IL-37 is increased in brains of children with autism spectrum disorder and inhibits human microglia stimulated by neurotensin

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Autism spectrum disorder (ASD) does not have a distinct pathogenesis or effective treatment. Increasing evidence supports the presence of immune dysfunction and inflammation in the brains of children with ASD. In this report, we present data that gene expression of the antiinflammatory cytokine IL-37, as well as of the proinflammatory cytokines IL-18 and TNF, is increased in the amygdala and dorsolateral prefrontal cortex of children with ASD as compared to non-ASD controls. Gene expression of IL-18R, which is a receptor for both IL-18 and IL-37, is also increased in the same brain areas of children with ASD. Interestingly, gene expression of the NTR3/sortilin receptor is reduced in the amygdala and dorsolateral prefrontal cortex. Pretreatment of cultured human microglia from normal adult brains with human recombinant IL-37 (1 to 100 ng/mL) inhibits neurotensin (NT)-stimulated secretion and gene expression of IL-1β and CXCL8. Another key finding is that NT, as well as the proinflammatory cytokines IL-1β and TNF increase IL-37 gene expression in cultured human microglia. The data presented here highlight the connection between inflammation and ASD, supporting the development of IL-37 as a potential therapeutic agent of ASD.

autism spectrum disorder | brain | inflammation | IL-37 | neurotensin

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by impaired social interactions and communication (1–3). ASD presently affects about 1 in 59 children and is estimated to reach 1 in 40 children in 2020 (4–6). The complexity of the factors involved in the clinical picture of ASD has hampered the development of effective treatments. While the pathogenesis of ASD is unknown, it appears to involve some immune (7–10), autoimmune (11, 12), or inflammatory (13) component. In fact, perinatal brain inflammation (14, 15) has been considered in the pathogenesis of neuropsychiatric disorders (16, 17), including ASD (13, 18).

A number of inflammatory molecules, such as interleukin-1β (IL-1β), tumor necrosis factor (TNF), and chemokine (C-X-C motif) ligand 8 (CXCL8), have been shown to be increased in the serum, cerebrospinal fluid (CSF), and brain of many patients with ASD (19–21). We had previously reported increased levels of neurotensin (NT) in the serum of children with ASD compared to non-ASD controls (22, 23). Our laboratory demonstrated that NT stimulates gene expression and secretion of the proinflammatory cytokine IL-1β and the chemokine CXCL8 from cultured human microglia (24). A number of other authors have reported the activation of microglia in the brains of children with ASD (24–26), supporting the presence of inflammation (27, 28).

Interleukin-37 (IL-37, previously known as IL-1F7) belongs to the IL-1 family of cytokines (29) and is a natural suppressor of inflammation (30–32). Five isoforms (a–e) have so far been identified with the "b" isoform being the most well studied (33). IL-37 is produced mainly by activated macrophages in response to Toll-like receptor (TLR) activation. An IL-37 precursor (pro–IL-37) is cleaved by caspase-1 into mature IL-37, some of which (~20%) enters the nucleus and the rest is released along with the pro–IL-37 outside the cells (34) where both are biologically active. Extracellular proteases can then process pro–IL-37 into a much more biologically active form as shown for the recombinant IL-37b with the N terminus Val46 (V46-218) (35).

Although no specific receptor for IL-37 has been identified, a number of studies showed that extracellular IL-37 binds to the alpha chain of the IL-18Rα (36, 37), but with much lower binding affinity than that of IL-18 (38). Moreover, IL-37 binds to an IL-18 binding protein (IL-18BP) (39), and to the decoy receptor 8 (IL-R8) (40) via which IL-37 inhibits innate inflammation (35, 41, 42) in vitro and in vivo (42).

In this report we investigated IL-37 gene expression in the amygdala and dorsolateral prefrontal cortex of children with ASD because there is extensive evidence from animals and humans connecting the amygdala to social behavior (43, 44). Moreover, the

Significance

IL-37, an antiinflammatory cytokine, is increased along with the proinflammatory cytokine IL-18 and its receptor IL-18R in the amygdala and dorsolateral prefrontal cortex of children with autism spectrum disorder (ASD). IL-37 inhibits neurotensin (NT)-stimulated secretion and gene expression of IL-1β and CXCL8 from cultured human microglia, the resident immune cells of the brain. Moreover, NT, IL-1β, and TNF increase gene expression of IL-37 in these microglia. These findings highlight the important role of NT in the activation of microglia and of IL-37 in the inhibition of inflammation, thus supporting the development of IL-37 as a treatment for ASD.

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Competing interest statement: T.C.T. is the inventor of US patents no. 7,906,153; no. 8,268,365, and no. 9,050,275 for the treatment of autism and neuroinflammatory conditions.

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amygdala communicates directly with the dorsolateral prefrontal cortex, which is important in behavioral processing (45).

