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TNF α -induced AMPA-receptor trafficking in CNS neurons; relevance to excitotoxicity?

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Abstract

Injury and disease in the CNS increases the amount of tumor necrosis factor α (TNF α) that neurons are exposed to. This cytokine is central to the inflammatory response that occurs after injury and during prolonged CNS disease, and contributes to the process of neuronal cell death. Previous studies have addressed how long-term apoptotic-signaling pathways that are initiated by TNF α might influence these processes, but the effects of inflammation on neurons and synaptic function in the timescale of minutes after exposure are largely unexplored. Our published studies examining the effect of TNF α on trafficking of AMPA-type glutamate receptors (AMPA-Rs) in hippocampal neurons demonstrate that glial-derived TNF α causes a rapid (<15 minute) increase in the number of neuronal, surface-localized, synaptic AMPARs leading to an increase in synaptic strength. This indicates that TNF α -signal transduction acts to facilitate increased surface localization of AMPARs from internal postsynaptic stores. Importantly, an excess of surface localized AMPARs might predispose the neuron to glutamate-mediated excitotoxicity and excessive intracellular calcium concentrations, leading to cell death. This suggests a new mechanism for excitotoxic TNF α -induced neuronal death that is initiated minutes after neurons are exposed to the products of the inflammatory response.

Here we review the importance of AMPAR trafficking in normal neuronal function and how abnormalities that are mediated by glial-derived cytokines such as TNF α can be central in causing neuronal disorders. We have further investigated the effects of TNF α on different neuronal cell types and present new data from cortical and hippocampal neurons in culture. Finally, we have expanded our investigation of the temporal profile of the action of this cytokine relevant to neuronal damage. We conclude that TNF α -mediated effects on AMPAR trafficking are common in diverse neuronal cell types and very rapid in their onset. The abnormal AMPAR trafficking elicited by TNF α might present a novel target to aid the development of new neuroprotective drugs.

Keywords

cytokine; astrocyte; AMPA; excitotoxicity; TNF

INTRODUCTION

The basic mechanisms of excitatory postsynaptic function

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and exerts its effects via several pre-synaptic and post-synaptic receptors with differing physiological properties. The ionotropic receptors (iGluRs) are ligand-gated ion channels that, on binding of glutamate, allow entry of cations into neurons. Thus, iGluRs are the major mediators of synaptic transmission. These receptors are further subdivided, based upon their biophysical and pharmacological properties, into *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptors (Hollmann and Heinemann, 1994; Chittajallu *et al.*, 1999). Each of these classes of receptor have differential localizations and contributions to the mechanisms of normal synaptic function. Proper control of synaptic strength is key to the normal function of the CNS including the formation and maintenance of memory. As such, the mechanisms underlying both basic synaptic transmission and plasticity are of interest to the neuroscience field. Synaptic transmission is an inherently regulatable process that is determined by changes in the release of glutamate from presynaptic terminals and the localization and activity of receptors on the postsynaptic membrane.

Recently, postsynaptic iGluRs have become a particular focus of numerous laboratories because it is apparent that receptor expression at the postsynaptic membrane is highly dynamic (Fig. 1) (reviewed in Carroll *et al.*, 2001; Malinow and Malenka, 2002; Song and Huganir, 2002; Brecht and Nicoll, 2003). Dominating this field are AMPARs, which mediate most fast excitatory synaptic transmission and are expressed postsynaptically. Recent evidence demonstrates that NMDA receptors (reviewed in Carroll and Zukin, 2002) and kainate receptors (Hirbec *et al.*, 2003) are also trafficked dynamically to affect synaptic function. Changes in postsynaptic responsiveness to presynaptically released glutamate might be mediated by alterations in either the conductance of pre-existing surface-expressed receptors or by a change in their number at the postsynaptic membrane. Although changes in channel conductance as a result of direct phosphorylation of iGluR subunits are important (Benke *et al.*, 1998), much data has led to the finding that the surface expression of AMPARs is highly regulatable and the amount of this surface expression is directly relevant to synaptic efficacy (reviewed in Malinow and Malenka, 2002; Collingridge *et al.*, 2004). In addition, the rapid mobility of AMPARs occurs in a constitutive fashion, with continual turnover of AMPARs at the synaptic membrane mediated by exocytosis and endocytosis (Lin *et al.*, 2000). However, AMPAR trafficking is also mediated by synaptic activity and subsequent receptor activation (Carroll *et al.*, 1999; Lissin *et al.*, 1999; Beattie *et al.*, 2000; Ehlers, 2000; Lin *et al.*, 2000). These activity dependent changes in AMPAR trafficking have been linked to the modulation of synaptic strength that occurs during some forms of long-term potentiation (LTP) (Malenka and Nicoll, 1999) and long-term depression (LTD) (Carroll *et al.*, 2001). The mechanisms underlying this regulation, both in the delivery of receptors to the postsynaptic membrane and their removal, have been fruitful areas of research.

