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Research Article

Amitriptyline Activity is Associated with Synaptic Marker Changes in the Hippocampus of Mice Exposed to Experimental Models of Depression

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Keywords Amitriptyline; GAP43; Depression

Abbreviations GAP43: axon Growth-Associated Protein 43; i.p.: intraperitoneal; TST: Tail Suspension Test; UCMS: Unpredictable Chronic Mild Stress

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Abstract

Study background: Tricyclic antidepressants are widely prescribed in the treatment of depression, although the mechanism of their therapeutic effects is poorly understood. Novel hypotheses suggest that antidepressants might act on synaptic plasticity and cytoskeletal remodeling. The aim of the present study was to evaluate in animals exposed to acute and chronic behavioural despair paradigms of depression, the treatment with amitriptyline, a widely used tricyclic antidepressant, on axon Growth-Associated Protein 43 (GAP43), a synaptic protein, in the mouse hippocampus.

Methods: The effect produced by peripheral administration of amitriptyline on hippocampal GAP43 expression was investigated by immunoblotting and immunofluorescence experiments.

Results: Animals exposed to the Tail Suspension Test (TST), a highly predictive model of antidepressant activity following acute treatment, did not show any variation in the GAP43 contents 6 and 24 h after testing. Acute administration of amitriptyline (10 mg/kg i.p.) increased hippocampal levels of GAP43. Conversely to TST, animals exposed to Unpredictable Chronic Mild Stress (UCMS), an animal model of depression, showed diminished GAP43 immunostaining in the hippocampal CA3 region. Chronic administration of amitriptyline not only counteracted the immobility induced by exposure to UCMS paradigm evaluated by the TST, but also reversed the decrease of the synaptic protein.

Conclusion: These findings suggest that depressive states might be associated to a reduction of synaptic protein expression. These synaptic changes might be involved in the mechanism of tricyclic antidepressant drugs and may contribute to their psychotherapeutic actions.

Introduction

Depression and anxiety are the most common mental health problems which affect a person's thoughts, behavior, feelings and physical wellbeing. Drugs used for treatment of major depressive disorders include Selective Serotonin-Reuptake Inhibitors (SSRIs), tricyclic antidepressants, monoamine oxidize inhibitors, and norepinephrine reuptake inhibitors. Although these antidepressant agents can produce a rapid increase of serotonin (5-HT) and/or Noradrenaline (NA) at synaptic levels, it usually takes at least 3 to 4 weeks to obtain an appreciable clinical effect [1,2]. The efficacy of antidepressants cannot be solely explained by their actions on the monoaminergic system and long-term effects are likely to occur. However, the molecular and cellular adaptations that underlie the therapeutic action of antidepressants have remained largely obscure. Neuronal plasticity is required for the adaptation of the brain to face a changing external environment. Currently, depression may be considered a consequence of stress-induced impairment in the plasticity of several brain areas [3]. In particular, the hippocampus, a region critically involved in motivation and emotion processing [4], has been shown affected in depressed patients [5-7] and in animals exposed to experimental models of depression [8,9]. Dysfunction of neuronal plasticity could therefore contribute to the pathophysiology of mood disorders and recovery could occur by induction of the appropriate plasticity or remodeling phenomena [10,11]. Several lines of evidence during the last decade have suggested that antidepressants may act by promoting this plasticity [12]. It has been demonstrated that the antidepressant group of Serotonin-Selective Reuptake Inhibitors (SSRIs), promotes different kinds of plasticity in the adult CNS, including increased hippocampal neurogenesis [13], LTP induction [14] or remodeling in the structure of pyramidal neurons [15,16]. However, it is worth noticing that despite the evidence indicates that fluoxetine might modulate some components of cytoskeletal and synaptic plasticity [17], it remains to be further clarified whether these effects are related to a selective antidepressant group of pharmacological compounds, or are common plasticity structural changes involved in the action of antidepressant categories further than SSRI. To address this point, we studied the effects of acute and chronic treatment with the tricyclic antidepressant amitriptyline on axon Growth-Associated Protein 43 (GAP-43), a marker of

neuronal plasticity, in the hippocampus of mice exposed to the Tail Suspension Test (TST), an animal model to evaluate antidepressant-like activity of drugs, and, to the Unpredictable Chronic Mild Stress (UCMS), animal model which emulate the behavioural despair paradigm of depression.