Results

Subject Characteristics. There was no statistical difference in age between ASD and non-ASD subjects. Samples were included if there was no statistical difference in RNA integrity number (RIN) and postmortem interval (PMI) as shown in *SI Appendix*, Table S1. The only inclusion criteria used were males, 1 to 12 y of age, because ASD is 4 times more common in males than females.

Increased Gene Expression of IL-37 and Its Putative Receptor IL-18R in the Amygdala and Dorsolateral Prefrontal Cortex of Children with ASD. IL-37 gene expression is increased in the amygdala ($P = 0.005$, Fig. 1*A*) and dorsolateral prefrontal cortex ($P = 0.004$, Fig. 2*A*) of patients with ASD, as compared to non-ASD controls.

We also measured gene expression of the proinflammatory cytokine IL-18 and its receptor IL-18R, which also binds to IL-37. IL-18 and IL-18R gene expression is increased in the amygdala ($P = 0.002$ and $P = 0.002$, respectively) of patients with ASD, as compared to non-ASD controls (Fig. 1 *B* and *C*). IL-18R gene expression ($P = 0.002$), but not IL-18, is also increased in the dorsolateral prefrontal cortex in patients with ASD (Fig. 2 *B* and *C*).

Gene expression of the proinflammatory cytokine TNF is also increased in both the amygdala ($P = 0.03$) and dorsolateral prefrontal cortex ($P = 0.006$) of patients with ASD as compared to non-ASD controls (Figs. 1*D* and 2*D*).

There Is a Significant Difference in Protein Expression of Only Neurotensin Receptor 3 (NTR3/Sortilin) in the Amygdala and Dorsolateral Prefrontal Cortex. NTR3/sortilin protein levels are significantly decreased in both amygdala ($P = 0.02$) and dorsolateral prefrontal cortex ($P = 0.03$) in patients with ASD as compared to non-ASD controls (Fig. 3).

There are no statistically significant differences in the gene expression levels of NT, NTR1, NTR2, or NTR3/sortilin in patients with ASD as compared to non-ASD controls either in the amygdala (*SI Appendix*, Fig. S1) or dorsolateral prefrontal cortex (*SI Appendix*, Fig. S2). The protein levels of NTR1 and NTR2 are also unchanged (*SI Appendix*, Fig. S3).

The amygdala samples analyzed for NT receptor protein levels were one less than in all other studies due to technical reasons.

IL-37 Inhibits NT-Stimulated Secretion and Gene Expression of IL-1β and CXCL8 from Human Microglia. Pretreatment of microglia with human recombinant IL-37 (1 ng/mL, 10 ng/mL and 100 ng/mL) for 24 h, and then stimulation with NT (10 nM) for 24 h, significantly inhibits secretion of IL-1β ($P = 0.02$, $P = 0.02$, and $P = 0.004$, respectively) (Fig. 4*A*) and CXCL8 ($P = 0.006$, $P = 0.001$, and $P = 0.03$, respectively) (Fig. 4*B*). Pretreatment of microglia with human recombinant IL-37 (1 ng/mL, 10 ng/mL, and 100 ng/mL) for 24 h, and then stimulation with NT (10 nM) for 6 h, significantly inhibits gene expression of IL-1β ($P = 0.02$, $P = 0.001$, and $P = 0.001$, respectively) (Fig. 4*C*) and CXCL8 ($P = 0.03$, $P = 0.001$, and $P = 0.02$, respectively) (Fig. 4*D*).

NT, IL-1β, and TNF Induce Gene Expression of IL-37 in Human Microglia. Stimulation for 6 h of human microglia by the microglia stimulant NT (100 nM) as well as the cytokines secreted by microglia, IL-1β (50 ng/mL) and TNF (10 ng/mL) increase ($P = 0.016$, $P = 0.03$, and $P = 0.016$, respectively) gene expression of IL-37 (Fig. 5). Lipopolysaccharide (LPS) used as a positive control also increases IL-37 gene expression (Fig. 5).

Discussion

Microglia are responsible for innate immunity of the brain (46, 47). Recent evidence indicates that brains of children with ASD have activated microglia (25, 26, 48, 49). The increased gene expression of TNF, IL-18, and IL-18R reported here supports the presence of inflammation in the amygdala and dorsolateral prefrontal cortex of children with ASD. We also show that the gene expression of IL-37 is increased in these same areas, but the reason for this increase is not clear. A speculation why this might occur is that IL-37 gene expression may be increased in an effort to suppress the

