Journal of Drug Metabolism and Toxicology

Mou et al., J Drug Metab Toxicol 2016, 7:1
DOI: 10.4172/2157-7609.1000197

Research Article Open Access

NRSF and CCR5 Established Neuron-glia Communication during Acute and Chronic Stresses

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Abstract

It has been reported that traumatic stress resulted in immune-suppression. Src kinase activation in the prefrontal cortex was believed to initiate cellular-reorganization at the recover stage of trauma. Herein, we reported that NRSF and CCR5 expression were consistently increased in the prefrontal cortex of SD rats when exposed to traumatic stress, in which CCR5 was activated mostly in neurons and targeted by astrocyte NRSF. Moreover, HPA axis activation could be acutely and sustainably triggered by traumatic stress and PSS at post-trauma respectively, both NRSF and CCR5 had inhibitory effect in the former event, while NRSF could block the scenario in the later event. Intriguingly, the effect of NRSF was mostly converged on multiple mechanisms that associated with GR activity, and the optimal preservation of neuroligin-1 formed neuron-astrocyte communication was achieved by NRSF. Therefore, the present results argue for the dichotomy of NRSF regulatory complexes, whose inhibition in HPA hyper-reactivity during acute and chronic stresses have significant potential for the development of therapeutic approaches in post-traumatic stress-related disorders.

Keywords: Neuron-glia communication; NRSF; CCR5; Stress; HPA axis

Abbreviations

NRSF: Neuron-Restrictive Silencer Factor; CCR5: C-C Chemokine Receptor Type 5; HPA axis: Hypothalamus-Pituitary-Adrenal Gland Axis; CRH: Corticotrophin-Releasing Hormone; AVP: Arginine–Vasopressin; ACTH: Adrenocorticotropin-Releasing Hormone; SD: Sprague Dawley; PSS: Predator Scent Stimulation; CORT: Corticosterone; DEX: Dexamethasone; CD: Cluster of Differentiation; IL-1, IL-8: Interleukin-1, 8; gp130: Glycoprotein 130; IGF-1: Insulin-Like Growth Factor Type 1; GR: Glucocorticoid Receptor

Introduction

Previously, we have reported that traumatic stress could induce immuno-depression, including decrease in splenocyte proliferation to concanavalin, reduced natural killer cell activity and production of a number of cytokines [1,2]. In adult rats, above immuno-depression reached the lowest level at 1 day of trauma, which began recovering after 3 day and fully recovered by 7 day, the two stages of alterations were mediated by IL-1β and IGF-1R, respectively. Src tyrosine kinases, Fyn in particular, were preferentially activated during traumatic stress and believed to reorganize IGF-1R signaling in the prefrontal cortex [1-6]. Moreover, it was reported that neurons communicate with glial cells in various ways, neuroligin-1 performed central function in the initial induction of synapse formation by binding to neurexin-1β [7-9]. When challenged with traumatic stress, contact of neurexin-1β and neuroligin-1 was under the control of Fyn, which was capable of enhancing the communication between neuron and astrocytes during the recovery from the immuno-suppression [1,4].

Many studies have shown that neuron-restrictive silencer factor (NRSF) is a transcription factor that functions as a critical molecule linking neuronal network formation and intrinsic homeostasis when it binds to the 21-nt DNA sequence, neuron-restrictive silencing element (NRSE) [10-15]. Some inflammation-related genes are reported to be under the regulation of NRSF, for example, CCR5, a chemokine receptor expressed in astrocytes, microglia and neurons, has been estimated to regulate neuron-astrocyte communication via calcium flux [16-23]; neuroligin-1 was also one of NRSF target genes. Then, it would be of interest to propose that NRSF could elicit neuron-astrocyte communication, by which restrain a plethora of stress-like behaviors and promote reorganization of maladaptive responses.

It is widely reported that stress responses involve the neuroendocrine system, in particular the hypothalamic–pituitary-adrenal (HPA) axis, which is activated by limbic and ascending brainstem and pontine pathways, and accompanied by a significant increase in the release of neuropeptides, CRH and AVP into the portal vessel system; the secretion of ACTH from the anterior pituitary, and glucocorticoids from the adrenal cortex [24-30]. Significantly, it was recognized that traumatic events could result in long-lasting psychopathological consequences [31], and related brain changes including a strong memory of the aversive event that is resistant to extinction, emotional numbing and deficit in declarative memory [32-36]. Since HPA activation was previously observed to be evoked rapidly by traumatic stress, and was most likely caused by IL-1β over-expression [2], therefore, it is conceivable that NRSF contributed neuron-glia communication is likely to build a complex network and achieve a distinct cellular outcome to sense and respond to HPA axis activation.

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Received December 28, 2015; **Accepted** January 04, 2016; **Published** January 10, 2016

Citation: Mou H, Zhao X, Li X, Liu J, Huang HW, et al (2016) NRSF and CCR5 Established Neuron-glia Communication during Acute and Chronic Stresses. J Drug Metab Toxicol 7: 197. doi:10.4172/2157-7609.1000197

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Materials and Method

Traumatic and chronic stress paradigm

Male SD rats (Animal Center of Chinese Academy of Sciences, 180–200 gm) were used in this study. The animals were housed in groups (five per cage) in a controlled environment on a 12 h light–dark cycle and allowed to acclimate for a minimum of 5 days before conducting experiments. The traumatic stress was performed as previously described [2]. Briefly, rats were anesthetized with pentobarbital sodium (35 mg/kg, i.p.), then were incised longitudinally to a length of 6 cm along the dorsal median line and 5 cm along the abdominal median line, and followed by dorso-myotomy and exploratory laparotomy. Five minutes after surgery, the wounds were sutured, and the animals were kept warm under standard housing conditions. No post-operative infection occurred.