Material and Methods

Animals

Male CD1 mice (20-22 g) from the Harlan Laboratories (Bresso, Italy) breeding farm were used. Mice were randomly assigned to standard cages, with four animals per cage. The cages were placed in the experimental room 24 h before behavioural test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence, Italy, under license from the Italian Department of Health and in compliance with the European Communities Council directive of 24 November 1986 (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals [18].

Behavioral testing

Animals were habituated to the experimental room and randomly assigned to each treatment group. Mice were investigated by observers blinded for treatment of the animals.

Tail suspension test: A piece of tape was adhered to the upper middle of the tail of each animal, creating a flap with the overlap of tape. Mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. The duration of the test was 6 min and immobility was also measured the first 2 min and the last 4 min. Immobility was defined as the absence of any limb or body movements, except those caused by respiration.

Unpredictable chronic mild stress (UCMS)

UCMS used in this study was designed as described previously [19]. In brief, UCMS-exposed mice were isolated in individual cages and subjected daily to various CMS procedures according to an unpredictable schedule for 4weeks. The UCMS protocol consists of the sequential application of a variety of mild stressors including restraint, forced swimming, water and/or food deprivation, and pairing with another stressed animal in wet sawdust, housing in wet sawdust, reversal of the light/dark cycle, and housing in constant illumination or darkness each for a period ranging from 10 min to 24 h in a schedule that lasts for 4 weeks. UCMS-induced modifications in mice were assessed using immobility time in the Tail Suspension Test (TST). Normal control mice were isolated in individual cages and undisturbed except for necessary housekeeping procedures.

Drug administration: Amitriptyline (Sigma, Milan, Italy) was dissolved in isotonic (NaCl 0.9%) saline solution immediately before use and injected, at doses ranging between 1-10 mg/kg i.p, 30 min before the tail suspension test in the acute response. For the chronic treatment amitriptyline was administered daily at the dose of 10 mg/kg i.p., for 2 weeks, since the day 14 after the beginning of the UCMS protocol.

Preparation of whole cell lysates fraction

The hippocampus samples were homogenized in an homogenization buffer containing 25 mMTris-HCl pH=7.5, 25 mMNaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mMNaPP, 4 mM PNFF, 1 mM Na3VO4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9,000 x g for 15 min at 4°C; the low speed pellet was discarded. Protein concentration was quantified using the BiCinchoninc Acid (BCA; Sigma-Aldrich, Italy) assay.

Western blot analysis: Membrane homogenates (50 μg) were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes (120 min at 100 V) using standard procedures. Membranes were blocked in PBST (PBS containing 0.1% Tween) containing 5% non fat dry milk for 120 min. Following washes, blots were incubated overnight at 4°C with specific antibody against GAP43 (1:1000) (Santa Cruz Biotechnology Inc, CA, and USA). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antisera (1:5000) and left for 1 h at room temperature. Blots were then extensively washed and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy) and signal intensity (pixels/mm2) quantified (ImageJ, NIH). Exposition and developing time used was standardized for all the blots. ß-actin (Santa Cruz Biotechnology, CA, USA) was used as loading control. Measurements in control samples were assigned a relative value of 100%. For each sample, the signal intensity was normalized to that of ß-actin.

Immunofluorescence

Mice were perfused through the left cardiac ventricle with 10 ml of a cold fixative (4% paraformaldehyde in 100 mM phosphate buffer). After perfusion, the brain tissues were quickly removed, post fixed for 18 h with the same fixative at 4°C, and transferred to 10%, then 20%, and then 30% sucrose solution. After pre-incubation in 5 mg/ml BSA/0.3% Triton-X-100/PBS, sections were incubated overnight at 4°C with primary antibody GAP43 (1:100 SantaCruz Biotechnology Inc, CA, USA). After rinsing in PBS containing 0.01% Triton-X-100, sections were incubated in secondary antibodies labelled with Alexa Fluor (1:400; Invitrogen) at room temperature for 2 h. Sections were cover slipped using Vector shield mounting medium (Vector Laboratories, Burlingame, CA). A Leica DF 350 FX microscope with appropriate excitation and emission filters for each fluorophore was used to acquire representative images. Images were acquired with x10 objective using a digital camera.GAP43 positive structures were quantified as relative area (immunoreactive area/total area of the field of view).