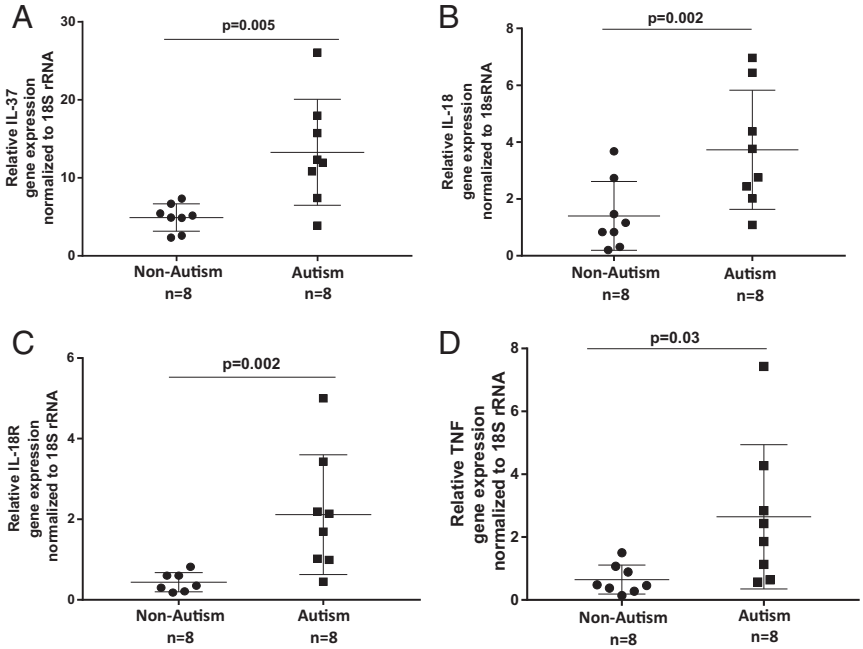

Fig. 1. Increased gene expression levels of IL-37, IL-18, IL-18R, and TNF in the amygdala of children with ASD. Gene expression levels of (*A*) IL-37, (*B*) IL-18, (*C*) IL-18R, and (*D*) TNF of ASD and non-ASD subjects were measured by qRT-PCR. Gene expression was normalized to 18S rRNA control gene. Measurements were repeated 3 times each.

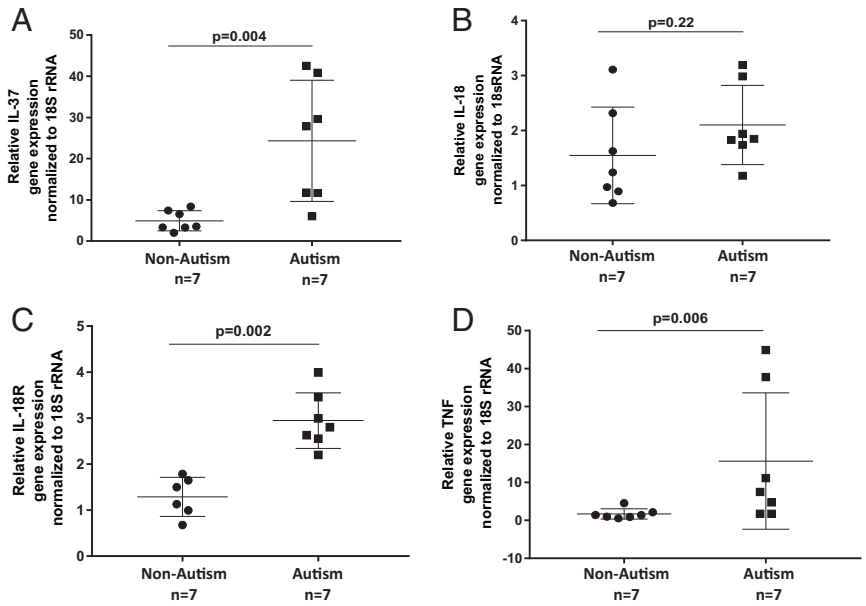

Fig. 2. Increased gene expression levels of IL-37, IL-18, IL-18R, and TNF in the dorsolateral prefrontal cortex of children with ASD. Gene expression levels of (*A*) IL-37, (*B*) IL-18, (*C*) IL-18R, and (*D*) TNF of ASD and non-ASD subjects were measured by qRT-PCR. Gene expression was normalized to 18S rRNA control gene. Measurements were repeated 3 times each.

inflammation in that part of the brain. Increased IL-37 gene expression was reported in the brain of patients after ischemic stroke and protected them from further inflammatory brain injury (50). Other studies also showed elevated serum IL-37 concentration in patients with sepsis (51) and in ankylosing spondylitis (52).

The findings presented in this paper are particularly important since ASD has been associated with dysfunctional connectivity within the amygdala, as well as between the amygdala and dorsolateral prefrontal cortex (53, 54). Bauman and Kemper (43) first identified neuropathologic changes in the amygdala of postmortem brains of patients with ASD. Children with ASD showed an initial excess of neurons in the basal amygdala with a reduction in adulthood, while normal controls have fewer neurons in childhood, but a greater number in adulthood (55). Moreover, amygdala in children with ASD had more growth compared to normotypic children, as evidenced by higher dendritic density than age-matched controls (56). There is extensive literature connecting the amygdala to social behavior (43, 44) and to pathophysiologic responses to stress (57).