The dynamics of AMPAR expression at the synaptic membrane are highly complex. In normal non-injury conditions, only AMPARs at the postsynaptic surface that are immediately apposed to the presynaptic bouton contribute to the synaptic response. The complement of receptors at the active postsynaptic site is modulated by a number of mechanisms (Fig. 1):

1. Internalization to intracellular stores.
2. Lateral diffusion to extrasynaptic sites on the plasma membrane where they are not activated by glutamate.
3. Delivery of new AMPARs from intracellular stores.

4. Lateral diffusion from extrasynaptic sites on the neuronal surface to the postsynaptic active site.
5. New synthesis and delivery of receptors from the endoplasmic reticulum.
6. Degradation of receptors via lysosomal pathways.

Increases in AMPAR surface expression have been correlated with the development of LTP through both the unsilencing of 'silent synapses' (synapses that lack AMPARs) (Isaac *et al.*, 1995; Liao *et al.*, 1995) and an increase in AMPARs at pre-existing synapses (Shi *et al.*, 1999; Hayashi *et al.*, 2000). Phosphorylation through kinases such as CaMKII and protein kinase A (PKA) is important in this regulation of AMPAR-mediated delivery to the postsynaptic membrane (Hayashi *et al.*, 2000; Lee *et al.*, 2000; Esteban *et al.*, 2003). Interaction with intracellular proteins is also important. The disruption of AMPAR binding to PDZ-domain-containing proteins and overexpression of such interacting proteins perturbs synaptic-surface expression (Hayashi *et al.*, 2000; Rumbaugh *et al.*, 2003). Additionally, the delivery of AMPARs to synaptic membranes requires interaction with the transmembrane protein stargazin or related transmembrane AMPAR-regulatory proteins (TARPs) (Chen *et al.*, 2000; Tomita *et al.*, 2003).

Endocytosis of receptors from the synaptic membrane is a dynamin-dependent process (Carroll *et al.*, 1999) that is regulated by phosphorylation and interaction with intracellular proteins. An increase in postsynaptic calcium levels has been linked with the physiological process of LTD (Mulkey and Malenka, 1992) and the regulated internalization of AMPARs (Beattie *et al.*, 2000). The calcium-sensitive phosphatase calcineurin is also linked to both these processes (Mulkey and Malenka, 1992; Beattie *et al.*, 2000), which indicates a role for AMPAR phosphorylation in the processes of receptor internalization and LTD. A strong body of literature also demonstrates that AMPAR-interacting proteins, including *N*-ethylmaleimide-sensitive fusion protein, glutamate-receptor-interacting protein and protein interacting with C-kinase are linked with the regulated internalization of AMPARs (reviewed in Braithwaite *et al.*, 2000; Passafaro *et al.*, 2001; Malinow and Malenka, 2002). TARPs might also regulate the internalization of AMPARs because TARP-AMPA dissociation appears to be necessary for the removal of AMPARs from the synaptic membrane (Tomita *et al.*, 2003).

The delivery to and removal of receptors from the post-synaptic membrane depends on several highly regulated mechanisms. These mechanisms have profound effects on the complement of receptors at the postsynaptic membrane, and enable the control of synaptic transmission and formation of synaptic plasticity. Transcriptional and translational control over the concentration of AMPAR subunit proteins have been shown to change the amount and subtype specificity of AMPAR surface expression over a time period of hours (Aronica *et al.*, 1997; Pellegrini-Giampietro *et al.*, 1997; Tanaka *et al.*, 2000). New data shows that this can be accomplished through the translation of messages that encode AMPARs in dendrites (Ju *et al.*, 2004). However, the speed of regulated changes in synaptic transmission and the requirement for a proper contingent of synaptic substructure proteins make it likely that trafficking mechanisms (and degradation) are a major contributor to the dynamic regulation of postsynaptic function (O'Brien *et al.*, 1998; Ehlers, 2000; Man *et al.*, 2000; Stein *et al.*, 2003; Tao *et al.*, 2003; Ehrlich and Malinow, 2004). Clearly, abnormalities in any of these trafficking systems will lead to dysfunction in synaptic transmission and development of neuronal disorders.

