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Plasticity based strategies for the treatment of depression

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Thèse de Doctorat de l'Université Paris Descartes – Paris V
Ecole Doctorale Médicament « de la molécule à la clinique »

Plasticity Based Strategies for the Treatment of Depression

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The work presented in this thesis is relevant to the following patent applications:

A European Patent Application n° EP 05290363605430 was filed by the UPMC and the CNRS on 17th February 2005, entitled « Intracellular inhibiting peptides » and naming Jocelyne CABOCHE and Peter VANHOUTTE as inventors. Regarding this invention, a PCT Application n° PCT EP 2006002068 all claiming priority of the European Patent Application has been filed on February 16th, 2006 and has been extended in USA, Japan and Canada.

These peptides provided by Drs Caboche and Vanhoutte were used in this thesis in a collaborative study.

A European Patent Application n° EP 08305636 was filed by the UPMC and the CNRS on 3rd October 2008, on “Peptide and non peptide inhibitors of Elk-1 in mood disorders”, naming Jocelyne CABOCHE, Peter VANHOUTTE, Bruno GIROS and Eleni TZAVARA as inventors. This application was PCT extended on October 2009 (PCT EP 2006002068).

The present thesis contains unpublished experimental data in direct relevance to the above patent.

Plasticity based targets for the treatment of depression

Abstract

Major depression is a devastating disease that affects up to 20% of world population and is classified today as a leading cause of disability-adjusted life years. Since late 50s with the serendipitous discovery of the first antidepressant agents, pathophysiology and therapeutics of depression are governed by the monoaminergic hypothesis. Monoaminergic-based treatment, although still in use today, was proven inefficient to treat a significant proportion of cases and presents a delayed onset of action.

Recent research has unveiled an array of new mechanisms through which antidepressant medication helps restore neuronal plasticity and neurotransmission that is disrupted in mood disorders and in animal models of depression. Glutamatergic transmission, in particular AMPA receptor, and signal transduction cascades have been implicated both in antidepressant action and the pathophysiology of depression, as here and now regulators that mediate persistent changes.

In this study, AMPA receptors positive modulators demonstrated antidepressant-like effects in a chronic model of depression and preliminary data suggest a faster onset of action than conventional antidepressants. The ERK/MAPK signaling pathway was also studied as a major integrator of synaptic plasticity modifications that links extracellular signals to gene expression regulation via its downstream molecular partners. We used a new class of inhibitors of the ERK pathway, whose design was based on the particular property of ERK to bind to its downstream targets via specific docking domains. A considerable amount of data provided evidence for an antidepressant action of selective inhibition of the ERK/Elk1 signaling complex in multiple animal models of depression.

Overall, the findings of this work reveal novel, promising targets for the treatment of depression.

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INTRODUCTION

Depressive disorders: diagnosis and therapeutics

Depressive disorders belong to the category of mood disorders defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM IV-TR) classification system as a group of diagnoses where disturbance in a person's mood is the primary symptom. The predominant feature of mood disorders is the experience of extreme feelings: inappropriate, exaggerated dysphoric and/or euphoric states that create significant distress or impairment in functioning. The term mood disorders encompasses a variety of affective problems that could be divided in bipolar disorders and depressive disorders.

Bipolar disorder, formerly known as manic depression, is characterized by intermittent episodes of mania or hypomania, usually interlaced with depressive episodes. Mania (or manic episode) is defined as a distinct period of abnormally and persistently elevated, expansive, or irritable mood lasting at least one week and consisting of three or more of the following: inflated self-esteem or grandiosity, decreased need for sleep, increased speed and/or volume of speech, disconnected racing thoughts or thoughts that seem to jump from topic to topic, distractibility, increase in goal-directed activity (either socially, professionally or sexually) or psychomotor agitation, excessive involvement in pleasurable activities that have a high potential for negative consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments). Hypomania refers to a mild form of mania. Bipolar disorders include bipolar I (one or more manic episodes or mixed episodes with or without major depressive episodes), bipolar II (recurrent intermittent hypomanic and depressive episodes), cyclothymia (recurrent hypomanic and dysthymic episodes) and bipolar disorder not otherwise specified (BD-NOS) sometimes called "sub-threshold" bipolar.

Depressive disorders include major depressive disorder (MDD), commonly called major depression, unipolar depression or clinical depression (one or more depressive episodes), dysthymia, i.e. low mood almost daily over a span of at least two years with less severe symptoms than major depression, and depressive disorder not otherwise specified (DD-

NOS). People with dysthymia are vulnerable to secondary episodes of major depression (sometimes referred as double depression).

Major depression is a common, severe, recurrent, debilitating and potentially life-threatening illness that affects up to 20% of world population. Among depressed patients only 50% present a complete remission, while 20-30% does not respond to a treatment at all and 15% commit suicide. A World Health Organization's (WHO) study classified depression as the leading cause of disability as measured by YLDs (Years Lived with Disability) and the 4th leading contributor to the global burden of disease in 2000. By the year 2020, depression is projected to reach 2nd place of the ranking of DALYs (Disability-Adjusted Life Years) calculated for all ages, both sexes. Today, depression is already the 2nd cause of DALYs in the age category 15-44 years for both sexes combined (World Health Organization, Geneva, 2001).

I. Clinical characteristics

a) Depressive Episode

At present, two established classification systems exist for the diagnosis of depressive disorders: the International Classification of Diseases (ICD-10) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). The ICD-10 is the classification used since 1994 by the World Health Organization (WHO) that has become the international standard diagnostic classification for most general epidemiological purposes. The DSM-IV-TR is the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders, with text revision added in 2000. The manual, published by the American Psychiatric Association, presents the criteria most widely used in the United States to classify mental disorders. The ICD-10 and DSM-IV-TR criteria for diagnosis of a depressive episode are similar but there are also some differences.

ICD-10

According to World Health Organization's ICD-10, a depressive episode is characterized by three core symptoms present for at least two weeks: depressed mood, anhedonia and reduced energy. The presence of somatic symptoms (weight gain/loss,

insomnia/hypersomnia, psychomotor retardation/agitation, feelings of guilt, cognitive dysfunctions and suicidal thoughts) further classifies an episode as:

- ✘ mild without somatic symptoms (2 core and 2 somatic symptoms),
- ✘ mild with somatic symptoms (2 core and 4 somatic symptoms),
- ✘ moderate without somatic symptoms (2 core and 3-4 somatic symptoms),
- ✘ moderate with somatic symptoms (2 core and >4 somatic symptoms),
- ✘ severe without psychotic symptoms (3 core and >4 somatic symptoms of severe intensity),
- ✘ severe with psychotic symptoms (3 core and >4 somatic symptoms of severe intensity, delusions, hallucinations, severe psychomotor retardation).

DSM IV-TR

According to American Psychiatric Association's DSM IV-TR, a major depressive episode is characterized by a depressive mood and/or a loss of interest or pleasure (anhedonia) as core symptoms for a period of time longer than two weeks. Among the following symptoms, at least five must be present for a diagnosis of major depression and to further classify both the episode itself and the course of the disorder:

1. Depressed mood. For children and adolescents, this may be irritable mood.
2. Anhedonia (a significantly reduced level of interest or pleasure in most or all activities).
3. A considerable loss or gain of weight. This may also be an increase or decrease in appetite.
4. Difficulty falling or staying asleep (insomnia) or sleeping more than usual (hypersomnia).
5. Psychomotor retardation or agitation.
6. Feeling fatigued, or diminished energy.
7. Thoughts of worthlessness or extreme guilt.
8. Diminished ability to think, concentrate, or make decisions.
9. Frequent thoughts of death or suicide (with or without a specific plan), or attempt of suicide.

b) Depression: a group of illnesses

If a depressive episode is recurrent, it is diagnosed as recurrent depressive disorder. The DSM-IV-TR recognizes six further subtypes of MDD, called specifiers, in addition to noting the length, severity and presence of psychotic features.

Subtypes	Symptoms	Prevalence
Melancholic depression	Pronounced anhedonia and depressed mood, excessive guilt, psychomotor retardation, weight loss, a worsening of symptoms in the morning hours, insomnia with early morning awakenings.	10% of depressed patients
Atypical depression	Mood reactivity (paradoxical anhedonia) and positivity, hyperphagia, hypersomnia, leaden paralysis, significant social impairment as a consequence of hypersensitivity to perceived interpersonal rejection.	
Psychotic depression	Major depressive episode (at least six criteria present) with delusions and hallucinations.	
Catatonic depression	Motionless (catatonic stupor) or peculiar and violent movements (catatonic excitement), mute or mimic.	Rare and severe
Postpartum depression	Sustained and sometimes disabling depression experienced by women after giving birth.	10-15% among new mothers
Seasonal affective disorder	Depressive episodes come on only in the autumn or winter and resolve in spring.	

The clinical picture of depression is complex. As evidenced from the above, symptomatology varies a lot among patients and is often associated to other psychiatric conditions or diseases (co-morbidity, see below). Many individuals have a single episode of depression in their lifetime, but more commonly depression occurs in repeated episodes and may become chronic. Like the outcome, etiology of depression is also considered of high heterogeneity. A genetic component has been strongly related to the disease with heritability ranging ~40%. Family, twin and adoption studies have suggested significant genetic influences for major depression, a predisposition for the disease that can develop under certain environmental circumstances. Environmental influence is also very important, with several life stressors impact on the development of depression. The age of onset and the course of illness (subtype, recurrent or not) are unpredictable and of a different etiopathophysiology. Patients' response to current treatments reflects the same heterogeneity and can vary from

none to complete remission with no specific reasons known today. No manual exists for antidepressant prescription, different classes of antidepressants are administered and the follow up of the treatment depends on the patients' response (tolerance, side effects, mood amelioration).

c) Comorbidity

In the past decade, large surveys conducted both in the community and in the primary care setting have established one simple but clinically important fact: depression that is not complicated by comorbidity is the exception, not the rule. Depression often co-occurs with other mental disorders as well as with medical/somatic diseases.

Anxiety disorders

Anxiety disorders are a cluster of several different forms of pathological fear and anxiety and are generally divided in generalized anxiety, phobic (agoraphobia, social phobia) and panic disorders. They also include obsessive-compulsive disorder and post-traumatic stress disorder. The experience of extreme anxiety may serve as a compounding stressor that facilitates further decompensation, leading (especially in patients with a genetic/familial diathesis) to major depression. Among patients in the community who meet criteria for major depression, approximately 50% are also suffering from an anxiety disorder (Hirschfeld R. 2001). Comorbid major depression and anxiety is especially common in the elderly (Devane et al. 2005) and typically present as either a diagnosis of both anxiety and depression meeting criteria or not (mild symptoms), either as one of the two disorders diagnosed (meet criteria) with subsyndromal levels of the symptomatology of the other.

Substance use disorders

Depression and substance use disorders coexistence raises the major question to whether one disorder is the cause of the other or do they share the same etiopathophysiology. Prevalence studies report rates of comorbidity of major depression with nicotine, alcohol, and illicit drug abuse ranging from 32% to 54% (Kessler et al. 1994, Rao et al. 1999, Glassman et al. 1990). Individuals with major depression are more likely to develop substance use disorders, and individuals with substance use disorders are at greater risk for the

development of major depression, compared to the general population (Brook et al. 2002). Depressive symptoms are commonly reported during acute and chronic withdrawal from drugs of abuse. Irritability, sleep difficulties, anxiety, and trouble with attention/concentration are common during withdrawal states. Neurobiological similarities, like corticotrophin-releasing factor (CRF) and hypothalamo-pituitary-adrenocortical (HPA) axis abnormalities and alterations in catecholamine, serotonin, GABA, and glutamate systems, between major depression and substance use disorders likely contribute to both symptom overlap and high rates of comorbidity (Markou et al. 1998). A growing amount of evidence indicates that the neurobiological alterations (depressive-like states) associated with acute withdrawal last for varying time periods and contribute to drug craving and relapse in substance use disorders (Sinha R. 2001).

Eating disorders and obesity

The appetite and metabolic abnormalities associated with depression and depression-related entities range from severe hypophagia and anorexia to binge eating and obesity. Major depression is the most commonly diagnosed comorbid disorder in both anorexia and bulimia nervosa (O'Brien and Vincent, 2003). A putative causal role for the anorexia/depression association can be starvation. Starvation and/or protein malnutrition can lead to elevations in corticotropin-releasing hormone (CRH), which may contribute to "melancholic" depression (Altemus & Gold, 1992). Furthermore, calorie-restricted dieting produces changes (lowering) in 5-HT functioning, which may also be a contributing factor in depressive symptomatology (Cowen et al. 1992).