Chronic stress paradigm was described previously [37-39], namely, rats were undergone traumatic stress, right afterwards were subjected to a cloth with the smell of cat for 14 days. Animal exposed to the cat odor but not undergone surgery was used as control group. All animals were weighed daily and returned to their home cage without further manipulation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Animal Care and Use Committee at Fudan University.

Prefrontal cortex injection was performed, a stainless steel guide cannula (0.5 mm in diameter) with an inserted cannula (0.25 mm in diameter) was implanted (Anterior: 3.0 mm; Lateral: 2.0 mm, relative to Bregma) and fixed onto the skull with dental cement. Maraviroc (a potent, selective small-molecule inhibitor of CCR5, 10 μM/day, MedChemexpress, Shanghai, China) dissolved in sterilized PBS were injected over 10 s via the cannula in a volume of 0.5 μl. Rats from the control group were injected with vehicle. At the end of each procedure, the entire injector system was left in place for an additional 10 min to minimize reflux. The position of the cannula was assessed by histological examination.

Recombinant adenovirus construction

Recombinant adenovirus expressing rat NRSF, CCR5 or negative dominant Fyn were constructed by inserting into the adenoviral shuttle vector pDE1sp1A (Microbix Biosystems, Inc. Canada), and the insert was then switched to the adenoviral vector through LR recombination. After homologous recombination in vivo with the backbone vector PJM17, plaques resulting from viral cytopathic effects were selected and expanded in 293 cells. Positive plaques were further purified and large-scale production of adenovirus was carried out by two sequential CsCl gradients and PD-10 Sephadex chromatography.

Cell cultures

For neuron cultures, rat fetuses were removed from pregnant rats on embryonic day 18. Cortices were dissected and collected in Hanks' balanced salt solution. Cells were dissociated and plated at a density of 10 cells per well into 24-well tissue culture plates pretreated with 0.1% polyethylenemine. Cells were maintained in serum-free Neurobasal medium containing B27 supplement (Gibco, Rockville, MD). After 3-4 days in culture, neurons sent out long processes. By 10 days, flow cytometry showed that MAP2 immuno-positive cells accounted for more than 95% of cells.

For astrocyte cultures, the dissociated cells were plated in untreated 24-well tissue culture plates. The culture medium was Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 U penicillin/50 μg/ml streptomycin, the adherent cells were purified after 24 h plating. When cultured for 2 weeks, then neuron-astrocyte co-culture was performed.

CRH, ACTH and CORT assay

Animals were fully anesthetized, brain was rapidly extracted and hypothalamus was dissected and sonicated using a tissue extraction reagent (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma). Homogenate was centrifuged (10 min, 14,000 g, 4°C). Supernatant was collected and stored at -20°C. Total protein was quantified using a Bradford assay. Cardiac blood withdrawn within 3 min of injection, afterwards, it was centrifuged (10 min, 14,000 g, 4°C) and serum collected. Content of CRH, ACTH and CORT were measured using a competitive immunoassay (Assay Designs, Inc., Ann Arbor, MI) as described in the manufacturer's protocol.

Immunofluorescent labeling

Rats were anesthetized with sodium pentobarbital (35 mg/100 g.kg, i.p.) and transcardially exsanguinated with 0.1 M PBS followed by perfusion of the fixation (4% paraformaldehyde in 0.1 M PBS, pH 7.4), each provided in a 7 ml/min flow rate. Serial sets of 20 μm coronal brain sections were collected on a freezing microstome (Leica, SM2000R). Frozen sections were subjected to anti-NRSF (ab70300, 1:1000, Abcam, Cambridge, CB) or anti-CCR5 (ab110103, 1:1000, Abcam), and Alexa Fluor 594-conjugated secondary antibody (CA21203S, 1:500, Invitrogen, Carlsbad, CA). Afterwards, sections were subsequently incubated with anti-GFAP or anti-tubulin (sc6170/sc8035, 1:1000; Santa Crutz, Santa Crutz, CA), and Alexa Fluor 488-conjugated secondary antibody (CA11055S, 1:500, Invitrogen). Data derived from each group were analyzed by Leika Q500IW image analysis system. For statistical analysis, fluorescent density is reported as the average density in 10 randomly selected areas in prefrontal cortex, after that, data was further analyzed with the aid of ImageJ analysis software.

DEX induced GR responses

Neurons were grown to 70–80% in 6-well plate, then transfected with pGRE-TK-luciferase. The transfectants were treated with 0.01–100 nm DEX for 12 h, and whole cell extracts were prepared. Luciferase activity was determined using extracts containing 10 units of β-galactosidase activity. Luciferase assay was performed according to the manufacturer's instructions (Promega).

Immunoprecipitation and Western Blotting and FACS

Proteins from tissue or cells were combined and diluted with 15 ml of buffer A (100 mM NaCl and 10 mM Tris, pH 7.4) and concentrated with a Centriplus™ YM-30 centrifugal filter column (Millipore) to 1 ml at a speed of 3500 g. The concentrated solution was transferred to a new tube with the addition of 0.1% digitonin (Sigma-Aldrich) and respective antibodies including anti-GR at the concentration of 1:200. The mixture was incubated at 4°C overnight with slow rotation. 60 μl of protein G-agarose beads (Invitrogen) were added, and the mixture was incubated for another 3h. Afterwards, the beads were washed and protein samples were eluted with SDS sample buffer (75 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol).