We had previously reported that NT stimulates gene expression and release of IL-1β and CXCL8 from cultured human microglia (24). Plasma levels of IL-1β and CXCL8 have been reported to be increased in children with ASD and were correlated with impaired communication and aberrant behavior (58). Elevated levels of IL-1β in neonatal blood from children with ASD were significantly associated with severity of the disease (59). In the present study, we show that NT, IL-1β, and TNF increase IL-37 gene expression in cultured human microglia. These findings are consistent with a previous report that IL-37 expression was increased following stimulation with IL-1β, IL-18, and TNF in mononuclear cells separated from peripheral blood (60, 61). We report no secretion of IL-37 protein in cultured human microglia. Similarly, it was previously shown that LPS stimulates gene expression, but apparently no protein secretion in human macrophages (35), implying that either no protein is produced or it is in some structural form (such as its dimer) not detectable by the ELISA used.

Our data show that IL-37 inhibits secretion and gene expression of IL-1β and CXCL8 from cultured human microglia stimulated by the neuropeptide NT. IL-37 has been reported to inhibit the generation of proinflammatory cytokines in vitro (62), as well as in vivo (63). Recombinant IL-37 also reduced secretion of inflammatory molecules from human blood M1 macrophages in vitro and mice in vivo (42). Increased gene expression of IL-37 was associated with suppression of IL-1β and IL-6 production from

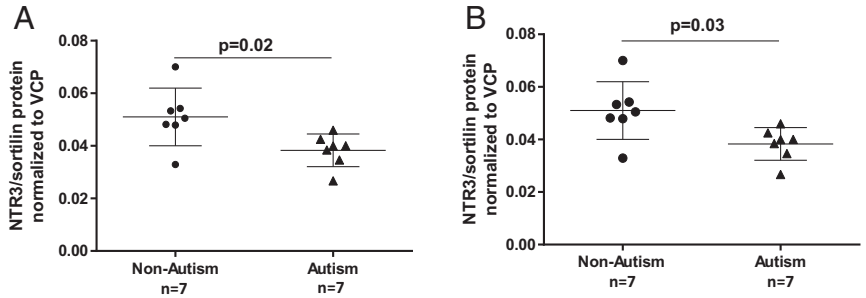

Fig. 3. Protein levels of NTR3/sortilin in amygdala and dorsolateral prefrontal cortex of children with ASD and non-ASD controls. Protein levels of NTR3/sortilin in (*A*) amygdala and (*B*) dorsolateral prefrontal cortex of 7 ASD and non-ASD subjects were quantified by Western blot analysis, followed by densitometry, using VCP as the loading control.

