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Role of NGF/TrkA signaling milieu in depressogenic induction of rat brain

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Abstract: *Objectives:* Stress-induced helplessness in rodents constitutes a well-defined model to investigate neurobiological mechanism of depression. In the present investigation, we investigated the correlation between stress induced helplessness and NGF, its receptor TrkA with two of its principal downstream signaling molecule ERK1, 2 and Akt. *Background:* Nerve Growth factor (NGF) regulates many physiological functions in the brain, alteration of which has been well associated with the pathogenesis of depression. *Method:* NGF level was measured by Sandwich ELISA and its cognate receptor TrkA and downstream molecules ERK 1, 2, Akt were assayed by Western blot. *Result:* Chronic stressed rats exhibited down regulation of NGF and TrkA along with ERK1, 2 and Akt. This parallels with the decreased escape behavior. The antidepressant drug Fluoxetine hydrochloride (FLX) treated rats exhibited significant increase in escape deficit in stress induced Learned helplessness. This was correlated with the restoration of NGF levels, its cognate receptor TrkA and expression of its down stream signaling molecules ERK1, 2 and Akt in hippocampus of rat brain. *Conclusion:* This supports the notion that pharmacological restoration of NGF and its receptor TrkA may be of therapeutic value for the treatment of depression.

Keywords: Depression, Neurotrophin, NGF, TrkA, ERK 1, 2, Akt, FLX

Introduction

Several studies have led to the formulation of the *Neurotrophic Hypothesis of Depression*, which postulates that low levels of NGF leads to a state of depression [1-2]. Neurotrophins are essential growth factors in the development of central nervous system [3]. The neurotrophin family includes nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They are initially synthesized as precursor proteins (pro-neurotrophins), to be secreted mostly in a mature, biologically active form [4-6].

Stress that impairs the hippocampal function influences the learning and memory. Experimental studies showed that chronic stress induce learning and memory impairment [7-9]. Although the neurobiological basis of depression remains largely unknown, experiments performed with animal models have led to novel hypotheses regarding how depression may occur. In particular, there is increasing evidence that Nerve growth factor (NGF) is involved in the pathophysiology and treatment of depression [2]. NGF plays an important role in the nervous system and the cholinergic function of the central nervous system (CNS) through out life. As essential modulators of neuronal activity and synaptic plasticity in the central and peripheral nervous system [10-11] neurotrophins have received increasing attention as their dysregulation might be responsible for the inappropriate adaptive neuronal response to stress with pathological consequences such as diminished dendritic branching and hippocampal volume reduction [12-14].

The macromolecule NGF is a key neurotrophic factor in the brain; it promotes neuronal cell survival [10], regulates dendrites [10-11] and induces plasticity changes in the brain [12]. TrkA belongs to receptor tyrosine protein kinase family and it is the primary signal transduction receptor for NGF; the biological effects of which act mainly through binding to it's specific receptor. Principal

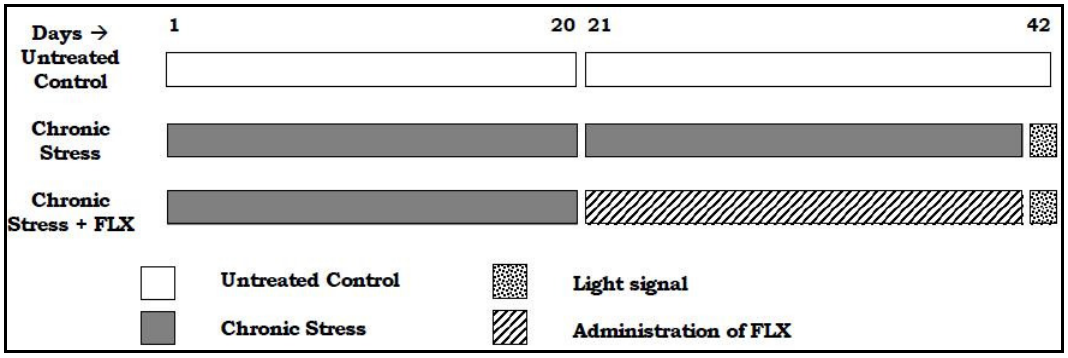
cellular response of NGF is the activation of ERK1, 2 and Akt for neuronal cell survival, differentiation, growth and plasticity [15-19].