Abnormal trafficking of AMPARs as a mechanism of neuronal dysfunction

The precise control of synaptic efficacy is important for normal function of the CNS and, as such, abnormalities in synaptic function can lead to neurological disorders and toxicity. Much research indicates that over-activation of iGluRs can lead to neurotoxicity, primarily through

excessive calcium influx that leads to the induction of apoptotic processes (Choi, 1992; Lee *et al.*, 1999; Mattson *et al.*, 2000; Weiss and Sensi, 2000; Mattson and Chan, 2003). This iGluR-mediated neurotoxicity is implicated in the neuronal loss that is associated with acute trauma to the CNS and disorders such as Alzheimer's disease and Parkinsonism. In addition, hyperactivity of iGluRs may lead to seizures such as occur in epilepsy without leading to neurotoxicity. By contrast, suboptimal iGluR activity can also lead to neurological deficits including schizophrenia (Tsai and Coyle, 2002) and might also underlie the cognitive deficits in disorders such as Alzheimer's disease (Kamenetz *et al.*, 2003). Addictive behavior involves inappropriate plasticity of glutamate-mediated transmission (Tzschentke and Schmidt, 2003), which, again, indicates that imbalances in iGluRs at synapses can have serious neurobiological consequences. Because the trafficking of AMPARs to the postsynaptic membrane is a major mechanism for normal neuronal function it is clear that abnormal trafficking might lead to these conditions of neuronal dysfunction. AMPARs are a major regulatory contributor to fast excitatory synaptic transmission. Although the majority of AMPARs are not permeable to Ca^{2+} , the major intracellular mediator of cell death, a subset of receptors at synaptic sites do not contain GluR2 subunits (GluR2-lacking AMPARs), which makes them Ca^{2+} -permeable (Ogoshi and Weiss, 2003). In addition, activation of AMPARs is required to relieve the voltage-dependent block of NMDA receptors, which are the major iGluRs that allow Ca^{2+} entry into neurons (Herron *et al.*, 1986). Therefore, abnormal trafficking of AMPARs can lead to excitotoxicity both directly and indirectly.

The trigger for abnormal AMPAR trafficking in neuronal dysfunction

Normally, trafficking of AMPARs in neurons is a constitutive process that is regulated by activity. In neuronal disorders an additional trigger must disrupt the trafficking process. Research indicates that a major contributory factor to AMPAR trafficking are cytokines released from glial cells including the inflammatory cytokine tumor necrosis factor α (TNF α) (Beattie *et al.*, 2002a). TNF α is involved intimately in inflammation, immune activation, differentiation and cell death, but its complex actions are increasingly implicated in diseases of the CNS (Perry *et al.*, 1995; Allan and Rothwell, 2001). TNF α and other cytokines are induced rapidly in response to tissue injury and infection and in the CNS (Perry *et al.*, 1995; Allan and Rothwell, 2001). TNF α administered to primary neurons in culture causes a dose-dependent cytotoxicity (Zhao *et al.*, 2001) and neuronal apoptosis (Reimann-Philipp *et al.*, 2001). However, some studies *in vitro* report neuroprotective effects of TNF α (Cheng *et al.*, 1994; Bruce *et al.*, 1996). Whether TNF α is damaging or protective appears to depend on many factors. TNF α present during acute injury appears to be damaging, but its long-term presence can be protective (Wilde *et al.*, 2000). The effect of TNF α on distinct cell types is influenced greatly by the presence of specific receptors on target cells. TNF α binds specifically to two distinct receptors, TNFR1 and TNFR2 which are coexpressed on neurons (Vitkovic *et al.*, 2000). The activation of TNFR1 appears to damage neurons whereas activation of TNFR2 is protective (Peschon *et al.*, 1998; Fontaine *et al.*, 2002; Yang *et al.*, 2002). Furthermore, the presence or absence of compounds that modify TNF α action also greatly influences possible neuroprotective and neurotoxic effects (Schubert *et al.*, 1997; Carlson *et al.*, 1998).

Therefore, there is a clear link between the actions of TNF α released by glia and neuronal AMPAR-trafficking events. TNF α is involved in the regulation of neuronal function and AMPAR localization, but the complex actions and temporal profile of a neuron's response to this cytokine determines its ultimate effects on neuronal pathology.

Interplay between TNF α and AMPAR trafficking after acute neural trauma

Several studies have demonstrated interplay between TNF α and AMPAR activity in neuronal disorders. Best characterized are the conditions of acute neural trauma and ischemic insults where there are both major inflammatory responses and neuronal damage. Acute neural trauma

and ischemic insults release glutamate from intracellular vesicles, which results in high extracellular concentrations that are thought to induce both necrotic and apoptotic neuronal cell death by activating iGluRs and increasing intracellular calcium levels (Choi, 1994). Neurotrauma also produces a complex ongoing cascade of inflammatory reactions that include the liberation of cytokines and chemokines, recruitment of immune cells from the periphery and activation of resident microglia (Bethea *et al.*, 1999; Popovich *et al.*, 2002). Considerable evidence indicates that this inflammatory response can contribute to secondary injury in trauma and stroke (Allan and Rothwell, 2001), but there is also evidence for a neuroprotective role for some aspects of the inflammatory cascade (Bruce *et al.*, 1996; Kim *et al.*, 2001; Shaked *et al.*, 2004).

