A Novel Role for Sema3A in Neuroprotection from Injury Mediated by Activated Microglia

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Microglia exist under physiological conditions in a resting state but become activated after neuronal injury. Recent studies have highlighted the reciprocal role of neurons in controlling both the number and activity of microglia. In this study, microglia derived from newborn rat cortices were cultured and activated by interferon-γ (IFN-γ) treatment, then exposed to recombinant Sema3A or conditioned medium derived from stressed embryonic cortical neurons. We found that activation of microglia by IFN-γ induced differential upregulation of the semaphorin receptors Plexin-A1 and Neuropilin-1. This result was confirmed by Northern blotting, reverse transcription-PCR, and Western blotting. Furthermore, recombinant Sema3A induced apoptosis of microglia when added to the in vitro culture, and a similar result was obtained on activated microglia when Sema3A was produced by stressed neurons. Using an in vivo model of microglia activation by striatal injection of lipopolysaccharide demonstrated a corresponding upregulation of Plexin-A1 and Neuropilin-1 in activated microglia and enhanced production of Sema3A by stressed adult neurons. These results suggest a novel semaphorin-mediated mechanism of neuroprotection whereby stressed neurons can protect themselves from further damage by activated microglia.

Key words: microglia; neuron; semaphorin; plexin; neuropilin; neuroprotection

Introduction

Microglia are resident immune effectors of the CNS. Under physiological conditions, they exist in resting conditions but become activated in response to neuronal injury. Activation alters both morphological and functional properties of microglia. It is a feature of many inflammatory and degenerative neurological diseases (Kreutzberg, 1996; Benveniste, 1997; McGeer and McGeer, 1999). The phagocytic, antigen-presenting, cytotoxic, and trophic effects of activated microglia are thought to facilitate repair (Elkabes et al., 1996; Polazzi et al., 2001; Harada et al., 2002). Activation has been associated with neuronal death in vitro. It may therefore also contribute to neuronal injury in the context of pathological processes (Chao et al., 1992; Giulian, 1999; Kingham et al., 1999; Liberatore et al., 1999; Streit et al., 1999; Golde et al., 2002). Furthermore, recent studies have highlighted the influence of neuron-derived signaling effects on microglial function, suggesting that controlled elimination of activated microglia may provide a method of limiting the inflammatory response (Neumann and Wekerle, 1998; Liu et al., 2001; Polazzi and Contestabile, 2003).

Semaphorins are a family of secreted and cell-bound signaling molecules defined by the presence of a common 500 aa Sema domain. They are best characterized in relation to axon guidance during development of the nervous system (Tessier-Lavigne and Goodman, 1996; Bagnard et al., 1998; Kolodkin, 1998). Neuropilin and plexin represent the two families of semaphorin receptors that interact to form a coreceptor complex for secreted semaphorins, including Sema3A. Although much remains to be learned about the biological activities and specificities of semaphorins, the functions of Sema3A are mediated primarily through binding to the Neuropilin-1 (Npn-1) and Plexin-A1 coreceptor complex (Takahashi et al., 1999; Tamagnone and Comoglio, 2000). Neuropilins lack a signaling-competent cytoplasmic domain and ensure semaphorin binding, whereas the transmembrane receptor plexin mediates the intracellular response (Kolodkin, 1998; Winberg et al., 1998; Tamagnone et al., 1999).

An emerging body of evidence suggests that semaphorins subserve diverse functions, many unrelated to axon guidance. These include immune modulation, organogenesis, angiogenesis, and cell migration (Behar et al., 1996; Comeau et al., 1998; Gagliardini and Fankhauser, 1999; Miao et al., 1999; Spriggs, 1999; Spassky et al., 2002; Cohen et al., 2003). Sema3A has also been implicated as a mediator of embryonic neuronal apoptosis. Taken with the recent identification of Sema3A in the adult and injured brain, this suggests a possible role for semaphorin in influencing neural repair (Shirvan et al., 1999; Fujita et al., 2001; Pasterkamp and Verhaagen, 2001). The observation that neurons secrete Sema3A and that Npn-1 and Npn-2 are expressed by activated microglia after middle cerebral artery occlusion suggests...
the potential for reciprocal interaction between neurons and microglia in association with tissue injury (Fujita et al., 2001).