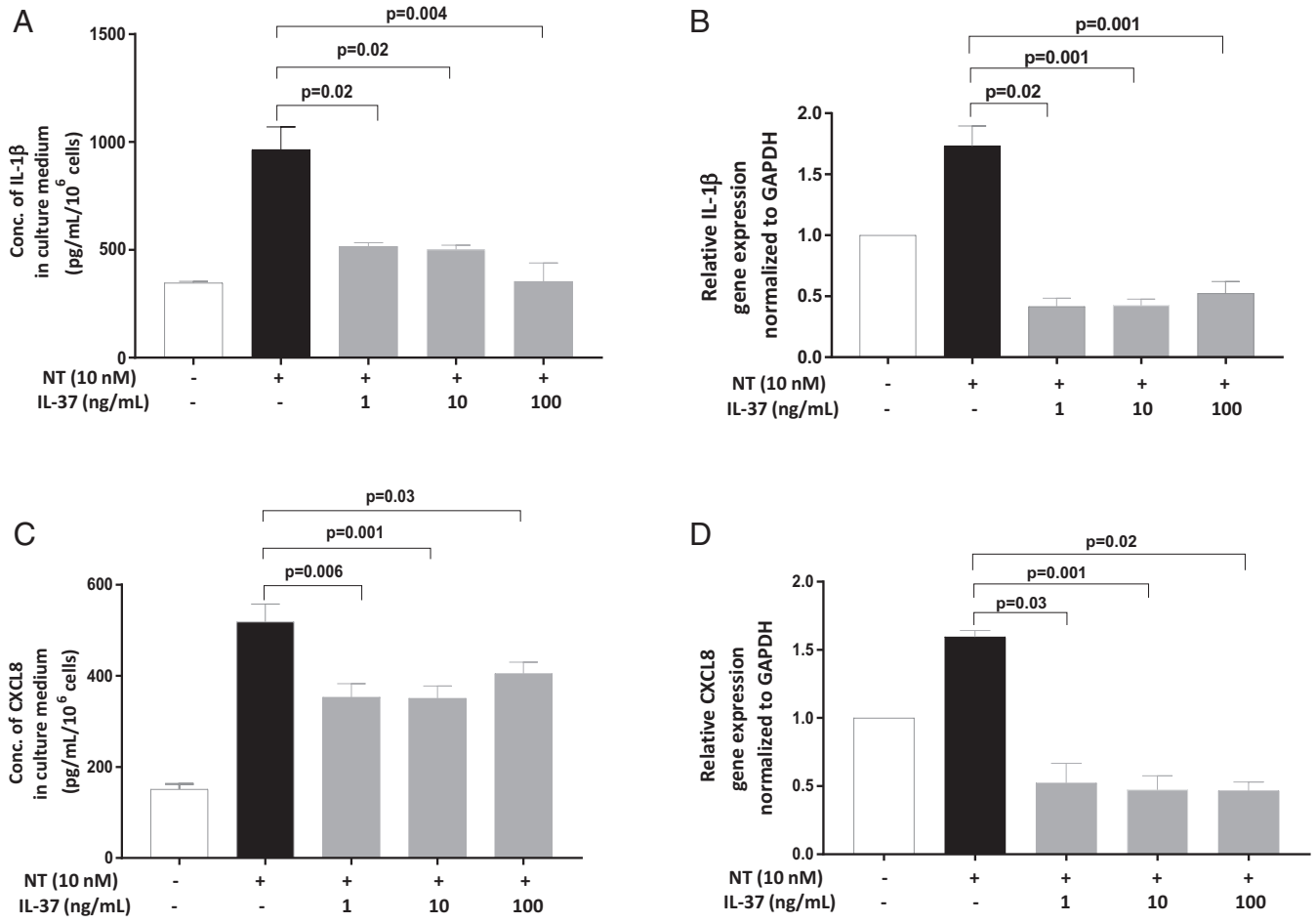

Fig. 4. IL-37 inhibits the secretion of IL-1β and CXCL8 stimulated by neurotensin. SV40 cells were pretreated with IL-37 (1 to 100 ng/mL) for 24 h and then stimulated with NT (10 nM) for 24 h to determine the secretion of IL-1β (*A*) and CXCL8 (*C*) by specific ELISAs. IL-37 inhibits the gene expression of IL-1β and CXCL8 stimulated by neurotensin. SV40 cells were pretreated with IL-37 (1 to 100 ng/mL) for 24 h and then stimulated with NT (10 nM) for 6 h to determine the secretion of IL-1β (*B*) and CXCL8 (*D*) by specific ELISAs. All conditions were performed in triplicates for each dataset and repeated 3 times ($n = 3$). Significance of comparisons is denoted by $P < 0.05$.

peripheral blood mononuclear cells from subjects with systemic inflammatory diseases (64–66). The precise inhibitory mechanism of action of IL-37 is presently not known. Extracellularly, the IL-37 monomer is the active form involved in reducing innate immunity (67); instead, homodimerization of IL-37 reduces its antiinflammatory activity (68). IL-37 may act by inhibiting the mammalian target of rapamycin (mTOR) (69) since this complex was reported to be involved in the stimulatory action of NT on human microglia (24). Another thought is that IL-37 inhibits inflammasome activation as reported in murine aspergillosis (70).

Because NT stimulates human microglia, we investigated expression of NT and its receptors (NTR1, NTR2, and NTR3/sortilin) in amygdala and dorsolateral prefrontal cortex. The present data show only that NTR3/sortilin protein levels are decreased in the amygdala and dorsolateral prefrontal cortex of children with ASD, suggesting possible end-product inhibition due to chronic stimulation by NT. Our laboratory had demonstrated that NT stimulates proliferation of cultured human microglia and triggers secretion of proinflammatory cytokines and chemokines through activation of NTR3/sortilin (24). NT receptors in the human brain transiently increase after birth (71) and are more concentrated in the amygdala (72).

The source of endogenous NT is unknown, but we suggest that it may be released locally in the amygdala and dorsolateral prefrontal cortex. NT may also enter the brain through the blood brain barrier (BBB) or the brain lymphatics, following which it stimulates microglia via activation of the NTR3/sortilin receptor (Fig. 6). The most likely cellular localization of NTR3/sortilin is the microglia because they have been reported to express only NTR3/sortilin (73). Activated microglia would then exhibit abnormal synaptic pruning and secrete IL-1β, CXCL8, and IL-18 gene expression of which along with its receptor IL-18R, are increased in the brain areas studied. These processes would result in neuronal damage and disrupted neuronal connectivity thus contributing to the symptoms of ASD. We further hypothesize that IL-37 is increased in these brain areas in an effort to inhibit the release of the proinflammatory molecules thus providing a potential treatment option for ASD (Fig. 6).

