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Lipopolysaccharide-induced Tau Phosphorylation and Kinase Activity: Modulation, but not Mediation, by Corticotropin-Releasing Factor Receptors

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Abstract

Clinical studies suggest that exposure to stress can increase risk for Alzheimer's disease (AD). Though the precise links between stress and vulnerability to develop AD remain unsettled, recent animal work suggests that stress may promote susceptibility to AD pathology by activating tau kinases and inducing tau phosphorylation (tau-P). Our previous findings indicate differential involvement of corticotropin-releasing factor receptors (CRFR1 and 2) in regulating tau-P in the hippocampus induced by acute restraint, an emotional stressor. To assess the generality of CRFR involvement in stress-induced tau-P and tau kinase activity, the present study extends our investigation to a well-characterized *physiological* stressor: immune challenge induced by bacterial lipopolysaccharide (LPS). Acute systemic administration of LPS (100 µg/kg) robustly increased hippocampal (but not isocortical or cerebellar) tau-P, peaking at 40–120 min post-injection and abating thereafter. Assessments of the genotype dependence of this effect yielded results distinct from the restraint model. Treatment with LPS increased phosphorylation in wild type, single and double CRFR knockouts with only subtle variation, which included a reliable exaggeration of tau-P responses in CRFR1-deficient mice. Parallel analyses implicate glycogen synthase kinase-3 and cyclin-dependent kinase 5 as likely cellular mediators of LPS-induced tau-P. Conversely, our data suggest that temperature-dependent fluctuations in tau phosphatase (PP2A) may not play a role in this context. Thus, neither the strict CRFR1-dependence of restraint-induced tau-P, nor the exaggeration of these responses in CRFR2 null mice, generalize to the LPS model. CRFR mediation of stress-induced hippocampal tau-P may be limited to emotional stressors.

Keywords

Alzheimer's disease; LPS; lipopolysaccharide; corticotropin-releasing factor; corticotropin-releasing; hippocampus; stress; tau phosphorylation; immunohistochemistry; western blot; telemetry

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive dementia and is pathologically defined by the presence of β -amyloid plaques and neurofibrillary tangles (NFTs). NFTs are

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insoluble intracellular inclusions composed of aggregated, hyperphosphorylated forms of the cytoskeletal protein, tau, which accumulate initially in the entorhinal cortex and CA1 hippocampal field in AD (Braak & Braak, 1991). Tau is a soluble protein integral for the stabilization of microtubule networks, and exists in multiple isoforms produced from a single gene by alternative mRNA splicing (Goedert *et al.*, 1989). The role of tau in normal cellular function and AD neuropathology is critically regulated by its capacity to be phosphorylated at multiple sites. A leading hypothesis holds that phosphorylation promotes detachment of tau from microtubules, destabilizing these cytoskeletal polymers and compromising axonal transport, leading to impaired neuronal function and/or viability (Stamer *et al.*, 2002; Roy *et al.*, 2005; Chevalier-Larsen & Holzbaur, 2006; Dixit *et al.*, 2008). Phosphorylated tau exhibits reduced ability to bind and stabilize microtubules, and can self-aggregate to form insoluble paired helical filaments (PHFs), bundles of which comprise NFTs. Severity of tangle pathology is positively correlated with cognitive deficit and neuronal loss in AD (Arriagada *et al.*, 1992; Gomez-Isla *et al.*, 1997), and the discovery that mutations in the tau gene underlie autosomal dominant forms of frontotemporal dementia suggests that pathological changes in tau are sufficient to produce neurodegeneration and cognitive impairment (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998).

Clinical data suggest that chronic stress exposure and stress-related psychiatric disorders can increase risk for developing AD (Wilson *et al.*, 2003; Wilson *et al.*, 2005). In support of this relationship, studies of transgenic mouse models demonstrate increased basal activity of stress-related tau kinases, increased A β production and plaque load, and deficits in hippocampal cell proliferation and contextual memory with stress exposure (Savage *et al.*, 2002; Dong *et al.*, 2004; Kang *et al.*, 2007). Furthermore, a host of investigations have documented that exposure to a variety of acute physiological stressors can activate tau kinases and induce tau phosphorylation in rodents (reviewed in (Rissman, 2009)). Our previous data demonstrate that acute physical restraint, a prototypic emotional stressor, can also dramatically activate tau kinases and hippocampal tau phosphorylation (tau-P), and that this effect is differentially dependent on the integrity of the two known receptors for corticotropin-releasing factor (CRF; (Rissman *et al.*, 2007), a major stress-related signaling system in brain and pituitary. Thus, while restraint-induced tau-P did not depend on stress-induced alterations in glucocorticoid secretion, it was blocked by genetic or pharmacologic disruption of signaling through the type 1 CRF receptor (CRFR1), and exaggerated in CRFR2-deficient mice. In the present study, we investigated whether this manner of regulation may generalize to an important physiological stressor: immune challenge induced by systemic injection of bacterial lipopolysaccharide (LPS). Widely used to model systemic infection or sepsis, the LPS challenge paradigm is of particular interest, as it presents a generalized inflammatory stimulus to the brain, characterized by microglial activation and pro-inflammatory cytokine and prostanoid production (Elmqvist *et al.*, 1997; Schiltz & Sawchenko, 2002). Chronic treatment with LPS can augment tau phosphorylation and pathology in murine models of AD and other tauopathies (Bhaskar *et al.*; Li *et al.*, 2003; Kitazawa *et al.*, 2005; Kitazawa *et al.*, 2008; Lee *et al.*, 2010).