There are several stress induced behavioral models of depression in animal. Among them Learned helplessness (LH) is a well accepted valid and reliable model of behavioral depression in which prior exposure to inescapable stress produces deficits in escape behavior [20-21]. This animal model offers an opportunity to understand the behavioral and neurochemical correlates of clinical depression, to test efficacy and screening of new antidepressant drugs. However, no evidences are yet available to establish the correlation between NGF and its receptor TrkA in hippocampus in stress induced behavioral model of depression. Therefore in the present study, we investigated changes of NGF and TrkA expression in the hippocampus if any along with its downstream signaling molecules ERK1, 2 in the MAPK pathway as well as Akt in the PI-3 kinase pathway in animal model of depression.

Material and Methods

Experimental Animals: Male Sprague-Dawley rats were used in present experiment. At the start of the experiment rats were of the same age (approximately 2 months) weighing 224±1.5 gm. All rats were individually housed in temperature controlled (22-24°C) room for at least 1 week prior to the experimentation, with ad libitum access to food and water. Rats were maintained on a 12h light / dark cycle (lights on at 7am). All experimental protocols were designed to minimize the number of animals and sufferings were approved by CPCSEA and the Institutional Animal Ethics Committee (IAEC) of RPM College, Uttarpara, Hooghly (W.B.). The stress protocols of Lin et al. and Valentine et al. [21-22] were followed with slight modification. Socially housed male rats were randomly assigned to 3 experimental groups: 1) Control group (n=10) : subjected to no footshock throughout the experiment; 2) Chronic stress group (n=10) : received 60 footshocks daily for first 20 days followed by next 20 days with alternating exposure to footshocks; 3) Recovery group (n=10) : exposed to footshocks daily for 20 days and received daily injections of FLX for consecutive 20 days and on day 42 exposed to light signal only to all of the rat groups (Figure 1).

Figure-1: Schematic representation of the 42-day experimental protocol. Untreated Control: rats were subjected to no footshocks. Chronic Stress: rats received footshocks daily for 20 days followed by 20 days of alternating exposure to footshocks. Chronic Stress + FLX: rats received footshocks daily for 20 days followed by a 20-day chronic antidepressant treatment instead of footshocks. On day 42 rats of chronic and recovery groups were exposed to the footshock box with the light only.