In the present study, we characterized the differential expression profile of neuropilin and plexin in resting and activated microglia. Activated microglia upregulate Npn-1 and Plexin-A1 in vitro and in vivo. We provide evidence that stressed neurons express Sema3A and mediate microglial apoptosis. The death effect of stressed neuron conditioned medium (SNCM) is blocked by inhibition of Npn-1 binding. Together, these findings suggest a novel mechanism whereby neurons exploit the microglial expression of Npn-1 and Plexin-A1, protecting themselves from potentially threatening effects of activated microglia by secreting Sema3A.

Materials and Methods

Microglial cell culture. Mixed glial cultures were prepared following the protocol of McCarthy and de Vellis (1980). Briefly, the forebrains of newborn rat pups were removed, and the meninges were stripped before mechanical and enzymatic dissociation. The resulting cell suspension was plated onto poly-l-lysine-coated 13 mm glass coverslips (1.5 million cells per well; Nunclon, Naper-ville, IL). Contaminating cells were removed by allowing the cell suspension to adhere to the surface for 10 min at 37°C before washing in two changes of medium. Adherent microglia were incubated for 24 h to allow them to become “quiescent” before culturing for an additional 24 h in conditioning medium (insulin-free Sato-supplemented DMEM containing bovine serum albumin (100 µg/ml), transferrin (100 µg/ml), progesterone (0.06 µg/ml), putrescine (16 µg/ml), selenite (0.04 µg/ml), thyroxine (0.4 µg/ml), and triiodothyronine (0.04 µg/ml)] with or without interferon-γ (IFNγ; 100 U/ml) to produce highly enriched populations of activated or resting microglia.

Neuronal cell cultures were prepared from the cortices of embryonic day 16 (E16) rat embryos (Wilkins et al., 2001). After enzymatic and mechanical dissociation, cells were plated onto freshly prepared poly-l-lysine-coated 13 mm glass coverslips (1.5 × 10⁴ neurons per coverslip) or plastic 6-well plates (2 million cells per well; Nunclon, Naperville, IL). Contaminating cells were removed by allowing the cell suspension to adhere to the surface for 10 min at 37°C before washing in two changes of medium. Adherent neurons were incubated for 24 h to allow them to become “quiescent” before culturing for an additional 24 h in conditioning medium (insulin-free Sato-supplemented DMEM) containing bovine serum albumin (100 µg/ml), transferrin (100 µg/ml), progesterone (0.06 µg/ml), putrescine (16 µg/ml), selenite (0.04 µg/ml), thyroxine (0.4 µg/ml), and triiodothyronine (0.04 µg/ml) with or without interferon-γ (IFNγ; 100 U/ml) to produce highly enriched populations of activated or resting microglia.

Preparation of SNCM. Neuronal cultures were prepared in poly-l-lysine-coated 6-well plates (2 million cells per well) and cultured for 1 or 5 days in 2% B27-supplemented medium. Cells were then washed in DMEM and cultured in conditioning medium (insulin-free Sato) for up to 72 h. After centrifugation at 1000 rpm for 5 min and passage through a 45 µm filter, the resulting supernatant was stored at −20°C and diluted 1:1 with fresh medium for experiments, unless stated otherwise.

Sema3A-conditioned medium was generated from 293T cells transfected with chicken Sema3A using myc-his tag and culture in 1% FCS-supplemented DMEM for 24 h (Koppel et al., 1997). Sema3A activity of conditioned medium was validated using the standard collapse unit assay on E15 rat dorsal root ganglia explants. At a 1:250 dilution, Sema3A-conditioned medium induced 50% growth cone collapse after 30 min (Koppel et al., 1997). For experimental procedures, Sema3A-conditioned medium was used at a 1:10 dilution with fresh medium (unless stated otherwise) for 24 h at 37°C. Control conditioned medium was derived from green fluorescent protein-transfected 293T cells.