MATERIALS AND METHODS

Animals

CRFR knockout mice (*CRFR1*^{-/-}, *CRFR2*^{-/-} or mice lacking both receptors, DBL) and littermate wild-type (*wt*) controls were bred from heterozygote breeder pairs of established lines (Smith *et al.*, 1998; Bale *et al.*, 2000) backcrossed to founder mice to achieve a pure C57BL/6 background. Genotype was determined by PCR, and males were used for experimentation at 15–22 weeks of age. Pregnant females used for generating CRFR1-deficient mice received drinking water supplemented with corticosterone (10 μ g/ml; Sigma-

Aldrich, St. Louis, MO) from embryonic day 12 to postnatal day 14 to prevent early mortality as a result of pulmonary dysplasia (Smith *et al.*, 1998). Because *CRFRI*^{-/-} mice exhibit adrenal cortical agenesis, experimental animals were reinstated on corticosterone for 21 d before testing to allow the normal nocturnal bias in appetitive behavior to approximate the circadian fluctuation in circulating hormone levels. To assess effectiveness of the replacement regimen, plasma corticosterone levels were determined by RIA from blood samples collected when the mice were killed (Radley *et al.*, 2009). The UCSD and Salk Institute Institutional Animal Care and Use Committees approved all experimental protocols.

LPS treatment

To assess the time course of LPS-induced tau-P, 12 week-old C57BL/6 male *wt* mice (Jackson Labs, Bar Harbor, ME) were adapted to daily handling and exposed to a single intraperitoneal (ip) injection of LPS (100 µg/kg) or no stress (NS) (n=3 each). The LPS amount given is considered a moderately high dose that is within the range commonly used to characterize CNS responses to proinflammatory challenge (e.g., (Elmqvist *et al.*, 1996), but lower than that at which indications of sepsis are encountered (Quan *et al.*, 1999). Animals were killed at various intervals ranging from immediately (0 min) to 240 min after injection. For CRFR regulation experiments, groups of *wt*, *CRFRI*^{-/-}, *CRFR2*^{-/-} or DBL (n=3 each) adapted to handling were exposed to a single ip injection of LPS (100 µg/kg in saline) and killed 40 min post injection. Separate controls for ip injection (vehicle-injected) were included.

Western blot analysis

Unanesthetized animals were killed by cervical dislocation, decapitated, and hippocampus, cortex and cerebellum were rapidly dissected and frozen. Tissue homogenates were prepared with a Dounce homogenizer using cold radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.4, 0.1%SDS, 1% NP40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM Na₃VO₄ and 1µM okadaic acid), as described previously (Rissman *et al.*, 2007). Before homogenization protease inhibitors were added (Pierce Biotechnology, Rockford IL). The samples were then centrifuged twice at 16,000 × g at 4°C for 30 minutes and the supernatant was collected. Protein concentrations were determined using BCA Protein Assay Kit (Pierce). Proteins were then heated in 4x LDS sample buffer (Invitrogen, Carlsbad, CA) at 70°C for 10 minutes. Ten micrograms of protein was then separated by 10% SDS-PAGE, transferred to PVDF membrane (Immobilon PVDF, Millipore). Blots were blocked for 30 min in 5% milk in TBST before primary antibodies were added and incubated at 4°C overnight. Primary antibodies were detected using either anti-mouse or anti-rabbit horseradish peroxidase secondary antibodies (1:1000 EMD Biosciences, La Jolla, CA) for one hour in 5% milk in TBST and developed with an enhanced chemiluminescence Western Blot detection kit (Pierce). Background subtraction was performed, and quantitative band intensity readings relative to β-actin controls were obtained using NIH Image J software (v. 1.44).

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde as described previously (Rissman *et al.*, 2007). 30 µm-thick frozen sections were cut on a sliding microtome and stored at -20°C in cryoprotectant solution (20% glycerol and 30% ethylene glycol in 0.1 M phosphate buffer). Antibodies against tau phosphorylated at the AT8 and PHF-1 sites were used to detect tau-P in free-floating sections containing hippocampus using reagents in a Mouse-on-Mouse Immunodetection Kit (Vector Laboratories, Burlingame, CA) to avoid detection of endogenous mouse IgG. Endogenous peroxidase was quenched with 0.3% hydrogen

peroxide, followed by 1% sodium borohydride to reduce free aldehydes. Reaction product was developed using a nickel-enhanced glucose oxidase method (Shu *et al.*, 1988).