A recent meta-analysis study found that obesity increases the risk of onset of clinically diagnosed depression among adults (55% increased risk) and that depressed people have a 58% increased risk to become obese (Luppino et al. 2010). Mechanisms underlying comorbid obesity and depression can be psychological and biological. Obesity increases body dissatisfaction and decreases self-esteem, both risk factors for depression. Disturbed eating patterns and eating disorders, as well as experiencing physical pain as a direct consequence of obesity, are also known to increase the risk of depression. A possible pathophysiologic mediator of the correlation between obesity and depression is inflammation, as weight gain has been shown to activate inflammatory pathways and inflammation in turn has been

associated with depression (Shoelson et al. 2007). The hypothalamic-pituitary-adrenal axis (HPA axis) might also play a role, as both diseases involve HPA-axis dysregulation (Walker BR 2001). Through HPA-axis dysregulation, obesity might cause development of depression. Finally, obesity involves increased risks of diabetes mellitus and increased insulin resistance (Lee et al. 2008), which could induce alterations in the brain and increase the risk of depression.

Diabetes and metabolic syndrom

Depression is common in both type 1 and type 2 diabetes (~25% of the diabetic population) and has significant effects on the course and outcome of this medical illness. Depression is associated with decreased metabolic control, poor medication and diet adherence, reduced quality of life, and higher healthcare expenditures. Likewise, poor diabetes control may worsen depression and impair antidepressant treatment response. Conventional antidepressants are effective and good glycemic control may contribute to improvements in mood and perceptions of well-being (Lustman and Clouse, 2005). Physiological functions disrupted in depression that influence normal glucose–insulin homeostasis include (a) dysregulation of HPA axis, (b) abnormal sleep physiology, (c) diminished activity levels, (d) inflammatory cytokine activation, (e) diminished central serotonin function associated with hyperphagia, and (f) central mechanisms mediated by depression-induced disruption in the insulin receptor-rich hippocampus (McIntyre et al. 2006).

Chronic pain

The true prevalence of depression in chronic pain varies with the population sampled (e.g., community, primary care, pain clinics, sports medicine, orthopedic clinics). Bair et al. (2003) performed a Medline database search examining depression or depressive disorders in pain and found that mean prevalence rates of concomitant major depression in pain patients was 52% in pain clinics, 38% in psychiatric clinics, 56% in orthopedic or rheumatology clinics, 85% in dental clinics addressing chronic facial pain, 13% in gynecologic clinics focusing on chronic pelvic pain, 18% in population-based settings, and 27% in primary care clinics.

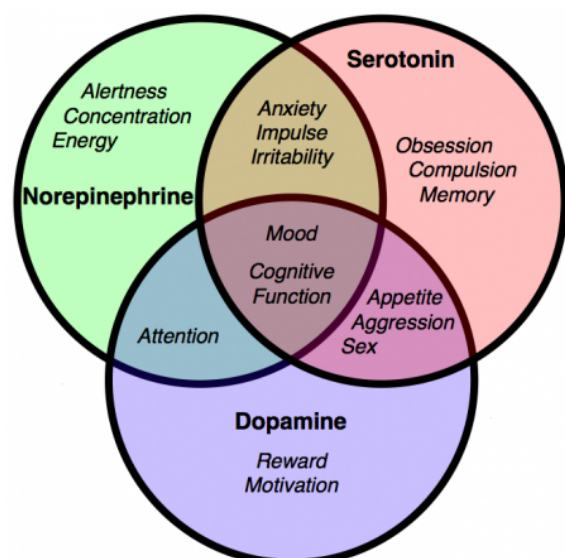
Cardiovascular diseases

Depression is common among patients with heart disease affecting the quality of life and the ability of patients with heart disease to engage in healthy behaviors and to avoid unhealthy ones. In addition, depression is associated with increased morbidity and mortality in those with established cardiovascular disease. Hypotheses regarding the mechanistic link between depression and cardiovascular focus on biological and/or psychosocial correlates of depression and poor cardiac outcome including (a) increased sympathetic nervous system (SNS) or hypothalamic–pituitary–adrenal (HPA) function, (b) SNS-related decreased heart rate variability, (c) dysregulation of inflammatory and immune functioning, (d) increased platelet/endothelial aggregation, (e) poor treatment compliance and/or unhealthy lifestyles, and (f) a cardio-toxic effect of antidepressant medications (Shimbo et al. 2005).

II. Current Antidepressant Treatment

Several treatments for depression are available today; almost all of them target the monoamine system. In this respect, no much progress has been done since middle 50's, when the serendipitous discovery that iproniazid (a drug administered for tuberculosis) improved also the mood of tuberculosis patients, gave rise to the first antidepressant treatment few years later. It was discovered that iproniazid was a monoamine oxidase (MAO) inhibitor, the enzyme that catalyses monoamines, MAO inhibition thus leading to increased amounts of monoamines (Delay et al, 1952). Some years later the antidepressant effects of imipramine, a weak anti-histaminic and mild anticholinergic drug, were also discovered accidentally in a search for a new antipsychotic (Kuhn, 1958). Imipramine was the first tricyclic antidepressant to be developed, acting by blocking serotonin and norepinephrin reuptake transporters (SERT and NET respectively) and antagonising histaminic and muscarinic receptors. Inhibition of SERT and NET leads to an increase in the monoamine amount in the synapse and is considered to be the base of the antidepressant effect. Antidepressant action was then related to an enhanced monoaminergic signaling.

These findings led to the hypothesis that depression results from reduced availability of monoamines, the so called monoaminergic hypothesis of depression. Monoaminergic systems (serotonin, 5-HT, norepinephrin, NE and dopamine, DA) differentially regulate aspects of emotionality and cognitive function in healthy states as it is schematically depicted in the figure below. In the same way, monoaminergic deficits are believed to contribute to different dimensions of the pathophysiology of depression underlying the heterogeneity of symptoms observed in patients.



Subsequently research on antidepressants flourished focusing on the development of compounds that increase specifically serotonin or norepinephrin leading to the selective serotonin or norepinephrin reuptake inhibitors (SSRIs and NRIs respectively) or in a combination of the two (SNRIs) (see table). Most of these

medicaments are still used today with success and constitute the major antidepressant treatment.

Antidepressant	Mode of Action	Substance (<i>Commercial Name</i>)	Side effects
Monoamine oxidase inhibitors (MAOIs)	Inhibit the action of monoamine oxidase (MAO)	Moclobemide (<i>Aurorix, Manerix, Moclodura</i>) Phenelzine (<i>Nardil</i>) Selegiline (<i>Selegiline, Eldepryl, Emsam</i>) Iproniazid (<i>Marsilid, Iprozid, Ipronid, Rivivol, Propilniazida</i>) Linezolid (<i>Zyvox, Zyvoxid</i>)	Hypertensive crisis if combined with food containing high levels of tyramine. Serotonin syndrome if combined with SSRIs.
Tricyclic antidepressants (TCAs)	Inhibitors of SERT and NET, affinity for muscarinic and H1 histaminic receptors	desipramine (<i>Norpramin, Pertofrane</i>) imipramine (& dibenzepin) (<i>Tofranil, Janimine</i>) amitriptyline (& butriptyline) (<i>Elavil, Endep, Tryptanol, Trepiline, Amyzol</i>)	Dry mouth, blurred vision, drowsiness, vertigo, sexual dysfunction, skin rash, weight loss/gain.
Selective serotonin reuptake inhibitors (SSRIs)	Inhibitors of SERT	fluoxetine (<i>Prozac</i>) paroxetine (<i>Paxil, Seroxat</i>) escitalopram (<i>Lexapro, Seroplex</i>) citalopram (<i>Celexa, Seropram</i>) sertraline (<i>Zoloft</i>)	Nausea, diarrhea, headaches, diminished libido, anorgasmia, erectile dysfunction.
Serotonin-norepinephrine reuptake inhibitors (SNRIs)	Inhibitors of SERT and NET	venlafaxine (<i>Effexor</i>) duloxetine (<i>Cymbalta</i>)	Nausea, dry mouth, headaches, vertigo.
Norepinephrine (noradrenaline) reuptake inhibitors (NRIs)	Inhibitors of NET	reboxetine (<i>Edronax</i>)	Dry mouth, constipation, headaches, drowsiness, vertigo, increased sweating, insomnia.

Another class of antidepressants, termed atypical, that also target the monoaminergic system, but in an indirect way was developed later. The class of atypical antidepressants consists of compounds that act indirectly on the recycling of monoamines modulating presynaptic receptors and synaptic monoaminergic content. Mianserin was the first to be discovered acting by blocking the presynaptic α_2 -adrenergic receptors (Leonard, 1978). The lack of specificity (α_1 and H1 antagonism) causes side effects like drowsiness and orthostatic hypotension. A stronger α_2 antagonist, mirtazapine, was developed later (Smith et al, 1990) that increases serotonin and norepinephrine release acting on presynaptic receptors. Tianeptine firstly appeared in the 80s with an unknown mode of action that indirectly increases serotonin uptake after acute or chronic administration. Finally, another class of antidepressants mostly used for sleep issues because of their strong sedative and hypnotic effects is the selective antagonist reuptake inhibitors (SARIs). This compounds act as 5-HT_{2A}

and α -1 adrenergic antagonists and inhibitors of SERT, NET and DAT with low affinity. Among atypical antidepressants, one can count bupropion, the only antidepressant targeting also the dopamine transporter and had firstly been used to treat bipolar disorder (Erfurth et al, 2002).

Atypical Antidepressants			
Noradrenergic and specific serotonergic antidepressants (NASSAs) also known as Tetracyclics (TeCAs)	Antagonists of presynaptic α -2 adrenergic receptors, 5-HT _{2A} , 5-HT _{2C} , 5-HT ₃ serotonergic receptors and H1 histaminic receptors	mirtazapine (<i>Norset, Remeron</i>) aptazapine (<i>CGS-7525A</i>) esmirtazapine (<i>ORG-50,081</i>) mianserin (<i>Bolvidon, Norval, Tolvon</i>) setiptiline (<i>Tecipul</i>)	Drowsiness, increased appetite and weight gain.
Norepinephrine-dopamine reuptake inhibitors (NDRIs)	Inhibitors of NET and DAT	bupropion (<i>Wellbutrin, Zyban</i>)	Dry mouth, nausea, insomnia, tremor, increased sweating, tinnitus.
Selective serotonin reuptake enhancer (SSRE)	Increases indirectly the serotonin reuptake	Tianeptine (<i>Stablon, Coaxil, Tatinol</i>)	Dry mouth, constipation, vertigo, drowsiness, orthostatic hypotension.
Selective antagonist reuptake Inhibitors (SARIs)	Antagonists of 5-HT _{2A} and α -1 adrenergic receptors and less potent inhibitors of SERT, NET, DAT	Etoiperidone (<i>Axiomin, Etonin</i>) Lubazodone (<i>YM-992, YM-35,995</i>) Nefazodone (<i>Serzone, Nefadar</i>) Trazodone (<i>Desyrel</i>)	Heaviness in head, nausea, vomiting, stomach pain, diarrhea/constipation, tiredness, nervousness, decreased concentration, confusion, nightmares, ringing in ears.

Two other classes of drugs used in severe cases are mood stabilizers and antipsychotics. Prescribed mostly for bipolar disorders (sudden changes of mood between manic and depressive states) and schizophrenia, they are also used to treat depression with hypomanic/manic or psychotic elements.

