet precise mechanisms that direct the expression of mood are unknown. Several theories have been postulated which implicate altered release of the neurotransmitter norepinephrine (NE) (Schildkraut, 1965) in the pathogenesis of depression. The classic Monoamine Theory of Depression proposes that symptoms of depression are due to an imbalance in the bioavailability of the monoamines, NE and serotonin (5-HT), within the central nervous system (CNS) (Schildkraut, 1965; Willner, 1985). Therefore, antidepressant drug-induced regulation of NE availability, as well as adrenergic receptors that regulate the release of NE, have been extensively investigated (Banerjee et al., 1977; Crews and Smith, 1978). A 'dysregulation hypothesis' has also been proposed which implicates an impaired negative feedback on presynaptic neurons due to dysfunctional presynaptic α_2-adrenergic receptors (Siever and Davis, 1984; Maes et al., 1999). These receptors are primary regulators of NE release from noradrenergic neurons and are classically known to inhibit stimulation-evoked NE release (Langer, 1981; Starke, 1981); they are, therefore, classified as autoinhibitory receptors. Alterations in α_2-adrenergic receptor function are associated with the mechanism of action of many antidepressant drugs (Crews and Smith, 1978; Svensson and Usdin, 1978; Smith et al., 1981). The precise mechanism governing these alterations, however, remains unclear.

In addition to the α_2-adrenergic agonist NE, a myriad of other mediators regulate NE release, including cytokines, such as tumor necrosis factor-alpha (TNF) (Soliven and Albert, 1992; Elenkov et al., 1992; Ignatowski and Spengler, 1994). TNF increases synaptic transmission from rat hippocampal neurons (Tancredi et al., 1992)

and inhibits stimulation-evoked NE release from the rat median eminence (Elenkov et al., 1992). In fact, TNF inhibits as well as facilitates the electrically-evoked release of [^{3}H]-NE from hippocampal slices isolated from naïve rats or from rats chronically administered antidepressant drugs, respectively (Ignatowski and Spengler, 1994; Ignatowski et al., 1996; Nickola et al., 2001). Following chronic administration (14 days) of the antidepressant drug desipramine, a NE reuptake blocker, to rats, or zimelidine, a selective 5-HT reuptake inhibitor, TNF no longer inhibits but rather facilitates [^{3}H]-NE release from electrically depolarized hippocampal slices. In addition, this transformation is dependent upon continual functioning of the α_2-adrenergic receptor (Ignatowski and Spengler, 1994; Nickola et al., 2001). Interestingly, the time required to achieve this transformation in the regulation of NE release by TNF concurs with the therapeutic effects of these drugs. Therefore, changes in TNF regulation of NE release directed by α_2-adrenergic receptor activation may represent a common mechanism of action of antidepressant drugs.

TNF is localized and synthesized within neurons of the locus coeruleus (the principal source of noradrenergic neurons in the brain) and localized within neurons in the hippocampus (a brain region replete in noradrenergic nerve terminals). The levels of TNF mRNA and protein are changed in these brain regions following acute and chronic administration of desipramine to rats (Ignatowski et al., 1997; Nickola et al., 2001). Therefore, it was of interest to investigate if the inhibition of TNF production in neurons by desipramine may be fundamental to the efficacy of this drug.

The present study was designed to investigate the cellular and behavioral roles that TNF plays in the mechanism of action of this antidepressant drug. The efficacy of

desipramine was examined using the forced swim test (FST), a behavioral model used to validate the efficacy of antidepressant drugs. At the cellular level, the regulation of [^{3}H]-NE release by TNF using field stimulation and superfusion of rat hippocampal slices was investigated following intracerebroventricular (icv) microinfusion of recombinant rat TNF (rrTNF), either alone or with simultaneous intraperitoneal (i.p.) administration of desipramine. Decreases in brain-derived TNF production induced by desipramine administration were mimicked with icv microinfusion of a polyclonal TNF antibody (pTNF-Ab), and regulation of [^{3}H]-NE release by TNF was investigated.

Methods

Intracerebroventricular (icv) microinfusion

Mini osmotic pumps (0.5 µl/hr, 14 days, Alza, Palo Alto, CA) and brain infusion cannulae (Alza) were assembled according to manufacturer's guidelines. The vehicle used for delivery of the infused compound was artificial cerebral spinal fluid (aCSF) of the following composition (mM): NaCl, 216.5; KCl, 3; $CaCl_2 \cdot 2H_20$, 1.4; $MgCl_2 \cdot 6H_2O$, 0.8; $Na_2HPO_4 \cdot 7H_2O$, 0.8; $NaH_2PO_4 \cdot H_2O$, 0.2). Gentamycin (0.1 mg/ml solution; Sigma, St. Louis, MO) was added to the aCSF to prevent bacterial growth. Rat albumin (fraction V, 1 mg/ml, Sigma) was added to stabilize the infused compound and to prevent proteins and charged molecules from binding non-specifically. Rats (male, Sprague-Dawley, 250-350 g), anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (3 mg/kg, i.p.) were secured on a stereotaxic platform. With bregma as the zero point, the following were the stereotaxic coordinates: A-P, -0.92 mm; lateral, 1.6 mm; vertical. 3.5 mm, for trephining the skull prior to cannulation of the right lateral cerebral ventricle. Compounds infused included the following: recombinant rat TNFα (rrTNF, R&D Systems, Minneapolis, MN); heat inactivated rrTNF (heated at 90ºC, 30 min, R&D), the control for rrTNF; polyclonal rabbit-anti-mouse TNFα antibody (pTNF-Ab, R&D) which exhibits cross reactivity to rat TNF; normal rabbit serum, the control for pTNF-Ab (Sigma); as well as aCSF alone, the vehicle. The concentration of rrTNF infused (1000 ng/24 hr at 0.5 µl/hr for 14 days) was chosen based on our previous studies that established a role for TNF in the CNS, and does not result in adverse side effects (Ignatowski et al., 1999). It should be noted that data from rats icv microinfused with aCSF alone and rats microinfused with

aCSF containing heat-inactivated rrTNF were pooled together due to non-significant differences between the two groups.

In our previous studies, we have demonstrated that a concentration of TNF between 1 and 30 ng/ml is required to affect neurotransmission in hippocampal slices *in vitro* (Ignatowski and Spengler, 1994). Therefore, icv microinfusion of 1000 ng/24 hr (0.7 ng/ min) appears to achieve an appropriate concentration within the synaptic cleft to affect neurotransmission. We have also shown that icv microinfusion of rrTNF at 30 ng/24 hr at 0.5 µl/hr for 14 days does not induce any significant behavioral or cellular changes from control rats (Ignatowski et al., 1999).

The dosage for the antibody infused is based on the EC_{50} value for inhibition of 0.3 pg/ml rrTNF in the WEHI-13VAR bioassay (Ignatowski et al., 1999) (see 'TNF bioassay' section). The viability of the pTNF-Ab, its control (normal rabbit serum), and rrTNF were confirmed in the bioassay following 14 days of incubation in aCSF at 37ºC. Rats microinfused icv with aCSF alone and rats microinfused with aCSF containing normal rabbit serum, the control for pTNF-Ab, were pooled due to non-significant differences between the two groups.