For Western Blot analysis, proteins were resolved in SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NY). The membrane was probed in the presence of various specific primary antibodies including

anti-NRSF, anti-GR (ab109022), anti-neuroligin 1 (ab56882, 1:1000, Abcam, Cambridge, CB). Then the membrane was incubated with secondary antibody conjugated with alkaline phosphatase, protein bands were detected by ECF substrate and scanned in the Storm 860 Imaging System. The band intensities were quantified and analyzed with the ImageQuant software (GE Healthcare).

Subcellular fractionation

Nuclear extracts were prepared from tissue or cells. Briefly, 1 ml of extraction buffer was added (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) together with the recommended amount of Complete. After three freeze-thaw cycles, cytoplasmic extracts were recovered by centrifugation at 15,000 × g for 5 min, and pellets were resuspended in buffer C (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 25% glycerol) together with the recommended amount of Complete. Following 30-min incubation at 4°C, nuclear extracts were recovered by centrifugation at 15,000 × g for 5 min. GR expression in the eluted solution was analyzed by Western blotting.

Real-time PCR

Total RNA was isolated from tissues or cells, mRNA was extracted by UNIzol reagent and treated with RNase-free DNase I (Takara, Japan). Reverse transcription using random hexamers was performed with Omniscript reverse transcriptase (QIAGEN, Los Angeles, CA). Using gene-specific primers, real-time PCR analysis was performed with the cDNA product from 50 ng RNA per well on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate along with a corresponding sample to which no reverse transcriptase was added (no reverse transcriptase control). PCR conditions for each primer pair were optimized in pilot experiments to amplify the desired product in the linear range of amplification. The general reaction conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression was normalized to the expression of 18s rRNA and quantified with the $2^{-\Delta\Delta}$Ct method, which computed the percentage change relative to control. Primers were as followed, NRSF (Forward: GCAAACTTGCTCCGAAGTGG, Reverse: GACAGGCACTAAGCCAACCT); CCR5 (Forward: GTATGTCAGCACCCTGCCAA, Reverse: GCAGCATAGTGAGCCCAGAA); CCR3 (Forward: GCTCCTGCCTCCACTGTATT, Reverse: TGGCCAAAACCCCACTCATT); CD14 (Forward: GCTGTTGCCTTTGACACTGG, Reverse: CGCATAGAAAAGCGCTGGAC); gp130 (Forward: TCCTCTCCTCACCCCATCAG, Reverse: ACGGACAATGGACACCCATC); IL-18 (Forward: ACAGCCAACGAATCCCAGAC, Reverse: ATAGGGTCACAGCCAGTCCT).

Statistics

This experiment was performed independently with the same parameters and normalized results were pooled. Image data was manually counted in 10 random selected fields under microscope combined with analysis using ImageJ software. One-way analysis of variance and post hoc Bonferroni multiple comparison test were performed using GraphPad Prism 5 software, differences with P-value less than 0.05 were considered statistically significant.

Results

Cellular NRSF expression during traumatic stress

Postsynaptic neuroligin 1 is proposed to activate presynaptic neurexin-1β and seem to induce local formation of presynaptic specializations [7-9]. In addition, neuroligin 1 was one of NRSF target genes, which played an important role in traumatic stress-induced immuno-modulation [4]. In the current study, traumatic stress was established in SD rats, by immunofluorescence, it was observed that NRSF expression was increased by about 2.7-fold that of control at day 3 (Figures 1A and 1B), the time-point at which immuno-suppression began to recover. Significantly, the up-regulation of NRSF was concentrated in astrocytes but not in neurons and microglia (Figures 1A, 1C and Supplementary Figure 1).

Neuron-astrocyte communication dependent NRSF expression

IL-1β and IGF-1R are thought to be activator or inhibitor in traumatic stress-induced immuno-suppression [2,5-6]. Furthermore, it was illustrated herein that, in neuron, astrocytes and neuron-astrocyte co-culture, NRSF expression remained unchanged following IL-1β or IL-1ra exposure. In contrast, NRSF expression could be initiated by IGF-1 in neuron-astrocyte co-culture, the effect was blocked by recombinant adenovirus expressing negative dominant Fyn, the data indicated that NRSF might contribute to the recovery process from traumatic stress [1] (Figures 2A-2C).

Expression of NRSF target genes during traumatic stress

The phenomenon of cross-sensitization between stress and neuroinflammation has been well characterized [40,41]. Then, by real-time PCR, we measured the expression of NRSF target neuro-inflammatory genes (CCR5, CCR3, CD14, gp130 and IL-8) in prefrontal cortex of stress-induced rats, and found that, among these genes, only CCR5 expression reached a maximum level at 3 days following traumatic stress; CD14, gp130 and IL-8 were substantially up-regulated at day 1, whereas, there was no change in CCR3 expression following traumatic stress (Figure 3A). When we measured genes expression by real-time PCR, it was revealed that, in neurons, there was no apparent change in the expression of any of above genes after exposure to IL-1β or IGF-1 (Figure 3B); in astrocytes, IL-1β elicited considerable up-regulation of gp130 and IL-8 expression (Figure 3C); in neuron-astrocyte co-culture, expression of gp130, IL-8 and CCR5 were significantly up-regulated by IL-1β and IGF-1 respectively (Figure 3D).