Mood Stabilizers	
lithium carbonate	Nausea, vomiting, diarrhea, ataxia
valproic Acid (VPA) (<i>Depakene</i>)	Indigestion and/or weight gain
lamotrigine (<i>Lamictal</i>)	Severe skin pathologies (Stevens-Johnson syndrome)

Atypical Antipsychotics	
risperidone (<i>Risperdal</i>)	Weight gain, extrapyramidal effects
olanzapine (<i>Zyprexa</i>)	Weight gain, sexual dysfunction, extrapyramidal effects, hyperlipidemia, increased risk for diabetes mellitus
clozapine (<i>Clozaril</i>)	Agranulocytosis, weight gain, sexual dysfunction, hyperlipidemia, increased risk for diabetes mellitus
quetiapine (<i>Seroquel</i>)	Sexual dysfunction, hyperlipidemia
ziprasidone (<i>Geodon</i>)	Sexual dysfunction, may increase ventricular arrhythmia
aripiprazole (<i>Abilify</i>)	Sexual dysfunction

Apart from pharmaceutical compounds, effective non-medication treatments for depression are also used today. Electroconvulsive therapy (ECT) dates back in the 1940s and was a first-line treatment for major depressive episode before the introduction of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA) (Sakel 1938, Fink et al. 1979). Today, ECT is still a relevant psychiatric treatment and is most often used for severe depression, usually only when other treatments have failed. Similar to ECT, transcranial magnetic stimulation (TMS) is a technique developed in the middle 90s, and has also been used to treat depression (Kirkcaldie et al. 1997). Clinical remission of depressive symptomatology has been observed with use of TMS but methodological limitations remain (Gershon et al. 2003).

Non-medication	
Electroconvulsive therapy (ECT)	General brain stimulation
Transcranial Magnetic Stimulation (TMS)	A magnetic field affects the brain by inducing electric currents and neuronal depolarization
Deep brain stimulation	Stimulation of subgenual cingulate gyrus, anterior capsule, inferior thalamic peduncle or nucleus accumbens has shown to have antidepressant effects in treatment-resistant depression
Phototherapy	Exposure to bright light has been found to be an effective means of treating seasonal affective disorder and an alternative treatment for antenatal depression
Psychotherapies	Learning new ways of coping with problems

As we see, there are a lot of compounds available to treat depression. However, all patients do not respond to pharmaceutical treatment in the same way (partially or not at all) and many of them experience heavy side effects, that impede treatment follow up and successful remission. Side effects comprise nausea, drowsiness, headaches, vertigo, weight loss/gain, diarrhea, sexual dysfunction, etc (see table), factors that cause malaise and suffering and can contribute to relapse. Moreover, all known antidepressant treatments have a delayed onset of action; mood improvement appears after several weeks of treatment, increasing suicide risk during that period. Therefore it is important to develop new therapeutic targets, based on a better understanding of the pathophysiology of depression. For this, several animal models are used today to study different clusters of symptoms similar or equivalent to human symptomatology.

Animal models of depression

Modeling depression in animals has proved a rather difficult task as mechanisms underlying this disease are not yet completely elucidated. Moreover, the heterogeneity of etiopathophysiology limits the design, application and relative strengths of depression models. The most prominent symptoms of depression are subjective feelings, which cannot be assessed in animals. Numerous attempts have been made to create animal models, or at least models of the symptoms of depression. Quantifiable correlates of human symptoms in experimental animals are necessary to model depressive states. Examples of measures that can be assessed in rodent behavioral models include motor responses to stress, reward-related responding and social interaction, with the rationale that they reflect levels of helplessness or despair, anhedonia, and social withdrawal, respectively, all relevant to human depression.

Models of depression are generally evaluated for their reliability or reproducibility, the extent to which they model the true disease process or its etiology in humans (construct or etiologic validity), their ability to reproduce in animals symptomatology of the illness in humans (face validity) and their ability to accurately predict outcome of treatments in humans (predictive or pharmacological validity) (McKinney WT. 2001, Willner P. 1984, 1997). A potential fourth criterion that has received considerably less attention is *pathological validity*, whereby depression-related physiological, molecular and cellular abnormalities in animals are validated by demonstrating identical changes in postmortem brain samples from depressed humans (Krishnan and Nestler, 2010).

Although the models attempt to produce specific behavioral or physiological features of depression, the features in the animal models likely come about through processes that are very different from those operative in human depression. Therefore, results need to be carefully interpreted for relevance that may be more specific to the model than for human depression.

I. Stress-based models

Exposure to stress is a main environmental risk factor associated with the occurrence of depression (Keller et al. 2007, Kendler et al., 1999; Kessler, 1997). Recent work has indicated that stress exposure may interact with genetic risk factors to increase susceptibility to depression (Caspi et al. 2003, Kaufman et al. 2006). For these reasons, many animal models have attempted to reproduce some core components of major depressive disorder through exposure to stress. Experimentally, the outcome of stress exposure is determined by several variables: the nature of the stress (physical vs. psychological), the severity of the stress, exposure parameters and the degree of control and predictability the animal has over stress exposure. Different neural circuits are activated by different types of stressors (Anisman and Matheson 2005, Herman and Cullinan 1997, Willner P. 1987).

These variables give rise to multiple types of animal models through which different components of depression are measured: etiology, symptomatology, genetic predisposition, environmental and/or developmental impact. Antidepressant efficacy and onset of action are also evaluated. Some of these models are presented below.

a) Models of construct or etiologic validity

Early-life stress

Experience of stressful events during early life has long been thought to contribute to the pathophysiology of emotional disorders. Recent research has begun to identify the long-term consequences of childhood trauma in neural function and emotional well-being. A growing body of evidence demonstrates that childhood trauma and neglect can exert a profound and pervasive influence on emotional behavior and risk for depression, anxiety disorders and substance abuse (de Wilde et al. 1992, Dube et al. 2001, Johnson et al. 2002, Kendler et al. 1995). Indeed, early life trauma may not only increase risk for these disorders in adulthood, but it may also precipitate illness onset, increase comorbidity among disorders and alter the efficacy of treatments (Holmes et al. 2005).

The most used paradigm of early-life stress is that of maternal deprivation. Animals are separated from their mother and littermates daily during the postnatal period. Previously

separated animals are then allowed to develop under normal conditions through adulthood, when phenotypic characteristics are evaluated. As adults, they show behavioral abnormalities, like increased anxiety and fear responses, reduced motor activity, reduced social motivation, reduced hedonic responding, sleep and appetite disturbances, and endocrine and neurochemical alterations in stress-relevant systems (Ladd et al. 2000, Levine 1957, Mintz et al. 2005, Plotsky and Meaney 1993, Dauge V 2003, Ruedi-Bettschen et al. 2005, 2006). Stress responsiveness of the hypothalamo-pituitary-adrenocortical (HPA) axis is a consistent finding, but traits in depression tests are more variable and appear to depend on animal strain (Pryce et al. 2005). Many of the behavioral changes in maternal separation models have analogy with symptoms of depression and the neuroendocrine changes are consistent with depression (Heim et al. 2004, Pryce et al. 2001, 2005).

Unpredictable chronic mild stress (UCMS)

The chronic mild stress paradigm is based on the initial discovery by Katz (1982) that exposure to severe predictable stressors leads to an anhedonic phenotype in rats. It was then established by Willner (1984, 1997) in a different form of a chronic mild stress procedure to mimic anhedonia in animals. Anhedonia is one of the core symptoms of depression and repeated uncontrollable stress has been associated to etiopathophysiology and development of the disease. Unpredictable chronic mild stress leads to a widely used paradigm today with strong construct, face and predictive validity. Multiple stressors during a long period of time (>3 weeks) result in a depressive-like state that develops gradually over time and that can be validated by a battery of behavioral tests in the end of the stress procedure. Behavioral and physiologic changes that have analogy with symptoms in humans, like anhedonia, resignation, decreased self-care, weight gain alterations and sleep disturbances, are induced by stress and some of them can be alleviated (completely or partially) by a chronic antidepressant treatment. These and other abnormalities, including increased hypothalamic–pituitary–adrenal (HPA) axis activation and immune system abnormalities, support face validity of this model (Willner P. 2005).

Social stress models

Social stress models utilize a conflict situation that results in one animal becoming or retaining dominant status and another ending up subordinate or “defeated”. A phenotypic trait produced in these models is social avoidance, which can be quantified and is suggested to model social withdrawal in human depression (Berton et al. 2006, Koolhaas et al. 1997, Van Kampen et al. 2002). Social defeat may also induce other behavioral or physiologic changes like increased anxiety and defensive behavior, decreased locomotor activity and sexual behavior, alterations in feeding and body weight, impaired immune function and hyperactivation of HPA axis (Bohus et al. 1993, Koolhaas et al. 1997, Martinez et al. 1998, Meerlo et al. 1996, Buwalda et al. 1999). Interestingly, anticipation of social defeat has also been shown to induce a marked activation of cortical cholecystokinergic neurons associated with anxiety-related behaviors in rats (Becker et al. 2001). These alterations are long-lasting and can be reversed by chronic but not acute antidepressant treatment (Berton et al. 2006, Huhman KL. 2006, Meerlo et al. 1996, 2002, Von Frijtag et al. 2002). Although, social defeat is considered as a model of high construct, face and predictive validities, some animals show resilience to this type of stress. This can be useful for investigating molecular substrates underlying vulnerability or resilience to stress (Krishnan et al. 2007).

b) Tests and models of high predictive validity

The forced swimming and tail suspension tests (FST, TST)

The forced swimming and tail suspension tests (FST, TST) are the most widely used models for screening antidepressant action. Even if these approaches have been rightly criticized for involving acute stress and acute antidepressant responses, they have permitted the rapid behavioral screening of novel chemical antidepressants and the phenotyping of genetically altered mutant mice. In both tests, animals are subjected to an acute stress from which they cannot escape (cylinder filled of water in FST, suspended by the tail in TST). A normal animal show an immediate burst of activity, try to escape, and then eventually adopt an “immobile” posture. Immobility time is recorded, reflecting the adoption of a passive response in a stress situation (resignation). All antidepressant agents reduce immobility time in these two tests. Although conceptually similar, the TST and FST do not show identical sensitivities to

pharmacologic agents or to strain differences, suggesting that responding in these tests may be determined by nonidentical substrates (Bai et al. 2001). Different mouse strains respond differently to basal immobility in the TST, indicating that this test is sensitive to genetic influence and is suggested to be related to variations in monoamine levels (Ripoll et al. 2003).

Learned helplessness (LH)

The learned helplessness paradigm consists of a stress-exposure period (several sessions) in which animals are exposed to inescapable stress: they are placed in a box where they receive electrical footshocks. In the test session, footshocks are delivered but the animal has the opportunity of actively escaping (active avoidance test). Animals previously exposed to inescapable stress show reduced ability to escape and this is restored by subchronic administration of several antidepressant treatments, like tricyclic antidepressants, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors, and electroconvulsive shock therapy (Martin et al. 1990, Sherman et al. 1982). This model has good predictive validity with few false positives. This model demonstrates the importance of controllability of the stressor as a key psychological component in inducing the behavioral deficit (Anisman and Matheson 2005). Animals that are helpless in this model also show several features similar to human depression, including decreased motor activity, weight loss, altered sleep, decreased motivation, and increases in stress hormones (Maier, 1984). LH models can identify vulnerable and resistant subgroups and can be a useful strategy for investigating mechanisms underlying differential susceptibility. Limitations of LH models include low reproducibility and the relevance of the induction methods has been questioned (Nestler et al. 2002).

Novelty Induced Hypophagia (NIH)

Novelty induced hypophagia (also called hyponeophagia or novelty-suppressed feeding) is an anxiety based model that compares feeding behavior in an anxiogenic versus a non-anxiogenic environment. Latency to consume food or drink is measured in their home cage (non-anxiogenic) and in a novel cage (anxiogenic). In the novel cage, the animal experiences conflict between the desire to approach and feed or drink, and the anxiety-induced avoidance of the novel environment. Before the test animals are either food-deprived or

habituated to consume a palatable food or drink. Latency to consume and total consumption is measured in the home cage. The next day (test session), animals are placed in a novel empty cage, brightly lighted (mild stressor) where they can find food or drink and latency to consume is recorded. Novelty increases latency to consumption showing anxiety taking over desire. Administration of anxiolytic agents (i.e. benzodiazepines) acutely or chronically reduce latency to consume, while antidepressant treatments reduce latency only when administered chronically. Hyponeophagia paradigms provide strong predictive validity for the anxiolytic effects of drug treatments and their onset of action (Dulawa and Hen, 2005).

c) Tests with high face validity

Face validity reflects depressive phenotype symptoms or behaviors equivalent to humans that can be evaluated in rodent models. Although subjective feelings are impossible to assess in animals, physical alterations (body weight), self-care (grooming) and hedonic sensitivity (anhedonia) can be easily measured.