Antibodies

Well-characterized antibodies against tau phosphorylated at specific sites, antibodies that record the activation state of tau kinases and phosphatases, and antisera against microglial and inflammatory markers were used. Details of each are provided in Table 1. Specificity of phospho-specific antibodies in mouse tissue was confirmed by pretreating sections from LPS-treated mice with alkaline phosphatase (40 mg/ml; Sigma-Aldrich), which eliminated detectable labeling (data not shown).

Telemetry

Under isoflurane anesthesia, a separate group of *wt* mice ($n=7$) was implanted intra-abdominally with telemetry transmitters (Mini-Mitter). Two days later, core body temperature was recorded continuously for 24 hr before and after ip injection of LPS, as above. Transmitter output was monitored by a receiver board placed beneath the animals' cages, which recorded core temperature to $\pm 0.1^\circ\text{C}$.

Statistical analyses

Optical density readings from Western blots were analyzed using either one- or two-way (genotype $\times$ injection) ANOVA using Prism 4 software (GraphPad, San Diego, CA). Data were plotted on histograms, and expressed as mean $\pm$ SEM percentage of control values.

RESULTS

Time course and regional specificity of LPS-induced tau-P

We first asked whether and over what time frame tau-P may be induced in response to an immune/inflammatory challenge, LPS treatment. Western analysis was used to examine tau-P at several AD-relevant N- and C-terminal sites (S^{202}/T^{205} (AT8), $S^{396}/404$ (PHF-1), and S^{422}) in hippocampal, cortex and cerebellar extracts from C57BL/6 mice killed at various intervals after a single injection of LPS (100 $\mu\text{g}/\text{kg}$, ip). In the hippocampus, relative to non stressed (NS) values, all sites exhibited significant (6–8 fold) increases in tau-P that were apparent at 20 min and were sustained through 120 min (AT8, S^{422}) or 240 min (PHF-1; see Fig. 1). Statistical analyses revealed a significant increase in tau-P with LPS treatment at the AT8 site (one way ANOVA, $F_{8,26} = 50.02$, $P < 0.0001$), the S^{422} site (one way ANOVA, $F_{8,26} = 47.41$, $P = 0.006$) and the PHF-1 site (one way ANOVA, $F_{8,26} = 52.36$, $P < 0.0001$). Posthoc analyses revealed that with the exception of the PHF-1 site, tau-P levels at the AT8 and S^{422} were reduced to levels that were not significantly different from those of unstressed controls by 180 min (PHF-1: $p < 0.01$, all others $p > 0.18$). Tau P levels immediately after the stressor (0 mins) were not significantly different from unstressed levels at the AT8 or S^{422} ($p = 0.23$), but was significantly increased at the PHF-1 site ($p = 0.006$). In the cortex and cerebellum, we observed consistently low levels of tau-P for all epitopes after LPS, and statistical analyses revealed no significant change in tau-P with LPS treatment (one way ANOVA, $F_{8,26} = 57.01$, $P = 0.36$). Increments in hippocampal tau-P were quite stable over 20–120 min after LPS for all epitopes, and the 40 min time point was selected for subsequent analyses.

Localization of tau-P and inflammatory responses

Groups of *wt* mice were injected with saline or LPS ($n=3$) as above, and perfused 1–3 hr later to allow immunolocalization of tau-P and inflammatory markers. Weak labeling for AT8-phosphorylated tau was detectable under control conditions primarily in hilar neurons

of the dentate gyrus and CA3 pyramidal neurons (Fig. 2). Also stained were scattered neurons in deeper portions of the granule cell layer of the dentate gyrus and presumed interneurons in the strata oriens and lacunosum-moleculare of Ammon's Horn. Labeling of the CA1 pyramidal layer was equivocal. LPS treatment provoked enhanced cellular labeling at each of these loci that was apparent at 1 hr, and persisted through 3 hrs after injection. Basal and stimulated PHF-1 staining displayed an essentially similar distribution and time course (data not shown).

Antisera against cyclooxygenase 2 (COX-2; a key enzyme in inducible prostanoid synthesis) and Iba-1 (a marker of microglia, the principal intrinsic immune effector cell type of the CNS) were used in the same material to confirm and characterize the inflammatory stimulus presented by LPS (Fig. 2). Iba-1-stained microglia displayed a progressive shortening and thickening of processes, characteristic of their activation, over the 3 hr post-injection period examined. COX-2 staining of cells exhibiting microglial morphology was observed under basal conditions, and increased in intensity at 2–3 hrs after treatment. The most salient effect of LPS injection of COX-2-immunoreactivity was an induction, again at 2–3 hr, in round and polygonal profiles associated with the vasculature, and shown previously to correspond to the nuclear regions of endothelial cells and perivascular macrophages, respectively (Schiltz & Sawchenko, 2002).