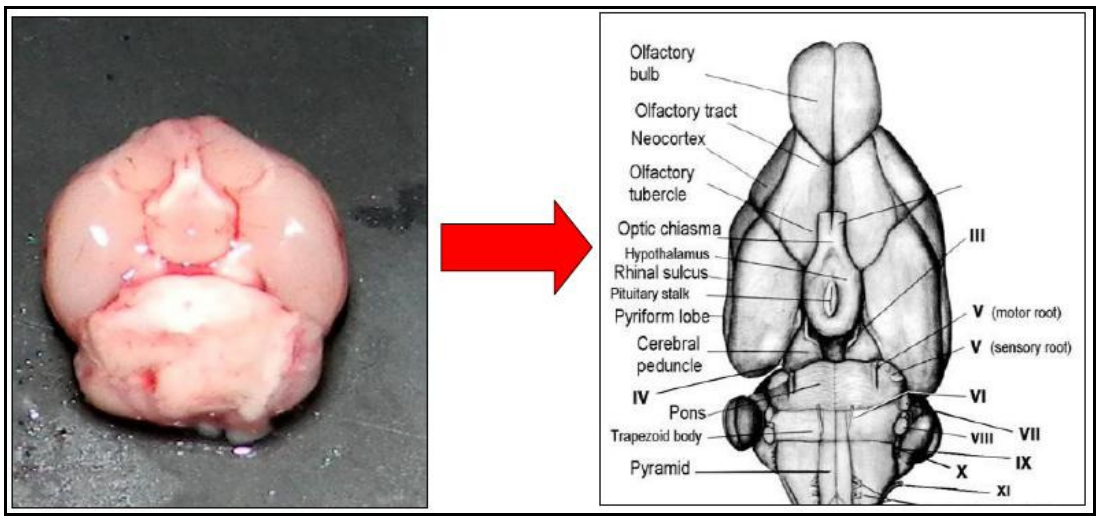


Stress Procedure: The footshock chamber consists of a box containing an animal space positioned on a metallic grid floor connected to a shock generator and scrambler. Rats in chronic stress group were placed in a box and received 60 inescapable footshocks (0.8mA intensity and 15 sec duration with interval of 45 sec.) with randomized starting time (between 9:00 and 17:00h) and intervals during a 30 to 120 min session to make the procedure as unpredictable as possible. A light signal (15 sec) preceded each footshock adding a "psychological" component to the stressor. On the last day, the chronic stress exposed rats were subjected to the light stimulus only, which was crucial as it provided a way to create a stress condition without the unwanted side effects of direct physical or painful

stimuli [22]. On day 42 rats were sacrificed using isoflurane anesthesia.

Administration of Fluoxetine hydrochloride (FLX): Fluoxetine hydrochloride (FLX) (Sigma Aldrich, St. Louis, MI, USA) was dissolved in 0.9% physiological saline and injected intra-peritoneally (i.p) at the dose of 10 mg/kg body weight. The dosage of FLX was based on studies demonstrating a reversal of shuttle box escape deficits, after injections of FLX [23] or exposure to chronic unpredictable shock [24]. Antidepressant drug was administered chronically from day 21 to day 41 once per day (Figure 2).

Figure-2: Isolation of different brain regions of rat.



Shuttle box Escape Testing: Shuttle box sessions were run by PC computer with custom software developed for the system (TSE Active Avoidance Systems GmbH, Bad Hamburg, Germany). At the start of each shuttle box session, animals were exposed to a 5 min habituation period in the same chamber where Inescapable shock (IS) or Escapable shock (ES) was applied. This was followed by 30 escape trials in which the arch gate separating the two halves of the shuttle box opened 5 sec prior to shock onset followed by randomized footshocks delivered at an intensity of 0.6 mA for 30 sec duration of escape latency. The test consisted of five fixed-ratio 1 (FR-1) trials during which one shuttle-crossing terminated shock. FR-1 trials were used to determine the normal motor function of the rats.

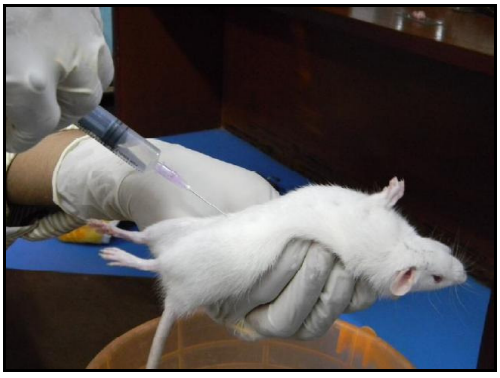
For escape testing, FR-1 trials were followed by 25 trials during which the rat had to cross from one side of the shuttle-box to the other, and then return, to terminate shock (fixed-ratio 2 or FR-2 trials). Shock terminated automatically if the response was not met within 30 seconds of the shock onset. A mean latency for the 25 FR-2 trials of ≥20 seconds are defined as learned helpless (LH) while mean latency of <20 seconds are defined as non learned helpless (NLH). Both FR-1 and FR-2 trials were presented with an average inter-trial interval of 60 sec. Crosses were automatically recorded by the PC whenever a micro-switch was activated by tilting of the pivoted grid floor after crossing. Shuttle box escape test was performed under red light conditions between 9:00 and 13:00h during the active period of the animals at least 16 hour after the last stress session and before the stress procedure of that day. The test was repeated 3 times on day1, day22 and day41. Animals of recovery group were exposed to shuttle box escape testing every third days starting after 6 days of drug treatment for a total of five shuttle box test session (day28, 31, 35, 38, 41). On testing days injections were given immediately after each shuttle box session.