Fur condition and splash test

Self-care is importantly decreased in depressive patients reflecting a resignation state, a very low self esteem and a major loss of interest. An equivalent hygienic behavior in animals is 'grooming', where animals clean themselves by licking. Tests are conceived to evaluate self-care in rodents (mice) include counting the grooming time (splash test) or quoting the condition of the fur.

Sucrose consumption

The most commonly employed procedure to evaluate stress-induced anhedonia is to quantify sweetened fluids (sucrose or saccharin) consumption. Non-stressed animals (rodents) naturally prefer sweetened solutions in a range of ~80% preference. Effectiveness of a chronic stress paradigm on hedonic sensitivity can be evaluated by testing sucrose preference at the end of the stress procedure. Animals exposed to chronic stress lose this preference reflecting anhedonia in human depression. Anhedonic measures after unpredictable chronic mild stress are sensitive to strain effects for both rats and mice (Nielsen et al. 2000, Pothion et al. 2004).

Stressor	Model	Measurement	Validity	Type
Inescapable acute stress	Tail suspension test (TST)	Resignation reflected by immobility time	predictive	Despair model
Inescapable acute stress	Forced swimming test (FST)	Resignation reflected by immobility time	predictive	Despair model
Inescapable repeated stress	Learned helplessness	Attempts to escape	predictive	Helplessness/hopelessness
Novelty stress	Novelty induced Hypophagia (NIH)	Latency to consume	predictive	Novelty anxiety
Unpredictable, multiple, chronic stressors	Unpredictable chronic mild stress (UCMS)	Anhedonia, physical alterations (weight, fur)	Face and construct	Uncontrollable stress
Chronic social defeat stress	Social defeat	Social behavior: interactions (submissive/dominant)	Face and construct	Social phobia
Early-life stress	Maternal deprivation	Behavioral changes in adulthood	Face and construct	Separation anxiety

II. Newer directions in animal models of depression

Currently, there is a shift away from traditional animal models to a more focused research dealing with an endophenotype-style approach, genetic models and incorporation of new findings from human neuroimaging and genetic studies (Cryan and Slattery, 2007). Stressful life events have been shown to play an important role in the development and manifestation of psychiatric illnesses (Kendler et al. 1999, Charney and Manji 2004, Anisman and Matheson 2005) but genetic influence is also very important and can vary the ability to cope with stress or not. Genetic modifications like global invalidation of a gene (knock out) or controlled region-specific regulation of the expression of genes (inducible knock out/knock in, cre-lox system) with protein products related to the pathophysiology of depression are widely used today to validate the role of proteins in depressive-like or resilient phenotypes.

Another approach, the selective breeding, consists of reproduction between animals of the same strain that share similar traits in a behavior tested, for example high resignation as measured in the tail suspension or forced swimming tests. This results in a breed with a depressive-like phenotype related to the selected genetic pool.

Finally, different strains have been found to differ in response to stressful stimuli. Two well studied mice strains, C57BL/6 and BALB/c mice show different sensibility and physiological response to stressful stimuli. BALB/c mice exhibit significantly greater anhedonia following both acute and chronic stress (Poulter et al. 2010), longer latencies to escape a shuttle box in which they have previously experienced an inescapable footshock (Shanks and Anisman 1988) and decreased responding for reward after exposure to stress (Zacharko and Anisman 1991). Physiological alterations might underlie these differences, as BALB/c mice have consistently shown stressor-provoked hyperactivation of the HPA axis (Anisman et al. 1998, Priebe et al. 2005, Prakash et al. 2006, Anisman et al. 2007). BALB/c mice have also been shown to exhibit basal and stress-induced differences in expression of GABA-A receptor subunits (Poulter et al. 2010) that have also been implicated in a mouse model of post-partum depression (Maguire and Mody 2008).

Pathophysiology of depression: Monoamines and beyond

I. Monoamines

As a basic understanding of depression the monoamine hypothesis was formulated in the mid 1960s based on the antidepressant efficacy of the tricyclic antidepressants (TCA) and the monoamine oxidase inhibitors (MAOIs), as discussed above. The observation that depressive symptoms were attenuated by a chronic pharmacological increase of the available amount of monoamines, led to the hypothesis that depression results from reduced availability or functional deficiency of monoaminergic transmitters in some cerebral regions. Moreover, pharmacological decrease of tryptophan, the serotonin precursor, using reserpine or dietary phenylalanine and tyrosine depletion, the dopamine/norepinephrine precursors, induce depressogenic effects in humans (Belmaker et al. 2008). Experimental acute monoaminergic depletion can induce relapse in patients with MDD in remission who used serotonergic antidepressants, in subjects with a family history of MDD or in drug-free patients with MDD in remission, but does not affect mood in healthy controls (Ruhe et al. 2007).

Although the monoaminergic hypothesis is still one of the major hypothesis underlying depression and gave rise to multiple antidepressant compounds, it seems insufficient to explain all symptomatology and/or antidepressant action. There is a profound heterogeneity in the phenotype of depression (i.e., its clinical presentation, age of onset, course of illness, and treatment response), signifying that depression encompasses many different disease states with distinct etiologies. Importantly, all patients do not respond to monoaminergic-based treatments. Moreover, all currently used antidepressant treatments have a delayed onset of action with elevation in mood occurring weeks after the beginning of the treatment. Paradoxically, antidepressant agents acutely increase the monoaminergic content with no effect in mood.

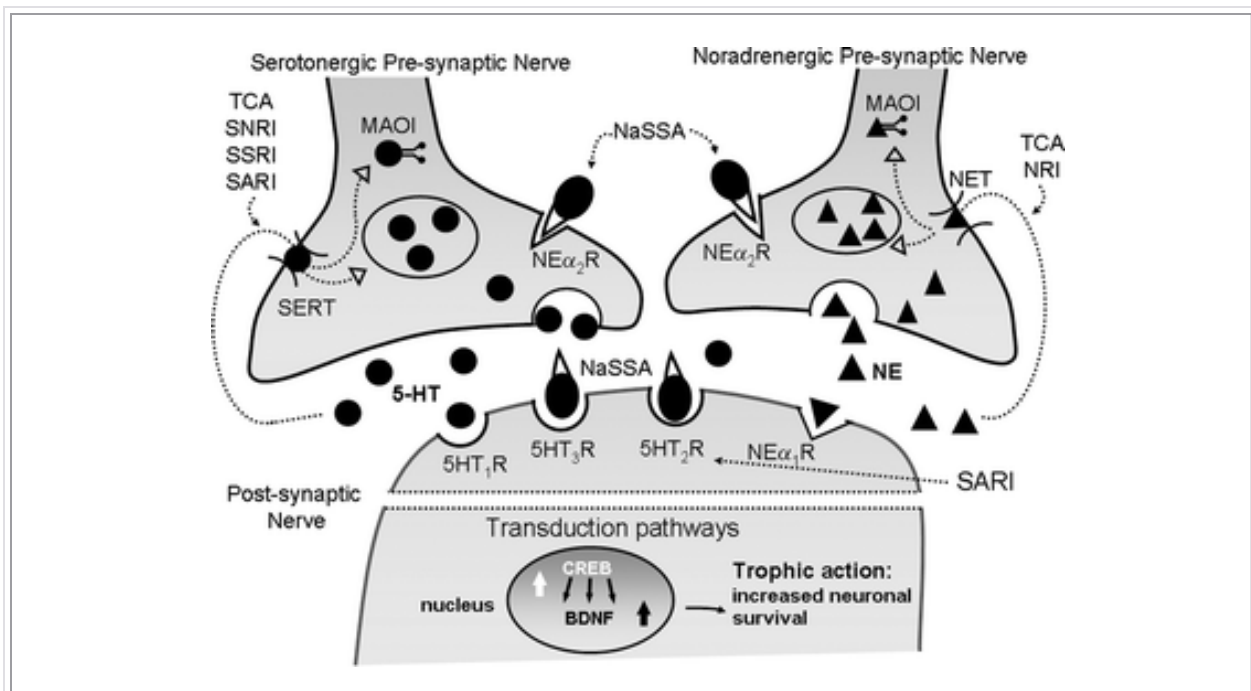


Figure 1: An example of some antidepressants effects on noradrenergic and serotonergic nerve endings. The figure shows the acute effect of some antidepressants on NE and 5-HT nerve endings. TCAs, NRIs, SSRIs, SNRIs, and SARIs modulate the reuptake of monoamines. NaSSA antidepressants are antagonists of some 5-HT receptor subtypes and also act as adrenergic alpha-2 receptors antagonists, thus controlling the monoaminergic release. This pharmacological action on 5-HT₂ and 5-HT₃ receptors, together with the effect on reuptake, promotes 5-HT action on other receptor subtypes, mainly 5-HT_{1A}, involved in the cerebral circuits deranged in depression. Chronic effects of antidepressants involve further mechanisms on signal transduction, second messengers' formation, and transcriptional control of specific genes, such as BDNF. Not indicated in the figure is the dopaminergic nerve ending. Some antidepressants have a triple action on 5-HT, NE, and DA synapses. Abbreviations: TCA TriCyclic antidepressant, SNRI serotonin and norepinephrine reuptake inhibitor, SSRI selective serotonin reuptake inhibitor, SARI serotonin antagonist reuptake inhibitor, NaSSA noradrenergic and specific serotonergic antidepressant, MAOI monoamine oxidase inhibitor, NET, norepinephrine transporter, SERT serotonin transporter, CREB cAMP response element binding protein, BDNF brain-derived neurotrophic factor (Lanni et al. 2009).

It is now thought that acute increases in the amount of synaptic monoamines induced by antidepressants produce secondary neuroplastic changes that are on a longer timescale and involve transcriptional and translational changes that mediate molecular and cellular plasticity (Pittenger and Duman, 2008). Neuronal plasticity or remodeling is a fundamental concept that underlies central nervous system function as it relates to the ability to acquire information and make the appropriate responses to the same or related future stimuli. This includes sensory, cognitive, emotional, social, as well as endocrine inputs and combinations of this information. Observations that structural alterations (neuronal atrophy and loss) occur in patients with mood disorders and in response to stress and that these are reversible

upon chronic administration of antidepressants led to the hypothesis that antidepressant action is mediated by neuroplastic changes. Increase of the synaptic amounts of monoamines activates intracellular pathways implicated in cellular survival, plasticity and resilience, as discussed later.

A major finding linking chronic antidepressant treatment to synaptic plasticity is the upregulation of the transcriptional factor CREB (cyclic-AMP-response-element-binding protein) in hippocampus and cerebral cortex. CREB is downstream of several serotonin and other stimulatory G-protein-coupled receptors and regulates among others the expression of the brain-derived neurotrophic factor (BDNF) gene (Figure 1). This suggests that effective treatments provide both trophic and neurochemical support, that serves to enhance and maintain normal synaptic connectivity, thereby allowing the chemical signal to reinstate the optimal functioning of critical circuits necessary for normal affective functioning.

Another example that relates monoaminergic antidepressant action to long-term changes is the calcium binding protein named p11. The serotonin 5-HT_{1B} receptor interacts with p11, a protein that enhances the efficiency of serotonin-1B receptor signaling. The 5-HT_{1B} receptor plays a crucial role on serotonin regulation as it serves both as a presynaptic autoreceptor on serotonergic neurons and as a postsynaptic heteroreceptor on non-serotonergic neurons (Maroteaux et al. 1992, Moret and Briley 2000, Morikawa et al. 2000). P11 was found to be downregulated in post mortem cingulate cortex samples from depressed patients and chronic treatment with SSRIs upregulates p11 in cerebral cortex (Svenningsson et al. 2006). A step forward in the understanding of the antidepressant action is the recently described functional link between BDNF and p11 (Warner-Schmidt et al. 2010).

Research in the past years has revealed several brain systems implicated in the etiopathology and the development of depression. Most of them are discussed below beginning with the well described hypothalamo-pituitary-adrenocortical axis as a primary physiologic response to stress, up to the neurotrophic hypothesis in depressive states and the newer concepts of signal transduction pathways and epigenetic modifications, which are believed to underlie the interface between genetic influence and environmental impact in the pathophysiology of the disease.