CCR5 expression during traumatic stress

By immunohistochemistry, we demonstrated that there was a dramatic increase in CCR5 expression in prefrontal cortex at day 3 following traumatic stress: the immuno-positive signals were enhanced by around 3.2-fold that of the control (Figures 4A and 4B). Meanwhile, we found that this robust enhancement was concentrated in neuron, wherein the relative densities of double staining were increased by around 2.6-fold that of the control (Figure 4C). In contrast, there was no detectable alteration in CCR5 expression in glial cells (Supplementary Figure 2). It was further revealed that CCR5 expression could be enhanced by IGF-1 in neuron-astrocyte co-culture (Figure 4D). In the similar culture system, we also demonstrated that CCR5 could be targeted by NRSF, whose promoter activity was exceptionally increased by IGF-1 (Figure 4E). Additionally, CCR5 luciferase activity was increased gradually in the presence of 5–50 mM KCl, indicating that CCR5 expression was in parallel with neuron depolarization (Figure 4F).

Contribution of CCR5 in the altered HPA axis during acute and chronic stresses

By competitive immunoassay, it was revealed that, after traumatic

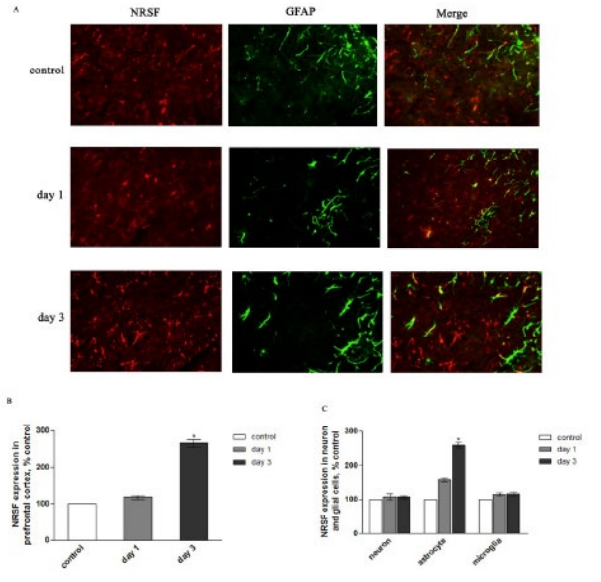

Figure 1: Cellular NRSF expression during traumatic stress. (A) SD rats were divided into three groups: control, day 1, and day 3 (1 and 3 days after trauma; n=5). Cross-sections of the frontal cortex were subsequently immuno-stained by anti-NRSF and Alexa594 antibodies, and anti-GFAP and Alexa488 antibodies, and analyzed by Leika Q500IW image analysis system (Leika Inc., Germany). Scale bars, 50 μm. Panels (B) and (C) show quantitative analysis of NRSF expression, and co-localization of NRSF with GFAP, tubulin and CD11b/c, respectively. Data were calculated as percentage of control; each value represents the mean ± S.E.M. of three independent experiments. *p<0.05 *vs.* control.

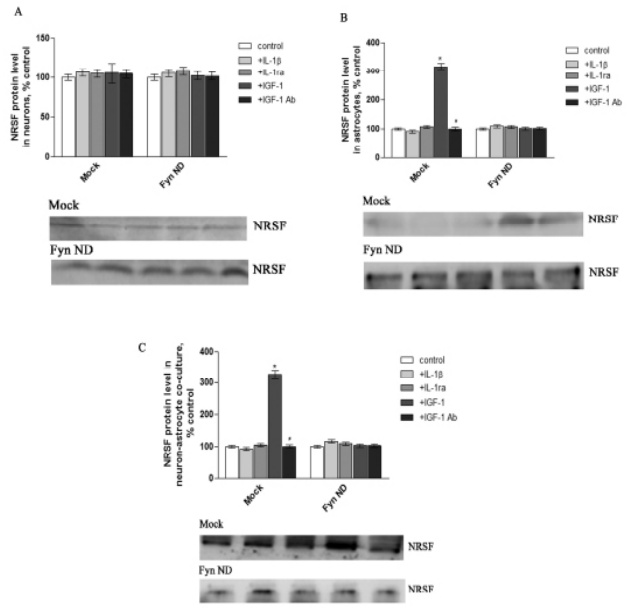

Figure 2: Neuron-astrocyte communication dependent NRSF expression. Neurons and astrocytes were separated from prefrontal cortex and cultured for the indicated times, then transfected with Mock or negative dominant Fyn; 48 h later, cells were exposed to IL-1β/IL-1ra or IGF-1/IGF-1Ab. NRSF expression in neurons (A), astrocytes (B), and co-culture of neurons and astrocytes (C) was determined by western blot analysis. Data were calculated as percentage of control; each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control, #p<0.05 *vs.* IGF-1.

stress, the operated rats had a greater enhancement in CRH release, and ACTH/corticosterone secretion at day 1. By day 3, these animals had a reduced HPA axis response. It is noteworthy that when exposure to PSS for 14 days, CRH release and ACTH/corticosterone secretion were persistently up-regulated in the operated rats. In the case of HPA axis activation, CCR5 could inhibit the effect exerted by trauma, however, it had a minor but not significant role in the animals subjected to PSS, the results were disappeared when CCR5 signaling was blocked by maraviroc treatment or CCR5 knockdown (Figures 5A–5E). In addition, there was no detectable alteration in body temperature in animals subjected to acute or chronic stresses (Figures 5F and 5G).