Drug administration schedule

All procedures were performed according to IACUC guidelines. Rats were maintained on a 12 hr light/dark cycle in LAF-accredited pathogen-free quarters with access to water and food *ad libitum*. Desipramine hydrochloride (desipramine, 10 mg/kg, i.p., Sigma) was dissolved in sterile saline and administered twice daily for 13 days (chronic) to rats. This dose of desipramine is a prototypical dose, displaying reliable

antidepressant activity in animal models predictive of antidepressant activity, such as the rat forced swim test (FST) (Araki et al., 1985; Kawashima et al., 1986). The drug was administered alone, simultaneous with icv microinfusion of aCSF, or simultaneous with icv microinfusion of aCSF containing appropriate compounds, in which case the drug administration was started on the morning after microinfusion surgery.

TNF bioassay

Brain regions were homogenized in RPMI-1640 (Mediatech, Herndon, VA) supplemented with 1% glutamine (Invitrogen, Grand Island, NY) and centrifuged at 14,000 x g (15 min at 4ºC). Supernatants from each homogenate were analyzed for bioactive TNF using the WEHI-13VAR fibroblast cytotoxicity assay as previously described (Ignatowski and Spengler, 1994). Results are expressed as the percentage of control values (pg/100 mg tissue weight) derived from a standard curve using recombinant human TNF (rhTNFα) (R&D). We have also tested rrTNF and recombinant murine TNF (rmTNF) in parallel with rhTNF in this bioassay. No differences were observed in standard curves generated with rhTNF or rmTNF (EC_{50} of 3-10 pg/ml in each case); however, the EC_{50} for rrTNF was 0.3 pg/ml. The lower limit of sensitivity of the assay using either rhTNF or rmTNF as the standard was 1 pg/ml. Since past studies have used rhTNF as the standard, this was the standard of choice in the present studies as well.

Field stimulated brain slices

The brains from rats sacrificed were isolated and the hippocampi were sliced transversely (0.4 mm thickness) (McIlwain tissue chopper, The Mickle Laboratory

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Engineering Co. Ltd., Goose Green, UK). The slices were placed in Krebs physiologic buffer solution of the following composition (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 10; ascorbic acid, 0.06; EDTA, 0.03 (Sigma). The slices were equilibrated for 10 min in Krebs buffer saturated with 95% O_2/5% $C0_2$ (Strate Welding, Buffalo, NY) at 37°C. At the end of the preincubation, [^{3}H]-NE (levo-[ring-2,5,6,-^{3}H]; Perkin Elmer, Boston, MA; specific activity 57.3 Ci/mmol) was added to a final concentration of 330 nM, and the slices were incubated for an additional 15 min, washed twice with fresh Krebs buffer, and transferred to 0.1 ml superfusion chambers (Brandel, Gaithersburg, MD). Each slice was positioned between two nylon mesh disks placed between two mesh platinum electrodes designed to stimulate neuron varicosities (field stimulation). Each tissue slice was superfused at a constant rate of 0.5 ml/min to ensure that the changes observed in radioactivity were actually changes in overflow of [^{3}H]-NE from the tissue. The slices were superfused with buffer for an initial 30 min period prior to, and during, the electrical field stimulation.

Neuron release of [^{3}H]-NE from hippocampal slices was studied by applying nine consecutive field stimulations (2 min periods) consisting of trains of squarewave pulses (26 V, 2 ms duration) every 16 min with the initial stimulation at 1 Hz and at 4 Hz for all subsequent stimulations. After the baseline response (the first 4 Hz stimulation) of [^{3}H]-NE release had been established, and during each subsequent stimulation, two experimental slices were perfused with Krebs buffer containing rmTNF (R&D) and two slices were perfused with rmTNF in the presence of the α_2-adrenergic antagonist yohimbine (0.1 µM, Sigma). The experimental tissue slices were allowed to equilibrate

in the presence of the drugs for 11.0 min prior to the onset of each stimulation. Two control chambers were perfused with Krebs buffer alone. After the second stimulation, TNF was added in 3-fold graded concentrations (0.01-10 ng/ml) in order to generate complete concentration-effect curves. When the effect of a constant concentration of a drug on a cumulative concentration effect of another compound was investigated, the constant drug was added to the two experimental chambers 16 min prior to the first electrical stimulation. Aliquots of the superfusate (2 ml) were collected at 4 min intervals, 1 ml from each collected sample was withdrawn, and 4 ml of Ultima Gold scintillation cocktail (Packard Instrument Co., Meriden, CT) was added to the remaining 1 ml of sample. Immediately following the last fraction collected, the tissue slices were removed from the chambers and solubilized in 0.2 ml 1 N NaOH. The evoked release of [^{3}H]-NE is expressed as the amount of labeled amine in each aliquot expressed as a percentage of the radiolabeled amine in the tissue immediately before the onset of field stimulation (fractional release). The labeled amine which was released in excess of the spontaneous efflux of tritium was greater than 90% unmetabolized [^{3}H]-NE when separated on Dowex columns. The stimulation-evoked release was calculated as the difference between the total release during the period of stimulation less the basal release. Complete concentration-effect curves were determined for TNF alone or in the presence of yohimbine. The EC_{50} values (concentration required for 50% of the maximum response) and the maximum responses for concentration-effect curves were obtained by using non-linear least squares regression to fit a curve (SigmaPlot, SPSS Inc., Chicago, IL). It should be noted that the curves graphed in each figure represent the average of individual curves in respective experiments. However, EC_{50} and maximum response

values reported in the Results section were calculated using SigmaPlot, and represent averaged values based on individual curves. Therefore, the graphed values do not exactly match the average calculated values.

Forced swim test (FST)

A modified FST (Detke et al., 1995) was used to validate the behavioral effects of desipramine. Swimming sessions were conducted by placing rats in a Plexiglas cylindrical tank containing 23-25^{o}C water. Two swimming sessions were conducted: an initial 15 min pre-test (prior to initiation of experimental paradigms), followed by a 5 min test prior to sacrifice (14 days later). The total duration of immobility, represented as rats remaining floating, only making movements necessary to keep their head above water, was measured during the 5 min test. Following swim sessions, rats were removed from the cylinder, dried, and placed in heated cages for 15 min before being returned to their home cages. A blinded, single rater performed scoring obtained from filming the sessions.

Statistics

One way analysis of variance (ANOVA) or Kruskal-Wallis one way analysis of variance on Ranks was carried out on the results, as indicated in figure legends. Overall statistical differences were analyzed using Bonferroni multiple comparisons (SPSS Inc). $P < 0.05$ was considered significant.

Results

Rats icv microinfused with rrTNF exhibited no observable changes in grooming patterns, and no obvious signs of aggression or sedation. No changes were observed in the hind paw temperatures in rats icv microinfused with rrTNF compared to rats icv microinfused with aCSF (data not shown). Rats that were chronically administered desipramine i.p., as indicated in the *Methods* section, either alone, or with simultaneous microinfusion of aCSF, exhibited a decreased rate of body weight gain (Fig. 1). Among the remaining groups of animals, icv microinfusion for 14 days of the compounds tested had no significant effect on the rate of weight gain during the entire duration of the experiments (Fig. 1).

TNF analysis

Brain tissue homogenates were assayed for levels of bioactive TNF using the WEHI-13VAR bioassay, with values ranging from 5-10 pg/100 mg tissue weight in naïve rats (data not shown). Increases in the levels of TNF (expressed as percent increase compared to rats microinfused with aCSF) in brain regions anatomically closest to the site of rrTNF icv microinfusion were as follows: hippocampus, 63,000 ± 2,700%; locus coeruleus, 77,800 ± 8,700%; hypothalamus, 38,000 ± 14,000%; parietal cortex 18,000 ± 2,000%; caudate nucleus, 5,000 ± 1,000%; and amygdala, 3,000 ± 700%. Bioactive levels of TNF in the systemic circulation (plasma) or in the spinal cord homogenates did not differ among rats microinfused with rrTNF, rats microinfused with aCSF and naïve rats. The number of rats used for all groups (naïve, aCSF-infused, and rrTNF-infused) was three per group.