II. Hypothalamo-Pituitary-Adrenocortical (HPA) axis

The HPA (Hypothalamo-Pituitary-Adrenocortical) axis is activated by both internal and external signals. Neurons of the paraventricular nuclei (PVN) in hypothalamus project to the median eminence where they secrete CRF (corticotrophin-releasing factor, also called CRH-corticotrophin-releasing hormone) into the hypophyseal portal system. CRF is then transported to the anterior pituitary and acts on corticotrophs to increase ACTH (adrenocorticotrophic hormone) secretion into plasma. Stimulation of corticotropin receptors in the adrenal cortex leads to glucocorticoids (cortisol in humans or corticosterone in rodents) release into the blood. Such activation involves several parasympathetic effects: elevated arterial pressure, heart rate, cardiac output and blood glucose levels, diversion of blood to muscle, suppression of the immune response and inflammation, inhibition of reproductive physiology and behavior (Sapolsky et al. 2000). Glucocorticoids exert a negative feedback to limit the magnitude and duration of release acting on hypothalamic cortisol receptors and decreasing CRF production to maintain homeostasis.

Stress activates HPA axis, a phenomenon that has been viewed as relevant for both the maintenance of homeostasis, as well as behavioral adaptation. Stress-induced increases in glucocorticoids and norepinephrine are required to supply the energy and to induce the high arousal states needed to cope with stressful stimuli. Persistent arousal, however, has been associated with anxiety, and continued hyperactivity of the HPA axis has been implicated in the pathogenesis of depression in humans (Holsboer F. 2001, Nemeroff CB. 1996).

a) Glucocorticoids and CNS

Hippocampus, medial prefrontal cortex and amygdala have been shown to have an indirect impact on PVN neurons modifying HPA activity. These regions are rich in glucocorticoid receptors. Hippocampal stimulation decreases glucocorticoid secretion in rat and human (Dunn and Orr 1984, Rubin et al. 1966) and hippocampal lesions or GR depletion increase corticosterone and/or ACTH release (Fendler et al. 1961, Knigge 1961, Knigge and Hays 1963, Sapolsky et al. 1984). Hippocampal regulation of the HPA axis appears to be both region- and stressor-specific, with a prominent role of ventral subiculum-caudotemporal CA1 (Herman et al. 1995, 1998, Mueller et al. 2004). Chronic stress and long-term corticosteroid treatment

are associated with hippocampal neuroimpairment and damage, decreased levels of hippocampal GRs and prolonged stress response (Sapolsky 1996, 2001, McEwen 1999, 2000, Duman 2004b).

The medial prefrontal cortex seems to regulate HPA response in a more complex manner: lesions of the anterior cingulate and prelimbic divisions enhance ACTH and corticosterone (CORT) secretion (Diorio et al. 1993) whether lesions in the right infralimbic cortex decrease CORT response (Sullivan and Gratton 1999). This can be explained by the anatomy of mPFC's efferents: the prelimbic part projects to the ventrolateral preoptic area, dorsomedial hypothalamus and peri-PVN region, areas implicated in stress inhibition (Hurley et al. 1991, Sesack et al. 1989), whereas infralimbic cortex projects to the anterior bed nucleus of the stria terminalis, medial and central amygdala and the nucleus of the solitary tract, all of which are implicated in stress excitation (Hurley et al. 1991, Sesack et al. 1989, Takagishi and Chiba 1991). Once activated by glucocorticoids, hippocampus and mPFC regulate negatively HPA response, through GABAergic interneurons in the peri-PVN area.

Amygdala is believed to activate HPA axis. Large amygdaloid lesions or lesions of the central or medial amygdaloid nuclei reduce ACTH and/or CORT secretion following stress (Allen and Allen 1974, Beaulieu et al. 1986, Dayas and Day 2002, Feldman et al. 1994, Van de Kar et al. 1991), whereas stimulation increases HPA output (Dunn and Whitener 1986, Matheson et al. 1971, Redgate and Fahringer 1973). Regulatory studies indicate that glucocorticoids positively increase CRH expression in the central amygdala (Makino et al. 1994), in marked contrast with known inhibitory effects of glucocorticoids on CRH production in the PVN (Beyer et al. 1988, Schafer et al. 1989).

b) HPA dysfunction in depression

When exposed to chronic stress, the HPA axis can show both response 'habituation' and response 'facilitation'. Habituation occurs when the same (homotypic) stressor is delivered repeatedly, and is characterized by progressive diminution of glucocorticoid responses to the stimulus (Bhatnagar et al 2002, Cole et al. 2000, Kant et al. 1985). HPA axis habituation is highly dependent on both the intensity and predictability of the stressful stimulus (Marti and Armario, 1997, Pitman et al. 1988). Facilitation is observed when animals repeatedly

exposed to one stimulus are hyperactive towards a novel (heterotypic) stressor (Akana et al. 1992, Kant et al. 1985). Importantly, facilitation can occur in the context of chronic stress-induced elevations in resting glucocorticoids levels, suggesting that this process involves a bypass or override of negative feedback signals. This bypass is thought to occur because of the deleterious effects of GCs on hippocampus, inhibiting the negative control of the last on CRF release.

The hypothalamic-pituitary-cortisol hypothesis of depression postulates that abnormalities in cortisol response to stress may underlie depression (Figure 2). Several studies showed impaired HPA function in patients with depression. After a psychological stressor, depressive patients had much higher cortisol levels in plasma during the recovery period but no difference in basal levels with non-depressed volunteers, while afternoon studies were more likely to reveal higher baseline cortisol levels in plasma, blunted stress reactivity, and impaired recovery in MDD patients (Burke et al 2005). In post-mortem brain of patients that committed suicide, CRF was increased in prefrontal cortex and CRH1 receptor's mRNA decreased as a possible response to CRF augmentation (Merali et al. 2004).

Currently, several novel approaches to the treatment of depression are being evaluated based on the present understanding of HPA axis dysregulation in mood disorders. The CRF-1 receptor antagonist R121919 has shown promise in the treatment of depression (Zobel et al. 2000) but was subsequently withdrawn from clinical trials due to hepatotoxicity. More recently, the glucocorticoid receptor antagonist, mifepristone (RU486), has been reported to be effective in the treatment of psychotic depression (Belanoff et al. 2002). These compounds may be effective in treating depression through the interruption of reverberating neuroendocrine loops involving the HPA axis and several areas of the brain (prefrontal cortex, amygdala, hippocampus, and hypothalamus) that become excessively activated in response to stress driven perhaps by hypersecretion of CRF. Such interruption of a positive feedback loop may allow the system to return to a more adaptive set point associated with remission of depression (Gold et al. 2002).

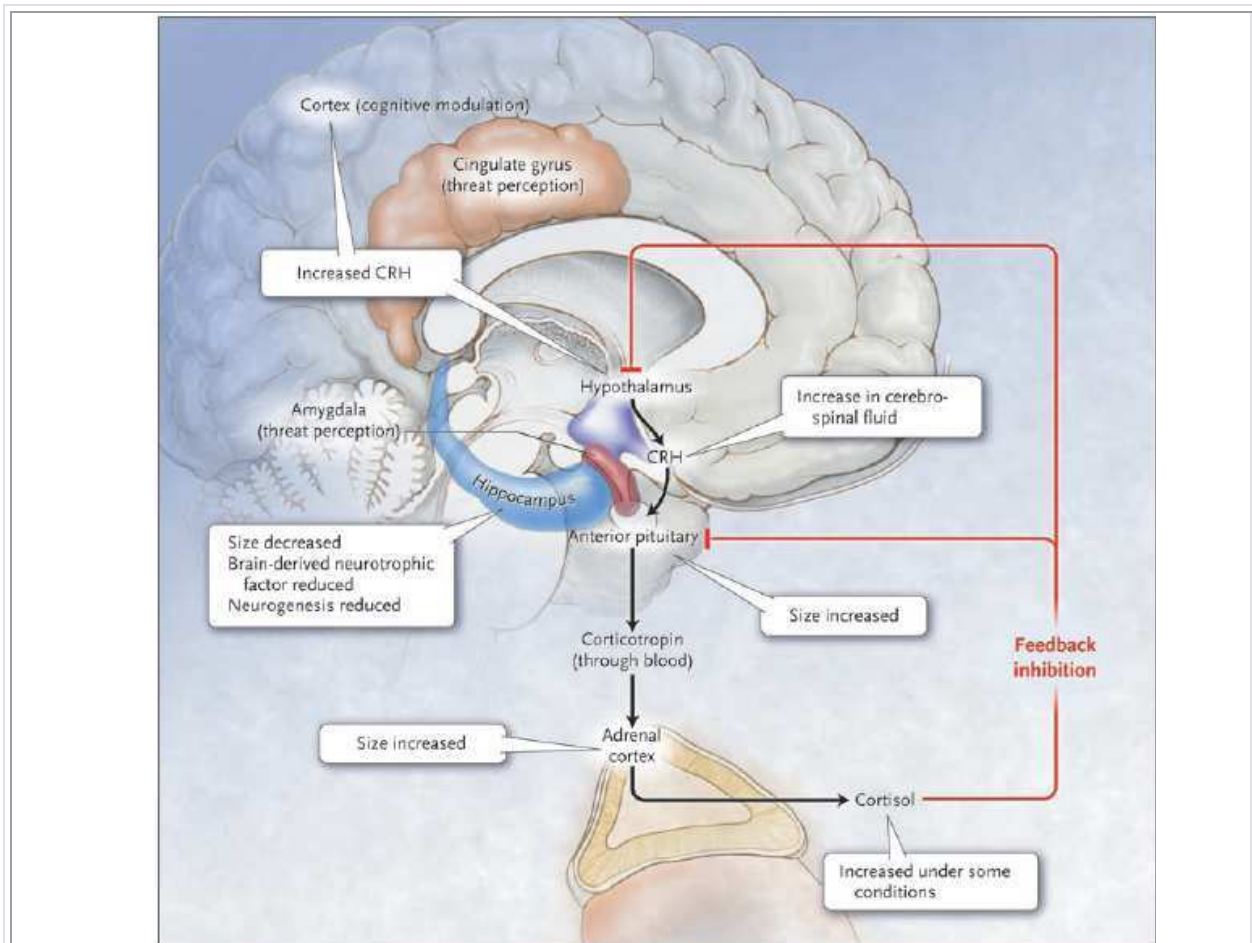


Figure 2: The Hypothalamic-Pituitary-Cortisol System in Depression.

Black arrows show that in response to stress, which is perceived by the brain cortex and amygdala and transmitted to the hypothalamus, corticotropin-releasing hormone (CRH) is released, inducing the anterior pituitary gland to secrete corticotropin into the bloodstream. Corticotropin stimulates the adrenal cortexes to secrete the glucocorticoid hormone cortisol. The red lines show that cortisol in turn, induces feed-back inhibition in the hypothalamus and the pituitary, suppressing the production of CRH and corticotropin, respectively. Findings in patients with depression that support the hypothalamic-pituitary-cortisol hypothesis include the following: cortisol levels are sometimes increased in severe depression, the size of the anterior pituitary and adrenal cortex are increased, and CRH levels in the cerebrospinal fluid and CRH expression in the limbic brain regions are increased. Hippocampal size and the numbers of neurons and glia are decreased, possibly reflecting reduced neurogenesis due to elevated cortisol levels or due to reduced brain-derived neurotrophic factor (Belmaker RH and Agam G, 2008).

c) Glucocorticoid receptors (GRs) signaling

Glucocorticoids have two types of receptors in the brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Expression of the MR is considerably more restricted than that of GR, but MRs have an approximately 5-10 fold greater affinity than GRs. This has led to the assumption that MR regulates basal HPA tone (receptors occupied in

low concentrations of GCs), whether GR is important in mediating glucocorticoid feedback following stress (when levels of circulating GCs are largely increased) (Reul and de Kloet 1985, 1986).

Both GR and MR are members of the nuclear hormone receptor superfamily (Beato et al. 1995). They have a modular structure, consisting of a DNA-binding domain (DBD), a ligand-binding domain (LBD) and two activating functions. In the absence of ligand, steroid hormone receptors are trapped in an inactive cytosolic complex together with heat shock proteins. Upon ligand binding, this complex dissociates and the receptor translocates into the nucleus to modulate transcriptional activity of target genes via different mechanisms (Reichardt and Schutz 1998). The classical mode of action of GR and MR involves binding of the receptor to specific response elements in the promoter of target genes (GRE-glucocorticoid response element) to induce their transcription. However, in the case of GR two transcriptional mechanisms have been further identified that can lead either to induction or to repression of transcription: DNA-binding (on GREs or negative GREs) and protein-protein interaction (cross-talk with other transcription factors) (Revollo and Cidlowski 2009, Figure 3).