Measurement of NGF protein levels in hippocampus by Sandwich ELISA: Endogenous NGF levels were measured in hippocampus using enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction (Chemicon, USA). Hippocampus were immediately

isolated after anesthesia is over. Briefly, hippocampus was homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1mM EGTA. Microtiter plates (96-well flat-bottom) were coated for 24h with the sample diluted 1:2 in sample diluent. The plates were then washed four times with sample diluent, and a monoclonal anti-NGF rabbit antibody diluted to 1:1000 sample diluent was added to each well and incubated for 3h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 2 h. After addition of streptavidin-enzyme, substrate was added followed by stop solution. The amount of NGF was determined by absorbance in 450 nm (Tecan Infinite M200). A standard curve was produced and it ranged from 7.8 to 500pg/ml of NGF. This curve was obtained from a direct relationship between optical density (OD) and NGF concentration. Total protein conc. was measured by Lowry's method using bovine serum albumin (BSA) as a standard.

Determination of expression of TrkA, ERK1, 2 and Akt protein in hippocampus by Western blot: The brains of chronic stress and control rats were removed for isolating the hippocampal tissues (Figure 3). 50-100 mg hippocampal tissue of each subject was lysed in 1 ml lysate (50 mmol/L Tis-HCl pH 7.4, 50 mmol/L NaCl, 1%Triton-X 100, 1 mmol/L EDTA, 100 µg/ml PMSF) and centrifuged at 15000 rpm for 15 minutes at 4°C to obtain the supernatant. The proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes.

Figure-3: Fluoxetine hydrochloride (FLX) was dissolved in 0.9% normal physiological saline and injected intra-peritoneally (i.p) at a volume of 2 ml/kg body weight. Drug was administered at 10 mg/kg/day.



The Western blot analysis was performed with the following primary antibodies like Trk-A (1:100 dilution, Santa Cruz Biotechnology, Inc), Akt (1:1000 dilution, Catalog No. sc-55523, Santa Cruz Biotechnology, Inc), ERK1, 2 (1:5000 dilution, Catalog No. 06-182, Upstate Biotechnology, Lake placid, NY) were used to detect the expression of the proteins. Respective secondary antibodies were used. Anti-β-actin monoclonal antibody (1:10000 dilution in 3% BSA, Sigma, USA) was used as the internal control. Immuno-reactive bands were visualized using the enhanced chemiluminescence (ECL) [Santa Cruz, C.A, USA]. The OD of each band was analyzed with the biology electrophoresis image analysis system (Smartview 2001, S/N: SV-0002202, Japan). The expression of studied proteins were determined by calculating the OD ratio of each band to β-actin protein.

Measurement of NGF and TrkA mRNA levels in hippocampus by RT PCR: Hippocampus were isolated and total mRNA was extracted from the 50-100mg tissue according to the instructions of TRIzol kit (Invitrogen, USA). NGF and TrkA mRNA in each extraction were determined by Real Time- Polymerase Chain Reaction (RT-PCR). GAPDH used as an internal control, was co-amplified with NGF and TrkA mRNAs. The primers were designed by AuGCT-technology Company (Beijing, China) according to the serial number from Genebank as follows:

NGF:
5'- AGCGTAATGTCCATGTTGTTCTAC - 3'(sense) and
5'-TGCTATCTGTGTACGGTTCTGC- 3'(antisense);

TrkA:
5'-CTTGCGCCGCATCCTGTCGT-3'(sense) and
5'-GCAGGCCGCGGAGGGTATTC- 3'(antisense).

The PCR products were observed after electrophoresis on 1.2% agarose gel and the density of each band was analyzed on the gel image analysis system (Smartview 2001, S/N: SV- 0002202, Japan). The level of the mRNA was determined by calculating the density

ratio of each band of NGF and TrkA mRNA to GAPDH mRNA.

Statistical Analysis: The Statistical Package for the Social Science (SPSS) 15.0 was utilized for statistical analyses. All data are expressed as mean ± standard error of n animals, and have been statistically analyzed with the student's t-test. *P* values less than 0.05 were considered statistically significant. Average escape latencies of FR-1 and FR-2 trials analyzed with a repeated measured ANOVA.