It is likely that inflammation and excitotoxicity together contribute to neuronal damage beyond the initial insult of acute trauma. For example, microglial activation can release glutamate (Barger and Basile, 2001), glutamate receptors on microglia and neurons can stimulate the release of cytokines including TNF α (Matute *et al.*, 2001), and TNF α can rapidly and dramatically increase the surface localization and trafficking of AMPA-type iGluRs (Beattie *et al.*, 2002a). Indeed, co-injection of TNF α with AMPAR agonists enhances necrotic cell death in the spinal cord and this enhancement is blocked by the specific AMPAR antagonist CNQX (Hermann *et al.*, 2001). Low extracellular potassium levels induce increased surface expression of AMPARs and increases AMPA-mediated currents in cerebellar granule cells (Ha *et al.*, 2002). These cells also show increased susceptibility to excitotoxicity, which indicates the importance of AMPAR trafficking in a different class of neurons. Together, these observations indicate that rapid changes in localization and/or sensitivity of AMPARs at the plasma membrane after injury might be mediated by cytokines in the inflammatory cascade, and that effects might contribute to the cascade of neuronal cell death after injury and ischemia.

AMPA antagonists are neuroprotective in models of spinal cord injury; NBQX is effective in limiting both gray and white matter damage after spinal cord contusions in rats (Wrathall *et al.*, 1997). Contusion injury to the cord rapidly increases levels of TNF α (Bethea *et al.*, 1999; Pan *et al.*, 1999), other inflammatory cytokines (McTigue *et al.*, 1998; McTigue *et al.*, 2000) and extracellular glutamate. Astrocytic damage can result in deficits in glutamate uptake and clearance from the extracellular space. Thus, initial damage might initiate secondary injury because the interplay between all of the major cellular players in the gray matter, neurons, microglia and astrocytes, leads to a cascade of increasing extracellular glutamate. This increase in glutamate release plus a rapid increase in surface AMPARs could dramatically raise the potential for neurotoxicity. The spread of this cascade to the white matter also puts myelinating oligodendrocytes and axon tracts at risk.

Oligodendrocytes also contain AMPARs (McDonald *et al.*, 1998; Park *et al.*, 2003) and are susceptible to cell death after spinal cord injury, ischemia and other insults. It has been proposed that TNF α and glutamate cooperate to kill oligodendrocytes by apoptosis in models of multiple sclerosis (MS) (reviewed in Matute *et al.*, 2001), but direct effects on AMPAR trafficking have not been tested. All the features of oligodendrocyte apoptosis that are observed *in vivo* in SCI (Crowe *et al.*, 1997; Beattie *et al.*, 2002b; Springer *et al.*, 1999) and models of MS can be mediated *in vitro* via AMPA and kainate receptors (Sanchez-Gomez *et al.*, 2003). Takahashi *et al.* (2003) support the idea that cytokines interleukin 1 β (IL1 β) and TNF α potentiate AMPAR-mediated oligodendrocyte cell death *in vitro* (Takahashi *et al.*, 2003). Although a cascade of inflammation and excessive cell-surface localization of AMPARs is attractive as a simple hypothetical pathway to neuron death, the situation is likely to be more complicated. First, TNF α and TNF α -receptor knock-out mice have both increased and decreased damage after CNS injury, depending upon the circumstances (Bruce *et al.*, 1996; Bethea *et al.*, 1999; Kim *et al.*, 2001). Second, although injections of glutamate and TNF α cause inflammatory-like changes in the spinal cord (Hermann *et al.*, 2001), others report that TNF α and its co-

inflammatory cytokine IL1 β have opposite effects on AMPAR-mediated cell death after injection into the brain (Allan, 2002).

It seems likely that there are at least two, somewhat distinct, stages of cell death after spinal cord and other CNS injuries: an acute, necrotic cell death, which is prominent in neurons and exacerbated by the effects of cytokines on AMPAR trafficking; and a longer-term, apoptotic wave of cell death that is prevalent in oligodendrocytes away from the primary lesion (Beattie *et al.*, 2002b). Although these changes are more likely to be mediated by long-term transcriptional and/or translational events, initial changes in AMPAR surface localization soon after injury might influence these long-term events, even if the alteration in AMPAR trafficking is transient. In addition, De *et al.* showed that the TNF α -induced increase in AMPARs and susceptibility to neuronal cell death depends on protein synthesis (De *et al.*, 2003). By contrast, decreased expression of iGluR subunits in both gray and white matter is observed in more chronic (e.g. 1 week after trauma) injuries (Grossman *et al.*, 1999; Park *et al.*, 2003). This might, of course, reflect the death of cells that express AMPARs early after injury, and that might have been affected by alterations in AMPAR trafficking.