Nuclear GR expression during acute and chronic stresses

It has been suggested that impaired release of glucocorticoids or GR desensitization might be important for long-lasting psychological changes [42-46]. Our present results showed that after traumatic stress, nuclear expression of GR was dramatically increased at day 1 following the operation, then returned to control levels at day 3 (Figure 6A). After challenged with PSS, nuclear GR expression was persistently enhanced, CCR5 manipulation could prevent the effect exerted by traumatic stress but not by PSS (Figure 6A). Of most interest, it was demonstrated that at day 1 following traumatic stress, the connection between NRSF and GR was strengthened (Figure 6B), moreover, in neuron-astrocyte co-culture, their connection could be specifically initiated by IL-1β (Figure 6C), the data indicated that nucleus GR distribution might interact with NRSF and involve in its transcription machinery.

Consequently, we transfected neurons with GR gene reporter luciferase, and analyzed the dose-response to DEX in neuron-astrocyte co-culture, it was demonstrated that, after administration of 0.1–100 nM DEX, GR luciferase activity increased gradually and in an NRSF-dependent manner. DEX also exhibited quite good efficacy in induction of nuclear GR expression, the result was apparently related to NRSF expression (Figures 6D and 6E).

Growth and organ weight during acute and chronic stresses

Animals exposed to PSS showed a 57.4 ± 5.6 g weight gain (Figure 7A), which was significantly different from that of the control animals (78.3 ± 9.5 g) and of those that underwent traumatic stress (77.4 ± 7.9 g); this effect could be improved by NRSF but not by CCR5 knockdown. Additionally, these animals showed a trend toward an increase in adrenal gland weight and a trend toward a decrease in pituitary weight, however, there was no statistical difference in this respect in the controls or animals subjected to traumatic stress (Figures 7B and 7C).

Contribution of NRSF in the altered HPA axis during acute and chronic stresses

By competitive immunoassay, it was revealed that, the mounted CRH release and ACTH/corticosterone secretion by traumatic stress could be improved when over-expression of NRSF. Notably, NRSF over-expression could also inhibit HPA axis response when operated rats were subjected to PSS (Figures 8A–8C). GR nuclear translocation displayed a similar pattern of change to that of CRH release and of ACTH/corticosterone secretion, and was also associated with NRSF expression (Figure 8D).

Contribution of NRSF in the altered HPA axis is dependent on neuron-astrocyte communication

The neurexin-1β/neuroligin-1 signaling pathway has been thought to steer the required synaptic cell adhesion and shape neuron-astrocyte

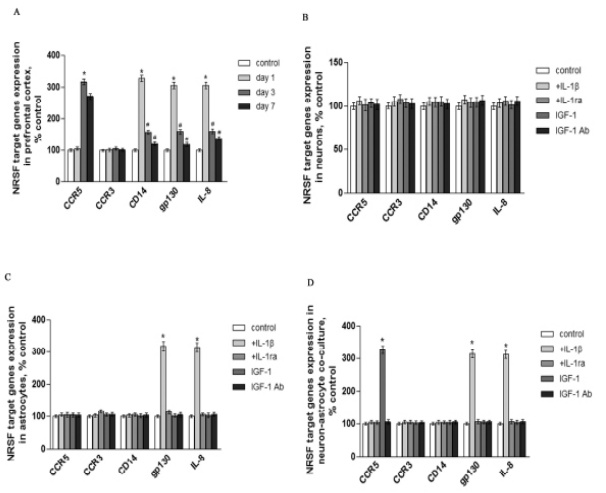

Figure 3: Expression of NRSF target genes during traumatic stress. SD rats were divided into three groups: control, day 1, day 3 and day 7 (1, 3 and 7 days after trauma; n=5). The prefrontal cortex was collected and homogenized, and expressions of NRSF target genes including CCR5, CCR3, CD14, gp130 and IL-8 were detected by real-time PCR (A). Neurons and astrocytes were separated from prefrontal cortex and cultured for the indicated times; afterwards, cells were exposed to IL-1β/IL-1ra or IGF-1/IGF-1Ab. The expressions of CCR5, CCR3, CD14, gp130 and IL-8 in neurons (B), astrocytes (C), and co-culture of neurons and astrocytes (D) was carried out by real-time PCR. Data were calculated as percentage of control; each value represents the mean ± S.E.M. of three independent experiments. *p<0.05 *vs.* control.