Results

Effects of chronic inescapable foot shocks and antidepressant treatment on escape latency and escape frequency: The mean FR-2 escape latencies were significantly higher ($F_{2,27}$=16.23; *p<0.001; Figure 4A) in the chronic stress group than normal controls. However, following FLX treatment chronic stressed rats in recovery groups showed escape latency similar to that of normal control indicating restoration of normal escape latency. Number of escape failures and avoidance responses altogether are represented as escape frequency. After FLX administration, escape frequency of chronic stress rat groups showed significant increase in escape frequency compared to only stressed group rats (t_{FLX} =3.96; *p<0.001; Figure 4B).

Figure-4A: FR-2 Escape latencies of the rats of 3 experimental groups. The FR-2 escape latencies were significantly decreased in case of chronic stress group rats ($F_{2,27}$=16.23;* p<0.001) than untreated controls.

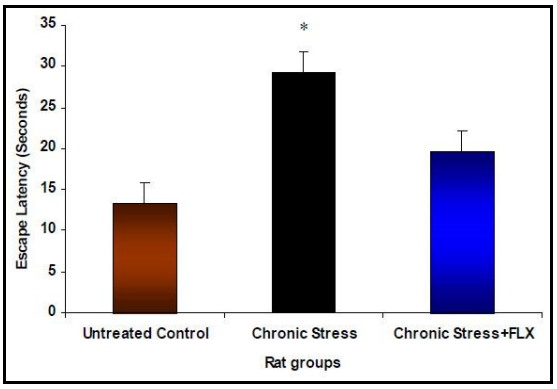
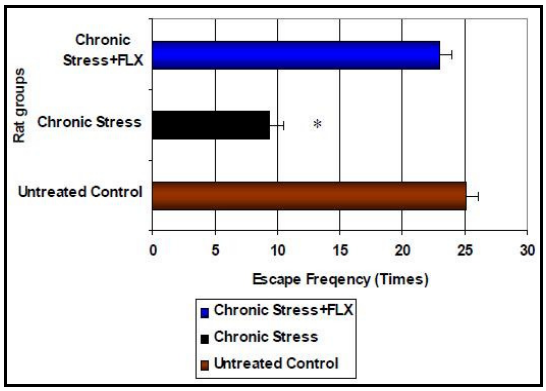
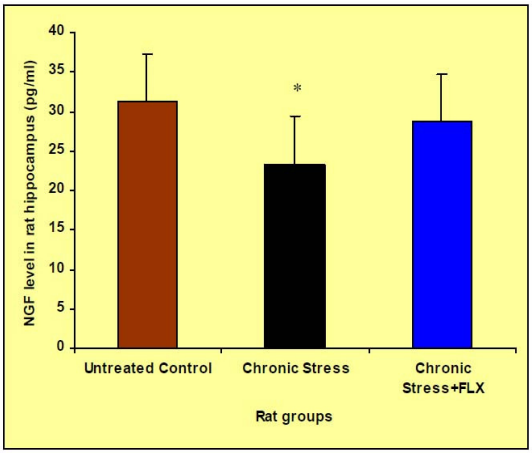


Figure-4B: FR-2 Escape Frequencies of the Rats of 3 Experimental groups. Escape frequency is significantly increased among FLX treated group compared to Chronic Stress group rat (t_{FLX}= 3.76; *p<0.001).



Effects of chronic inescapable foot shocks and antidepressant treatment on NGF level in Hippocampus of rat brain: Chronic stress procedure significantly reduces the NGF protein levels in the hippocampus of the LH rats compared to normal controls (t =30.01; df=18; *p<0.001; Figure 5). NGF level was significantly restored in FLX (t=17.57; df=18; *p<0.001; Figure 5) treated groups.