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Effect of continual icv microinfusion of rrTNF on the fractional release of [^{3}H]-NE

In agreement with our previous findings (Ignatowski and Spengler, 1994), present studies demonstrate that exogenous TNF inhibited the electrically stimulated release of [^{3}H]-NE from hippocampal slices isolated from naïve rats (max. response elicited by exogenous TNF at 4 Hz stimulation, 32.39 ± 1.29%; EC_{50}, 0.28 ± 0.08 ng/ml). This inhibition was significantly augmented in the presence of the α_2-adrenergic antagonist yohimbine (0.1 μM) *in vitro* (max. response, 55.14 ± 2.95%, $p < 0.05$; EC_{50}, 0.24 ± 0.07 ng/ml, NS), as indicated by an increase in the maximum response to exogenous TNF alone. Depolarization-evoked release of [^{3}H]-NE from hippocampal slices isolated from rats microinfused icv with aCSF, when incubated with exogenous TNF in the superfusion system, demonstrated no significant change in the maximum response or the EC_{50} of TNF-mediated inhibition of [^{3}H]-NE release as compared to naïve rats, in the absence (max. response 24.12 ± 1.59%, NS; EC_{50}, 0.30 ± 0.13 ng/ml, NS) or presence of yohimbine (0.1 μM) *in vitro* (max. response 64.75 ± 1.86%, NS; EC_{50}, 0.28 ± 0.12 ng/ml, NS) (Fig. 2). The inhibition produced by TNF in the presence of yohimbine (0.1 μM) *in vitro* from hippocampal slices isolated from rats infused with aCSF was significantly increased (max. response, 64.75 ± 0.93%, $p < 0.05$; EC_{50}, 0.18 ± 0.12 ng/ml, NS) when compared to values obtained in the absence of *in vitro* yohimbine (max. response, 24.12 ± 1.59%; EC_{50}, 0.34 ± 0.14 ng/ml). Inhibition of [^{3}H]-NE release is depicted on the top half of graphs 3 and 4 since this represents the control, or normal physiologic response.

Field stimulation of hippocampal slices isolated from rats microinfused with rrTNF (icv, 1000 ng/24 hr at 0.5 μl/hr for 14 days) demonstrated a pronounced enhancement of the maximum inhibition of [^{3}H]-NE release by exogenous TNF in the superfusion system

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(70.29 ± 2.12%, $p < 0.05$), compared to rats microinfused with aCSF, with no change in the EC_{50} (0.22 ± 0.13 ng/ml, NS) (Fig. 2). In the presence of yohimbine (0.1 μM) *in vitro*, the maximum response was decreased to values similar to that seen with hippocampal slices isolated from rats microinfused with aCSF (25.37 ± 1.33%, NS from rats microinfused with aCSF; $p < 0.01$ compared to microinfusion of rrTNF alone; EC_{50}, 0.17 ± 0.14 ng/ml, NS from rats microinfused with aCSF) (Fig. 2).

The percentage of stimulation-evoked [^{3}H]-NE release from hippocampal slices in excess of spontaneous efflux at both 1 Hz and 4 Hz frequencies of stimulation is presented in Table 1. Field stimulation of hippocampal slices from rats microinfused icv with aCSF alone demonstrated a frequency-dependent increase in stimulation-evoked release of [^{3}H]-NE, similar to that which occurs in hippocampal slices from naïve rats. *In vitro* blockade of the α_2-adrenergic receptor with yohimbine (0.1 μM) significantly increased the fractional release of stimulation-evoked [^{3}H]-NE in a frequency dependent fashion, at both 1 Hz and 4 Hz, with an increase in the 4 Hz/1 Hz ratio, demonstrating a greater effect of the antagonist at the higher frequency of stimulation. Fourteen days of icv microinfusion with rrTNF (1000 ng/24 hr at 0.5 μl/hr) significantly decreased the fractional release of [^{3}H]-NE from hippocampal slices at both 1 Hz and 4 Hz, with a significant decrease in the 4 Hz/1 Hz ratio compared to rats microinfused with aCSF (Table 1). *In vitro* blockade of the α_2-adrenergic receptor with yohimbine (0.1 μM) significantly increased the fractional release of [^{3}H]-NE in a frequency-dependent fashion, more effective at the higher frequency of stimulation (Table 1).

Effect of a decrease in brain-derived TNF levels

After chronic administration of desipramine (10 mg/kg, i.p., twice daily for 14 days), the regulation of [^{3}H]-NE release by TNF *in vitro* is transformed from inhibition to facilitation of electrically-evoked release of [^{3}H]-NE (Ignatowski and Spengler, 1994). We have previously demonstrated that desipramine administration causes a significant decrease in TNF mRNA accumulation (approx. 18%; Nickola et al., 2001) as well as in the expression of TNF protein in neurons (Ignatowski et al., 1997). Collectively, these data support the hypothesis that a sustained decrease in TNF levels within the brain, due to continued desipramine administration, causes a transformation in the regulation of NE release by TNF. This is clearly demonstrated when hippocampal slices isolated from these rats are exposed to exogenous TNF in the superfusion system.

If the efficacy of desipramine as an antidepressant is due to a decrease in the levels of TNF in the brain, then, an increase in TNF levels induced by microinfusion, simultaneous with desipramine administration, should counteract, or oppose the decrease in TNF levels induced by desipramine, and therefore, prevent its efficacy. To test this hypothesis, rats were chronically administered desipramine (10 mg/kg, i.p., twice daily) and simultaneously microinfused with rrTNF (icv, 1000 ng/24 hr at 0.5 µl/hr for 14 days). Desipramine administration was started 24 hr after surgery and continued during the entire duration of icv microinfusion. After this duration, rats were decapitated, hippocampi isolated and sliced, and the regulation of NE release by exogenous TNF was analyzed.

As stated earlier, chronic administration of desipramine alone to rats (10 mg/kg, i.p., twice daily for 14 days) causes a transformation in presynaptic sensitivity to TNF (Ignatowski and Spengler, 1994). A similar transformation was evident in hippocampal

slices isolated from rats receiving aCSF microinfusion simultaneous with i.p. desipramine administration (Fig. 3). Interestingly, in agreement with our hypothesis, this transformation was prevented when rrTNF was microinfused (icv, 1000 ng/24 hr at 0.5 μl/hr for 14 days) simultaneous with chronic (i.p.) desipramine administration. In the same paradigm, but in the presence of yohimbine (0.1 μM) *in vitro*, TNF inhibition of NE release was similar to that observed with slices isolated from rats microinfused with aCSF alone, producing a maximum inhibition of 24.99 ± 3.26%, (NS compared to aCSF microinfused alone; $p < 0.05$ compared to rrTNF microinfused icv with simultaneous desipramine administration, i.p.). No change was observed in the EC_{50} value (0.25 ± 0.15 ng/ml, NS compared to hippocampal slices isolated from aCSF microinfused rats). In addition, TNF inhibition of NE release from hippocampal slices isolated from rats microinfused with rrTNF (icv, 1000 ng/24 hr at 0.5 μl/hr for 14 days) (max. response, 70.29 ± 2.12%; EC_{50}, 0.22 ± 0.13 ng/ml) (Fig. 2) did not differ from that obtained from slices isolated from rats microinfused with rrTNF with simultaneous chronic (i.p.) desipramine administration (max. response, 66.95 ± 2.60%; EC_{50}, 0.13 ± 0.05 ng/ml) (Fig. 3).