The fact that IL-37 gene expression is increased both in the brain of children with ASD and in human microglia activated by NT provides associations that could be important in the pathogenesis of ASD. However, there are a number of limitations concerning this study. There is no available information of the cognitive or functional level before death, or the presence of any comorbidities in the subjects with ASD. Our in vitro data were generated using human microglia from normal adult brains that do not reflect how microglia from patients with ASD would behave, especially since ASD is a complicated disease (74). Moreover, the human microglia we used are immortalized (SV-40 microglia) and are already fixed in a proinflammatory phase and may not be able to generate

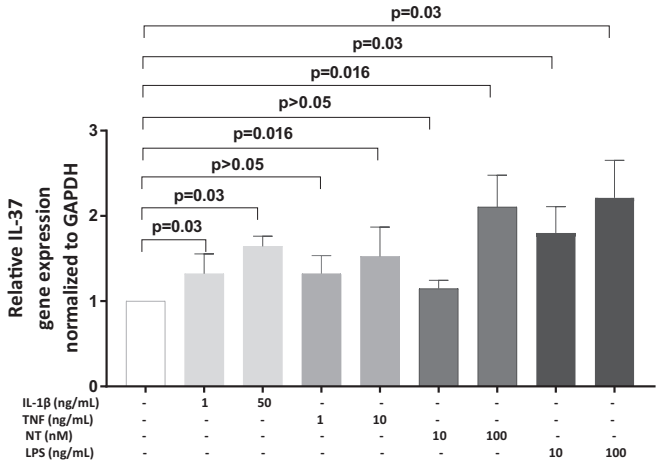

Fig. 5. NT, IL-1β, and TNF induce IL-37 gene expression. SV40 cells were stimulated with NT (100 nM), IL-1β (50 ng/mL), and TNF (10 ng/mL) for 6 h to measure gene expression of IL-37 by qRT-PCR. Gene expression was normalized to GAPDH control gene. All conditions were performed in triplicates for each dataset and repeated 3 times ($n = 3$). Significance of comparisons is denoted by $P < 0.05$. LPS was used as a positive control.

maximal responses as compared to normal primary microglia. However, we had previously shown that the response to NT was similar whether immortalized or primary cells were used (24). A clinically relevant concentration of NT is not presently known, but we reported that serum levels of NT were ∼0.5 ng/mL in children with ASD (23). Concentrations of either NT or IL-1β would be expected to be higher when used in vitro especially with immortalized cell lines.

Conclusion

The lack of reliable biomarkers (75) and specific pathogenesis (76) for ASD has prevented effective treatments. The present results provide a plausible pathogenetic process linking NT, originally characterized by Leeman and coworkers (77), to focal inflammation of the brain. Use of IL-37 could have a major advantage over drugs targeting IL-1β because IL-37 could inhibit secretion of not only IL-1β, but other proinflammatory cytokines and chemokines. IL-37 could, therefore, be developed as a treatment approach for ASD.

Materials

Human recombinant IL-37, isoform b, N terminus Val46 was obtained from R&D Systems (7585-IL catalog no., Minneapolis, MN). NT and LPS were purchased from Sigma-Aldrich (St. Louis, MO). RNeasy Mini (Qiagen Inc., Valencia, CA), and iScript cDNA synthesis kits were purchased from Bio-Rad (Hercules, CA). Taqman gene expression primers were purchased from Applied Biosystems (Foster City, CA). ELISA kits for IL-1β and CXCL8 were purchased from R&D Systems. Rabbit anti-human primary antibodies for IL-1β and β-actin were purchased from Cell Signaling Technology (Danvers, MA) and mouse anti-human ASC and cleaved IL-1β were obtained from Santa Cruz Biotechnology (Dallas, TX).

Methods

Human Brain Samples. Postmortem human brain tissues of deceased Caucasian male children (3 to 14 y old) with ASD ($n = 8$) and non-ASD ($n = 8$) were obtained from the NIH NeuroBioBank at the University of Maryland, Baltimore, MD (https://neurobiobank.nih.gov/ (application approved October 30, 2015/biospecimen availability confirmed). This work involves retrospective analysis of brain samples of deceased children with no identifiers and did not require institutional review board approval as this was obtained by University of Maryland Neurobiobank at the source. Samples were obtained from dorsolateral prefrontal cortex (Brodmann areas 46, 9) and amygdala (Brodmann areas 6, 12) (*SI Appendix*, Table S1). The only inclusion criteria used were males, 1 to 12 y of age, who had died in car accidents. Unfortunately, there is no available information of how diagnosis of ASD was reached, what the level of cognitive or functional level was before death, or the presence of any comorbidities. Controls were selected without any known brain disease or trauma and were matched to the subjects with ASD to the extent possible as shown in *SI Appendix*, Table S1.