The glucocorticoid feedback can be fast (non-genomic) or delayed (genomic) through GR-like and/or MR receptors. In hypothalamus, Tasker's team has shown that GCs acting on GR-like membrane receptors rapidly inhibit the parvocellular neurosecretory neurons of PVN via retrograde endocannabinoid release (Di et al. 2003, Tasker et al. 2006). In hippocampus corticosterone pulses rapidly enhance glutamate release and excitatory transmission through pre- and post-synaptic membrane MRs (Karst et al. 2005, Joels et al. 2008, Olijslagers et al. 2008). Non-genomic effects contribute to fast behavioral effects, while genomic effects facilitate suppression of temporary raised excitability, recovery from the stressful experience and storage of information in the memory for future use (de Kloet et al, 2008). Upon activation MR and GR translocate from the different cell compartments (pre- or post-synaptic terminals, dendrites, dendritic spines or spine organelles) to the nucleus through a microtubule-based transport. Once in the nucleus, they both bind to GREs, but GR is much better capable to interact with transcription factors such as activating protein (AP-1) and nuclear factor kappa B (NFkB) (Pearce and Yamamoto, 1993). GR monomers interact with transcription factors (co-regulators) driven by catecholamines and other stress factors

activating membrane-signaling cascades. These coregulators may either potentiate transcription as coactivators, or suppress function as corepressors. In addition, coregulator activity seems to be tightly controlled by a host of posttranslational modifications that allow rapid changes in signaling sensitivity (Han et al. 2009).

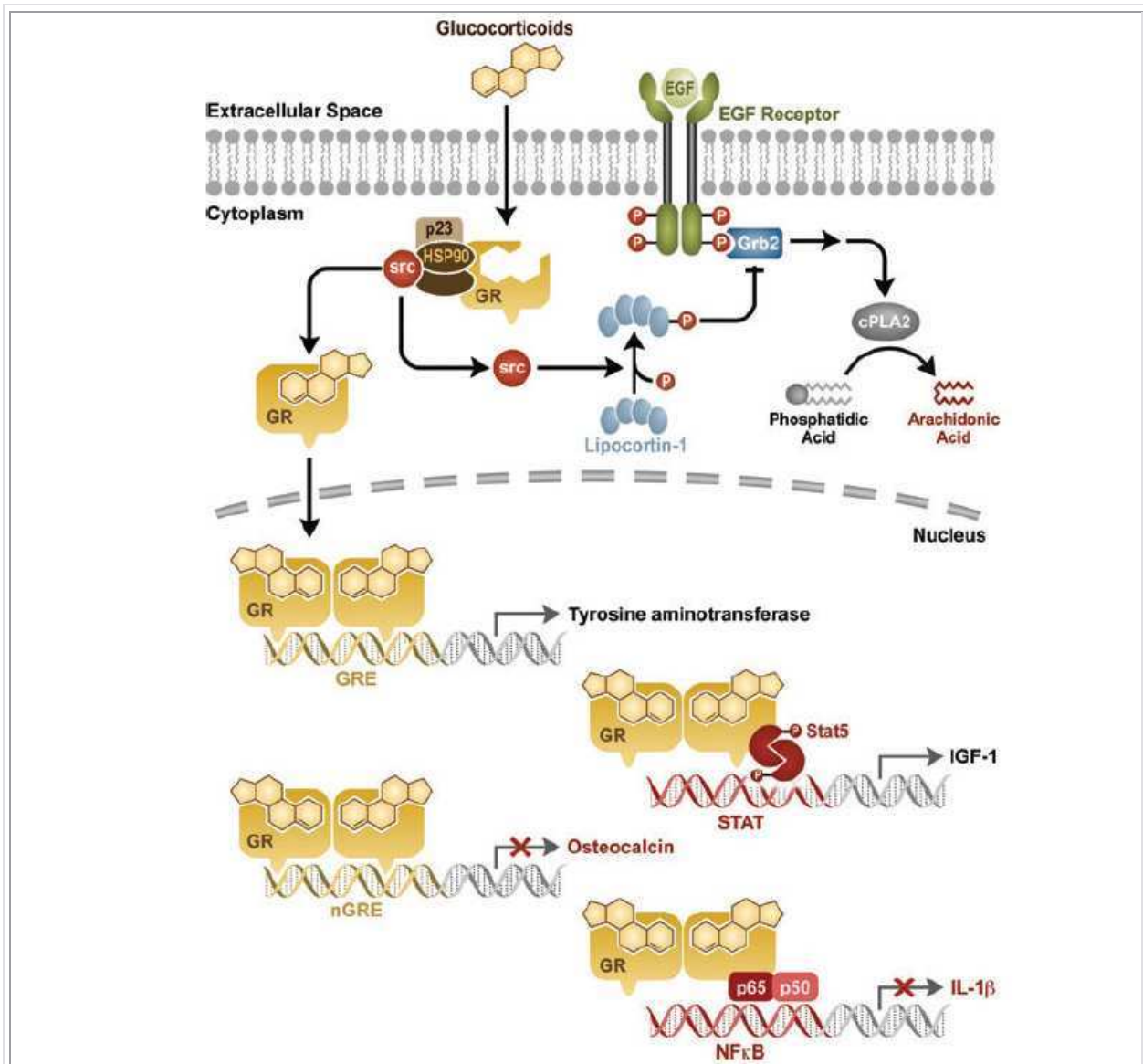


Figure 3. Glucocorticoid signaling results in changes in gene expression and rapid nongenomic events in the cytoplasm. Ligand binding to the GR leads to the dissociation of a cytoplasmic complex interacting with GR. Ligand-bound GR rapidly translocates into the nucleus, where it can activate gene expression by directly binding to GREs in the DNA, or associate with transcription factors, such as STAT5. GR can also promote gene silencing by directly interacting with nGREs in the DNA, or by protein–protein interactions with transcription factors, such as NFκB. In the cytosol, the dissociation of the protein complex interacting with GR leads to the release of src kinase, which phosphorylates lipocortin-1. Phosphorylated lipocortin-1 displaces Grb2 from the activated EGF receptor to inhibit the activation of cPLA2 and the creation of arachidonic acid (Revollo and Cidlowski 2009)

III. Inflammation and depression: cytokines

Activation of immune responses and the release of inflammatory cytokines have been related to the pathophysiology of major depression. Patients with major depression have been found to exhibit increased biomarkers of inflammation in both periphery and brain. Specifically, increased concentrations of innate immune cytokines, including interleukins IL-1 and IL-6, tumor necrosis factor TNF- α and their soluble receptors have been found in the peripheral blood and/or CSF of depressed patients (Raison et al. 2006). Aside from evidence of increased inflammatory markers in depressed patients, data from laboratory animals and humans indicate that the administration of innate immune cytokines, including interferon (IFN)- α , leads to multiple behavioral changes that overlap with major depression, like depressed mood, anhedonia, fatigue, psychomotor slowing, disrupted sleep, cognitive impairment, anxiety and suicidal ideation (Dantzer et al. 2008).

Cytokines signals access the brain by molecular active transport, by passage through the BBB or through the visceral afferent nerve fibers and ultimately interact with pathophysiologic pathways relevant to mood regulation. Once cytokine signals reach the brain, they are further transduced and amplified in part through activation of inflammatory intermediaries such as NF- κ B. There is also a cytokine network within the brain that includes glial elements (astrocytes and microglia), which produce cytokines and chemokines, and multiple cell types, including neurons, which express cytokine receptors. Cytokines can influence virtually every pathophysiologic pathway relevant to depression, including neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and regional brain activity (Raison et al. 2006, Figure 4).

One mechanism by which cytokines may contribute to depression is through their effects on the glucocorticoid receptor (GR). Cytokines have been shown to decrease GR expression, block translocation of the GR from cytoplasm to nucleus, and disrupt GR-DNA binding through nuclear protein-protein interactions. In addition, cytokines have been shown to increase the expression of the relatively inert GR beta isoform. Specific cytokine signaling molecules that have been shown to be involved in the disruption of GR actively include p38 mitogen-activated protein kinase, which is associated with reduced GR translocation, and signal transducer and activator of transcription (STAT)5, which binds to GR in the nucleus.

Nuclear factor- κ B (NF- κ B) also has been shown to lead to GR suppression through mutually inhibitory GR-NF- κ B nuclear interactions. Interestingly, several antidepressants have been shown to enhance GR function, like activation of protein kinase A (PKA). Antidepressants and PKA activation have also been found to inhibit inflammatory cytokines and their signaling pathways, suggesting that drugs that target both inflammatory responses and the GR may have special efficacy in the treatment of depression (Pace and Miller 2009).