A significant increase in stimulation-evoked [^{3}H]-NE release at both 1 Hz and 4 Hz was evident in hippocampal slices isolated from rats chronically administered desipramine, with a significant decrease in the 4 Hz/1 Hz ratio (Table 1) compared to slices isolated from rats microinfused with aCSF. Following *in vitro* blockade of the α_2-adrenergic receptor with yohimbine (0.1μM), stimulation-evoked [^{3}H]-NE release was significantly increased at both 1 Hz and 4 Hz, with no change in the 4 Hz/1 Hz ratio. Fourteen days of icv microinfusion of rrTNF (1000 ng/24 hr at 0.5 μl/hr) along with chronic desipramine administration (i.p, 10 mg/kg, twice daily) to the same animal significantly decreased the

percentage of stimulation-evoked [^{3}H]-NE release from hippocampal slices at both 1 Hz and 4 Hz, with a significant increase in the 4 Hz/1 Hz ratio (Table 1) compared to hippocampal slices isolated from rats microinfused with aCSF alone. *In vitro* blockade of the α_2-adrenergic receptor with yohimbine (0.1 μM) significantly increased the percentage of [^{3}H]-NE released only at 4 Hz, with a significant increase in the 4 Hz/1 Hz ratio, as compared to rats receiving microinfusion of rrTNF with chronic desipramine administration (i.p.).

Levels of bioactive TNF in the brains of rats icv microinfused with rrTNF and administered i.p. desipramine were similar to those values observed in rats microinfused with rrTNF alone (data not shown).

Effect of decreasing the levels of TNF in the brain on presynaptic sensitivity

Results from our laboratory suggest that decreases in TNF generation in the hippocampus during chronic desipramine administration are associated with changes in α_2-adrenergic responses, such that activation of the α_2-adrenergic autoreceptor, along with neuron sensitivity to TNF, supports an increase in electrically-depolarized NE release from superfused hippocampal slices (Ignatowski and Spengler, 1994; Ignatowski et al., 1997). To establish this, we microinfused a pTNF-Ab into the right lateral cerebral ventricle of rats, to mimic the decrease in the levels of TNF observed during chronic desipramine administration. Presynaptic sensitivity to TNF was assessed following 14 days of continual icv microinfusion of pTNF-Ab. No change was observed in presynaptic sensitivity to TNF in hippocampal slices isolated from rats icv microinfused with aCSF-

containing normal rabbit serum for 14 days, compared to hippocampal slices isolated from rats icv microinfused with aCSF; therefore, data from these two groups were pooled (Fig. 4). Microinfusion of aCSF to rats produced no significant change in the maximum response or the EC_{50} value of TNF inhibition of [^{3}H]-NE release in the absence (max. response 21.88 ± 1.66%, NS; EC_{50}, 0.29 ± 0.11 ng/ml, NS) or presence of yohimbine (0.1 μM) *in vitro* (max. response 68.89 ± 0.99%, NS; EC_{50}, 0.18 ± 0.12 ng/ml, NS) (Fig. 4) as compared to naïve rats. However, similar to values obtained from naïve rats, inclusion of yohimbine (0.1 μM) *in vitro* did significantly ($p < 0.05$ for comparison of max. responses) enhance TNF-induced inhibition of NE release (Fig. 4). Similar to that which was observed following chronic desipramine administration, field stimulation of hippocampal slices isolated from rats icv microinfused with a pTNF-Ab demonstrated a transformation in presynaptic sensitivity to TNF, such that exogenous TNF concentration-dependently facilitated [^{3}H]-NE release (max. response 30.24 ± 9.03%, $p < 0.001$ compared to aCSF microinfused; EC_{50}, 0.32 ± 0.25 ng/ml, NS compared to aCSF microinfused) (Fig. 4). In the presence of yohimbine (0.1 μM) *in vitro*, the TNF concentration-effect curve was reversed back to inhibition of [^{3}H]-NE release, similar to that observed in hippocampal slices isolated from rats icv microinfused with aCSF, producing a maximum inhibition of 48.09 ± 6.46% (NS compared to slices isolated from aCSF microinfused rats), with no change in EC_{50} (0.23 ± 0.18 ng/ml, NS compared to slices isolated from aCSF microinfused rats) (Fig. 4).

The concentration of the TNF antibody (TNF-Ab) used for icv microinfusion is a pharmacological dose of 17 times a 1/30,000 dilution, based on Ab-induced blockade of the EC_{50} value (0.3 pg/ml) of rrTNFα in the WEHI bioassay. This concentration of TNF-

Ab reaches concentrations appropriate to block TNF in neighboring brain regions due to complete CSF turnover every 1-2 hours in the rat brain, such that the half-life of most solutes is 0.75 h (White et al., 1994). Systemic plasma levels of TNF were not altered by administration of TNF-Ab by this approach (data not shown). The levels of TNF in the cervical and thoraco-lumbar spinal cord homogenates were also not changed in rats microinfused with aCSF or with pTNF-Ab. This establishes that the effects of pTNF-Ab microinfusion into the brain are due to the blockade of TNF activity in neighboring brain regions of the right lateral cerebral ventricle. Bioactive levels of TNF (expressed as percent inhibition from rats infused with aCSF alone, including rats microinfused with aCSF-containing normal rabbit serum) in brain regions anatomically closest to the site of pTNF-Ab microinfusion were: hippocampus, 85.04 ± 2.80% inhibition; locus coeruleus, 58.29 ± 15.52% inhibition, hypothalamus, 44.10 ± 7.85% inhibition; parietal cortex, 29.53 ± 12.73% inhibition; caudate nucleus, 75.04 ± 10.18% inhibition; and amygdala, 45.62 ± 33.80% inhibition.

Fourteen days of icv microinfusion with pTNF-Ab (17 times 1/30,000 dilution) did not significantly change the percentage of stimulation-evoked [^{3}H]-NE release from hippocampal slices at both 1 Hz and 4 Hz, compared to hippocampal slices isolated from aCSF microinfused rats (Table 1). However, i*n vitro* blockade of the α_2-adrenergic receptor with yohimbine (0.1 μM) demonstrated a significant increase in the percentage of [^{3}H]-NE released at 4 Hz (Table 1).

Effect of increasing as well as decreasing TNF levels in the brain on the immobility time in the FST

The effect of continual icv microinfusion of rrTNF (1000 ng/24 hr at 0.5 μl/hr for 14 days) on the total duration of immobility in the FST is shown in Figure 5. Rats that were icv microinfused with rrTNF for 14 days demonstrated a significant increase in the duration of immobility in the 5 min FST (195.30 ± 1.45 sec, $p < 0.05$) compared to rats icv microinfused with aCSF alone (153.40 ± 3.26 sec).