CRFR involvement in LPS-induced tau-P

As LPS can induce tau-P at AD-relevant epitopes in the hippocampus in a similar manner as seen following restraint stress, we next asked whether the differential CRFR involvement we have documented in restraint-induced tau-P extends to a “physiological” stressor, LPS. Mice deficient in CRFR1, CRFR2 or both receptors were sacrificed 40 min after treatment with LPS. Because our time course data reliably identified LPS-induced tau-P only in the hippocampus among regions examined, analyses were restricted to this region. LPS treatment resulted in enhanced hippocampal phosphorylation at the AT8 and PHF-1 sites in *wt*, single and DBL CRFR knockouts with significant variation (PHF-1, one way ANOVA, $F_{7,22} = 32.05$, $P=0.006$; AT8, one way ANOVA, $F_{7,23} = 57.04$, $P<0.0001$). Posthoc analyses revealed statistically significant enhancement of tau-P responses at the PHF-1 and AT8 sites in CRFR1-deficient mice ($P<0.001$, each), relative to *wt* and CRFR2 or DBL knockout groups. Although significant increase in tau-P was observed in *wt*, single and DBL CRFR knockouts with LPS treatment at the S⁴²² locus (one way ANOVA, $F_{7,23} = 47.41$, $P=0.001$), posthoc analyses revealed no statistically significant differences were seen in response between genotypes (all $P>0.20$). Phosphorylation responses at the N-terminal epitope, AT8, were quite variable in the hippocampus of CRFR2 and DBL mice, though means of these groups did not differ reliably from those of *wt* controls (Fig 3). Overall, these findings raise the intriguing possibility that CRFR mediation of stress-induced tau-P may be limited to emotional insults.

Activation of specific tau kinases in LPS treated mice

To identify potential mediators of LPS-induced tau-P, we applied antibodies specific to active and inactive states of kinases implicated in tau-P to the same hippocampal extracts used in the preceding analyses (Fig. 4). Of the four kinase mechanisms examined, the most robust effects of LPS were seen on phosphorylation (activation) of glycogen synthase kinase-3 (GSK-3) isoforms and on levels of the cyclin-dependent kinase-5 (CDK5) activator proteins, p25 and p35. Variations in these effects as a function of CRFR status were relatively subtle, with one notable exception.

Statistical analyses revealed that, relative to *wt* controls, LPS-induced phosphorylation of the active (pY²¹⁶) forms of GSK-3 α and β tended to be marginally (20–25%), though

significantly, greater in each of the CRFR mutant groups (GSK-3 α pY²¹⁶ one way ANOVA, $F_{7,23} = 51.63$, $P < 0.0001$; GSK-3 β pY²¹⁶ one way ANOVA, $F_{7,23} = 124.80$, $P < 0.0001$). Posthoc analyses identified that tau-P responses in CRFR mutants were significantly greater than *wt* animals (all $P < 0.05$ except the *wt* versus *CRFR2*^{-/-} comparison for GSK3 α , $P = 0.15$). We also observed significant alterations in the *inactive* (pS⁹) form of GSK-3 α and β (one way ANOVA, $F_{7,23} = 66.87$, $P < 0.0001$; one way ANOVA, $F_{7,23} = 47.29$, $P < 0.0001$, respectively). Interestingly, posthoc analyses revealed that the stimulated increase in phosphorylation of CRFR1 knockouts greatly exceeded the modest responses observed in all other genotypes (all $P < 0.001$). No such variation was noted for the pS⁹ form of GSK-3 β , which displayed small and consistent LPS-induced increments across groups.

Among other kinases, levels of CDK5 activator protein, p35, were markedly and comparably increased after LPS treatment in each of the genotypes examined (one way ANOVA, $F_{7,23} = 2104$, $P < 0.0001$). Lesser, though still highly reliable, upregulation of the truncated p35 product, p25, was also observed in all groups (one way ANOVA, $F_{7,23} = 66.87$, $P < 0.0001$), with double CRFR knockouts displaying a reduced response, relative to *wt* controls ($P < 0.001$). The pT¹⁸³/Y¹⁸⁵ forms of the 46 and 54 kDa *c-Jun* N-terminal protein kinases (JNK46/54) were unresponsive to LPS (JNK 46, one way ANOVA, $F_{7,23} = 56.71$, $P = 0.32$; JNK 56 one way ANOVA, $F_{7,23} = 36.87$, $P = 0.29$), as was the activated (pT²⁰²) form of the 44 kDa mitogen-activated protein kinase, ERK1 (one way ANOVA, $F_{7,23} = 49.70$, $P = 0.54$). Although ERK2 (pY²⁰⁴) displayed variable upregulation in *wt* animals, CRFR mutants were unresponsive, and we observed no significant changes with LPS treatment (one way ANOVA, $F_{7,23} = 24.17$, $P = 0.15$).