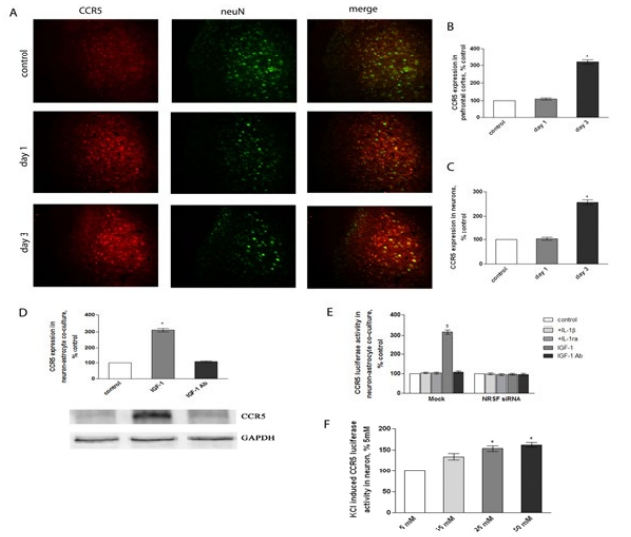

Figure 4: CCR5 expression during traumatic stress. SD rats were divided into three groups: control, day 1, and day 3 (1 and 3 days after trauma; n=5). Cross sections of frontal cortex were subsequently immuno-stained by anti-CCR5 and Alexa594 antibodies, and anti-tubulin and Alexa488 antibodies, and were analyzed by Leika Q500IW image analysis system. Scale bars, 50 μm (A). Panels B and C show quantitative analysis of CCR5 expression, and co-localization of CCR5 with neuN, respectively. Neurons and astrocytes were separated from prefrontal cortex and cultured for the indicated times: (D) in neurons, CCR5 activity in the presence of 5–50 mM of KCl was detected by luciferase assay; (E) in co-culture of neurons and astrocytes, IGF-1 induced CCR5 expression was measured by western blot analysis; (F) in co-culture of neurons and astrocytes, after transfection of NRSF or NRSF siRNA, CCR5 activity was measured by luciferase assay. Data were calculated as percentage of control; each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control or 5 mM.

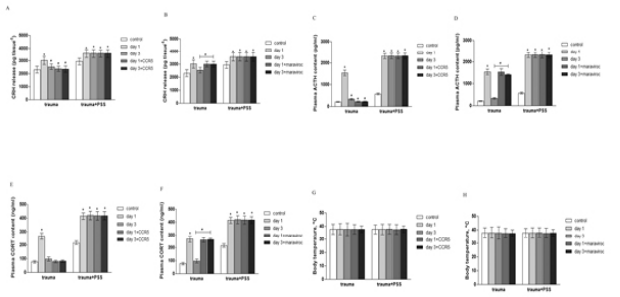

Figure 5: Contribution of CCR5 in the altered HPA axis during acute and chronic stresses. SD rats underwent traumatic stress, or were subjected to predator scent exposure following trauma (trauma+PSS) for 14 days, and CCR5 was over-expressed using adeno-virus or whose activity was inhibited by frontal cortex injection of maraviroc. (A, B) Hypothalamus was sonicated using a tissue extraction reagent (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma), the homogenate was centrifuged (10 min, 14,000 *g*, 4°C) and the supernatant was collected; (C-E) cardiac blood was centrifuged (10 min, 14,000 *g*, 4°C) and CRH release, ACTH and CORT were measured using a competitive immunoassay (Assay Designs, Inc., Ann Arbor, MI). Total protein was quantified using a Bradford assay. (F, G) the core temperature of the rats was recorded daily. Data were normalized and calculated, each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control, #p<0.05 *vs.* day 1.

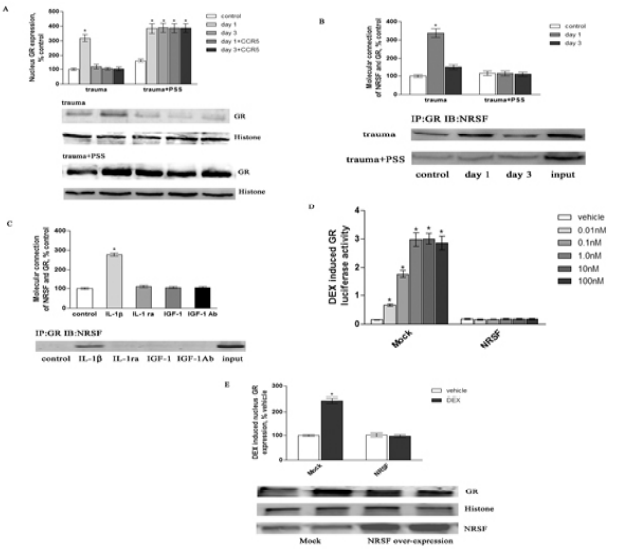

Figure 6: Nucleus GR expression during acute and chronic stresses. SD rats were underwent traumatic stress, or were subjected to predator scent exposure following trauma (trauma+PSS) for 14 days, and CCR5 was over-expressed by adeno-virus, the nucleus fraction was separated from prefrontal cortex, and GR expression was determined by western blot analysis (A). SD rats underwent traumatic stress, were subjected to predator scent exposure following trauma (trauma+PSS) for 14 days. (A) Prefrontal cortex was collected and homogenized, connection of NRSF and GR was determined by immunoprecipitation, in which anti-GR was used as the immunoprecipitated antibody, and anti-NRSF as the immunoblot antibody (B). In a co-culture of neurons and astrocytes: (C) after exposure to IL-1β/IL-1ra or IGF-1/IGF-1Ab, the interaction between NRSF and GR was determined by immunoprecipitation, in which anti-GR was used as the immunoprecipitated antibody, and anti-NRSF as the immunoblot antibody; (D) after transfection of Mock or NRSF, the dose response of GR activity to DEX was measured by luciferase assay; (E) DEX-induced nuclear GR expression was determined by western blot analysis. Data were calculated as percentage of control; each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control or Mock.