The FST is an established behavioral model used to validate the efficacy of antidepressant drugs (Detke et al., 1995; Lucki et al., 1997). As demonstrated previously, rats chronically administered desipramine demonstrate a decrease in the time of immobility in the FST (Kawashima et al., 1986). Chronic desipramine administration (10 mg/kg, i.p., twice daily, 14 days) to rats, including animals icv microinfused with aCSF and administered desipramine i.p. (10 mg/kg, twice daily, 13 days), significantly decreased the time of immobility (93.00 ± 6.18 sec; $p < 0.01$) compared to rats icv microinfused with aCSF alone (153.40 ± 3.26 sec) (Fig. 5). Continual icv microinfusion of rrTNF with simultaneous i.p. desipramine administration (10 mg/kg, twice daily, 13 days) significantly increased the time of immobility (192.00 ± 2.65 sec, $p < 0.05$) compared to i.p. desipramine administration (93.00 ± 6.18 sec), thereby preventing the efficacy of desipramine established in this model. Continual icv microinfusion of pTNF-Ab significantly decreased the time of immobility (105.75 ± 5.95 sec, $p < 0.05$ compared to rats microinfused with aCSF) in the 5 min FST, mimicking the decreased time of immobility that is seen in rats chronically administered desipramine i.p. (93.00 ± 6.18 sec) (Fig. 5).

Discussion

The present study was designed to investigate the role of TNF in the mechanism of action of the antidepressant drug desipramine. There are numerous reports implicating this cytokine in the pathogenesis of depression and in the mechanism of action of drugs used to treat this disorder. Individuals with endogenous depression exhibit increased serum levels of TNF (Mikova et al., 2001), and volunteers administered TNF exhibit symptoms of depression (Mittelman et al., 1992; Reichenberg et al., 2001). Interestingly, one day of desipramine administration depletes neuron localized TNF mRNA and protein in brain regions that are implicated in the expression of mood (Ignatowski et al., 1997; Nickola et al., 2001), remaining decreased with continual administration (chronic). Although neuron-localized TNF protein and mRNA are attenuated following only one day of desipramine administration (Ignatowski and Spengler, 1994; Nickola et al., 2001), the therapeutic efficacy of desipramine is evident after chronic administration.

The other key target of antidepressant drug action is the α_2-adrenergic receptor. The density and sensitivity of α_2-adrenergic receptors are increased in clinically depressed patients (Callado et al., 1998; Garcia-Sevilla et al., 1999) and are decreased after chronic antidepressant administration (Crews and Smith, 1978; Svensson and Usdin, 1978; Smith et al., 1981). We hypothesize that homeostatic expression of TNF in neurons is pivotal to proper noradrenergic function, and therefore mood. We believe that desipramine-induced acute loss of TNF initiates alterations or transformations in α_2-adrenergic receptor number and/or sensitivity, which are completed with the continued loss of TNF, a time frame that corresponds with the efficacy of this drug.

In the present study, rats are administered desipramine chronically (10 mg/kg, i.p., twice daily for 13 days). While the data presented do not demonstrate a dose-effect relationship for desipramine, the dose employed produces reliable antidepressant effects in rats. During chronic administration of desipramine, rats exhibit a decrease in the rate of body weight gain, characteristic of desipramine administration (Fig. 1) (Willner et al., 1981; Nobrega and Coscina; 1987; Shen et al., 1999). However, icv microinfusion of a pTNF-Ab, which produces similar effects as desipramine, does not affect the rate of weight gain. It appears that this characteristic loss in weight gain induced by desipramine is not necessary for its efficacy as an antidepressant. The present data also demonstrate that icv microinfusion of rrTNF does not affect the rate of weight gained (Fig. 1).

Neuron exposure to TNF dictates their response to subsequent α_2-adrenergic receptor activation (Renauld et al., 2004), and vice-versa (Nickola et al., 2000). Therefore, subsequent to sustained loss of neuron-associated TNF with chronic desipramine administration (Ignatowski et al., 1997; Nickola et al., 2001) the dynamic equilibrium between TNF production and α_2-adrenergic receptor activation is altered. In addition, during the process of superfusion (washes away neuromodulators from neurons that no longer receive input from the cell bodies) this equilibrium may be temporarily modified. The addition of TNF *in vitro* during superfusion of isolated hippocampal slices may re-establish the dynamic equilibrium and the true response of the α_2-adrenergic receptor.

We hypothesize that the sustained decrease in TNF induced by chronic desipramine administration alters the G-protein repertoire, changing the response of the neurons to mediators acting on G-protein coupled receptors. A change in the availability of G-proteins, therefore, would allow the α_2-adrenergic receptor to couple with opposing

G-proteins, hence facilitating [^{3}H]-NE release *in vitro*. In fact, TNF-induced facilitation of [^{3}H]-NE release is switched back to inhibition by TNF when the α_2-adrenergic receptor is blocked with yohimbine (Fig. 3).

Following icv microinfusion of rrTNF with simultaneous i.p. desipramine administration, the release of [^{3}H]-NE decreases at both 1 Hz and 4 Hz (Table 1), opposite to that after desipramine administration alone. The present data also demonstrate that concomitant microinfusion of rrTNF with desipramine administration prevents the transformation in TNF-regulation of NE release, from inhibition to facilitation, which occurs during desipramine administration alone. Therefore, a decrease in TNF levels in the brain induced by chronic desipramine administration is necessary for subsequent alteration in the inhibitory function of the α_2-adrenergic receptor.

The decrease in the levels of TNF induced by desipramine was mimicked during icv microinfusion of a polyclonal antibody to TNF followed by examining the regulation of NE release by exogenous TNF (Fig. 4). In hippocampal slices isolated from these rats, presynaptic sensitivity to TNF is transformed such that TNF facilitates [^{3}H]-NE release, similar to rats chronically administered desipramine. Additionally, the restoration in TNF inhibition of NE release during α_2-adrenergic blockade *in vitro* demonstrates a functional association with α_2-adrenergic activation. However, the fractional release of [^{3}H]-NE is not affected (Table 1). Therefore, the increase in fractional release of [^{3}H]-NE induced by chronic desipramine administration may not be required for its efficacy as an antidepressant. Chronic zimelidine administration also transforms presynaptic sensitivity to TNF, but without affecting the fractional release of

[^{3}H]-NE (Nickola et al., 2001). The diverse effects on the fractional release of [^{3}H]-NE may also explain the unique characteristics among different antidepressant drugs.

If the transformation in presynaptic sensitivity to TNF is in fact due to desipramine-induced decreases in the levels of this cytokine in the brain, then rats icv microinfused with rrTNF (for 14 days) would display the opposite response as that to desipramine administration. Microinfusion (icv) of rrTNF enhances TNF-induced inhibition of [^{3}H]-NE release *in vitro* (Fig. 2). The attenuation in TNF-inhibition of [^{3}H]-NE release, with blockade of the α_2-adrenergic receptor *in vitro* suggests that increased TNF levels *in vivo* induce a more inhibitory conformation of the α_2-adrenergic receptor, possibly due to increased $G\alpha_i$- protein content (Scherzer et al., 1997; Hotta et al., 1999). This conditions presynaptic sensitivity to TNF, demonstrated by a net increase in inhibition of [^{3}H]-NE release by TNF *in vitro*.

