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IMMUNOLOGY AND INFLAMMATION


The authors note that their conflict of interest statement was omitted during publication. The authors declare the following: “T.C.T. has been awarded, under an agreement with Tufts University, the following patents: US 9,050,275 - Methods of Screening for and Treating Autism Spectrum Disorders and Compositions for Same and US 9,176,146 - Methods of Treating Autism Spectrum Disorders and Compositions for Same. A Provisional Patent Application was also filed by Tufts University as US 62/396,546 - Compositions and Methods of Autism Treatment. T.C.T. is also the Scientific Director of Algonot, LLC (Sarasota, FL) that has developed the flavonoid-containing trademarked dietary supplement NeuroProtek, for which Tufts receives royalties. No portion of the work described in this paper was funded by Algonot.”

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Neurotensin stimulates sortilin and mTOR in human microglia inhibitable by methoxyluteolin, a potential therapeutic target for autism

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We had reported elevated serum levels of the peptide neurotensin (NT) in children with autism spectrum disorders (ASD). Here, we show that NT stimulates primary human microglia, the resident immune cells of the brain, and the immortalized cell line of human microglia-SV40. NT (10 nM) increases the gene expression and release (P < 0.001) of the proinflammatory cytokine IL-1β and chemokine (C-X-C motif) ligand 9 (CCL9), chemokine (C-C motif) ligand 2 (CCL2), and CCL5 from human microglia. NT also stimulates proliferation (P < 0.05) of microglia-SV40. Microglia express only the receptor 3 (NTR3)/sortilin and not the NTR1 or NTR2. The use of siRNA to target sortilin reduces (P < 0.001) the NT-stimulated cytokine and chemokine gene expression and release from human microglia. Stimulation with NT (10 nM) increases the gene expression of sortilin (P < 0.0001) and causes the receptor to be translocated from the cytoplasm to the cell surface, and to be secreted extracellularly. Our findings also show increased levels of sortilin (P < 0.0001) in the serum from children with ASD (n = 36), compared with healthy controls (n = 20). NT stimulation of microglia-SV40 causes activation of the mammalian target of rapamycin (mTOR) signaling kinase, as shown by phosphorylation of its substrates and inhibition of these responses by drugs that prevent mTOR activation. NT-stimulated responses are inhibited by the flavonoid methoxyluteolin (0.1–1 μM). The data provide a link between sortilin and the pathological findings of microglia and inflammation of the brain in ASD. Thus, inhibition of this pathway using methoxyluteolin could provide an effective treatment of ASD.

Autism spectrum disorders (ASD) are neurodevelopmental disorders (1, 2). The prevalence of ASD is now estimated to be 1 in 45 children (3). Unfortunately, there is still no distinct pathogenesis (4) even though a number of neuropathological defects have been reported in the brains of children with infantile autism (5). Microglia, the highly plastic resident immune cells of the brain (6, 7), have been shown to be activated in the brains of patients with ASD (8–11). Microglia activation and proliferation could lead to focal inflammation of the brain and “choking” of normal synaptic connectivity (12, 13). Microglia express membrane receptors for several neuropeptides, allowing them to communicate with neurons, astrocytes (14), and mast cells (15), known to be involved in allergic and inflammatory processes (16).

Various stimuli, such as the bacterial lipopolysaccharide (LPS) (14, 17), have been shown to switch microglia into the M1 phenotype, denoted by the release of proinflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF) (18), as well as the chemokines (C-C motif) ligand 2 (CCL2) and CCL5 (8, 19), also found to be increased in brains of deceased patients with ASD. Immune dysfunction (18, 20–22) and inflammation of the brain (23–25) are now invoked in the pathogenesis of ASD. However, the stimuli that promote these inflammatory processes in the brain are presently unknown.

Our laboratory had reported increased serum levels of the peptide neurotensin (NT), but not substance P or β-endorphin (26), in children with ASD (26, 27). NT is found in the brain (28, 29) and is primarily secreted from neurons (29) and astrocytes (30). NT responses are mediated through three receptors: NTR1 (31) and NTR2 (32, 33), which belong to the G protein-coupled seven-transmembrane receptor family (34), and NTR3, also known as sortilin (35). NTR3/sortilin is a type I sorting protein [part of the Vps10p domain single-transmembrane receptor family (31)], a multifaceted receptor mainly expressed in the CNS during embryonic development (36).