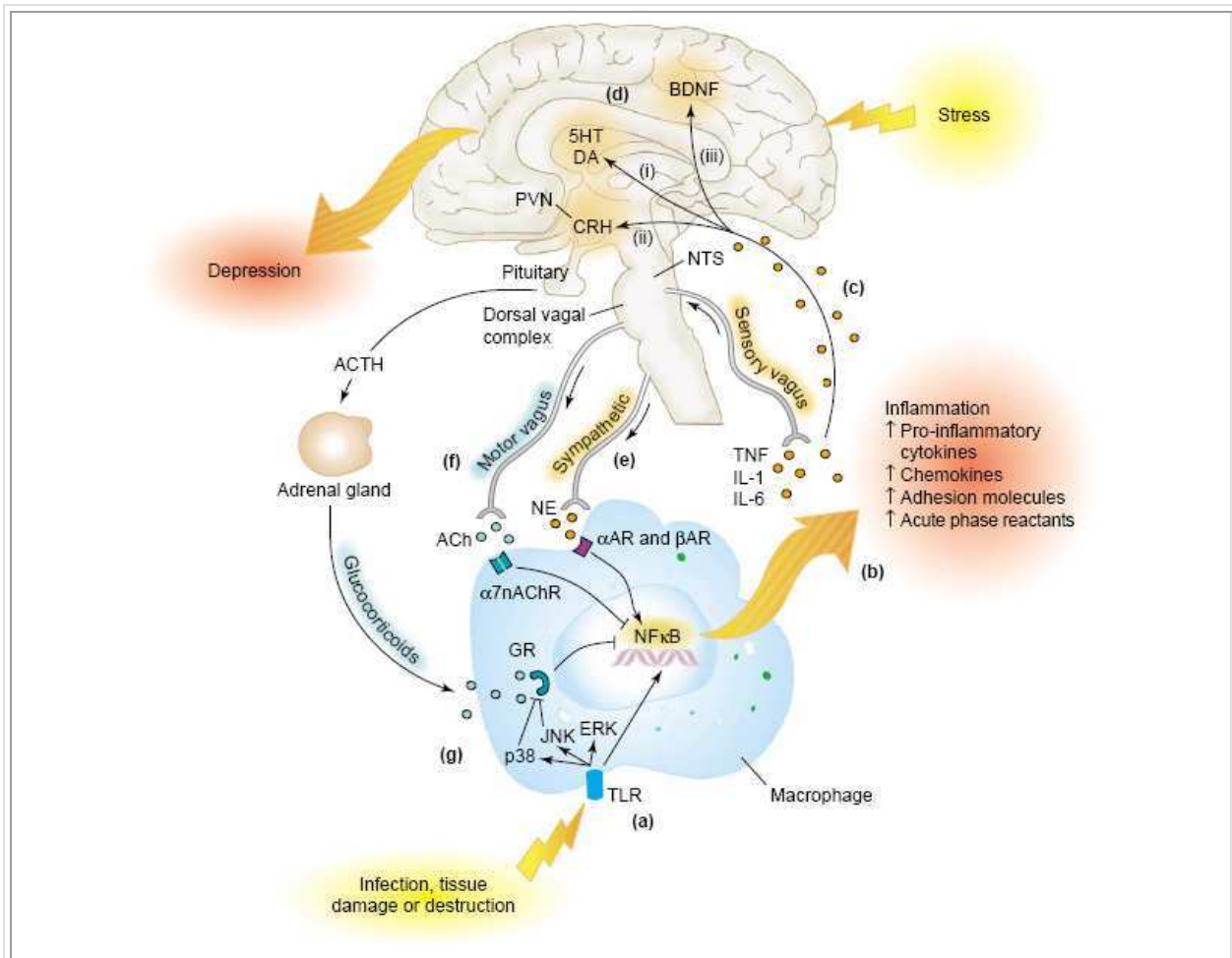


Figure 4: Stress–immune interactions and depression. (a) Activation of NF- κ B through Toll-like receptors (TLR) during immune challenge leads to an inflammatory response including (b) the release of the proinflammatory cytokines TNF- α , IL-1 and IL-6. (c) These cytokines, in turn, access the brain via leaky regions in the blood–brain barrier, active transport molecules and afferent nerve fibers (e.g. sensory vagus), which relay information through the nucleus tractus solitarius (NTS) [Maier and Watkins 1998]. (d) Once in the brain, cytokine signals participate in pathways (indicated in orange) known to be involved in the development of depression, including: (i) altered metabolism of relevant neurotransmitters such as serotonin (5HT) and dopamine (DA) [Gao, et al. 2002, Dunn et al. 1999]; (ii) activation of CRH in the paraventricular nucleus (PVN) and the subsequent production and/or release of ACTH and glucocorticoids (cortisol) [Besedovsky and del Rey 1996, Silverman et al. 2005] and (iii) disruption of synaptic plasticity through alterations in relevant growth factors [e.g. brain-derived neurotrophic factor (BDNF)] [Lu et al. 2004, Madrigal et al. 2002]. (e) Exposure to environmental stressors promotes activation of inflammatory signaling (NF- κ B) through increased outflow of proinflammatory sympathetic nervous system responses [release of norepinephrine (NE), which binds to α (aAR) and β (bAR) adrenoceptors] (orange). (f) Stressors also induce withdrawal of inhibitory motor vagal input [release of acetylcholine (ACh), which binds to the α 7 subunit of the nicotinic acetylcholine receptor (α 7nAChR)] (blue) [Bierhaus et al. 2003, Pavlov and Tracey 2005]. (g) Activation of the mitogen activated protein kinase pathways, including p38 and Jun amino-terminal kinase (JNK), inhibit the function of glucocorticoid receptors (GR), thereby releasing NF- κ B from negative regulation by glucocorticoids released as a result of the HPA axis in response to stress (blue) [Wang et al. 2004, McKay and Cidlowski 1999]. (Raison et al. 2006).

IV. Circadian system: melatonin

The circadian pacemaker or biological clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, on top of the optic chiasma. The circadian clock uses several internal and external synchronizers that are able to modify the period and the phase of circadian rhythms. The light/dark cycle is the dominant synchronizing agent for circadian rhythmicity, with light presented in the evening stimulating the human circadian pacemaker to phase-delay its rhythms, and light stimulus given in the morning producing a phase advance (Moore RY. 1997). Circadian pacemaker regulation is also determined by neurotransmitter function and by the phase shifting effects of various chemical or pharmacological components, including melatonin (N-acetyl-5-methoxytryptamine) (Brainard et al. 2002). Secretion (primarily from the pineal gland) and action of melatonin is tightly related to seasonal cycles (longer peak in winter, shorter in summer) and to light-dark cycles (high at night, low during the day), and is thus considered to be the 'hormone of darkness' and the body's chronological pacemaker or 'Zeitgeber' (Pandi-Perumal et al. 2006). The amplitude and duration of the nocturnal melatonin peak translates photoperiodic information through activation of the MT1 and MT2 G-protein coupled melatonin receptors located in the SCN. Activation of MT1 receptors directly inhibits firing of neurons in the SCN, hence regulating the amplitude of the circadian rhythmicity, and possibly facilitating sleep promotion, whereas melatonin-mediated activation of MT2 receptors is responsible for inducing phase shifts and hence involved in the entrainment of circadian rhythmicity (Hattar et al. 2003).

The complex relation between the endogenous circadian pacemaker and the appearance of depressive symptoms is far from being elucidated (Turek FW. 2007). Depression seems to be related with a disruption in the central circadian clock function. Circadian amplitude reduction of temperature, thyroid-stimulating hormone, norepinephrine and motor activity seems to be the most relevant chronobiological abnormalities in depression (Tsujimoto et al. 1990). A phase advance of the rhythm of cortisol, adrenocorticotropin, prolactin and growth hormone secretion has also been noted in depressed patients (Van Cauter et al. 1996). Literature regarding melatonin levels in depression remains controversial. Reduced blood melatonin concentrations and a trend toward a phase delay of melatonin rhythms have been

reported in several affective disorders (Claustrat et al. 1984, Crasson et al. 2004, Paccierotti et al. 2001), suggesting that antidepressant efficacy could be related to melatonin secretion through monoaminergic mechanisms (Palazidou et al. 1992). However, the lack of melatonin disturbances noted in other studies (Carvalho et al. 2006) would point out to the increase in melatonin being related to the pharmacological effect of antidepressants and not to the improvement of depressive symptoms. Administration of melatonin to depressed patients has been shown to generally improve sleep, with little effect on depressive symptoms, and does not substantially enhance the effect of existing antidepressant therapies in patients with treatment-resistant depression (Dalton et al. 2000). It appears then that melatonin is not sufficient on its own to achieve a robust clinical antidepressant efficacy.

Agomelatine, a naphthalene analog of melatonin, is a newly developed selective agonist of the melatonergic MT1 and MT2 receptors (Millan et al. 2003), that shows a longer half-life and a comparatively greater affinity than melatonin for MT1 and MT2 receptors both in the SCN and in other brain areas (Delagrangé and Boutin 2006). Agomelatine also shows serotonin 5-HT_{2C} receptor antagonism (Millan et al. 2003). Blockade of the 5-HT_{2C} receptors is suggested to be implicated in the antidepressant profile of agomelatine, as increased sensitivity of 5-HT_{2C} receptors have been noted in depressed patients, and a correlation between the therapeutic actions of antidepressants and a reduced number of 5-HT_{2C} receptors has also been reported (Riedel et al. 2002). Agomelatine has shown antidepressant and anxiolytic properties in a battery of animal models of depression ranging from response to acute stress to chronic stress protocols (Pandi-Perumal et al. 2006, Millan et al. 2005, Papp et al. 2006, Zupancic and Guilleminault 2006, Rainer et al. 2011). Agomelatine also seems to reverse stress-induced biochemical abnormalities in the brain, including cell proliferation and neurogenesis in hippocampus, protein expression and activation of biological markers related to depression (Morley-Fletcher, 2011, Dągryte et al. 2011, Rainer et al. 2011, Paizanis et al. 2010).

Agomelatine addresses one of the core symptoms in depression by restoring disturbed sleep in depressed patients and may improve sexual dysfunction. The drug has been shown to effectively treat patients with major depressive disorder, with a particularly robust efficacy in more severely depressed subgroups. The good safety profile of agomelatine, due to its

unique receptor profile, predicts a higher patient acceptability and a better compliance (Kasper and Hamon, 2009).

V. Hypothalamic feeding peptides - Neuropeptides

Hypothalamic peptides are best known for their prominent role in the regulation of feeding behavior, but recent studies have demonstrated that these peptides also contribute to emotional behavior (Nestler and Carlezon, 2006). The anhedonic and lethargy symptoms and significant changes in body weight that occur in many depressed patients suggest the involvement of hypothalamic mechanisms in a subtype of depression.

Orexigenic (pro-appetite) peptides, like orexin (hypocretin), melanin-concentrating hormone (MCH), ARP (agouti-related peptide) and NPY (neuropeptide Y) have been implicated in the antidepressant action in a controversial manner. Experimental treatments with orexin and antagonism of MCH₁ receptors promote antidepressant-like effects, while over-expressing MCH is correspondingly pro-depressant (Lutter et al. 2008, Shimazaki et al. 2006). Well-known for having a role in stress responses, NPY has been intensively examined in animal models of depression and in clinical studies. In depressed subjects and suicide victims, lower levels of NPY in cerebrospinal fluid, plasma, and prefrontal cortex have been reported (Caberlotto and Hurd 2001, Widdowson et al. 1992). Experimental decreases in NPY levels promote depressive-like behavioral responses in animals and stimulation of NPY neurotransmission produces antidepressant-like and anxiolytic-like effects (Sajdyk et al. 2004).

Anorexigenic peptides, including melanocortin (α MSH) and cocaine- and amphetamine-regulated transcript (CART) may also be targeted for treating depression.

VI. Glutamatergic hypothesis of depression

The amino acid glutamate is the major excitatory neurotransmitter in the mammalian CNS and it exerts its physiological effects through two types of receptors: the metabotropic glutamate receptors (mGluRs) and the ionotropic ligand-gated ion channels. The ionotropic receptors are separated into three subgroups based upon their pharmacology: AMPA, NMDA and kainate (KA) receptors.

The clinical findings of abnormalities in glutamate function in depression reported in the majority of studies consist of increases in glutamate levels in the cerebrospinal fluid (CSF) (Levine et al. 2000), in plasma (Kim et al. 1982, Altamura et al. 1993, Mauri et al. 1998, Mitani et al. 2006) and in the prefrontal cortex of post-mortem MDD brain (Hashimoto et al. 2007). On the other hand, reduction of NR1/NMDA receptors has been reported in a study of post-mortem MDD brains in the superior temporal cortex (Nudmamud-Thanoi and Reynolds, 2004), and reduction of mRNA levels of the NR2A and NR2B subunit of the NMDA receptors and the GluR1, GluR3, and GluR5 subunits of the AMPA receptors in the perirhinal cortex (Beneyto et al. 2007) and of NR1 in the dentate gyrus of hippocampus (Law and Deakin 2001). There is a positive correlation between plasma glutamate levels and severity of depressive symptoms in patients with MDD (Mitani et al. 2006) and 5-week treatment with antidepressants significantly decreased the levels of glutamate in sera (Maes et al. 1998). With substantial evidence of increased glutamate transmission in depressive states and its contribution to stress-related neurotoxicity, glutamate receptors are potential targets for antidepressant action (Sapolsky et al. 2000, Zarate et al. 2003) as discussed later in this manuscript.

VII. Neurotrophin hypothesis: BDNF

The “neurotrophin hypothesis of depression” is based on observations of atrophy and altered volume of certain brain regions of patients suffering from depression. Many studies in major depressed/suicidal subjects have demonstrated altered brain structure, such as reduction in cell number, density, cell body size, neuronal and glial density in frontal cortical or hippocampal brain areas and decrease in parahippocampal cortex cortical/laminar thickness (Sheline et al. 2003, Campbell et al. 2004, Dwivedi Y, 2009). Cognitive difficulties in

major depression include impairment of concentration and attention, capacities that are regulated by the dorsolateral prefrontal cortex (Goldman-Rakic, 1996). Abnormalities in this region found in depressed patients (Baxter et al. 1989, Harvey et al. 2005) and in post-mortem tissue (Rajkowska et al. 1999, 2007) have been related to impaired ability to think or concentrate. Another core symptom in depression is a deficit in explicit memory found in depressed patients with both first and recurrent episode (Zakzanis et al. 1998), a cognitive capacity well established to depend on hippocampus and medial temporal lobe function (Squire et al. 2004). Structural imaging studies have revealed decreased hippocampal size in patients with major depression (MacQueen et al. 2003). These morphometric changes have been related to disrupted hippocampal function and capacity for neuroplasticity, which could contribute to several cognitive aspects of depression. Functions of hippocampal circuitry include control of learning and memory, in cooperation with prefrontal cortex, but also negative regulation of the HPA axis through its projections to hypothalamus. HPA axis dysregulation has been well established in depression, as formerly discussed.

Stress has also been reported to cause damage and atrophy of neurons in certain brain regions and most notably in hippocampus, which expresses high levels of receptors for glucocorticoids (Sapolsky 1996, McEwen 1999, 2000, Sapolsky 2001, Duman 2004). Alterations of hippocampal structure and function in response to stress provided the rationale for analysis of neurotrophic factors.

a) BDNF protein

Brain-derived neurotrophic factor (BDNF) is a member of the structurally and functionally homologous neurotrophin family. It is the most widely distributed trophic factor in the brain, and is involved in growth, differentiation, maturation, and survival of immature neurons. Moreover, BDNF plays an important role in synaptic plasticity, augmentation of neurotransmission, and regulation of receptor sensitivity in mature neurons (Numakawa et al. 2010).