Glial-derived TNF α rapidly increases AMPAR surface localization in hippocampal neurons

The intimate physical apposition of glia and neurons has profound implications for the mechanisms underlying both synaptic plasticity and neural damage caused by several brain insults. For example, astrocytes often respond to activity with an increase in intracellular calcium levels, and can release a variety of chemical substances that can transiently modulate neural processes (Bezzi *et al.*, 2001). Recently, it has been shown in retinal ganglion neuron cultures that astrocytes are required for normal synaptogenesis and synaptic stability, and that the required, unidentified factor is a diffusible extracellular signal (Pfrieger and Barres, 1997; Ullian *et al.*, 2001). These experiments investigated the effects of astrocytes on synapse number and function over the course of days and did not address whether glia are required for more rapid modulation of synaptic strength.

The possibility that TNF α might influence the surface expression of AMPARs and, thereby, synaptic strength is indicated by the observations that TNF α enhances the responses of brainstem neurons to excitatory afferent inputs (Nagler *et al.*, 2001) and greatly potentiates the cell death induced by injection of the AMPAR agonists into the spinal cord. The latter effect is effect blocked by an AMPAR antagonist (Mauch *et al.*, 2001). Because neurons are exposed to a large increase in TNF α after injury (Perry *et al.*, 1995; Allan and Rothwell, 2001), we sought to determine how this might change synaptic function during and immediately after TNF α exposure. To address this, our group utilized the model system of primary dissociated hippocampal neurons in culture. We demonstrated that bath application of exogenous TNF α causes a rapid (<15 minute), significant increase in the number surface-localized AMPARs containing the GluR1 subunit (Beattie *et al.*, 2002a). The increase in surface-expressed GluR1 was visualized and measured by immunofluorescence microscopy and confirmed using electrophysiological recordings. There was no change in the amount of surface localization of the NMDA receptor subunit NR1 at this timepoint (Beattie *et al.*, 2002a). It was also determined, by colocalization of GluR1 with the synaptic marker synaptophysin, that the delivery of surface AMPARs increased at both synaptic and extrasynaptic sites. The rapid timeframe of AMPAR delivery indicates a trafficking effect rather than a change in protein translation. However, new data showing the close apposition of the endoplasmic reticulum and mRNA with the base of dendritic spines indicates that protein production for the synapse could be faster than believed previously (reviewed in Glanzer and Eberwine, 2003; Ju *et al.*, 2004; Zukin *et al.*, 2004). In support of a trafficking mechanism for this action, it was demonstrated recently that a pool of AMPARs in recycling endosomes supplies the synapse with receptors as little as 20 minutes following NMDA-receptor-mediated stimuli that induce LTP (Lu *et*

al., 2001; Park *et al.*, 2004). These data also indicate that these newly delivered AMPARs are likely to come from preexisting, intracellular pools (Eshhar *et al.*, 1993).

Because TNF α can be produced by both neurons and glia (Allan and Rothwell, 2001), especially after injury to the CNS, we asked which of these cell types are the source of TNF α in our hippocampal cultures. First, we looked microscopically to examine the staining pattern of TNF α in hippocampal neurons and astrocytes. Both cell types stained but astrocytes displayed dense perinuclear staining that suggested TNF α in biosynthetic pathways of these cells (Fig. 2), whereas neurons displayed diffuse staining. To more definitively test the hypothesis that TNF α is supplied by astrocytes in our cultures, we prepared astrocyte-conditioned media from neuron-depleted cultures and applied it to mixed cultures. An increase in both surfaced-localized GluR1-subunit-containing AMPARs and synaptic strength was observed on neurons after exposure of mixed cultures to the conditioned media for 15 minutes (Fig. 3). Specifically removing soluble TNF α from this astrocyte-conditioned media using either an anti-TNF α antibody or a soluble form of the TNFR1 receptor blocked the increase in surface AMPARs and synaptic strength. Furthermore, treatment of astrocyte cultures with GM6001, a metalloproteinase inhibitor that inhibits release of soluble TNF α from the cell surface, prevented the effect of the conditioned media on hippocampal neurons. In complimentary acute, electrophysiological experiments in hippocampal slices, we found that blocking TNF α signaling with soluble TNFR1 blocks the enhancing effect of TNF α on synaptic strength (Beattie *et al.*, 2002a).

These data, which show the rapid response of increased AMPAR surface localization through the bath application of exogenous and astrocyte-derived TNF α , raise further questions. How long does the response last with continuous TNF α application? Are both TNF α receptors, TNFR1 and TNFR2, responsible for the signaling that causes this activity? Do neurons from areas other than the hippocampus respond similarly to TNF α ? These questions underscore the importance of future, more detailed, studies of the effects of TNF α . Clearly, TNF α affects the trafficking of AMPARs and is a major contributing factor to damage in several neuronal disorders, especially those in which inflammatory responses occur. There is, however, much evidence that TNF α has differing roles, either exacerbating neuronal damage or protecting neurons, depending upon the situation. With this complex scenario of TNF α as potential friend and foe to the injured neuron, it is vital to study the role of TNF α in AMPAR trafficking in different neuron classes and that we understand the temporal aspects of its action. We have, therefore, begun more detailed studies of the action of TNF α on AMPAR trafficking. Our new studies, which are discussed below, compare TNF α -induced AMPAR trafficking in hippocampal and cortical neurons.