Frozen brain tissues were sectioned (30-μm thickness) using a cryostat. The characteristics of the deceased subjects are listed in *SI Appendix*, Table S2. Brain areas were available from the same subjects, which allowed direct comparisons of outcome measures between regions within the same subjects. Samples were provided from males only because ASD is 4 times more common in males than females, and to avoid any additional gender and hormonal variabilities. The deceased children whose brain samples were analyzed were unrelated to those whose serum was obtained.

RNA Isolation from Brain Tissue. Total RNA was extracted from frozen brain tissue specimens from ASD and non-ASD subjects using the mirVana miRNA Isolation Kit (Ambion, Life Technologies, Carlsbad, CA) after frozen tissue section homogenization. Reverse transcription (RT) was performed with 500 to 1,000 ng of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA). RNA purity (A260/280 ratios) was calculated and RINs for all samples were reported by the brain bank.

Quantitative Real-Time Reverse Transcription PCR. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA) to assess the gene expression of IL-37 (Hs00367201_m1), IL-18 (Hs01038788_m1), IL-18R (Hs00175381_m1), and TNF (Hs00174128_m1). All qPCR studies were conducted using inventoried Taqman gene expression probes from Invitrogen, which were validated by the vendor and publicly available. Samples were run for 45 cycles using the Applied Biosystems 7300 Real-Time PCR System. Normalization of gene expression to 18SrRNA (4310893E) and comparison of gene expression between groups was calculated according to the 2 -ΔΔCt method by Schmittgen (78).

For all mRNA studies, tissue samples were included if RIN was above 5.0. However, due to evidence showing that RIN values are not always the most accurate predictors of RNA quality in human postmortem brain samples (79), PMI, and pH measures were used as indicators of tissue quality, as these factors have been reported to correlate with protein levels (80). Furthermore, cause of death was used as an additional indicator of tissue integrity (81).

NT Receptor Protein Measurements. Protein levels of NTR1, NTR2, and NTR3/sortilin were determined by Western blotting analysis. Initially, brain tissues were homogenized using lysis radioimmunoprecipitation (RIPA) buffer in the presence of a protease inhibitor mixtures (Sigma-Aldrich), followed by sonication using a Polytron (Brinkmann Instruments, Westbury, NY). The total protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA) and BSA was used as standard. The total cellular protein (10- or 20-μg aliquots) was separated using 4 to 12% Mini-Protean TGX Precast gels under SDS denaturing conditions (Bio-Rad) and electrotransferred onto nitrocellulose membranes (Bio-Rad). Blocking was carried out with 5% BSA in Tris-buffered saline containing 0.01% Tween-20 (Sigma). The membranes were probed with the following primary antibodies: NTR1, NTR2, and NTR3/sortilin (Sigma-Aldrich) using valosin-containing protein (VCP) for the loading control (Cell Signaling Technology). All proteins were visualized with horseradish-peroxidase (HRP)-conjugated secondary antibodies and then by enhanced SuperSignal West Pico chemiluminescence (Thermo Fisher Scientific).

Human Microglia Cell Culture. The immortalized human microglia-SV40 cell line derived from primary human microglia was purchased from Applied Biological Materials Inc. (ABM Inc.; Richmond, BC, Canada) and cultured in Prigrow III medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in type I collagen-coated T25-flasks (ABM Inc.). Microglia-SV40 maintain their phenotype and proliferation rates for over 10 passages, during which all experiments were performed using multiple microglia thaws and subcultured cells. Experiments were carried out in type I collagen coated plates (BD PureCoat ECM Mimetic Cultureware Collagen I peptide plates, Becton Dickinson, Bedford, MA) (82). Cell viability was determined by trypan blue (0.4%) exclusion.

IL-1β and CXCL8 Secretion from Human Microglia. Microglia (0.5×10^5 cells/well) were seeded in 12-well, type I collagen or poly-L-lysine–coated plates (Becton Dickinson) for 24 h. Pretreatment with human rIL-37 (1 to 100 ng/mL) for 24 h and then stimulation with NT (10 nM) or LPS (10 ng/mL) (Sigma-Aldrich) for 24 h was carried out. Supernatant fluids were collected and IL-1β (DY201) and CXCL8 (DY208) secretion from human microglia-conditioned culture medium was quantified by using commercially available ELISA kits