The time courses of LPS effects on GSK-3, CDK5-p25 and ERK2 were similar to those shown in Figure 1 for stress-induced tau-P (data not shown). We also observed no significant changes in total levels of GSK-3, CDK-5 or JNK in *wt* or CRFR mutant mice in any brain region (data not shown).

Involvement of phosphatases?

Phosphatases participate along with kinases in regulating tau-P in a push-pull manner, with PP2A acknowledged as the dominant tau phosphatase (Goedert *et al.*, 1992; Wang *et al.*, 1996; Benneicib *et al.*, 2000; Gong *et al.*, 2000; Planel *et al.*, 2001; Planel *et al.*, 2004). Because reduced PP2A activity associated with decreased core body temperature has been implicated in stress-induced increments in tau-P, (Planel *et al.*, 2001), we probed for alterations in levels of the catalytic subunit of PP2A (PP2A-c) over 4 hrs after LPS treatment, and observed no reliable fluctuations, relative to controls (Fig. 5). A separate group ($n=7$) of mice implanted with telemetry transducers were subjected to the same LPS challenge and displayed a monophasic, $\sim 2^{\circ}\text{C}$ rise in core temperature (Rudaya *et al.*, 2005), without the early hypothermic component characteristically seen in some species' (e.g. rats') responses to LPS (Serrats *et al.*, 2010). These findings fail to support a major involvement of a hypothermia-dependent mechanism in LPS-stimulated increases in tau-P.

DISCUSSION

The present findings extend the range of acute stressors capable of inducing hippocampal tau-P to include the prototypic immune/inflammatory insult presented by systemic LPS administration. This response shows at least partial overlap with those elicited by other stressors, notably restraint, in its capacity to phosphorylate AD-relevant epitopes, the cellular distribution of phosphorylation responses and its association with activation of specific tau kinases. It does not, however, share a strict dependence on the integrity of CRFR1 for its expression, as seen with restraint; CRFR signaling appears to play, at most, a modulatory role in LPS-induced tau-P.

Relationship to Previous Studies

Initial studies describing the stress-induced tau-P phenomenon employed rather extreme insults, such as starvation, heat shock or swimming in ice-cold water (reviewed in (Rissman, 2009). These challenges are considered “physiological” stressors, which are distinguished from the category of emotional stressors based on the nature of the sensory input that registers the challenge, the extent to which affective responses are invoked, and the global pattern of activational responses induced within the brain (Sawchenko *et al.*, 1996; Herman & Cullinan, 1997; Sawchenko *et al.*, 2000; Dayas *et al.*, 2001). Our previous work demonstrating that a representative emotional stressor, restraint, was capable of robustly inducing tau-P, indicated a potential relevance of the phenomenon to daily life stresses experienced by humans, and resonated with epidemiological work associating increased stress exposure and/or sensitivity with a greater propensity to develop AD (Wilson *et al.*, 2003; Wilson *et al.*, 2006; Wilson *et al.*, 2007). However, the fact that acute stress-induced tau-P is consistently reported to be a transient phenomenon (reviewed in (Rissman, 2009), has left open stern questions as to its pathological relevance. Potential insight into this issue came with the finding that daily restraint exposure repeated over 14 consecutive days gives rise to cumulative and persistent increases in tau-P, a portion of which is sequestered in an insoluble, and potentially pre-pathogenic, form (Rissman *et al.*, 2007). Importantly, effects of both acute and repeated restraint stress on hippocampal tau-P are blocked by genetic or pharmacologic disruption of CRFR1 signaling, and exaggerated in CRFR2-deficient mice (Rissman *et al.*, 2007). Moreover, DBL mice display effects like those of CRFR1 mutants, suggesting CRFR1 mediation of the phenomenon, downstream of a CRFR2-dependent restraining influence.