NTR3/sortilin has been shown to be expressed in murine microglia through which NT stimulates IL-1β, CCL2, and TNF gene expression (37). However, rodent microglia have major biochemical and pharmacological differences compared with primary human microglia (38). Moreover, animal models do not reflect human inflammatory processes (39). A subset (1–5%) of ASD cases has gene mutations in regulatory proteins upstream of the signaling complexes termed the “mammalian target of

**Significance**

Human microglia, the resident immune cells of the brain, express only the neurotensin (NT) receptor-3/sortilin. NT significantly increases microglia synthesis and release of proinflammatory cytokine IL-1β and chemokine (C-X-C motif) ligand 8 (CCL8), chemokine (C-C motif) ligand 2 (CCL2), and CCL5 via NTR3/sortilin. A soluble form of this receptor is secreted from stimulated microglia and is increased in the serum of children with autism spectrum disorders (ASD). These responses and the NT-stimulated increases in microglia numbers are mediated via mammalian target of rapamycin (mTOR) activation and are inhibitable by the natural flavonoids luteolin and methoxyluteolin.

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These mutations in mice lead to a behavioral phenotype resembling autism (41), and targeting the mTOR pathway has been shown to reverse autism-like behavior (42, 43). The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway also regulates the activation of both microglia (44) and mast cells (45), which may cross-talk to exacerbate inflammation of the brain (15).

There are no clinically available drugs addressing the core symptoms of ASD. The natural flavonoid luteolin has potent antioxidant and antiinflammatory properties (46). It also inhibits activation of microglia (17, 47–49). Luteolin also reverses autism-like behavior in mice (50). Two clinical studies further reported that a luteolin-containing dietary formulation significantly improved attention and sociability in children with ASD (51, 52). Its structural analog containing dietary formulation significantly improved attention and sociability in children with ASD by investigating whether NT stimulation (10 nM) for 24 h increases the surface-associated NTR3/sortilin in primary human microglia (Fig. 3B) and in the microglia-SV40, which appears to be colocalized with the filamentous actin-binding protein ionized calcium-binding adaptor molecule-1 (Iba-1) (Fig. 3C). We hypothesized that surface NTR3/sortilin may also be secreted extracellularly. Stimulation of human microglia-SV40 with NT (10 nM) for 24 h (P < 0.0001) or 48 h (P < 0.05) increases levels of soluble NTR3/sortilin (Fig. 3D). There is no apparent significant difference in the total cellular NTR3/sortilin levels in human microglia stimulated with NT (10 or 100 nM) after 24 or 48 h, compared with control cells (Fig. 3E).

NT Stimulates Proinflammatory Cytokine and Chemokine Release from Human Microglia via NTR3/Sortilin. Even though human microglia do not express NTR1 and NTR2, we pretreated microglia-SV40 with the NTR1 selective nonpeptide antagonist SR48692 or the dual NTR1/NTR2 antagonist SR142948A (10–1,000 nM for 1 h), before stimulation with NT (10 or 100 nM). Pretreatment with these receptor antagonists did not affect IL-1β, CXCL8, CCL2, and CCL5 mediator release from human microglia-SV40. To determine whether NTR3/sortilin mediates the NT-stimulated proinflammatory cytokine and chemokine secretion of numerous proinflammatory cytokines and chemokines (Fig. S1), which were then measured by specific ELISAs. NT stimulation at physiological doses (10 or 100 nM) for 24 h increases secretion of the proinflammatory cytokine IL-1β and chemokine (C-X-C) motif ligand 8 (CXCL8), CCL2, and CCL5 (P < 0.001), compared with controls, from both primary human microglia and microglia-SV40 (Fig. 1 A–D). LPS (10 or 100 ng/mL) used as a positive control also increases (P < 0.0001) the secretion of all mediators, including IL-6 and TNF, from both human microglia cell types after 24-h stimulation (Fig. S2). NT stimulation (10 or 100 nM) significantly increases (P < 0.0001) the gene expression of IL-1β, CXCL8, CCL2, and CCL5 after 12 h in both primary human microglia and microglia-SV40 (Fig. 2 A–D). LPS (10 or 100 ng/mL; P < 0.0001) also increases the synthesis of these mediators from human microglia after 12-h stimulation.