For caspase inhibition experiments, 50 µM of the pan-caspase inhibitor Z-VAD-FMK (R & D Systems, Abingdon, UK) was added to the incubation medium for 24 h. Function-blocking anti-Npn-1 antibody (a gift from A. Kolodkin, Johns Hopkins, Baltimore, MD) was used at 100 µg/ml.

Figure 1. Characterization of the activation state of microglia. Microglia were purified from mixed glial cultures, plated for 24 h to become quiescent, and cultured for an additional 24 h with (right) or without (left) IFNγ, representing activated and resting microglia, respectively. A–D, Representative photomicrographs of microglia under phase-contrast microscopy (A, B) or immunolabeled using an antibody against the microglial marker β-isolectin (C, D) show the distinct morphology of resting (A, C) and activated (B, D) microglia. E–H, Resting microglia are characterized by ramified morphology in contrast to an amoeboid appearance after activation. Immunomicrographs show OX6-positive activated microglia after treatment with IFNγ (6, H) with corresponding phase-contrast images (E, G). I, Western blot demonstrating upregulation of inducible nitric oxide synthase (iNOS) after IFNγ-mediated activation of microglia (representative of three independent experiments). Equal loading is confirmed by actin. R, Resting microglia; A, activated microglia.
Immunocytochemistry. Immunocytochemistry was used to identify cell phenotypes. Cells were stained “live” or after fixation in 4% paraformaldehyde. The primary antibodies against oligodendrocyte cell-surface markers were galectocerebroside (GaC; 1:4) and A2B5 (1:4) (both derived from hybridoma lines; European Collection of Cell Cultures, Salisbury, UK). Intracellular markers were stained after permeabilization of fixed cultures with 100% methanol for 10 min at −20°C; β4-isoelectrin (1:50), OX6 (1:200), and neuronal β3-tubulin (1:200) (all from Sigma); and astrocyte GFAP (1:200; Dako, High Wycombe, UK). Fluorescence-conjugated secondary antibodies were used to visualize primary antibody staining, and Hoechst 33258 (1:5000 bisbenzamide; Sigma) was added for the final 10 min of incubation for nuclear identification. Analysis was undertaken using a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling kit (Boehringer, Bagnolet, France).

Lipopolysaccharide lesion model. Adult rats were killed and perfused with 4% paraformaldehyde 24 h after basal ganglia microinjection of lipopolysaccharide (LPS; 5 µg; Sigma) to simulate a lesion model. After fixation in 4% paraformaldehyde and dehydration in 70%, 80%, and 95% ethanol and then in 100% ethanol, brains were embedded in paraffin and cut serially into 5-µm sections. Sections were stained with hematoxylin and eosin (H&E) for histological examination (Hamamatsu, Japan) and with immunohistochemistry (IHC; see later) for immunocytochemical examination. Immunohistochemistry was performed on 5-µm sections of paraffin-embedded tissue.

Reverse transcription-PCR. Total RNA was isolated from microglia conditioned for 24 h with or without IFN-γ, postnatal day 1 (P1) rat cortices, or cortical neuron cultures using Trizol (Invitrogen, Paisley, UK). Gene-specific 32P-labeled probes were synthesized (from Amersham Biosciences, Uppsala, Sweden). Gene-specific 32P-labeled probes were synthesized (from Amersham Biosciences, Uppsala, Sweden). Gene-specific 32P-labeled probes were synthesized (from Amersham Biosciences, Uppsala, Sweden).