The present study sought to extend this analysis to acute LPS injection, a potent physiological stressor that presents a generalized inflammatory stimulus to brain. Interest in this particular model is enhanced by the finding that chronic (i.e., intermittently repeated) LPS treatment exacerbates tau pathology in a transgenic murine AD model (Kitazawa *et al.*, 2005). We found rapid and robust LPS-induced hippocampal tau-P at two of three AD-relevant epitopes tested (PHF-1, AT8), which is somewhat unexpected in light of immediate-early gene-based functional mapping studies that do not identify the hippocampus as a prominent site of LPS-induced cellular activation (Sagar *et al.*, 1995; Elmquist *et al.*, 1996). Nevertheless, repeated LPS treatment has been shown to increase tau-P at the AT8 and AT180 (T²³¹/S²³⁵), but not the PHF-1, sites, and exacerbate tau pathology, in a mouse AD model, with the tau kinase CDK5 (but not GSK-3 or JNKs) implicated as mediating the effect (Kitazawa *et al.*, 2005). Our data from an acute LPS model are partially consonant with these results, though we do not find tau-P to be restricted to N-terminal epitopes, and do not find a singular association with enhanced CDK5 activity. These differences may be attributable to the use of acute versus repeated LPS treatment paradigms, as recent data demonstrate that modulation of CDK5 can influence tau-P at PHF-1 loci (Piedrahita *et al.*, 2010). Although we did not find increased tau-P at the S⁴²² site, this epitope has been found to be only marginally responsive to other stressors (Rissman *et al.*, 2007), and the two representative N- and C-terminal sites we probed (AT8 and PHF-1) responded robustly in all genotypes tested.

Cellular Mediators

Acute LPS-induced increases in hippocampal tau-P were observed in *wt* mice as well as mice deficient in either or both CRFRs. Phosphorylation responses at the AT8 site were quite variable among individual subjects in the CRFR2 and double knockout groups (Fig. 3). The basis for this variability is not clear, though it is not likely to be explained by differences in such factors as treatment efficacy or extract integrity, as other measures of tau-P and kinase status derived from the same extracts displayed no marked inconsistencies.

The sole reliable variation in LPS-induced tau-P observed across genotypes was an enhancement of this response in CRFR1 null mice, relative to *wt* controls.

Parallel monitoring of the activation state of tau kinases was used to identify candidate cellular effectors of LPS-induced tau-P (Fig. 4). Implicated factors include the different GSK isoforms, GSK-3 α and β , which are both capable of phosphorylating tau at multiple sites (Liu *et al.*, 2003). The activated (pY²¹⁶) forms of these factors were prominently increased in LPS-challenged mice, with all CRFR-deficient groups displaying somewhat greater responses than *wt* animals. Generally more muted were tendencies for enhanced phosphorylation at the S⁹ GSK-3 site associated with inhibition of kinase activity (Cohen & Frame, 2001), though this locus of GSK3 α displayed a very prominent and selective response. Similarly, while levels of CDK5, did not vary with treatment status, its activator proteins, p25 and (particularly) p35 (Patrick *et al.*, 1999), were comparably upregulated by LPS across genotypes. While implicating CDK5 and GSK-3 mechanisms as participating in LPS-induced tau-P, our analysis of tau kinases identify no clear correlate that might explain the enhanced phosphorylation responses observed in CRFR1-deficient mice, likely indicating an involvement of additional factors in shaping this response.

Work by Planel and colleagues in stress models ranging from starvation, to glucoprivation, to a range of different anesthetic treatments observe marked inhibition of tau phosphatase activity that is highly correlated with reductions in core body temperature, and have proposed a central role for temperature-dependent reductions in phosphatase activity in stress-induced tau-P, as well as tau pathology in AD (Planel *et al.*, 2001; Planel *et al.*, 2007). Because LPS treatment yields a biphasic temperature response in rats (initial hypothermia followed by protracted fever (Romanovsky *et al.*, 1998; Zhang *et al.*, 2003), we hypothesized that this might generalize to mice, raising the possibility that an early temperature-dependent inhibition of PP2A-c could be mechanistically involved in tau-P responses. However, as indicated by others, we find that LPS-treated mice exhibit a monophasic fever, with no hypothermic component (Rudaya *et al.*, 2005). Accordingly, PP2A-c levels were unchanged over the time course studied, suggesting that a temperature-dependent mechanism is unlikely to have contributed to LPS-induced tau-P in our paradigm (Fig. 5).

CRFR Involvement

Previous work supporting involvement of the CRFR signaling system in stress-induced increases in tau-P (Rissman *et al.*, 2007) and brain interstitial fluid levels of amyloid- β (Kang *et al.*, 2007) supported a broad involvement of this signaling system in experiential effects on AD pathogenesis, and justified examination of whether such involvement might extend to other, non-experiential stress models. The present results fail to support such an involvement in tau-P responses to a systemic inflammatory stimulus. Rather, evidence was found for a restraining role of CRFR1 signaling in this context.