Results

NT Induces Expression of Proinflammatory Cytokines and Chemokines in Human Microglia. To evaluate whether NT can switch human microglia to the proinflammatory M1 phenotype, we first used a human cytokine and chemokine array blot to identify any cytokines and chemokines differentially expressed in control and NT-stimulated human microglia-SV40. NT increases expression of numerous proinflammatory cytokines and chemokines (Fig. 1A), which were then measured by specific ELISAs. NT stimulation at physiological doses (10 or 100 nM) for 24 h increases secretion of the proinflammatory cytokine IL-1β and chemokine (C-X-C) motif ligand 8 (CXCL8), CCL2, and CCL5 (P < 0.001), compared with controls, from both primary human microglia and microglia-SV40 (Fig. 1 A–D). LPS (10 or 100 ng/mL) used as a positive control also increases (P < 0.0001) the secretion of all mediators, including IL-6 and TNF, from both human microglia cell types after 24-h stimulation (Fig. S2). NT stimulation (10 or 100 nM) significantly increases (P < 0.0001) the gene expression of IL-1β, CXCL8, CCL2, and CCL5 after 12 h in both primary human microglia and microglia-SV40 (Fig. 2 A–D). LPS (10 or 100 ng/mL; P < 0.0001) also increases the synthesis of these mediators from human microglia after 12-h stimulation.

Human Microglia Express Only NTR3/Sortilin, Which Increases in Gene and Surface Protein Localization in Response to NT. We next investigated the expression of the three types of NT receptors, NTR1, NTR2, and NTR3/sortilin, in both primary human microglia and the human microglia-SV40 cell line. The high-affinity NTR1 and low-affinity NTR2 are undetectable; however, NTR3/sortilin gene expression is detectable, and significantly increases (P = 0.0001) after 12 or 24 h of NT treatment (10 or 100 nM) (Fig. 3A). Protein levels of NTR1 and NTR2 are also undetectable by Western blot analysis.

To determine changes in the cellular localization of NTR3/sortilin after NT stimulation, differential interference contrast (DIC)/confocal immunofluorescence microscopy was carried out. Immunodetectable NTR3/sortilin in control primary human microglia and microglia-SV40 reveals a cytosolic distribution. Stimulation with NT (10 nM) for 24 h increases the surface-associated NTR3/sortilin in primary human microglia (Fig. 3B) and in the microglia-SV40, which appears to be colocalized with the filamentous actin-binding protein ionized calcium-binding adaptor molecule-1 (Iba-1) (Fig. 3C). We hypothesized that surface NTR3/sortilin may also be secreted extracellularly. Stimulation of human microglia-SV40 with NT (10 nM) for 24 h (P < 0.0001) or 48 h (P < 0.05) increases levels of soluble NTR3/sortilin (Fig. 3D). There is no apparent significant difference in the total cellular NTR3/sortilin levels in human microglia stimulated with NT (10 or 100 nM) after 24 or 48 h, compared with control cells (Fig. 3E).
release, human microglia were subjected to siRNA down-regulation of NTR3/sortilin levels. Human microglia-SV40 were transfected with two different scrambled and targeted NTR3/sortilin siRNAs.

Gene expression analysis by quantitative real-time (qRT)-PCR revealed >95% knockdown of NTR3/sortilin in siRNA-transfected cells after 48 h, compared with control siRNA-transfected or unstimulated microglia (Fig. 4A). Protein levels of NTR3/sortilin in microglia-SV40 transfected with NTR3 siRNA are abolished, whereas the control cells treated with scrambled siRNA retain normal expression levels after 48 or 72 h (Fig. 4B). Stimulation by NT of microglia in which NTR3/sortilin levels were down-regulated significantly decreases ($P < 0.001$) IL-1$\beta$, CXCL8, CCL2, and CCL5 release, compared with control siRNA-transfected or unstimulated microglia (Fig. 4B).