Figure 2. Differential expression of plexin and neuropilin RNA dependent on the activation state of microglia. A, B, RNA isolated from microglia cultured with or without IFN-γ for 24 h was analyzed for expression of plexin and neuropilin isoforms by Northern blot (A) and RT-PCR (B). Both techniques show upregulation of Plexin-A1 and Neuropilin-1 after activation (representive gel of three independent experiments). C, Immuno-blot of entire cell lysates from resting and activated microglia blotted for antibodies against Plexin-A1 and Plexin-A2 show upregulation of Plexin-A1 after activation. Equal loading is demonstrated by actin. Results are based on three independent experiments. R, Resting microglia; A, activated microglia.
conjugated secondary antibodies with enhanced chemoluminescence (Renaissance ECL reagent; NEN, Boston, MA).

Cell counting. Cells were viewed using a Leitz microscope. Five consecutive random fields (using a grid) were counted for each coverslip. Results are expressed as mean ± SEM from at least three different coverslips and at least three separate experiments, unless stated otherwise. Statistical analysis (ANOVA with Newman–Keuls post hoc) was conducted using Prism 2.0 software.

Results
Activated microglia express Plexin-A1 and Npn-1
Enriched populations of resting and activated microglia from neonatal rat cortex mixed glial cultures were characterized. Microglia (identified by β4-isolectin-positive staining) represented 95.5 ± 1.3% (n = 3) of cells at 24 h after plating; 3.1 ± 0.5% were GFAP+ astrocytes, and the remainder were A2B5+ oligodendrocyte precursor cells. Cells were allowed to become quiescent in 2% B27-supplemented medium for 24 h before culture for an additional 24 h with or without IFNγ to produce resting or activated microglia. Resting microglia adopted a characteristic ramified morphology compared with the amoeboid appearance of activated microglia, which were OX6 positive with increased inducible nitric oxide synthase expression (Fig. 1).

Initial studies to examine the effect of activation on gene expression in microglia using differential display identified an unknown cDNA, the expression of which was significantly upregulated after activation (data not shown). Homology with corresponding human and mouse sequences identified this transcript as Plexin-A1 (Fig. 2). In view of the fact that, together with Npn-1, Plexin-A1 forms part of the high-affinity receptor complex for Sema3A, we next studied the expression profile of other members of the semaphorin receptor family in resting and activated microglia by Northern analysis (Fig. 2A). Upregulation of Npn-1 (and Plexin-A1) after microglial activation was confirmed by reverse transcription-PCR (RT-PCR) and by Western blot analysis (Fig. 2B, C). The other members of the neuropilin–plexin family were not differentially expressed after microglia activation.

Dose-dependent death effect of Sema3A on microglia
After the demonstration of increased Plexin-A1 and Npn-1 in activated microglia, we next examined the influence of Sema3A on both resting and activated mi-
microglia. We exposed resting and activated microglia to Sema3A-conditioned medium for 24 h, followed by fixation and immunocytochemistry. Contingent on the activation state, microglia showed a dose-dependent death effect, and, in particular, activated cells were more susceptible (Fig. 3A). No effect on survival was observed using control conditioned medium. β-Lectin and Hoechst counts were comparable, consistent with the purity of the cultures; >98% were β- lectin positive cells at this time point. Interestingly, at a 1:10 and 1:20 dilution, there was a significant differential death effect, and activated microglia were more susceptible to death at each dilution (p < 0.01 and p < 0.05, respectively).

Morphological analysis of Hoechst-labeled nuclei and DNA ladder assay (data not shown) demonstrated characteristic fragmented condensed nuclei and DNA cleavage after Sema3A exposure. Moreover, this, together with the finding that the addition of the pan-caspase inhibitor Z-VAD-FMK significantly reduces the death effect of Sema3A after microglia (p < 0.001) (Fig. 3C), indicates that Sema3A causes apoptosis of microglia. The addition of anti-Npn-1 function-blocking antibody to microglia cultures significantly inhibited the death effect of Sema3A (p < 0.001) (Fig. 3D). Control experiments in non-Sema3A-treated cells showed no discernible effect of anti-Npn-1 on microglial survival (data not shown).