Time-course of TNF α effect

Is the increase in surface-localized AMPARs induced by TNF α treatment maintained throughout the timecourse of TNF α application? To address this question, we treated hippocampal neurons in culture with TNF α for 15 minutes. To observe and quantify surface localized AMPARs, we immunofluorescently labeled non-permeabilized neurons with an antibody that recognizes an extracellular epitope of GluR1, and analyzed surface GluR1 fluorescence images captured by digital microscopy. Like our previous data, which showed an ~200% increase in cell-surface AMPARs (Beattie *et al.*, 2002a), treatment with TNF α for 15 minutes dramatically increased surface-localized GluR1 to $195 \pm 10\%$ of that observed in untreated, control neurons (Fig. 4A,B,D). Treatment of neurons with TNF α for 30 minutes increased in surface-localized GluR1 to $149 \pm 10\%$ of that in untreated, control neurons (Fig. 4A,C,D). However, at 30 minutes there is a decrease in surface-localized GluR1 from the peak response at 15 minutes (Fig. 4D). These data indicate that, following TNF α exposure surface-localized GluR1 reaches a maximum at ~15 minutes, and although surface GluR1 levels fall

by after 30-minute-exposure, AMPAR surface localization is still abnormally high. This implies that neurons might have mechanisms to sense and compensate for excess surface AMPARs. The molecular pathways that underlie these mechanisms might be important in protecting against excitotoxicity.

TNF α rapidly increases surface expression of AMPARs in primary cortical neurons in culture

So far, our studies have been confined to the well-established culture system of primary hippocampal neurons. This begs the question: is the phenomenon of induction of surface localized AMPARs by TNF α restricted to hippocampal neurons or is it a more general phenomenon? To begin to address this question, we have cultured primary neurons derived from the cerebral cortex and exposed mature neurons to TNF α for 15 minutes, and observed and quantified surface GluR1 by immunofluorescent microscopy as described above. Our data show an increase in surface-localized GluR1 of $147 \pm 11\%$ in neurons treated with TNF α for 15 minutes compared with untreated, control neurons (Fig. 5). These data indicate that other neuron types respond to TNF α in a manner that is similar to hippocampal neurons. However, we observed ~50% fewer surface-localized AMPARs following treatment with TNF α for 15 minutes compared with hippocampal cultures. This may reflect either a difference in sensitivity to TNF α between neuron types (perhaps because of different expression and type of TNF α receptors) or a lower available pool of GluR1. At present we have only tested the 15-minute-treatment with TNF α in cortical neurons, so cannot comment on a possible peak response in this class of neurons. Planned future time-course experiments will address this.

These data demonstrate that two diverse classes of neurons are sensitive to exposure to TNF α and indicate that this cytokine may predispose other neuron types to increased sensitivity to excitotoxicity. In support of this data, neurons of the spinal cord are also sensitive to TNF α and show increased excitotoxicity when exposed to TNF α , which is blocked by the AMPAR antagonist CNQX (Hermann *et al.*, 2001). Understanding the mechanism by which TNF α induces increased AMPAR surface localization will allow a better understanding of this phenomenon and might lead to preventative therapies that treat the earliest stages of excitotoxicity.

CONCLUSIONS

Our research has brought us to the fascinating, complex intersection between the nervous and immune systems. In this rapidly blurring border zone, a cytokine whose historical home is outside the CNS, currently finds itself at home. TNF α finds itself seated securely in the brain, with specific receptors on neurons and glia for company, and with a role in maintaining normal synaptic function. But this 'new resident' of the brain is likely to be an unwelcome tenant in the CNS during acute injury when it becomes abnormally abundant and destroys the delicate chemical and electrical balance between neurons and glia. Past research has focused on the long-term apoptotic signaling characteristics of TNF α in an attempt to understand the mechanisms of this carnage. We have begun to investigate the rapid effects of TNF α on the surface localization of an ionotropic glutamate receptor whose precise regulation is central to normal synaptic function and neuron viability.

We have tested the response of AMPAR trafficking to TNF α exposure. This does not in itself directly address changes in the neuron's excitotoxic vulnerability. However, as discussed above, a large body of literature links excessive iGluR activation and increased calcium-induced excitotoxicity. Indeed, co-stimulation of AMPARs and TNF α receptors in the spinal cord (with agonists that alone are non-toxic) induces dramatic excitotoxicity (Hermann *et al.*, 2001). Thus, we predict that our planned future studies to examine the excitotoxic sensitization of various neuronal cultures with TNF α will correlate abnormal AMPAR trafficking and neuron death. An important test that goes beyond correlation and towards a better

understanding of the mechanism that underlies excitotoxicity will be to test the effect of TNF α on neurons when cell-surface delivery of AMPARs is blocked. If this experiment reduces excitotoxic death either *in vivo* or *in vitro* during extended exposure to TNF α , an exciting new drug target for neuroprotection will have been uncovered.