To ensure that the effect of down-regulated NTR3/sortilin was not due to any involvement in intracellular mediator transport or release, we investigated the level of proinflammatory mediator gene expression in NT-stimulated scrambled and targeted NTR3/sortilin siRNA-treated microglia-SV40. Gene expression of IL-1$\beta$, CXCL8, CCL2, and CCL5 significantly decreases ($P < 0.001$) after NT stimulation in microglia with down-regulated NTR3/sortilin levels, compared with scrambled siRNA-treated or unstimulated microglia (Fig. S3).

**NT Activates PI3K/mTOR Signaling in Human Microglia That Is Blocked by Luteolin and Methoxyxalutelin.** To investigate the signaling pathway involved in the stimulation of human microglia-SV40 in response to NT, a phosphoarray blot to detect the phosphorylated (p) signaling proteins was used. Protein levels of phosphorylated substrates that are up-regulated in microglia-SV40 after NT stimulation include the downstream mTOR substrates, p70 ribosomal 6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) proteins (Fig. S4A). Western blot analysis was then performed to detect the total and p-levels of mTOR, as well as p70S6K and 4EBP1 proteins after stimulation by NT (10 nM) from 0 to 60 min. NT increases the levels of pmTOR, as well as p70S6K and 4EBP1 proteins after stimulation by NT (10 nM) from 0 to 60 min. NT increases the levels of pmTOR (Ser2448 and the downstream mTORC1 substrate pp70S6KThr389 within 30 min (Fig. S4A).

We next investigated whether the natural flavonoids luteolin and methoxyluteolin affect mTOR signaling by comparing their inhibitory effect with the inhibitory effect of various mTOR inhibitors in microglia stimulated by NT. We used the first-generation allosteric mTOR inhibitor, rapamycin; the small-molecule ATP-competitive kinase inhibitor of mTORC1 and mTORC2, KU-0063794 (KU); and the dual PI3K/mTOR inhibitor, PF-04691502 (PF). Human microglia-SV40 were serum-starved overnight and preincubated with rapamycin, 0.5 $\mu$M KU/PF, or luteolin and methoxyluteolin (0.1–10 $\mu$M) for 12 h before NT stimulation (30 min), which significantly decreases levels of pmTORSer2448 and p70S6KThr389 compared with those levels in microglia stimulated by NT alone (Fig. 5B–D). NT did not increase the levels of total or p4EBP1 proteins in stimulated microglia-SV40; however, the levels p4EBP1 in unstimulated microglia decrease in the presence of mTOR inhibitors, whereas the flavonoids had no effect on these inhibitors (Fig. 5E).

Phospho-ELISAs were also performed on microglia stimulated with NT and/or pretreated with the PI3K/mTOR inhibitors or luteolin and methoxyluteolin to quantify levels of pAKT Ser473, pmTORSer2448, and pp70S6KThr389 to assess activation of mTOR. Levels of pAKT or pmTOR or p70S6K proteins

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**Fig. 3.** NTR3/sortilin gene expression and cellular localization in HM, and its release in response to NT. (A) Primary HM (2.5 × 10$^5$ cells) and immortalized HM-SV40 were stimulated with NT for 12 or 24 h to measure gene expression levels of NTR3/sortilin in control and NT-stimulated (10 or 100 nM) microglia by qRT-PCR. All conditions were performed in triplicate for each dataset and were repeated three times ($n = 3$). Results were normalized against the endogenous GAPDH and expressed relative to the mean of the control for the gene of interest, with significance of comparisons denoted by *$P < 0.05$, **$P < 0.001$, or ***$P < 0.0001$. Microglia-SV40 (5 × 10$^5$ cells per four-well chamber–coated slide) were stimulated with NT (10 nM) for 24 h, and then fixed and permeabilized to stain for nuclei using DAPI (blue), with specific antibodies for Alexa 488-NTR3/sortilin (green) or Alexa 594-lba-1, a microglial marker protein (red), whereas rabbit IgG was used for the negative control. The cell surface and cytosolic distribution of NTR3/sortilin protein is shown in control and NT-stimulated primary HM (B) and immortalized HM-SV40 (C), where colocalization with lba-1 is also apparent (white arrows). Qualitative analysis was done using images from triplicates, and representative images are shown. (D) HM-SV40 (1 × 10$^5$ cells) were stimulated with NT (10 nM) for 24 or 48 h to measure release of soluble NTR3/sortilin in culture media by ELISA. All conditions were performed in triplicate for each dataset and repeated three times ($n = 3$). Significance of comparisons is denoted by *$P < 0.05$, or **$P < 0.001$, or ***$P < 0.0001$. (E) HM-SV40 (1 × 10$^5$ cells) were stimulated with NT (10 or 100 nM) for 24 or 48 h to measure total cellular NTR3/sortilin levels by Western blot analysis, where TREM-2 served as a microglial marker protein and $\beta$-actin as the loading control. All conditions were performed in triplicate for each dataset and were repeated three times ($n = 3$), and a representative image is shown.
NT-induced proinflammatory mediator release from human microglia via NTR3/sortilin. Immortalized HM-SV40 (1 × 10⁶ cells) were transfected with two different predesigned and validated siRNAs targeting human NTR3/sortilin (NTR3#1 and NTR3#2) or scrambled controls (Sc#1 and Sc#2) for 48 and 72 h before evaluation of NTR3/sortilin gene levels by qRT-PCR (A) and protein levels by Western blot analysis (B). Control and siRNA-transfected microglia-SV40 (5 × 10⁶ cells) were stimulated with NT (10 nM) for 24 h to measure release of IL-1β (C), CXCL8 (D), CCL2 (E), and CCL5 (F) by ELISA. All conditions were performed in triplicate for each dataset and were repeated three times (n = 3). Significance of comparisons is denoted by ***P < 0.0001.