Data analysis

Behavioural experiments: Immobility time (s) is the mean value (±SD) of five to six animals per group. Analysis of variance followed by Bonferroni/Dunn post hoc test was used for statistical analysis.

Western blotting: The mean of band intensities obtained from 6 to 8 independent experiments are expressed as the mean ± s.e.m. and the differences between groups determined by one-way ANOVA followed by Tukey post hoc test, with significance accepted at $p < 0.05$.

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Immunofluorescence: Immunoreactive areas are mean values (±SD) of three to four separate experiments. Individual experiments consisted of three to five tissue sections of each of the five to six animals per group. In each section, immunoreactive area was measured in three fields of the CA3 of hippocampus. Differences among mean immunoreactive areas or mean relative areas were statistically analyzed by one or two-way ANOVA followed by Bonferroni test.

Results

Effect of amitriptyline on the mouse Tail Suspension Test (TST)

The i.p. administration of amitriptyline dose-dependently decreased the immobility time values recorded in the TST in comparison with saline-treated control mice. Amitriptyline, at 1 mg/kg was devoid of any effect. While not significant, at 5 mg/kg a trend to decreased immobility time was observed, whereas at the dose of 10 mg/kg amitriptyline induced a statistically significant antidepressant effect (P<0.001; Figure 1). The dose of 10 mg/kg was, then, used throughout the experimental paradigms.

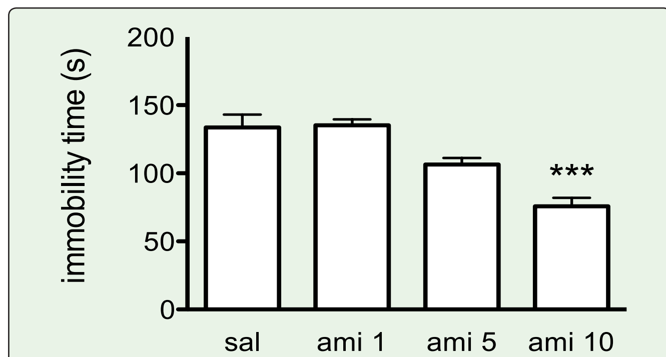

Figure 1: Effects of amitriptyline (1, 5 or 10 mg/kg) on the immobility time (s) in the mouse tail suspension test. Vertical lines represent S.E.M. ***p<0.001 compared to saline-treated mice. Sal: saline; Ami: amitriptyline.

Increased of GAP43 by acute amitriptyline administration

Hippocampus of mice was examined for protein expression levels of GAP43.In the total cell lysates protein preparation, GAP43 was unmodified in the hippocampus 6 and 24 hours after acute TST, while amitriptyline administration significantly increased GAP43 levels, in comparison with control saline-treated mice (P<0.01; Figure 2).

Administration of amitriptyline prevents the development of depressive-like behavior in UCMS mice

UCMS has been proposed as a classical chronic stress model of depression and anxiety [20]. Development of depressive-like behavior was established after 4 weeks of mild stress treatment by detection of the immobility time in the TST. The effect of chronic treatment with amitriptyline on behavioral function was then examined. After a 4-week period of UCMS paradigm application, a significant reduction of the immobility time emerged in the UCMS exposed animals in comparison with unstressed control mice (P<0.01). A significant difference between the amitriptyline treated group and the UCMS-exposed saline-treated control group in the immobility time of the animals was observed. After 2 weeks of drug treatment at the active dose of 10 mg/kg, the immobility time of the animals significantly reduced in comparison with saline treated UCMS mice, returning to control values measured in animals not exposed to chronic stress (P<0.01; Figure 3).