Deriving from the dependence of the neuroendocrine stress response on anterior pituitary CRFR1 expression, it is also noteworthy that both *CRFR1*^{-/-} and double knockout mice exhibit a dramatically impaired capacity to mount pituitary-adrenal stress hormone responses (Bale *et al.*, 2002; Bale & Vale, 2004). This argues strongly against glucocorticoids, a dominant class of stress mediators, as playing a major role in LPS-induced tau-P in brain. Current evidence implicates signaling by proinflammatory cytokines and the brain's intrinsic immune effector cells, microglia, as mediating the capacity of LPS stimulation to induce tau-P and exacerbate of AD and other tauopathies (Kitazawa *et al.*, 2005; Lee *et al.*, 2010). It is not unreasonable to expect that CRFR signaling might participate in this context, as manipulation of either or both receptor(s) is widely reported to modulate peripheral immune responses, including LPS-induced production of pro-

inflammatory cytokines and prostanoids (Zhang & Li, 2009); (Tsatsanis *et al.*, 2007), as well as both central and peripheral inflammatory disease processes (Kiank *et al.*, 2003; Stevens *et al.*, 2003; Wang *et al.*, 2007). Accordingly, there are indications that CRFRs are expressed by cell types, including microglia, that participate in CNS inflammatory responses (Stevens *et al.*, 2003). It remains to be determined whether and how the modulation of tau-P responses to LPS as a function of CRFR status null mice may reflect alterations in inflammatory signaling mechanisms.

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Abbreviations used

CRF	Corticotropin Releasing Factor
AD	Alzheimer's disease
LPS	lipopolysaccharide
HPA	hypothalamic-pituitary adrenal axis
NFT	neurofibrillary tangles
PHF	paired helical filament
tau-P	tau phosphorylation
ip	intraperitoneal

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Figure 1. Time course of LPS-induced tau-P

Western blots of hippocampal extracts from *wt* mice that were unstressed (NS) or treated with 100 μ g/kg LPS and killed 0, 20, 40, 60, 90, 120, or 240 min post injection. Levels of tau-P were low in NS animals, but rose either immediately after stress (PHF-1) or within 20–40 mins post injection (AT8, S⁴²²). Tau-P at the PHF-1 site were elevated through 240 min post-stress, but returned to baseline by 180 mins at the AT8 and S⁴²² sites. Due primarily to the peak seen at the AT8 site, the 40 min timepoint was chosen for subsequent analyses. β -actin was used as a loading control.

Figure 2. Hippocampal localization of LPS-induced tau-P and inflammatory responses
Immunoperoxidase staining for AT8-phosphorylated tau- (top), Iba-1- (middle) and COX-2- immunoreactivities in saline-injected controls (Sal) and at 1–3 hrs after systemic LPS challenge. LPS increases tau-P principally in hilar neurons of the dentate gyrus and CA3 pyramidal cells. Less prominent responses are seen in a few cells in deep aspects of the dentate granule cell layer (gr). Among inflammatory markers, a progressive retraction and thickening of the processes of Iba-1-positive microglial cells is apparent over 1–3 hrs after LPS treatment. COX-2 cellular labeling is more prominent at 2–3 hrs post injection, at which time enzyme expression is induced in vascular-associated cells. Scale bars: 100 μ m (top); 50 μ m (middle and bottom).

Figure 3. LPS-induced tau-P in wt and CRFR null mutant mice

Tau-P responses in hippocampal extracts from *wt*, *CRFR1*^{-/-}, *CRFR2*^{-/-} and DBL knockout mice killed 40 mins after ip injection of saline (C) or 100 µg/kg LPS. Because of the variability seen in responses of some of the knockout groups, blots from all three animals are shown for these groups, while representative blots are shown for the control conditions. With the exception of the S⁴²² site that showed no change between groups (all, $P > 0.05$), levels of tau-P were significantly increased in CRFR1 knockout mice in comparison to CRFR2 and DBL knockout mice (all, $P < 0.05$), but not *wt* ($P = 0.09$). Somewhat variable tau-P responses are seen in CRFR mutant mice, particularly among the CRFR2 and DBL groups. Data are presented as mean $\pm$ SEM integrated optical density. β -actin was used as a loading control. *, differs significantly from (saline-injected) *wt* controls, $P < 0.001$; †, significantly different from LPS-treated *wt* group, $P < 0.05$.

Figure 4. LPS effects on tau kinase activity and their CRFR dependence

Organized like Fig. 3 to show various indices of tau kinase activity in *wt* and CRFR-deficient mice after ip injection of saline (C) or LPS. Again, blots from all three animals are shown for the mutant groups, while representative blots are shown for the control conditions. Histograms show mean ± SEM integrated optical density values. Levels of the activated (pY²¹⁶) form of GSK-3α and β were markedly increased in all genotypes in response to LPS treatment, with CRFR mutant groups showing marginally, though reliably, more robust responses than *wt* mice. Levels of the inactive (pS⁹) GSK-3 isoforms generally exhibited lesser LPS-responsiveness, except for GSK-3α in CRFR1 knockout mice. In addition, LPS provoked increases in levels of the CDK5 activator proteins, p25 and p35, that were comparable across genotypes. Levels of activated JNK and ERKs were relatively unresponsive to LPS. β-actin was used as a loading control. *, differs significantly different from unstressed (C) *wt* condition, $P < 0.001$, †, differs significantly from LPS-stimulated *wt* value, $P < 0.01$.