**Discussion**

The impetus for this present study came from our previous reports that serum NT levels are increased in children with ASD (26, 27). Unlike the previous studies that used murine microglia (55), the present findings show that NT stimulates primary microglia obtained from human brains and also an immortalized human cell line, microglia-SV40. Human microglia express only NTR2. NTR3/sortilin was previously shown to be expressed in NTR3/sortilin from NT-stimulated microglia, we measured soluble NTR3/sortilin in the serum of children with ASD, compared with unstimulated healthy controls. There is a significant positive correlation between serum NTR3/sortilin and circulating NT, levels of these proteins were measured in the same group of patients with ASD and controls. There is a significant positive correlation between serum NTR3/sortilin and NT levels (Spearman’s r = 0.3940, P = 0.0283) (Fig. 9A, Inset).
proinflammatory cytokine/chemokine release from NT-stimulated human microglia-SV40, implying that these NT responses are mediated via NTR3/sortilin.

The difference in the localization of immunodetectable NTR3/sortilin from the cytosol in nonstimulated microglia to the cell surface after stimulation with NT led us to wonder whether sortilin may be secreted extracellularly. The colocalization of NTR3/sortilin with Iba-1, which interacts with actin and is often used to detect microglia (60), allowed us to speculate that Iba-1 may participate in movement of NTR3/sortilin to the cell surface. The extracellular domain of NTR3/sortilin, known as soluble sortilin (61), is shown here to be secreted from NT-stimulated cultured human microglia. Soluble sortilin is also increased in the serum of children with ASD, compared with normal healthy children. Because there is a strong positive correlation of the soluble receptor with serum NT levels in these same patients with ASD, serum NTR3/sortilin may bind to serum NT and limit its biological activity much like the soluble IL-1 receptor binds to circulating IL-1β (62).

Our experiments next explored the signaling pathways involved following NT stimulation of human microglia mediated by NTR3/sortilin by the use of a kinase array blot that detects phosphorylated substrates; this array shows increased mTOR signaling, following which it was determined that activation of the mTOR pathway is necessary for proinflammatory mediator expression in NT-stimulated human microglia. These findings provide evidence why some patients with ASD who have gene mutations in one negative regulatory protein of mTOR, the phosphatase and tensin homolog (63, 64), develop inflammation of the brain that is linked to ASD pathogenesis (65, 66).