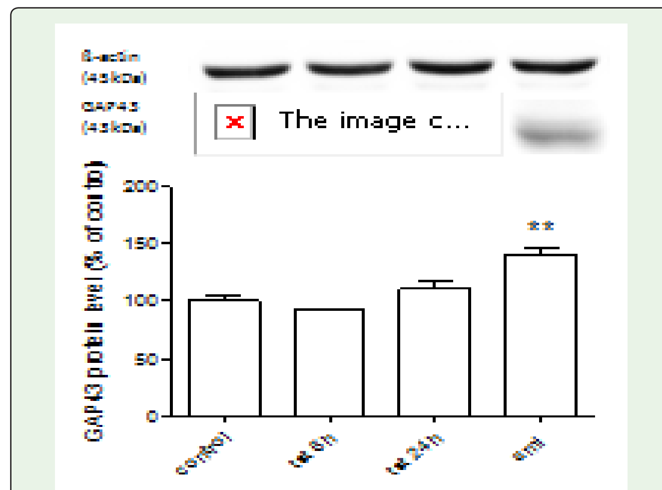

Figure 2: Expression of GAP43 in the hippocampus of mice 6 and 24 hours after exposition to an acute session of Tail Suspension Test (TST). Acute administration of amitriptyline (ami, 10 mg/ kg i.p.) increased GAP43 levels. The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Vertical lines represent S.E.M. **p<0.01 compared to vehicle-treated mice.

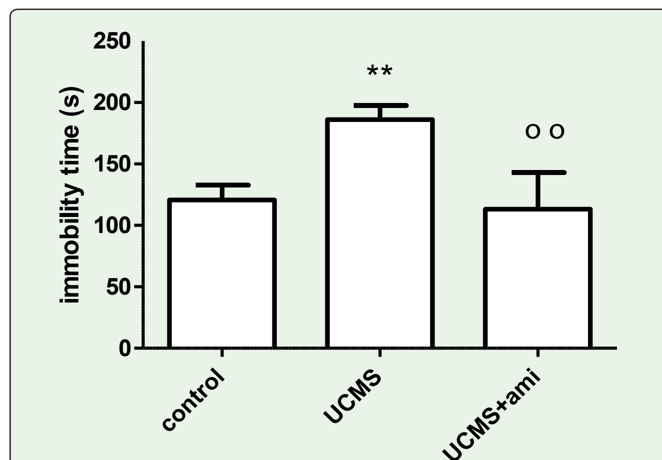

Figure 3: Effects of chronic amitriptyline treatment on the immobility time in the mouse tail suspension test in UCMS exposed mice. UCMS reduced immobility time while amitriptyline treatment increased the immobility time in UCMS-exposed mice. Vertical lines represent S.E.M. **p<0.01, **p<0.01 compared to vehicle-treated mice.

Treatment with amitriptyline reversed the synaptic alteration induced by exposure to UCMS paradigm

Persistence of depressive behavior in UCMS animals for 4 weeks was accompanied by diminished GAP43 positive neuronal structures in the CA3 region of hippocampus (Figures 4A-4C). Quantitative analysis of the immunolabeled structures showed a reduction for GAP43 respect to control group (P<0.05). Full reversion of GAP43 reduction by chronic administration of amitriptyline was found at day 28, as illustrated by immunofluorescence images. The effect of amitriptyline on this synaptic protein was confirmed by quantitative analysis of the immunolabeled area (P<0.05; Figure 4D).