The surprising and exciting observation that TNF α signaling feeds into the control of AMPAR trafficking leads to more questions, some of which we list below.

1. Which TNF α receptor (TNFR1 or TNFR2) activates AMPAR trafficking?
2. What other receptors and surface proteins are trafficked differentially by TNF α signaling and are particular subtypes of AMPARs affected preferentially? The existence of GluR2-lacking, calcium-permeable AMPARs is gaining acceptance. Indeed, there is evidence of decreased expression of GluR2 subunits in various neurological disorders including epilepsy and amyotrophic lateral sclerosis (ALS). If GluR2-lacking AMPARs are included in the TNF α -induced surface-localized receptor population, it would have implications for excitotoxicity. In acute neurotrauma and in diseases such as epilepsy, there are high concentrations of glutamate in synaptic and extrasynaptic locations. Calcium-permeable AMPARs in extrasynaptic sites would provide a dangerous source of calcium influx even in the absence of depolarization, thus, short-circuiting the need for NMDARs to induce long-term changes that depend on calcium signaling. In addition, calcium influx through calcium-permeable AMPARs might contribute to calcium-induced apoptosis.
3. How long does the trafficking change last in culture and does it happen *in vivo*?
4. Is TNF α and abnormal AMPAR trafficking part of longer-term, more gradual neuron death, such as occurs in neurodegenerative diseases? Correlative data from studies in epilepsy and ALS, discussed above, indicates that this may be the case. What long-term changes occur as a result of the rapid rise in calcium concentration, which, we believe, is maintained over 30 minutes in hippocampal neurons?
5. What are the main sources of TNF α after injury and during neurological disease? Here, we show that astrocytes are a potential source, but what of microglial activation and neurons themselves, and TNF α from the blood supply? As discussed above, TNF α crosses the blood–brain barrier after acute trauma.
6. Does TNF α signaling contribute in the short or long-term to an increase in AMPAR protein production? Even though our observations of very rapid AMPAR trafficking indicates that translation of new protein does not contribute, new data discussed above shows that the synapse may have a very rapid protein translation machinery nearby. Thus, more careful studies controlling for protein translation are planned. Regardless of the method by which more AMPARs get to the surface, it is clear that, at least over 30 minutes exposure to TNF α , there is an increase in potentially active AMPARs at the cell surface. Thus, questions of how this might initiate long-term changes in neuron health are of great interest.

We believe that there is enough precedent to believe that these questions will lead to a uniquely useful view of the underlying mechanisms of excitotoxicity. We are hopeful that our future studies, based on these questions, will provide the neuro-science community with new drug targets to reduce neuron damage and death that occurs after acute trauma and during neurodegenerative diseases.

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Fig. 1. Glutamate receptor trafficking in the dendritic spine during normal and abnormal synaptic function

AMPA receptors (AMPA) can be removed rapidly from the synapse on dendritic spines by either endocytosis (1) or lateral movement to extrasynaptic surface locales (2). Endocytosed AMPARs can be trafficked to lysosomes for degradation (6) or recycled back to the synapse (3). AMPARs can also be transported into the synapse after synthesis in either somatic or local dendritic endoplasmic reticulum (5) and then exocytosed to the synapse (3). Lateral movement of extrasynaptic receptors to the synapse (4) can also replenish receptors at this location. *, recent work shows that NMDA receptors (NMDAR) also are trafficked into and out of the synapse though possibly at a different timecourse than AMPARs (reviewed in Carroll and Zukin, 2002). The increase in surface receptors following either increased delivery via pathways 3 and 4 or a reduction in removal via pathways 1, 6 and 2 might increase the possibility of excitotoxicity during acute trauma and neuronal overactivation during epilepsy.

Fig. 2. Perinuclear TNF α staining in astrocytes in culture

(A) Astrocytes stained for TNF α . Often, staining is punctate and perinuclear in appearance. Astrocyte cultures lacking neurons were grown as described (Beattie *et al.*, 2002) and stained as described below. Co-staining with a GFAP-specific antibody (R&D Systems; Cat. No. AF-510-NA; 1 $\mu\text{g ml}^{-1}$) confirmed these cells as astrocytes (data not shown). (B) Neurons also frequently exhibit TNF α immunoreactivity that is more evenly distributed and less punctate than observed in astrocytes. Absence of co-staining with a GFAP-specific antibody (R&D Systems; Cat. No. AF-510-NA; 1 $\mu\text{g ml}^{-1}$) confirmed these cells as neurons (data not shown). Staining for TNF α used a goat anti-TNF α antibody (R&D Systems; Cat. No. AF-510-NA; 1 $\mu\text{g ml}^{-1}$) applied to fixed, permeabilized cells for 1 hour at room temperature. Cells were then washed three times in blocking solution (PBS, 1% BSA, 0.8 $\mu\text{g ml}^{-1}$ saponin). The signal was visualized by application of a donkey anti-goat antibody conjugated to Cy3 (Jackson ImmunoResearch), at a dilution of 1:800 for 45 minutes at room temperature. The cells were then washed three times in PBS and mounted.