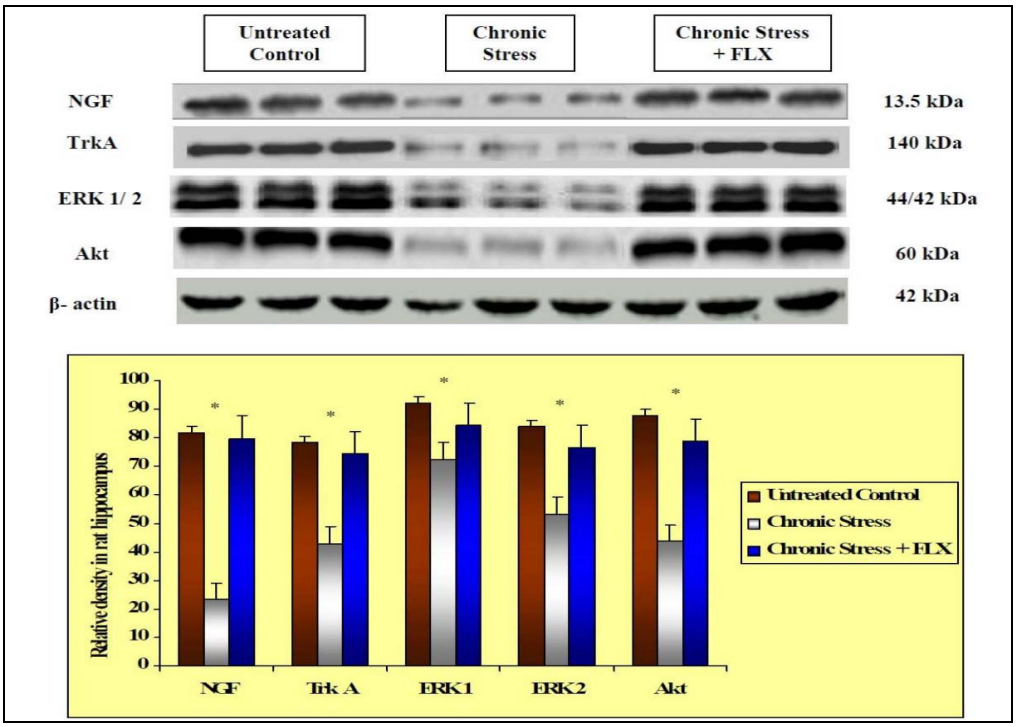
Figure-5: Hippocampal NGF level in 3 different rat groups. NGF levels were measured (pg/ml) by sandwich ELISA on day 42. Data were expressed as mean ±SEM, n= 10.*p<0.001 versus untreated control.



Effects of chronic inescapable foot shocks and antidepressant treatment on NGF, TrkA, ERK1, 2 and Akt expression in Hippocampus of rat brain: The molecular weight of TrkA, ERK 1, 2, Akt and β-actin were 140 kDa, 44 kDa, 42 kDa, 56 kDa and 42 kDa respectively. The expression levels of TrkA, ERK1, 2 and Akt proteins were normalized against the internal control β-actin. The results showed considerable expression of NGF with its receptor TrkA and its downstream signaling