Our findings also suggest that mTOR signaling is involved in the transcriptional regulation of proinflammatory cytokine and chemokine synthesis in human microglia. This effect may be mediated via the activation of nuclear factor-kappa B (NF-κB) (67) and the signal transducer and activator of transcription (STAT) pathways (68), critical for transcription of proinflammatory cytokines and chemokines (69). An important finding is that the flavonoids luteolin and methoxyluteolin significantly inhibit gene expression of all the proinflammatory mediators, as well as the activation of mTOR, after stimulation by NT. Not only did the flavonoids inhibit NT-stimulated microglial responses but they also inhibited LPS-stimulated proinflammatory cytokine and chemokine synthesis in microglia, as previously shown in murine microglia (48, 70). Greater concentrations of flavonoids are required to inhibit cytokine or chemokine protein release, compared with gene expression. This apparent discrepancy may be due to some initial secretion before gene expression is fully inhibited by the lower flavonoid concentrations. Alternatively, there may be differential inhibition of gene expression, involving the inhibition of nuclear transcription targets (NF-κB or STAT), compared with cytokine or chemokine protein secretion. Instead, mediator trafficking and secretion may involve inhibition of specific target proteins involved in vesicle fusion, such as soluble N-ethylmaleimide-sensitive factor attachment protein complexes (71, 72).

The source of the increased levels of serum NT in children with ASD is not known. The highest levels of serum NT we had measured before were present in those children with ASD who had gastrointestinal symptoms (27). NT is also found in the gut (29, 73) and increases permeability of the intestinal lumen (74). NT may enter the blood and reach the brain by stimulating perivascular mast cells (75, 76), which disrupt both the gut–blood barrier (77, 78) and the blood–brain barrier (BBB) (79–81) (Fig. 9B). Microglia-derived IL-1β, CXCL8, CCL2, and CCL5 in response to NT can augment the activation of perivascular mast cells, further disrupting the BBB (82–84), perhaps initiating a feedback mechanism. Microglia are stimulated by mast cell-derived histamine (85) and tryptase (86). Hence, communication...
Fig. 6. Microglia proinflammatory mediator release in response to NT is attenuated by the PI3K/mTOR inhibitors and the flavonoids Lut and Methut. HM-SV40 (5 × 10^6 cells) were pretreated with the dual PI3K/mTOR (PF, 0.1 μM) and the mTOR (Rap and KU, 0.1 μM) inhibitors or flavonoids (Lut and Methut, 5 μM (Upper) or 0.1–10 μM (Lower)) for 2 h, and then stimulated with NT (10 nM) for 24 h in serum-free medium to measure release of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by ELISA. All inhibitors were dissolved in water or DMSO with a final concentration <100 nM. Results were normalized against the endogenous gene GAPDH and are expressed relative to vehicle control (1). *P < 0.05, **P < 0.001, and ***P < 0.0001.

Fig. 7. Microglia proinflammatory mediator gene expression in response to NT is attenuated by the PI3K/mTOR inhibitors and the flavonoids Lut and Methut. HM-SV40 (5 × 10^6 cells) were pretreated with the dual PI3K/mTOR (PF, 0.1 μM) and the mTOR (Rap and KU, 0.1 μM) inhibitors or flavonoids (Lut and Methut, 0.1 μM (Upper) or 0.1–10 μM (Lower)) for 2 h, and then stimulated with NT (10 nM) for 12 h in serum-free media to determine changes in gene levels of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by qRT-PCR. All inhibitors were dissolved in water or DMSO with a final concentration <0.1%. Results were normalized against the endogenous gene GAPDH and are expressed relative to vehicle control (1). *P < 0.05 and ***P < 0.0001.
or poly-L-lysine-coated plates (Becton Dickinson) for 24 h before stimulation with NT (1–1,000 nM) or LPS (10–1,000 ng/mL) (Sigma-Aldrich) was carried out and was isolated after 6, 12, or 24 h using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed with 300 ng of total RNA using the Script cDNA Synthesis Kit (Bio-Rad). For qRT-PCR, 300 ng of total RNA using the Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run with each experiment. Gene expression was normalized to GAPDH, which was used as an endogenous control. The gene expression of receptors of NT (NTR1, NTR2, and NTR3) and the cell-type-specific antigens for the microglial lineage, CD11b and CD86, were also determined.

Protein levels of NTR3/sortilin in culture medium from human microglia after stimulation by NT (10 nM) for 24 or 48 h were measured using the SORT1 ELISA (LifeSpan BioSciences, Inc.). Initially, human microglia were seeded in six-well, type I collagen- or poly-L-lysine-coated plates for 24 h before stimulation with NT (1–100 nM) or LPS (10–1,000 ng/mL) and were stimulated with NT (10 nM) for 24 or 48 h. Total RNA was isolated as described above, and qRT-PCR was performed using Taqman gene expression assays to assess the expression of IL-1β, CXCL8, CCL2, and CCL5 in microglia after NT stimulation.