Fig. 3. Astrocyte-conditioned media increases surface expression of AMPARs and synaptic strength via TNF α

(A) Examples of surface AMPAR staining in untreated and conditioned-media-treated neurons. (B) Quantification of effects of conditioned media on surface AMPAR staining. (* $P < 0.01$; untreated $100 \pm 9\%$, $n = 45$; conditioned media $152 \pm 9\%$, $n = 37$). (C) Examples of miniature EPSCs before and after application of conditioned media (calibration bars, 20 pA, 500 mseconds). (D) Mean change in miniature EPSC frequency and amplitude in cells treated with either control or conditioned media. ($n = 7$, untreated; $n = 8$ conditioned-media-treated cells; * $P < 0.01$; % initial mEPSC frequency: conditioned media $185 \pm 25\%$; normal media $76 \pm 5\%$; % initial mEPSC amplitude: conditioned media $117 \pm 14\%$; normal media $96 \pm 2\%$). (E) Examples of surface AMPAR staining in an untreated cell and a cell treated with conditioned media containing TNFR1. (F) Quantification of effects of conditioned media containing TNFR1, anti-TNF α antibody and the matrix metalloproteinase inhibitor GM6001. ($n = 31-45$ for each group; untreated $100 \pm 9\%$; TNFR1 and conditioned media $113 \pm 13\%$; anti-TNF α antibody and conditioned media $80 \pm 10\%$; GM6001 and conditioned media $78 \pm 9\%$). Figure reproduced, with permission, from Beattie *et al.* (2002).

Fig. 4. TNF α increases surface expression of AMPARs in primary hippocampal neurons in culture (A–C) Examples of surface AMPAR staining in untreated (A), and neurons treated with TNF α for 15 minutes (B) and 30 minutes (C). (D) Quantitation of the effects of TNF α on surface AMPAR staining ($n = 50$ – 70 for each group pooled from at least three different experiments; $*P = 0.08$; $**P = 0.4$; untreated, $100 \pm 14\%$; 15 minute TNF α , $195 \pm 10\%$; 30 minute TNF α , $149 \pm 10\%$). Arrows point to processes where very defined punctate staining appears after TNF application (B,C). Experimental methods: After treatment of 17–25-day-old neuron cultures (see Beattie *et al.*, 2002 for culture methods) with 6 nM TNF α at 37°C for the times indicated, neurons were chilled on ice, washed with cold PBS, and surface AMPARs visualized by indirect immunofluorescence using a rabbit antibody to the extracellular N-terminus of GluR1 (Ab-1%, Oncogene Research Products) at a dilution of 1:20 for 1 hour at 4°C. Neurons were then washed with cold PBS and fixed with 4% paraformaldehyde/4% sucrose in PBS. The non-permeabilized cells were then blocked with 3% BSA, 1% goat serum in PBS and a donkey anti-rabbit secondary Ab conjugated to Alexafluor 568 (Molecular Probes) was applied at a dilution of 1:800 for 45 minutes at room temperature, followed by thorough washing with PBS and mounting on slides with Fluoromount G (Electron Microscopy Services). Neurons were visualized and images captured with immunofluorescence microscopy as described in Beattie *et al.* (2002). For individual experiments, images for all conditions were analyzed using identical acquisition parameters and untreated and treated cells from the same culture preparation compared. Images from each experiment were obtained using a threshold equal to the average background fluorescence in untreated, control cells. The total area of fluorescently labeled surface AMPARs was measured automatically by Metamorph software and divided by the total cell area (determined using a lower threshold level to measure background fluorescence produced by fixed cells). For each experiment, the fluorescence of all cells was normalized by dividing by the average fluorescence of the untreated control cells. Each experimental manipulation was repeated a minimum of three times using different culture preparations. n represents the number of microscope fields examined. Statistical significance between individual experimental groups and the control group was determined using Student's t -test. Error bars represent s.e.m.

Fig. 5. TNF α rapidly increases surface expression of AMPARs in primary cortical neurons in culture

(A–B) Examples of surface AMPAR staining in untreated (A) and cortical neurons treated with TNF α for 15 minutes (B). (C) Quantification of effects of TNF α on surface AMPAR staining ($n = 70$ for each group pooled from four experiments; $*P = 0.01$; untreated, $100 \pm 11\%$; 15 minute TNF α , $147 \pm 11\%$). Cortical neurons were prepared, treated and visualized as described in Fig. 4.