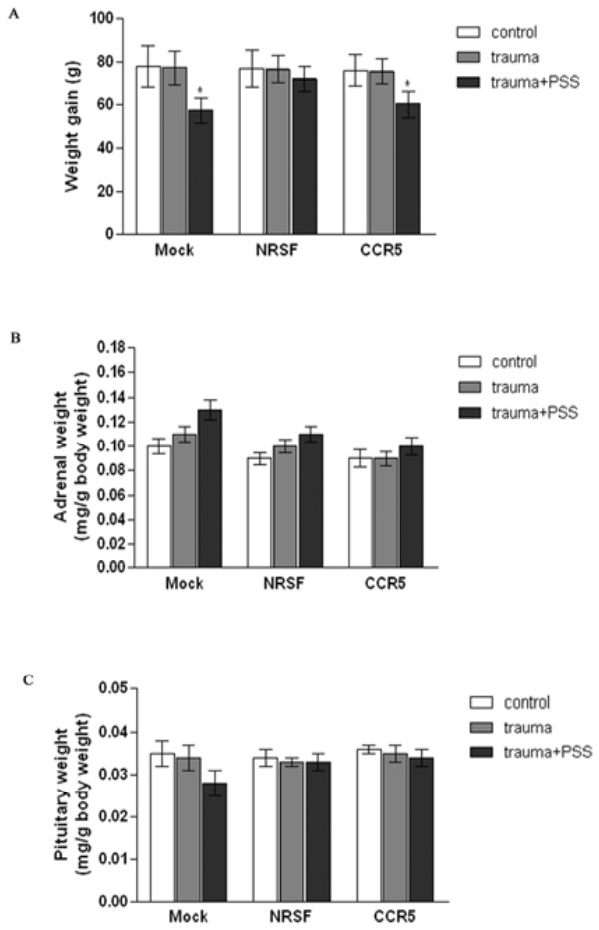

Figure 7: Growth and organ weight during acute and chronic stresses. SD rats underwent traumatic stress, were subjected to predator scent exposure following trauma (trauma+PSS) for 14 days. NRSF or CCR5 were over-expressed by adeno-virus: (A) animals were weighed daily; (B and C), the weights of the adrenal and pituitary glands were recorded.

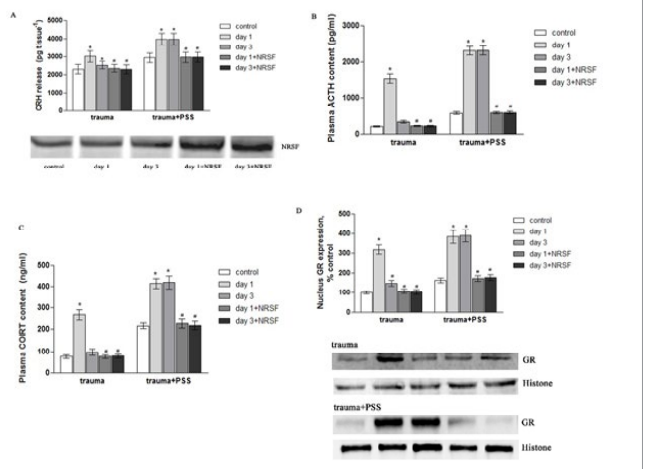

Figure 8: Contribution of NRSF in the altered HPA axis during acute and chronic stresses. SD rats underwent traumatic stress, were subjected to predator exposure following trauma (trauma+PSS) for 14 days, and NRSF was over-expressed by adeno-virus: (A) hypothalamus was sonicated using a tissue extraction reagent supplemented with a protease inhibitor cocktail, the homogenate was centrifuged (10 min, 14,000 *g*, 4°C) and the supernatant collected; (B and C) cardiac blood was centrifuged (10 min, 14,000 *g*, 4°C), and CRH release, ACTH and CORT were measured using a competitive immunoassay. Total protein was quantified using a Bradford assay. (D) Nucleus GR expression was determined by western blot analysis. Data were calculated, each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control, #p<0.05 *vs.* day 1/3.

communication in the brain [4]. In our experiment, it was shown that neuregulin-1 expression was strongly upregulated at day 3 following traumatic stress, the response could be further augmented in the presence of PSS (Figure 9A). Particularly, the optimally activation of HPA axis by NRSF, including CRH release, ACTH/corticosterone secretion, and GR nucleus translocation, could be modulated by neuregulin-1, indicating that the effect of NRSF was preferentially due to astrocyte activation (Figures 9B–9D).

Discussion

NRSF is a zinc finger transcription factor, that binds to a 21-nt DNA sequence NRSE [13], and is fundamental for establishment of a homeostatic response and neurogenesis [11,14]. In the present study, we found that NRSF expression was increased at the recovery stage of traumatic stress, whose expression was concentrated in astrocytes and displayed with Fyn-dependent manner. Especially, the up-regulation of NRSF could be initiated by IGF-1, and mostly occurred when neuron-astrocyte communication was intact. We already reported that, IGF-1 and Fyn could deliver trans-synaptic signaling and age-dependently implicate in the inhibition of traumatic stress-induced immuno-suppression [6], then, NRSF was proposed to drive a neuronal network to promote immune surveillance in the CNS and probably form an efficient complex for the defense system to stress-like events.

Well characterized, multiple functions of NRSF have been attributed to its extensive regulatory components. By screening inflammation-related genes, we found that during traumatic stress, parallel to up-regulation of NRSF, CCR5 was profoundly increased in neurons, whose expression was only initiated in the presence of astrocytes or neuron depolarization. Comparably, other genes including CCR3, CD14, gp130 and IL-18 appeared to be not related with NRSF or traumatic stress. We therefore hypothesized that in the presence of acute and chronic stress, astrocytes were potentially modulated by NRSF, which evoked neuron activation and the subsequent CCR5 expression.