Figure 5. LPS effects on tau phosphatase and body temperature

To assay whether temperature-induced changes in PP2A activity might be involved in the tau-P responses seen with LPS, we used western blot analysis of relative levels of PP2A-c and telemetry assays in LPS treated mice over 240 mins. We observed a monophasic hyperthermic response (~2°C rise in core temperature) and no reliable change in PP2A-c levels were seen over the time course, suggesting that temperature-dependent alterations in phosphatase activity are not integrally involved in LPS-stimulated increases in tau-P.

Table 1

Antibody Characteristics

Antibody	Immunogen	Specificity	Source	Host	Application	Reference
AT8	Human phospho tau S ²⁰² /T ²⁰⁵	H.M.R Tau pS ²⁰² /T ²⁰⁵	Pierce Endogen Cat # MN1020B	Mouse	IHC, WB	(Mercken <i>et al.</i> , 1992; Rissman <i>et al.</i> , 2007)
pTau-S422	Phospho tau peptide pS422	H.M.R Total P38 MapK	Millipore Cat # AB9664	Rabbit	WB	(Tanaka <i>et al.</i> , 1998; Rissman <i>et al.</i> , 2007)
PHF-1	Human phospho tau S ³⁹⁶ /404	H.M.R Tau pS ³⁹⁶ /404	Dr. P. Davies	Mouse	IHC, WB	(Greenberg & Davies, 1990; Greenberg <i>et al.</i> , 1992; Rissman <i>et al.</i> , 2007)
Phospho-GSK-3β	Phospho Peptide GSK pY ²¹⁶	H.M.R GSK pY ²¹⁶	BD Transduction Cat # 612313	Mouse	IHC, WB	(Kitazawa <i>et al.</i> , 2005; Rissman <i>et al.</i> , 2007)
Phospho-GSK-3β	Phospho peptide GSK S ⁹	H.M.R GSK pS ⁹	Cell Signaling Cat # 9336	Mouse	IHC, WB	(Kitazawa <i>et al.</i> , 2005; Rissman <i>et al.</i> , 2007)
GSK-3	Human GSK-3	H.M.R Total GSK-3α,β	EMD Biosciences Cat # 05-412	Mouse	IHC, WB	(Phiel <i>et al.</i> , 2003; Rissman <i>et al.</i> , 2007)
Phospho-p44/42 MAPK (Erk1/2)	Phospho human Erk1/2 T ²⁰² /Y ²⁰⁴	H.M.R pERK 1/2	Cell Signaling Cat # 9106	Mouse	IHC, WB	(Zimmermann & Moelling, 1999) (Rissman <i>et al.</i> , 2007)
MAPK (ERK 1/2)	Human ERK 1/2 P44/42	H.M.R Total ERK 1/2	Cell Signaling Cat # 4695	Rabbit	IHC, WB	(Miyaji <i>et al.</i> , 2009) (Rissman <i>et al.</i> , 2007)
Phospho-SAPK/JNK	Phosphopeptide JNK T ¹⁸³ /Y ¹⁸⁵	H.M.R JNK 1/2	Cell Signaling Cat # 9255	Mouse	IHC, WB	(Kitazawa <i>et al.</i> , 2005; (Rissman <i>et al.</i> , 2007)
JNK	Human JNK	H.M.R Total JNK	Cell Signaling Cat # 9252	Rabbit	WB	(Kitazawa <i>et al.</i> , 2005; (Rissman <i>et al.</i> , 2007)
PP2A	Human PP2A aa. 153–309	H.M.R PP2A C subunit	BD Transduction Cat # 610555	Mouse	WB	(Planel <i>et al.</i> , 2001; Rissman <i>et al.</i> , 2007)
CDK5	AA16, human cdk5	H.M.R Total CDK5	Santa Cruz Cat # sc-173	Rabbit	WB	(Kitazawa <i>et al.</i> , 2005; Rissman <i>et al.</i> , 2007)
CDK5 p35/p25	C terminus of human p35	H.M.R p35/p25	Santa Cruz Cat # sc-820	Rabbit	WB	(Kitazawa <i>et al.</i> , 2005; Rissman <i>et al.</i> , 2007)
Iba-1	Synthetic Iba-1 peptide	H.M.R	Wako Cat # 019-19741	Rabbit	IHC, WB	(Imai & Kohsaka, 2002; Serrats <i>et al.</i> , 2010)
Cox-2	AA 584–604 rat COX-2	M.R	Santa Cruz Cat # sc-1746	Goat	IHC, WB	(Schultz & Sawchenko, 2002; Serrats <i>et al.</i> , 2010)
β-actin	C-terminus of β-actin	Various, β-actin	Sigma Cat # sab2100037	Rabbit	WB	(Rissman <i>et al.</i> , 2007)

H, Human; M, mouse; R, rat; IHC, immunohistochemistry; WB, Western blot