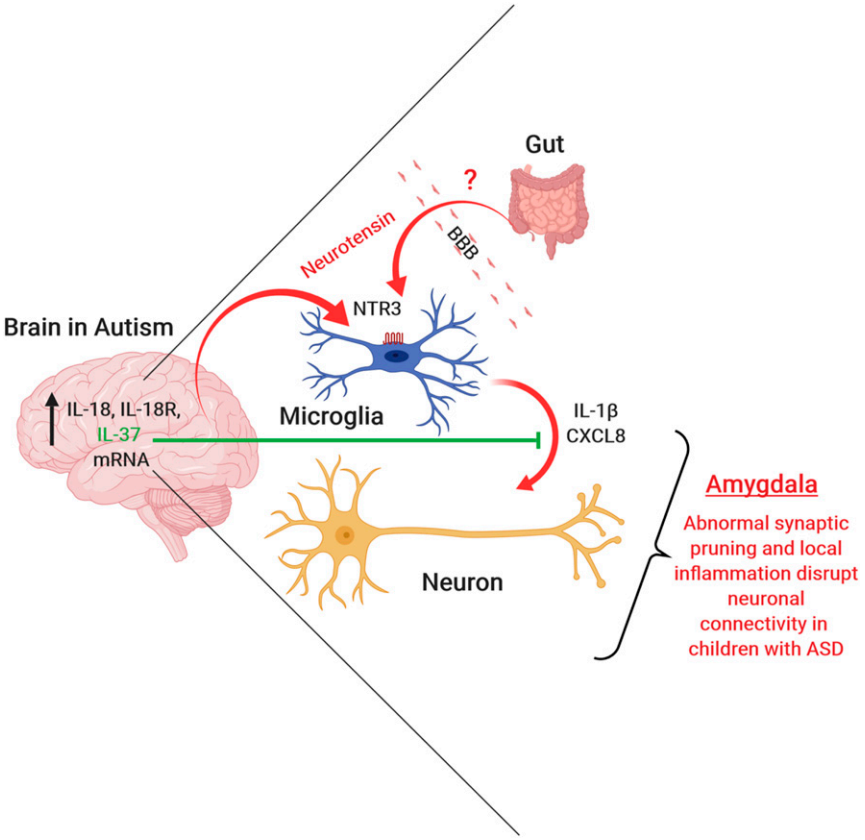

Fig. 6. Diagrammatic representation of the proposed interactions and sites of action of IL-37. Increased expression of IL-18 and IL-18R indicates inflammation in the amygdala and dorsolateral prefrontal cortex. Neurotensin released in these areas or entering the brain through the BBB stimulates microglia primarily in the amygdala through activation of the NTR3/sortilin receptor. Activated microglia now exhibit abnormal synaptic pruning and secrete IL-1β and CXCL8, which contribute to focal inflammation resulting in direct neuronal damage and disrupted neuronal connectivity that contribute to the symptoms of ASD. IL-37 is increased in these brain areas in an effort to inhibit the release of the proinflammatory molecules thus providing a potential treatment option for ASD.

(R&D Systems) as per manufacturer's instructions. For all experiments, the control cells were treated with equal volume of culture medium and the minimum detectable level for all mediators by ELISA was 5 pg/mL.

IL-1β and CXCL8 Gene Expression in Human Microglia. Microglia (1×10^5 cells per well) were seeded in 6-well, type I collagen- or poly-L-lysine–coated plates (Becton Dickinson) for 24 h before pretreatment with human rIL-37 (1 to 100 ng/mL) for 24 h and then stimulation with NT (10 nM) or LPS (10 ng/mL) (Sigma-Aldrich) for 6 h was carried out. Cell pellets were collected and total mRNA was extracted with an RNeasy Mini kit (Qiagen Inc.) in accordance with the manufacturer's intructions. An iScript cDNA synthesis kit (Bio-Rad) was used for reverse-transcription of each mRNA sample. qRT-PCR was performed using Taqman gene expression assays (Applied Biosystems). Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems). Relative mRNA levels were determined from standard curves run with each experiment. The gene levels of IL-1β (Hs01555410_m1) and CXCL8 (Hs00174103_m1) were measured and expression was normalized to GAPDH (4310884E) endogenous control (Applied Biosystems).

Statistical Analysis. All conditions were performed in triplicate, and all experiments were repeated at least 3 times ($n = 3$). Results from cultured cells are presented as mean ± SD. Comparisons were made between control and stimulated cells using the unpaired, 2-tailed, Student's t test with significance of comparisons denoted by the horizontal lines and by $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$ (83). Analysis of human brain samples is presented as a scattergram with symbols representing individual data points and the horizontal lines representing the mean for each group. Normality of distribution was checked with the Shapiro–Wilk's test. Depending on whether data were normally distributed or not, comparison between the non-ASD and the ASD groups was performed using either paired t test or Wilcoxon matched-pairs signed rank test. Significance of comparisons is denoted by $P < 0.05$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***). The analysis was performed by using the GraphPad Prism version 7.0 software (GraphPad Software, San Diego).

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