Increased serum NTR3/sortilin levels in ASD and the proposed scheme by which NT may contribute to inflammation of the brain. (A) Levels of soluble NTR3/sortilin and circulating NT were measured by ELISA in the serum of children with ASD, compared with age- and sex-matched healthy controls. Significance of comparisons is denoted by \( P < 0.0001 \). (Inset) Positive correlation between serum sortilin and NT levels is shown using the Spearman rank correlation test. The black line is a linear regression of the data. (B) Diagrammatic representation of how serum NT could derive primarily from the gut and increase permeability of the intestinal lumen and the BBB by stimulating perivascular mast cells. NT in the brain could then stimulate microglia via NTR3/sortilin, which is elevated in the serum of children with ASD. Proinflammatory mediator release from microglia through activation of mTOR signaling kinase thus may contribute to inflammation of the brain and the pathogenesis of ASD. The flavonoid Methlut inhibits these processes and could be a novel treatment of ASD.
for 6, 12, or 24 h. For select experiments, microglia were pretreated with P13K/mTOR inhibitors before stimulation with NT or LPS for 12 h in serum-free media. All conditions were performed in triplicate, and all experiments were repeated at least three times (n = 3). Results from cultured cells were presented as mean ± SD. Comparisons were made between (i) control and stimulated cells and (ii) stimulated cells with and without siRNA pretreatment using the unpaired, two-tailed, Student’s t test, with significance of comparison denoted by the horizontal lines and by *P < 0.05, **P < 0.01, and ***P < 0.0001. Comparisons were also made between (i) all conditions with stimulated cells and with inhibitors using one-way ANOVA followed by post hoc analysis by Dunnett’s multiple comparison test, for which significance is denoted by horizontal lines and indicated values of P < 0.001 or P < 0.0001 and (ii) all of the inhibitors/fluoronoids among themselves using one-way ANOVA followed by post hoc analysis by Tukey’s multiple comparison test, with those conditions for which there is significance denoted by the horizontal brackets and by the corresponding probability value (P < 0.05, **P < 0.01, and ***P < 0.001). Analysis of human serum samples is presented as a scattergram with symbols representing individual data points and horizontal lines representing the mean for each group. Normality of distribution was checked with the Shapiro–Wilks’s test. Comparison between the healthy control and ASD groups was performed using the Mann–Whitney U test and the Wilcoxon matched pair test. Correlations between serum cortisol and NT levels were examined using the Spearman rank correlation test. Significance of comparisons is denoted by P < 0.0001.

The analysis was performed using GraphPad Prism version 5.0 software (GraphPad Software). Representative images for Western blots were scanned and analyzed using ImageJ (NIH; https://imagej.nih.gov/ij/) and confocal images were analyzed using Fiji imaging software.

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Fig. S1. Identification of proinflammatory mediators differentially expressed in control and NT-stimulated human microglia (HM)-SV40. Immortalized HM-SV40 (10 × 10⁶ cells) were stimulated with NT (10 nM) for 24 h, and the cultured medium/supernatants were subjected to the human cytokine and chemokine array blot to probe for the release of proinflammatory mediators. Increased levels of several proinflammatory cytokines and chemokines (blue), including IL-1β, CXCL8, CCL2, and CCL5 (red), were denoted in NT-stimulated microglia after qualitative comparisons with those levels from control cells were made. All conditions were performed in a single blot as shown (n = 1).