The autoinhibitory role of the α_2-adrenergic receptor is evident upon examining the frequency-dependent release of [^{3}H]-NE from field stimulated tissue slices (Table 1). Alpha$_2$-adrenegic blockade demonstrates a greater effect on release at 4 Hz compared to 1 Hz stimulation. The amount of [^{3}H]-NE is increased in the vicinity of the α_2-adrenergic autoreceptor at the higher frequency, and consequently exerts elevated autoinhibition (Table 1) (Ignatowski and Spengler, 1994). The fractional release decreases at both frequencies in slices isolated from rats icv microinfused with rrTNF (Table 1) (Ignatowski et al., 1999). The 4 Hz/1 Hz ratio also decreases, indicating a greater decrease at the higher frequency. However, α_2-adrenergic blockade is evident only at 4 Hz. Therefore, this receptor remains functional following icv microinfusion of rrTNF, and the decrease in fractional release is not solely due to an increase in the α_2-adrenergic autoinhibitory

receptor. It is interesting to note that the decrease in [^{3}H]-NE release induced by rrTNF icv microinfusion mimics the chronic constriction injury (CCI) model of neuropathic pain, which involves an increase in the amount of TNF in the brain (Ignatowski et al., 1999; Covey et al., 2000). Furthermore, icv microinfusion of rrTNF enhances CCI-induced thermal hyperalgesia (physical manifestation of neuropathic pain) in injured rats, and induces thermal hyperalgesia in naïve animals (Ignatowski et al., 1999). Centrally mediated pain is an altered mood state associated with increased levels of TNF in the hippocampus and locus coeruleus (Ignatowski et al., 1999; Covey et al., 2000). Interestingly, pain is alleviated by antidepressants (Bryson and Wilde, 1996).

The efficacy of desipramine and/or the effect of an increase in the level of brain TNF were analyzed using the FST (Fig. 5). The FST is a behavioral model that assesses the efficacy of an antidepressant drug as a reduction in immobility (Detke et al., 1995). Chronic administration of desipramine (10 mg/kg, i.p., twice daily) significantly decreases the duration of immobility in the FST. Interestingly, icv microinfusion of a pTNF-Ab mimics chronic desipramine administration in the FST. Furthermore, TNFα knockout mice, similar to an antidepressant drug-like effect, exhibit a decrease in the time of immobility in the FST (Yamada et al., 2000). Collectively, these data strongly suggest that decreasing the levels of TNF in the brain is integral to antidepressant drug efficacy.

In contrast to rats administered desipramine or icv microinfused with pTNF-Ab, rats icv microinfused with rrTNF exhibit an increase in the duration of immobility. Interestingly, the decrease in immobility in rats chronically administered desipramine was prevented by simultaneous icv microinfusion of rrTNF with i.p. desipramine. It can,

therefore, be reasonably concluded that icv microinfusion of rrTNF opposes desipramine-induced decreases in neuron-associated TNF that are required to change presynaptic sensitivity to TNF and hence, elicit antidepressant efficacy. This is confirmed in rats that were icv microinfused with pTNF-Ab, which also mimics the effects of desipramine as seen in the FST**. T**hese data demonstrate that decreases in the levels of TNF are necessary, not only for desipramine-induced changes in presynaptic sensitivity, but also for the efficacy of this drug.

Given the commonalities observed in presynaptic sensitivity to TNF with desipramine and zimelidine (Ignatowski and Spengler, 1994; Nickola et al., 2001), antidepressant drugs may be effective, not because they are 'noradrenergic' or 'serotonergic' agents per se, but because they universally modify intra-neuron TNF expression, and the levels of this cytokine modulate the α_2-adrenergic receptor, both on serotonergic and noradrenergic neurons.

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Footnotes

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Figure Legends

Fig. 1. The effect of icv microinfusion of rrTNF (1000 ng/24 hr at 0.5µl/hr for 14 days), as well as chronic administration of desipramine to rats (10 mg/kg, i.p., twice daily for 13 days) on body weight. The data are expressed as the change in grams from original individual body weight. Each point represents the mean ± S.E.M. with the number of rats given in parentheses. Statistical significance for body weights was evaluated with ANOVA, followed by post hoc tests using Bonferroni multiple comparisons. Asterisks indicate groups that differed significantly from rats microinfused with aCSF alone at day 14, * $p < 0.05$. [DMI = rats administered desipramine]. Note: the 'DMI' group includes rats administered desipramine either alone or with simultaneous aCSF-microinfusion; these rats were pooled due to non-significant differences between the two groups.

Fig. 2. The effect of icv microinfusion of rrTNF (1000 ng/24 hr at 0.5µl/hr for 14 days) on the subsequent regulation of NE release from hippocampal slices by TNF *in vitro*. TNF concentration-effect curves for field stimulation (4 Hz) of isolated hippocampal slices obtained from rats icv microinfused for 14 days with aCSF, or from rats icv microinfused for 14 days with rrTNF, and the effect of (0.1 µM) yohimbine *in vitro*. Note: the 'aCSF microinfusion' group includes rats icv microinfused with aCSF alone and rats microinfused with aCSF containing heat-inactivated rrTNF; these rats were pooled due to non-significant differences between the two groups. Statistical significance for maximum responses was evaluated with ANOVA, followed by post hoc tests using Bonferroni multiple comparisons; * $p < 0.05$ compared to aCSF alone; ** $p < 0.01$

compared to rrTNF w/10^{-7}M yohimbine. Each point represents the mean ± S.E.M. with the number of rats given in parentheses.

Fig. 3. The effect of sustained high levels of TNF in the brain on subsequent regulation of NE release by TNF *in vitro*, both with and without i.p. antidepressant drug administration. TNF concentration-effect curves for field stimulation (4 Hz) of hippocampal slices isolated from rats icv microinfused for 14 days with aCSF, rats administered desipramine (10 mg/kg, i.p., twice daily) for 13 days (including aCSF microinfusion), and rats icv microinfused for 14 days with rrTNF along with administration of i.p. desipramine (13 days), as represented by solid lines. Dashed lines represent the effect of *in vitro* blockade of the α_2-adrenergic receptor with the specific antagonist yohimbine (0.1 μM) in respective paradigms. Note: the 'aCSF microinfusion' group includes rats icv microinfused with aCSF alone and rats microinfused with aCSF containing heat-inactivated rrTNF; these rats were pooled due to non-significant differences between the two groups. Similarly, the 'chronic desipramine' group includes rats administered i.p. desipramine alone, and rats icv microinfused with aCSF given simultaneous i.p administration of desipramine. Data were pooled together from these groups due to non-significant differences. Statistical significance for maximum responses was evaluated with ANOVA, followed by post hoc tests using Bonferroni multiple comparisons: * $p < 0.05$, ** $p < 0.001$ as compared to aCSF alone; *** $p < 0.05$, $^{\#}$ $p < 0.001$ as compared to rrTNF + i.p. desipramine. Each point represents the mean ± S.E.M. with the number of rats given in parentheses.

Fig. 4. The effect of sustained decreases in the levels of TNF in the brain on the subsequent regulation of NE release by TNF *in vitro*. TNF concentration-effect curves for field stimulation (4 Hz) of hippocampal slices isolated from rats icv microinfused for 14 days with aCSF or with pTNF-Ab, as represented by solid lines; and the effect of *in vitro* blockade of the α_2-adrenergic receptor with the specific antagonist yohimbine (0.1 μM), as represented by dashed lines. Note: the 'aCSF microinfusion' group includes rats icv microinfused with aCSF alone and rats microinfused with aCSF containing normal rabbit serum, the control for pTNF-Ab; these rats were pooled due to non-significant differences between the two groups. Statistical significance for maximum responses was evaluated with ANOVA, followed by post hoc tests using Bonferroni multiple comparisons: * $p < 0.05$, ** $p < 0.001$ as compared to aCSF alone; # $p < 0.001$ as compared to pTNF-Ab alone. Each point represents the mean ± S.E.M. with the number of rats given in parentheses.