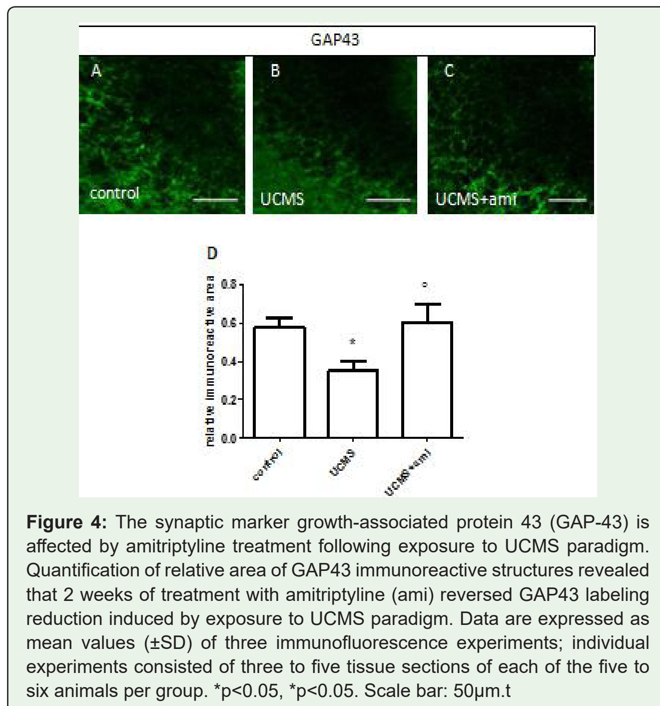

Figure 4: The synaptic marker growth-associated protein 43 (GAP-43) is affected by amitriptyline treatment following exposure to UCMS paradigm. Quantification of relative area of GAP43 immunoreactive structures revealed that 2 weeks of treatment with amitriptyline (ami) reversed GAP43 labeling reduction induced by exposure to UCMS paradigm. Data are expressed as mean values (±SD) of three immunofluorescence experiments; individual experiments consisted of three to five tissue sections of each of the five to six animals per group. *p<0.05, *p<0.05. Scale bar: 50μm.t

Discussion

In the present work, we show that acute and chronic treatment with amitriptyline alters the expression of the synaptic protein GAP43, in the hippocampus, one of the major components of the limbic system that is implicated in the pathophysiology and treatment of mood disorders [21]. Mice pretreated with the antidepressant drug were exposed to the Tail Suspension Test (TST), a widely used and highly predictive paradigm to detect antidepressant-like activities sensitive to acute drug administration [22]. The antidepressant-like effect of amitriptyline was dose-dependent and our results indicated that the maximum antidepressant-like activity is obtained when amitriptyline was given at the dose of 10 mg/kg. Several studies involving patients with major depression have described a reduction of hippocampal volume [23] and, in addition, some preclinical studies have reported that stress exposure is associated to alterations in axonal components and, down regulation of neurogenesis in the sub granular layer of the adult dentate gyrus [24]. It is known that chronic stress induces structural remodeling of excitatory axo-spinous synaptic connectivity and differentially regulates synaptic protein expression in the hippocampus [25]. Regarding the antidepressant action on these plastic phenomena, a well-established effect is to stimulate the synthesis of neurotrophic factors [26,27] and to promote neurogenesis in the hippocampus [28]. On the contrary, the effect of antidepressant treatment on synaptic plasticity remains largely unknown. In our study we observed that an acute 6-min session of TST was unable to alter the levels of GAP43 in the hippocampus neither 6 nor 24 hours after the behavioral test. Despite the lack of influence by the behavioural despair paradigm on the levels of the synaptic marker an increased GAP43 protein expression was observed after acute amitriptyline administration. UCMS exhibits high predictive, face and construct validity as an animal model of depression [29]. Consistent with previous studies [30], we have shown that chronic amitriptyline treatment prevented the UCMS-induced increase in the immobility time in the TST. In addition, when studying the synaptic protein GAP43 in the CA3 region of the hippocampus, we found that the UCMS paradigm induces a large decrease of the synaptic marker. These results are in line with those reported by Thome et al. [31] and Kuroda and McEwen [32] showing, diminished GAP-43 mRNA levels in stressed rats and support the hypothesis that brain plasticity might be disturbed in experimental models of depression [33]. Here, we demonstrated that a behaviorally treatment with the antidepressant amitriptyline fully reverses the reduction of synaptic protein GAP43 induced in the CA3 by exposure to the UCMS paradigm. The increase of GAP43 observed after amitriptyline administration could contribute to the final effect on synapse remodeling since this protein has been described to play important roles in the regulation of cytoskeletal organization in the nerve ending of cultured cells, as well as in the formation of new synapses and in the enhanced axonal sprouting observed after brain injury in adult mice. All these results are in accordance with those reported by Reines et al. 2008, in which chronic treatment with fluoxetine, an antidepressant belonging to the SSRI group, reverses the reduction of synaptic proteins by exposure to the LH paradigm. Our results demonstrate that synaptic protein are increased by different groups of antidepressants regardless their cellular mechanism of action and indicate that synaptic plasticity might represent a common pharmacological target strongly involved in the behavioral efficacy of antidepressants. To summarize, the results presented in this paper suggest that mechanisms involved in the stabilization of amitriptyline-induced synaptic changes might become interesting targets to be further investigated with regard to the pharmacological treatment of depression.

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