Fig. S2. Proinflammatory mediator release from HM stimulated by LPS. Primary HM (5 × 10⁴ cells) and immortalized HM-SV40 (5 × 10⁴ cells) were stimulated with LPS (1–100 ng/mL) for 24 h to measure release of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by specific ELISAs. All conditions were performed in triplicate for each dataset and were repeated three times (n = 3). Significance of comparisons is denoted by *P < 0.05, **P < 0.001, or ***P < 0.0001. Conc., concentration.
Proinflammatory mediator gene expression in NT-stimulated human microglia pretreated with control scrambled (Sc) and NTR3/sortilin siRNA. Control and siRNA-transfected microglia-SV40 (2 × 10^6 cells) were stimulated with NT (10 nM) for 24 h, and the culture medium was concentrated to measure release of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by qRT-PCR. All conditions were performed in triplicate for each dataset and were repeated three times (n = 3). Results were normalized against the endogenous GAPDH and expressed relative to the mean of the control for the gene of interest, with significance of comparisons denoted by **P < 0.001 or ***P < 0.0001.
Fig. S4. NT stimulation of HM-SV40 involves activation of mTOR signaling, which is inhibited by luteolin (Lut) and methoxyluteolin (Methlut). (A) Immortalized HM-SV40 \(10 \times 10^6\) cells were serum-starved overnight and then stimulated with NT (10 nM) for 30 min before cellular lysates were harvested to probe for the phosphorylated levels of intracellular signaling kinases, as shown using a human phosphoarray blot. Increased levels of several phosphoproteins (blue), including components of the mTOR signaling pathway (red), were denoted in NT-stimulated microglia after qualitative comparisons with control cells were made. All conditions were performed in the single blot shown \((n=1)\). HM-SV40 \(1 \times 10^6\) cells were serum-starved and pretreated with PI3K/mTOR inhibitors \((0.5 \mu M)\) and the flavonoids (Lut and Methlut, 5 \(\mu M)\) overnight, and then stimulated with NT (10 nM) for 30 min to measure the protein levels of the mTORC2 substrate pAKTSer473 \((B)\), pmTORser2448 \((C)\), and the downstream mTORC1 substrate pp70S6KThr389 \((D)\) using specific phospho-ELISA kits. All conditions were performed in triplicate for each dataset and were repeated three times \((n=3)\). Significance of comparisons was determined for stimulated cells and for those cells with inhibitors/flavonoids, as denoted by the horizontal lines \((P < 0.0001)\), and also among all of the inhibitor/flavonoid treatments shown by the horizontal brackets and by corresponding \(*P < 0.05, **P < 0.001, \text{and} \ ***P < 0.0001\). Rap, rapamycin.

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Fig. S5. Microglia proinflammatory mediator release in response to LPS is attenuated by the PI3K/mTOR inhibitors and the flavonoids Lut and Methlut. HM-SV40 (5 × 10⁴ cells) were pretreated with the dual PI3K/mTOR (PF, 0.5 μM) and the mTOR inhibitors (Rap and KU, 0.5 μM), as well as the flavonoids [Lut and Methlut, 5 μM (Upper) or 0.1–10 μM (Lower)] for 30 min, and then stimulated with NT (10 nM) for 24 h in serum-free media to measure release of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by ELISA. All inhibitors were dissolved in water or DMSO with a final concentration <0.1%. All conditions were performed in triplicate for each dataset and were repeated three times (n = 3). Significance of comparisons was determined for stimulated cells and for those cells with inhibitors/flavonoids, as denoted by the horizontal lines (*P < 0.0001), and also among all of the inhibitor/flavonoid treatments shown by the horizontal brackets and by corresponding *P < 0.05, **P < 0.001, and ***P < 0.0001.
Fig. S6. Microglia proinflammatory mediator gene expression in response to LPS is attenuated by the PI3K/mTOR inhibitors and the flavonoids Lut and Methlut. HM-SV40 (2.5 x 10^5 cells) were pretreated with the dual PI3K/mTOR (PF, 0.5 μM), the mTOR (Rap and KU, 0.1 μM) inhibitors, and the flavonoids (Lut and Methlut, 0.1 μM) for 2 h, and then stimulated with NT (10 nM) for 12 h in serum-free media to determine changes in gene levels of IL-1β (A), CXCL8 (B), CCL2 (C), and CCL5 (D) by qRT-PCR. All inhibitors were dissolved in water or DMSO with a final concentration <0.1%. All conditions were performed in triplicate for each dataset and were repeated three times (n = 3). Results were normalized against the endogenous gene GAPDH and are expressed relative to the mean of the gene of interest. Significance of comparisons was determined for stimulated cells and for those cells with inhibitors/flavonoids, as denoted by the horizontal lines (P < 0.0001), and also among all of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding *P < 0.05, **P < 0.001, and ***P < 0.0001.