Fig. 5. The effect of increasing as well as decreasing the levels of TNF in the brain on the duration of immobility in the FST. Following 14 days of icv microinfusion of rrTNF (1000 ng/24 hr at 0.5 μl/hr), rrTNF along with i.p. desipramine for 13 days (10 mg/kg, twice daily) (rrTNF w/i.p. DMI), pTNF-Ab, or chronic i.p. desipramine (13 days, DMI) administration, the time of immobility for each paradigm was scored in the 5 min test. Note: the 'chronic desipramine' group includes rats administered i.p. desipramine only and rats microinfused icv with aCSF and simultaneously administered desipramine i.p.; these rats were pooled due to non-significant differences between the two groups. Statistical significance for swim tests was evaluated with Kruskal-Wallis one way

analysis of variance on Ranks, followed by post hoc tests using Bonferroni multiple comparisons. Each value represents the mean ± S.E.M. with the number of rats given in parentheses. Asterisks indicate groups that differed significantly from rats microinfused with aCSF alone, * $p < 0.05$; # represents groups significantly different from chronic i.p. desipramine administered rats, # $p < 0.05$.

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TABLE 1. Stimulation-evoked (1 Hz or 4 Hz) [^{3}H]-NE release from hippocampal slices isolated from rats icv microinfused with aCSF, rrTNF, rrTNF along with i.p. desipramine, or pTNF-Ab and the effect of *in vitro* yohimbine (0.1 µM). The data are expressed as the percent release of the total [^{3}H] pool in the tissue at the time of stimulation in excess of spontaneous efflux. Each value represents the mean ± S.E.M (number of determinations indicated in parentheses). Statistical significance was evaluated with ANOVA, followed by post hoc tests using Bonferroni multiple comparisons. Asterisks indicate groups that differed significantly from rats microinfused with aCSF alone, * $p < 0.05$; # represents groups significantly different from own experimental paradigm, in the absence of yohimbine: # $p < 0.05$. [DMI = desipramine; yoh = yohimbine]

	1 Hz	4 Hz	4 Hz/1 Hz
aCSF (5)	1.11 ± 0.16	2.52 ± 0.4	2.32 ± 0.26
aCSF w/yoh (5)	2.01 ± 0.27 *	6.15 ± 0.21 *	2.92 ± 0.23 *
rrTNF (3)	0.57 ± 0.05 *	1.04 ± 0.02 *	1.84 ± 0.12 *
rrTNF w/yoh (3)	0.61 ± 0.23	2.79 ± 0.05 #	5.95 ± 0.91 #
Chronic DMI (4)	2.97 ± 0.07 *	4.98 ± 0.37 *	1.56 ± 0.08 *
Chronic DMI w/yoh (4)	6.56 ± 0.38 #	10.67 ± 0.47 #	1.68 ± 0.15
rrTNF + DMI (3)	0.75 ± 0.37 *	1.59 ± 0.32 *	2.83 ± 0.57 *
rrTNF +DMI w/yoh (3)	1.07 ± 0.37	2.72 ± 1.35 #	3.69 ± 0.89 #
pTNF-Ab (4)	0.84 ± 0.57	2.5 ± 0.89	3.32 ± 0.99
pTNF-Ab w/yoh (4)	1.31 ± 0.95	4.65 ± 1.96 #	4.92 ± 1.11

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Figure 1

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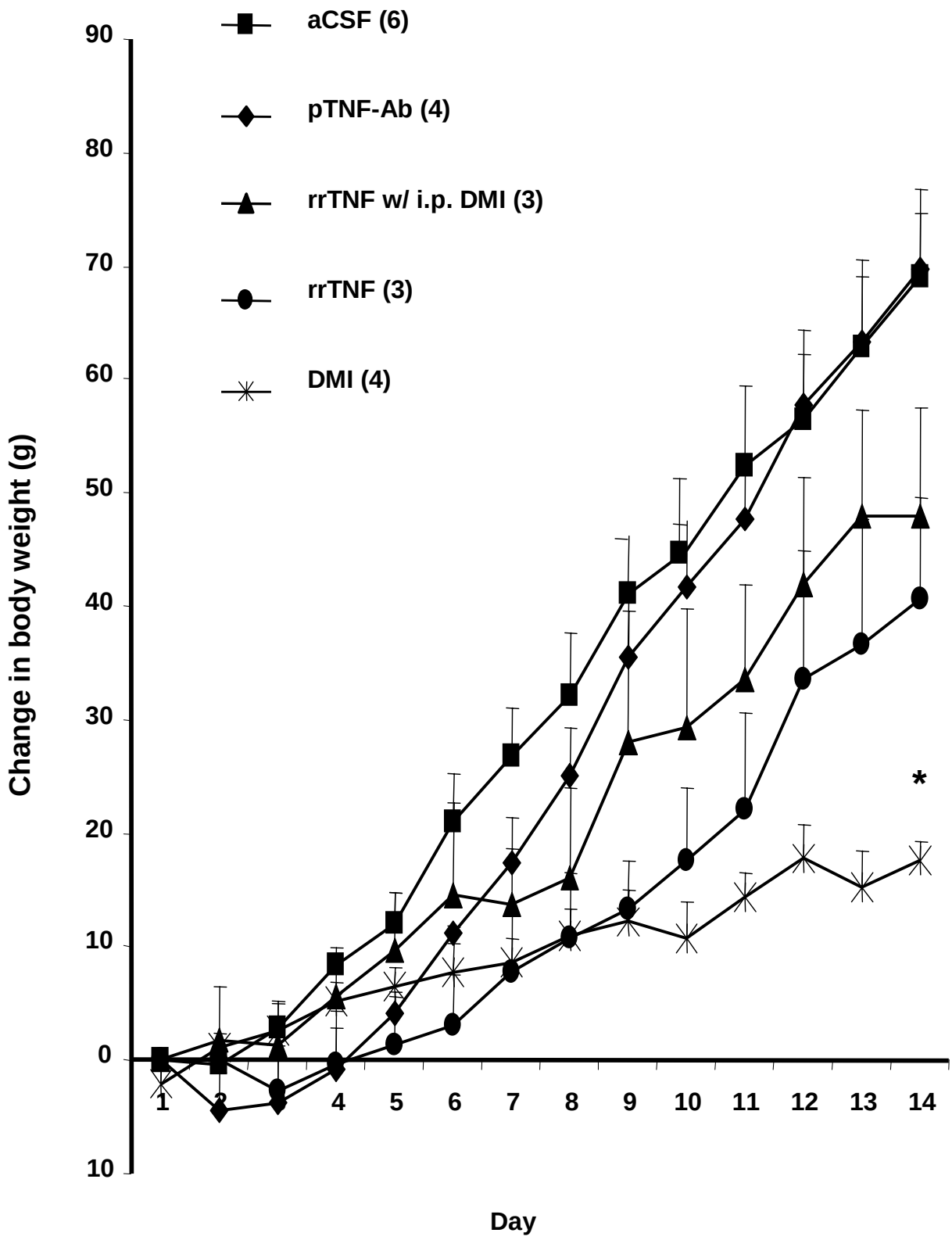