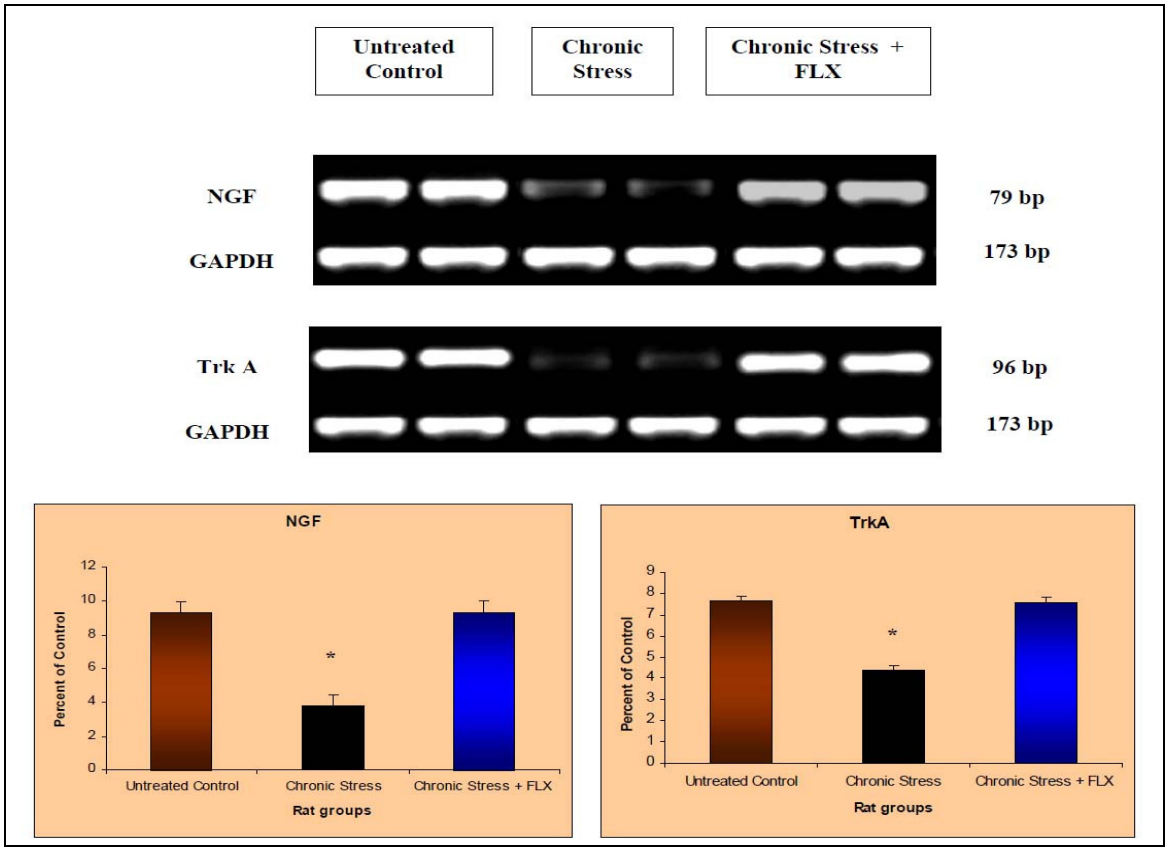
molecules ERK1, 2 and Akt in the hippocampus. Following chronic stress, expression of receptor TrkA, ERK1, 2 and Akt were decreased significantly (t_{NGF}=60.79; df=19; *p<0.001; t_{TrkA}=16.61; df=19; *p<0.001; t_{ERK1}=13.50; df=19; *p<0.001; t_{ERK2}=22.86; df=19; *p<0.001; t_{Akt}=33.70; df=19; *p<0.001; Figure 6) compared to the untreated control group. However, chronic stressed rats after FLX treatment (recovery group) showed significant recovery of their expressions (t_{NGF}=54.34; df=19; *p<0.001; t_{TrkA}=12.72; df=19; *p<0.001; t_{ERK1}=5.50; df=19; *p<0.001; t_{ERK2}=10.82; df=19; *p<0.001; t_{Akt}=12.62; df=19; *p<0.001; Figure 6) like untreated normal controls, indicating the drug mediated recovery of the NGF receptor and its down stream signaling molecules in hippocampus of rat.

Figure-6: Representative Western blots showing the expression of NGF, Trk A, ERK1, ERK2 and Akt in hippocampus of rat brain in 3 Untreated Control subjects, 3 Chronic Stress subjects and 3 Chronic Stress + FLX treated subjects. kDa indicates kilodalton. Lane 1-3: Untreated Control group, Lane 4-6: Chronic Stress group and lane 7-9: Chronic Stress + FLX group. *p<0.001 vs the Untreated control



Effects of chronic inescapable foot shocks and antidepressant treatment on NGF and TrkA mRNA levels in hippocampus of rat brain: Alterations in the mRNA expression of NGF and TrkA due to chronic inescapable footshocks and antidepressant drug treatment are depicted below. The lengths of NGF, TrkA and GAPDH amplified fragments were 79 bp, 96 bp and 173 bp respectively and the bands were clear. The levels of NGF and TrkA mRNA were normalized against GAPDH mRNA levels as an internal control. GAPDH mRNA expressions were never altered in both control and stressed subjects (t_{GAPDH}=0.116; df=19; p=0.52). Compared with the untreated control group, the levels of NGF and TrkA mRNA were significantly reduced (t_{NGF}=18.90; df=19; *p<0.001; t_{TrkA}=19.58; df=19; *p<0.001; Figure 7) in chronic stress-induced rat group and the differences were statistically significant. GAPDH mRNA expressions were never altered in both antidepressant treated and depressed rats (t_{GAPDH}=0.121; df=19; p=0.36). Compared with the stress-induced rat group, the levels of NGF and TrkA mRNA were significantly increased (t_{NGF}=18.90; df=19; *p<0.001; t_{TrkA}=19.58; df=19; *p<0.001; Figure 7) after chronic administration of FLX in recovery group and the differences were statistically proved. There was no significant alterations in mRNA expressions between untreated control and FLX treated groups (t_{NGF}=0.11; df=19; p=0.02; t_{TrkA}=0.20; df=19; p=0.80).