It is known that traumatic stress leads to HPA axis activation via the release of pro-inflammatory cytokines [2]. Herein, we demonstrated that traumatic stress caused a rapid and temporal increase in release of hypothalamic CRH, secretion of ACTH from the anterior pituitary, and glucocorticoids from the adrenal cortex, which could be inhibited by CCR5 over-expression. Above HPA hyper-reactivity was also found in predator scent exposure followed by traumatic stress, which accompanied by persistent GR nuclear translocation, and a trend toward an increase in adrenal gland weight and decrease in pituitary weight, NRSF but not CCR5, had a crucial defensive effect in this case. It is established that both acute and chronic stress could selectively recruit cortical glucocorticoid signaling to inhibit HPA axis responses, based on our observation, NRSF and CCR5 might be critical for activating the HPA axis in the presence of two broad types of stressors: those having a predominant emotional component and those that represent a direct challenge to homeostasis [27]. In addition, we demonstrated that GR activity could be modulated by dexamethasone, a glucocorticoid hormone, and molecular connection of NRSF and GR

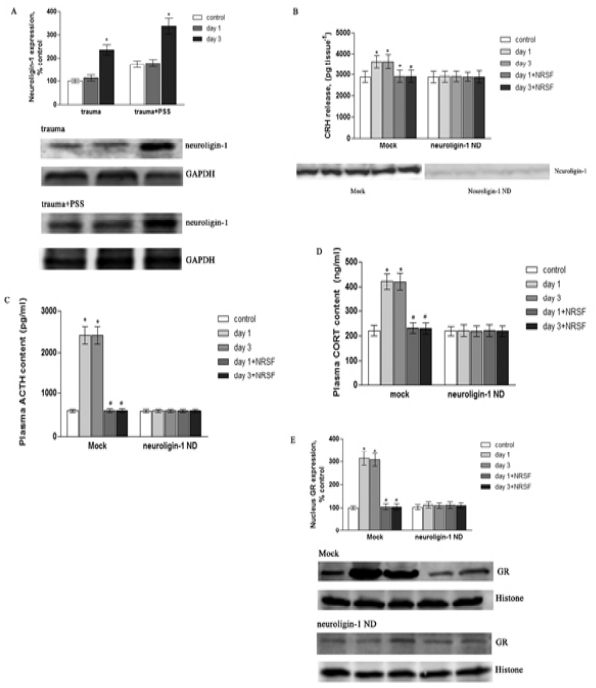

Figure 9: Contribution of NRSF in the altered HPA axis is dependent on neuron-astrocyte communication. SD rats underwent traumatic stress, were subjected to predator scent exposure following trauma (trauma+PSS) for 14 days, the prefrontal cortex was collected and homogenized, and neuroligin-1 expression was determined by western blot analysis (A). SD rats were infected with Mock or negative dominant neuroligin-1, then exposed to traumatic stress, or subjected to predator scent exposure following trauma (trauma+PSS) for 14 days, and NRSF was over-expressed by adeno-virus: (B) hypothalamus was sonicated using a tissue extraction reagent supplemented with a protease inhibitor cocktail and the homogenate was centrifuged (10 min, 14,000 *g*, 4°C) and the supernatant collected; (C and D) cardiac blood was centrifuged (10 min, 14,000 *g*, 4°C), and CRH release, ACTH and CORT were measured using a competitive immunoassay (Assay Designs, Inc., Ann Arbor, MI), in this part, animals exposed to PSS but not undergone traumatic stress were used as control group. Total protein was quantified using a Bradford assay. (E) GR expression in the nucleus was measured by western blot analysis. Data were calculated as percentage of control; each value represents the mean ± SEM of three independent experiments. *p<0.05 *vs.* control, #p<0.05 *vs.* day 1/3.

was triggered by IL-1β at the early stage of traumatic stress, thereby, the data indicated that when challenged with acute and chronic stresses, GR could be activated by ligand binding, the newly formed complex was translocated into cell nucleus and in close association with NRSF evoked multiple mechanisms, by which exclusively trigger modality-specific prefrontal output neurons activation and restraint of GR activation and HPA axis responses.

Neuroligin-1 is well described as a synaptic cell-adhesion molecule that is expressed in non-neuronal cells and usually functions to form synapses between neurons and non-neuronal cells by contacting neurexin 1β-expressing neurons [47-51]. We previously reported that neuron-glial signaling was optimally activated and clustered within neurexin-1β/neuroligin-1 cargos, which might resolve the dichotomy of neural cell activities during traumatic stress [4]. Herein, we demonstrated that neuroligin-1 was evolutionarily facilitated in response to traumatic stress and PSS at post-trauma, optimal preservation of neuroligin-1 related neuron-glia communication was achieved by NRSF. Accordingly, our data support the idea that two signals delivered by NRSF and CCR5 might be recruited during stress-like events, NRSF acted as a "negotiator" and primarily enabled inhibition of stress responses through direct or indirect neuron-glia communication.

In summary, we reported that exposure to traumatic stress increased NRSF and CCR5 expression in prefrontal cortex, and their over-expression coincided with the recovery from immuno-suppression. Specifically, CCR5 was mostly activated in neurons and targeted by astrocyte NRSF via neuron-astrocyte communication, and their robust increases could profoundly abrupt activation of HPA axis. Intriguingly, it was NRSF but not CCR5 that could inhibit long-lasting HPA activation during PSS at post-trauma, and converged into a mechanism that related with GR nucleus distribution. The effect of NRSF was preferentially shaped by neuroligin-1-formed neuron-astrocyte communication, the regulatory networks had significant potential for the development of therapeutic approaches for post-traumatic stress related disorders.

Acknowledgement

This research was supported by grants from National Nature Sciences of China (81471370), natural science from Minhang District of Shanghai (2011MHZ21) and National Key Basic Research Program of China (2013CB531900).

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