**Stressed neurons produce Sema3A**

We next examined neuronal expression of semaphorin (Fujita et al., 2001). E16 rat cortical neurons were cultured in 2% B27-supplemented medium for 5 d and stressed by replacement of medium with insulin-free Sato for up to 3 d. Neuronal cultures were phenotypically characterized at 5 d in vitro. βIII-Tubulin + neurons represented 94.6 ± 1.6% (n = 3) of the cells; the remainder were predominantly astrocytes, with 0.4% O4 + oligodendrocyte precursors and 0.2% GalC + oligodendrocytes. Immune characterization at 3 d after supplement replacement revealed 75% neuronal death. RT-PCR demonstrated Sema3A expression by remaining neurons at 24, 48, and 72 h after the induction of neuronal stress (Fig. 4A).

**SNCM mediates microglia death**

We next asked whether SNCM could influence microglial survival. SNCM was derived from E16 rat cortical neurons, cultured for 5 d in 2% B27-supplemented medium, followed by an additional 3 d in Sato-only-supplemented medium. Analysis of cultures 24 h after the addition of SNCM to both resting and activated microglia revealed a significant death effect by SNCM (Fig. 4B). Activated microglia were significantly more susceptible (p < 0.05).

To determine whether death induced by SNCM is attributable to Sema3A present in conditioned medium, we used function-blocking anti-Npn-1 antibody. Anti-Npn-1 (α-Npn-1) resulted in a significant reduction in SNCM-mediated microglial death (p < 0.01), suggesting that Sema3A is in part responsible for SNCM-mediated microglial cell death (Fig. 4C). A limited effect was also observed in the resting microglia. This is likely to be attributable to the presence of some activated microglia as a result of culturing, even within the resting cultures.

**In vivo expression of Sema3A, Plexin-A1, and Npn-1 in adult brain**

To determine whether the identification in vitro of differential Plexin-A1 and Npn-1 expression contingent on the microglia activation state also occurs in vivo, we studied adult rat brains that were killed at 24 h after the induction of focal injury using LPS. Immunohistochemical characterization confirmed that LPS injection resulted in an acute inflammatory response characterized by microglial activation (Fig. 5A–E). Contralateral control lesions of vehicle alone did not produce an acute inflammatory response (Fig. 5A, B, D). Immunohistochemistry for OX42 demonstrated ramified resting microglia in control lesion cortex and amoeboid-appearing activated microglia in LPS lesions. Significantly, Plexin-A1 and Npn-1 immunoreactivity was present only in LPS-mediated inflammatory lesions, colocalized with activated microglia (Fig. 5F–I). Sema3A expression was upregulated in the LPS-treated region compared with control lesions (Fig. 6A–D). Furthermore, Sema3A expression was evident in NeuN-positive neurons (Fig. 6E–G). Western blot analysis of brain sections after LPS or vehicle lesions confirmed significantly enhanced Sema3A expression in LPS-treated tissue (Fig. 6H, I). Consistent with our in vitro findings, in vivo nuclear morphological analysis provides evidence that Sema3A-mediated microglial death is apoptotic (Fig. 7).

**Discussion**

Here, we show for the first time that Sema3A can mediate neuronal–microglia interactions. We demonstrate that, contingent on the activation state, microglia differentially express the semaphorin receptors Plexin-A1 and Npn-1 in vitro and in vivo. We confirm neuronal expression of Sema3A in vitro and in vivo and provide evidence that stressed neurons are able to mediate microglial death by production of Sema3A.
Differential expression of Plexin-A1 and Npn-1 by microglia