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Figure 2

JPET #67835

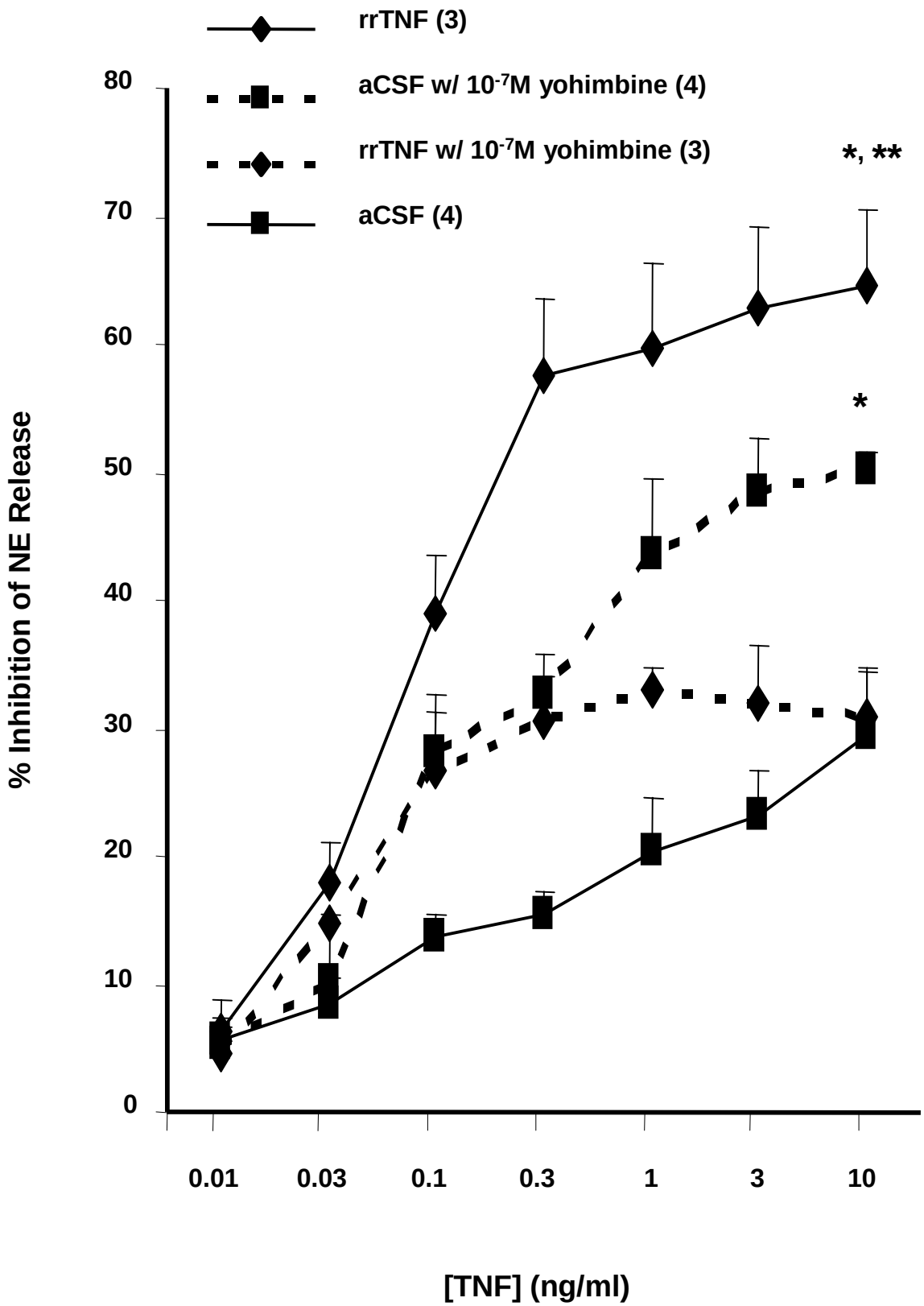

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Figure 3

JPET #67835

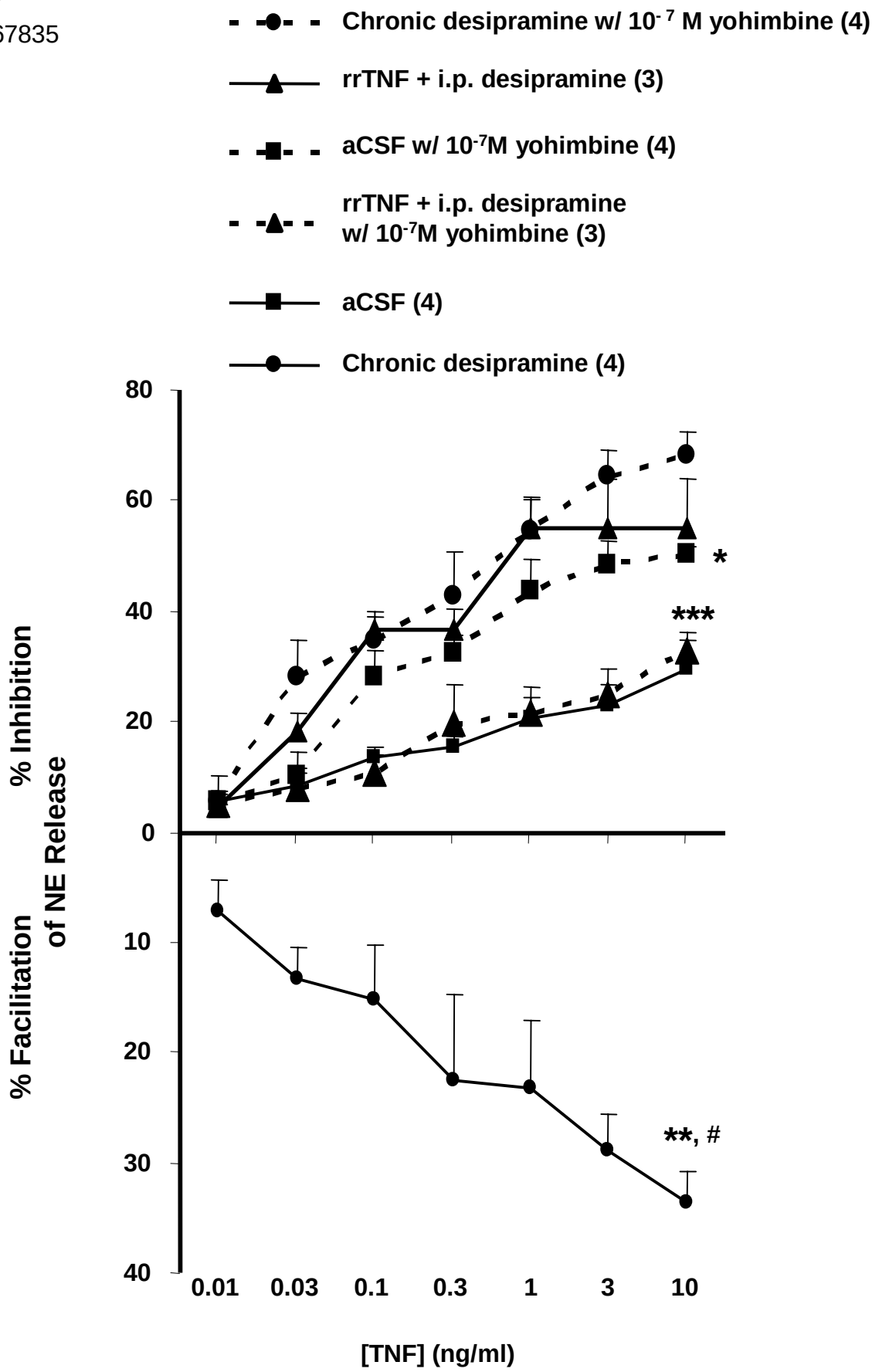

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Figure 4

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