Figure-7: mRNA levels of NGF and TrkA in hippocampus of 3 experimental rat groups are compared together. Expression profiles of NGF and TrkA are significantly reduced after chronic stress and increased after administration of FLX. Data were expressed as mean ±SEM, n= 10.



Discussion

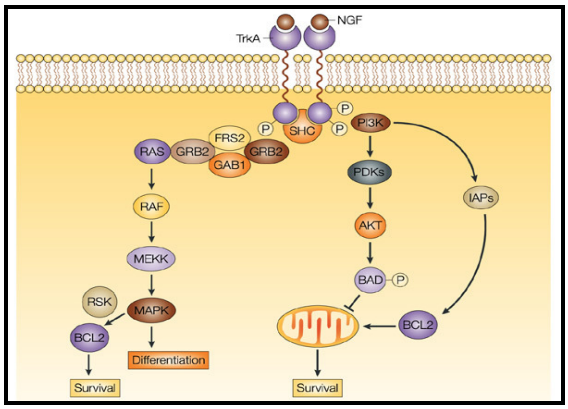
The magnitudes of escape latency in rats, exposed to chronic stress was significantly higher (*p<0.001) than the untreated control group indicating significant increase in behavioral depression of chronic stressed rats (Figure 4A and 4B). This corroborates well with the previous findings using learned helplessness model of behavioral depression. NGF seems to be one of the key molecular mediators of synaptic plasticity [12, 25-26], the expression of plasticity is strictly dependent on neuronal activity [25]. Researchers have already reported that NGF has an antidepressant like effect [26] and administration of antidepressant increases NGF expression in animal model of depression [26-28].

The present study analyzes NGF protein and mRNA levels along with its cognate receptor TrkA in the hippocampus after chronic exposure of inescapable footshocks and antidepressant treatment. It showed significantly decreased NGF protein and mRNA, TrkA mRNA levels after chronic stress compared to untreated control rats correlating well with the decreased escape latency (indicator of behavioral depression) in stressed rats [21, 26]. This correlation of hippocampal NGF/TrkA level with development of depression as observed in the present study corroborates well with previous studies [26]. This unique correlation of hippocampal NGF and TrkA levels with depression is further strengthened as chronic FLX treatment almost normalizes the levels of both hippocampal NGF and TrkA in stressed rats, correlates well with the escape behavior.

From previous evidences, it is clearly indicated that the interaction between NGF and its cognate receptor TrkA enhances the intra-cellular signal transductions either through MAPK or PI-3 kinase pathway through the activation of principal downstream signaling molecules [29-32]. The

interaction between NGF and TrkA in the mechanism of learning and memory is now a subject of widespread attention. Previous studies reported that the interaction between NGF/TrkA signaling is very important for spatial memory formation (Figure 8) [26]. In the present investigation, decreased expression of NGF receptor TrkA and its down stream signaling molecules ERK1, 2 and Akt in hippocampus following chronic stress and its recovery with FLX treatment correlated with the development of behavioral depression.

Figure-8: Schematic representation of NGF/ TrkA Signaling cascades. Key factors (Receptor TrkA, Akt and ERK) of this signaling pathway are marked with red arrows.



Therefore, the results of the present investigation corroborates well with hippocampal NGF hypothesis of stress behavior and further confirms that pharmacological recovery of this neurotrophin receptor and its signaling pathways may alleviate stress induced behavioral alterations.

Acknowledgements

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