The profile of neuropilin and Sema3A has been analyzed in a cerebral model of ischemia, demonstrating microglial expression of Npn-1 and Npn-2 (Fujita et al., 2001). Our findings confirm and extend these observations, highlighting the differential expression profile of Plexin-A1 and Npn-1 between resting and activated microglia. Importantly, the other members of the plexin–neuropilin family were not found to be differentially expressed after microglia activation. We provide evidence in vitro and in vivo for upregulation of both Plexin-A1 and Npn-1 after activation of microglia. Recent reports also implicate semaphorin-mediated neuronal and neuronal progenitor apoptotic cell death (Gagliardini and Fankhauser, 1999; Shirvan et al., 1999, 2002; Bagnard et al., 2001). We therefore examined whether semaphorin has a similar effect on microglia. Exposure to Sema3A-conditioned medium resulted in dose-dependent death of microglia, with activated microglia proving significantly more vulnerable. Death was apoptotic as determined by nuclear morphology, caspase inhibition, and DNA ladder assay analysis. The addition of anti-Npn-1 antibody significantly attenuated the death effect, consistent with the conclusion that Sema3A can mediate apoptosis of microglia.

Microglia–neuron interaction: a role for semaphorin

An emerging concept is the idea of cross talk between microglia and neurons, an interaction that can be regarded as harmful or beneficial depending on context. In vitro studies demonstrate microglia neurotoxicity through various mechanisms including nitric oxide and NMDA-mediated toxicity (Piani et al., 1991; Chao et al., 1992; Kingham et al., 1999). Activated microglia have been implicated in demyelinating and neurodegenerative diseases (Itagaki et al., 1989; Lucchinetti et al., 2000; McGeer and McGeer, 2003). There is some experimental support for the proposal that blocking microglia activation may be neuroprotective, for example through the production of trophic factors including NGF and NT3 (Mallat et al., 1989; Thanos et al., 1993; Elkabes et al., 1996; Rabchevsky and Streit, 1997; Lim et al., 2000; Wu et al., 2002; Zhang et al., 2003). Furthermore, the observation that TNF-α null mice show increased neuronal death after ischemia in association with reduced microglia recruitment is consistent with microglia-mediated neuroprotection.
Studies highlighting the reciprocal relationship between microglia and neurons emphasize the dynamic nature of microglia–neuron interaction (Zietlow et al., 1999; Polazzi and Contestabile, 2003). The precise identity of the protein signals underlying neuron-mediated apoptosis of activated microglia remains unclear (Polazzi and Contestabile, 2003).

Increasing recognition of the importance of semaphorin-mediated signaling in roles other than developmental axon guidance, such as the response to injury, offers a possible biological explanation for the sequence of microglial neuropilin and plexin expression, followed by an apoptotic response to Sema3A. Adult semaphorin expression contributes to the nonpermissive environment of glial scars (Pasterkamp et al., 1999; Niclou et al., 2003). In addition, reciprocal expression in the ischemic adult brain of neuronal Sema3A and microglial neuropilin suggests semaphorin-mediated microglia–neuron interaction (Fujita et al., 2001). Our observation that the effect of medium conditioned by stressed neurons on microglia is significantly blocked by anti-neuropilin antibody suggests that neuron-derived Sema3A mediates microglial apoptosis. Additional evidence is provided by the upregulation of neuronal Sema3A, and Plexin-A1 and Npn-1, by activated microglia after LPS-mediated activation in the adult rat brain. Together, these findings indicate that semaphorin is capable of mediating cell death (Shirvan et al., 1999; Bagnard et al., 2001).

Tanaka and Koike (2002) have identified upregulation of a novel microglia gene (mrf-1) after neuronal death that is also implicated in cross talk between microglia and damaged neurons. Our study identifies a novel mechanism of microglia–neuron interaction that may inform the emerging concept of neuronal control of microglia. Because activated microglia are implicated in many disease processes, a biological context for these in vitro and in vivo findings can be readily envisaged (Chao et al., 1992; Giulian, 1999; Kingham et al., 1999; Liberatore et al., 1999; Streit et al., 1999; Neumann, 2001).

In summary, we demonstrate differential expression by activated microglia of the semaphorin receptors neuropilin and plexin. Stressed neurons mediate death of microglia by a semaphorin-dependent mechanism indicating reciprocal regulation of microglia. This may represent a mechanism for containing the inflammatory response to tissue injury whereby the survival of stressed but viable neurons is ensured without compromising the response.
moyal of microglia of neurons that have already sustained a lethal injury.

References


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