The BDNF gene is comprised of at least eight promoters, each of which initiates transcription of alternative 5' exon spliced onto a common 3' exon, encoding the entire open reading frame of the BDNF protein. Additionally, BDNF mRNA has two distinct polyadenylation sites

resulting in production of distinct populations of mRNA, with either short or long 3' untranslated regions (3'UTRs). At least 18 distinct transcripts are generated through the complexity of the BDNF gene that all lead to the same protein (Liu et al. 2006, Aid et al. 2007). Different sequences of mRNA show different distributions and/or translational efficacies, and different transcript expressions are regulated by different stimuli. The promoter of the BDNF gene contains a cyclic-AMP response element (CRE) and has been shown to be induced by CREB (Shieh et al. 1998, Tao et al. 1998). Phosphorylation of CREB has been shown to increase in brain in response to TrkB activation (Shaywitz and Greenberg 1999) as well as after chronic antidepressant administration (Thome et al. 2000), as discussed later.

BDNF is translated as a precursor protein (proBDNF) and then proteolytically cleaved (processed) to generate a small mature protein (mBDNF). Both precursor and mature proteins bind to the same receptors (TrkB and p75), but with different affinities. ProBDNF binds preferentially to p75, while mBDNF has a high affinity for TrkB receptor. Trk receptors contain a tyrosine kinase domain that exerts trophic effects, whereas p75 belongs to the tumor necrosis factor family and has a death domain that plays a role in apoptosis as well. BDNF has therefore the yang and yin of the action on neurons depending on processing of proBDNF and differential affinity of proBDNF and mBDNF for TrkB and p75 receptors (Lu et al. 2005). Binding of BDNF to TrkB leads to activation of the receptor through phosphorylation, which induces several intracellular signaling pathways, i.e. mitogen-activated protein kinase (MAPK), phospholipase C-gamma (PLC γ), and phosphatidylinositol 3-kinase (PI3K) pathways (Huang and Reichart 2003). Mice lacking TrkB in the hippocampal neuron progenitor cells demonstrated increased anxiety-like behavior and decreased sensitivity to antidepressants (Li et al. 2008, Bergami et al. 2008).

b) BDNF in depression

Numerous studies have documented that both acute and chronic stress paradigms decrease the expression of hippocampal BDNF mRNA in animals and that chronic antidepressant treatment inverses this effect (Smith et al. 1995, Duman and Monteggia 2006, Martinowich et al. 2007). The upregulation of BDNF is observed with different classes of antidepressants, including selective serotonin reuptake inhibitors (SSRIs) and norepinephrine selective

reuptake inhibitors (NSRIs), monoamine oxidase inhibitors (MAOIs), atypical antidepressants and electroconvulsive seizures (ECS). The most clinically effective treatments, ECS and MAOIs, have the greatest effect on the induction of BDNF (Nibuya et al. 1995). Other treatments, like AMPAkinetics, NMDA antagonists, transcranial magnetic stimulation and exercise, that are known to have antidepressant efficacy also increase the expression of BDNF in hippocampus (Lauterborn et al. 2003, Marvanova et al. 2001, Muller et al. 2000, Neepker et al. 1996, Adlar et al. 2004, Russo-Neustadt et al. 2000, 2004). Central administration or direct hippocampal infusions of BDNF protein can produce antidepressant effects in rodents (Siuciak et al. 1997, Shirayama et al. 2002, Hoshaw et al. 2005).

Impaired neurogenesis in hippocampus has been proposed to mediate deleterious effects of stress on hippocampal structure and depressive-like behavior in mice. Important experimental observations link antidepressant action to the stimulation of neurogenesis in hippocampus (Santarelli et al. 2003), but equally compelling contradictory reports indicate that increased neurogenesis may occur but is not indispensable for depression and antidepressant action (Hanson et al. 2011).

In human post-mortem studies of suicide victims with depression, BDNF expression has quite consistently been reported to be reduced in hippocampus and prefrontal cortex (Dwivedi et al. 2003, Karege et al. 2005, Dunham et al. 2009). Importantly, BDNF expression was unchanged or even increased in the hippocampus of suicide victims with antidepressants, which suggests that antidepressants increase the level of BDNF (Chen et al. 2001). There is evidence that antidepressants and electroconvulsive therapy (ECT) increase hippocampal volume in patients with depression (Frodl et al. 2008, Nordanskog et al. 2010). As regards blood BDNF levels, drug naïve patients with depression often showed decreased BDNF in contrast to patients treated with antidepressants where increased BDNF levels were found (Shimizu et al. 2003). Recent meta-analyses confirmed such findings and significant correlation was found between changes in BDNF level after antidepressant medications and changes in depression scores (Sen et al. 2008, Brunoni et al. 2008).

Growing evidence from molecular genetic studies has also suggested that genetic variations in the BDNF gene confer susceptibility to mood disorders. There are at least two functional polymorphisms in the BDNF gene that have been extensively studied in relation to

neuropsychiatric diseases: the Val66Met polymorphism and the BDNF-LCPR. The met polymorphism at codon 66, compared to that of Val, decreases BDNF trafficking and correlates with a lower hippocampal volume (Egan et al. 2003, Chen et al. 2004, Chepenik et al. 2009). The BDNF-linked complex polymorphic region (BDNF-LCPR) is located 1kb upstream of the coding exon (putative promoter region) and contains three different dinucleotide repeats in succession, yielding a total of 23 novel allelic variants (Okada et al. 2006). Among the four common alleles, the 'A1 allele' was found to be associated with reduced transcriptional activity and associated with a risk of bipolar disorder.

c) BDNF in distinct brain systems and networks

i) Prefrontal cortex

Although hippocampal levels of BDNF and CREB are related to antidepressant action and are considered beneficial for the hippocampal function, this is not the case for all brain regions related to depression. The hippocampus and prefrontal cortex (PFC) are implicated in learning and memory, attention and impulse control, which suggests they may mediate cognitive aspects of depression, such as memory impairments and feelings of hopelessness, guilt, doom, and suicidality (Chamberlain and Sahakian 2006, Nestler et al. 2002). Volumetric changes and BDNF expression in the PFC in depression are similar to those described in the human hippocampus. Abnormal high levels of ventromedial PFC activity and abnormal low levels of dorsolateral PFC activity in resting brain activity have been associated with depression (Yu and Chen 2011). Both BDNF and TrkB levels are significantly decreased in the PFC of suicide patients compared to controls and antidepressant therapy restores brain BDNF levels to the normal range (Castren E 2004). Repeated stress causes dendritic shortening and decreases BDNF and TrkB levels in PFC, as well as decreased phosphorylation of ERK2 and CREB (Brown et al. 2005). Antidepressant treatment increases BDNF and TrkB expression and phosphorylation of CREB and ERK2 without altering stress-induced decrease of total levels of ERK2 or CREB (Qi et al. 2008). The dorsolateral PFC includes portions of the middle and superior frontal gyri on the lateral surface of the frontal lobes, receives input from specific sensory cortices, and has dense interconnections with premotor areas, the frontal eye fields, and lateral parietal cortex. DLPFC has primarily been associated with "cognitive" or "executive" functions, whereas ventromedial PFC is largely ascribed

“emotional” or “affective” functions. vmPFC projects to the hypothalamus and the periaqueductal gray, which mediate the visceral autonomic activity associated with emotion, and the ventral striatum, which signals reward and motivational value. In addition, vmPFC has dense connections with the amygdala, which is involved in threat detection and fear conditioning (Yu and Chen 2011).

ii) Striatum

The ventral striatum (nucleus accumbens, NAc) and amygdala, and related brain areas, are important in emotional memory, and could as a result mediate the anhedonia, anxiety, and reduced motivation that predominate in many depressed patients (Davidson et al. 1999). In the ventral tegmental area-nucleus accumbens (NAc) dopaminergic reward circuit BDNF and CREB actions are opposite to that in PFC and hippocampus. Elevated CREB activity in the NAc produces depressive-like phenotypes in rodents including reduced reward experience or “anhedonia” and increased immobility in the forced swim test, symbolizing behavioral “despair” (Barrot et al. 2002, Pliakas et al. 2001). Local BDNF infusion into NAc exerts a prodepression-like effect in the forced swim test, and blockade of BDNF function in NAc exerts an antidepressant-like effect (Eisch et al. 2003). Exposure to stress increases BDNF expression in the NAc and selective BDNF depletion in the VTA protects from the depressive effects produced by the social defeat stress contrarily to deleterious effects of stress on hippocampal BDNF expression (Berton et al. 2006, Berton and Nestler, 2006). The relationship of VTA-NAc pathway to mood disorders requires further study, but it seems plausible that disturbances in this pathway would be related to abnormalities in hedonic tone and motivation which are central features of mania and depression (Nestler et al. 2002). This is supported by the finding of decreased striatal response to happy stimuli associated with level of anhedonia in depressed subjects as well as the observation of increased striatal activity in mania (Keedwell et al. 2005).

iii) Amygdala

Stress-induced changes in BDNF are transient in amygdala. Both acute and chronic stress increases spine synapse formation in amygdala (Vyas et al. 2002) and chronic stress enhances amygdala-dependent unlearned fear and fear conditioning (Conrad et al. 1999). Intermittent water immersion stress and social defeat stress increases BDNF expression in

basolateral and central nucleus of the amygdala 1h or 2h respectively after stress, while acute social defeat or repeated restraint stress decreases BDNF 24h after stress (Pizarro et al. 2004, Fanous et al. 2010). In humans, increased volume and baseline amygdala activity have been observed in adult bipolar patients (Mah et al. 2007).

In summary, the diverse roles of BDNF in depression depend on its location in the neural circuitry, namely, in the hippocampus and prefrontal cortex BDNF inhibits depressive symptoms and mediates antidepressant action whereas in the NAc and amygdala likely facilitates depression- and anxiety-like symptoms. Of course, these various brain areas cannot be thought of as distinct, they operate in a series of highly interacting parallel circuits, which perhaps begins to formulate a neural circuitry involved in depression.

d) Hippocampal atrophy: Glucocorticoids - Glutamate – BDNF

The hippocampus has a very high concentration of glutamate and expresses both MR and GR corticosteroid receptors. MR receptor activation in the hippocampus is associated with reduced calcium currents, whereas activation of GR receptors causes increased calcium currents and enhanced responses to excitatory amino acids. Very high levels of GR receptor activation markedly increases calcium currents and lead to increased NMDA receptor throughput that could predispose to neurotoxicity. Indeed, a growing body of data has implicated glutamatergic neurotransmission in stress-induced hippocampal atrophy and death.

Hippocampal atrophy is mostly observed in the CA3 pyramidal neurons, and has been linked to HPA dysregulation (decreased hippocampal negative control on glucocorticoids release) and to impaired spatial memory (Conrad CD, 2006). The stress-induced atrophy of CA3 neurons (i.e., decreased number and length of the apical dendritic branches) occurs after chronic stress and/or upon exposure to high levels of glucocorticoids, suggesting that activation of the HPA axis likely plays a major role in mediating the stress-induced atrophy (Sapolsky R.M. 1996b, 2000, McEwen B.S. 1999). Chronic corticosterone administration (3 weeks) also induced a reduction in total dendritic length specific to fourth and sixth branch orders of pyramidal CA1 hippocampal neurons (Morales-Medina et al. 2009).

There are at least two lines linking altered HPA axis and BDNF dysfunction. First, stress-induced hyperactivity of the HPA axis and resultant increase in glucocorticoid level reduce the BDNF expression. Second, GR, through which glucocorticoid exerts its effects, directly influences the function of the specific receptor of BDNF, TrkB. Many studies demonstrated reduced expression of BDNF in the hippocampus of animals with various kinds of acute and chronic stress (e.g. restraint, footshock, social isolation, social defeat, swim stress, etc.) and early environmental stress (e.g. maternal deprivation) (summarized by Duman and Monteggia, 2006). As mentioned above, stress activates the HPA axis and increases the glucocorticoid level, which in turn decreases BDNF expression in the hippocampus (Hansson et al. 2003, Smith et al. 1995). Recent studies suggest that stress, such as immobilization and social defeat, reduce the expression of BDNF via the mechanism of histone remodeling (Tsankova et al. 2006, Fuchikami et al. 2009). Although many studies have shown that stress and glucocorticoid regulates expression of BDNF, there is little information on whether excessive glucocorticoid impacts BDNF function.

Kumamaru et al. (2008) showed that in immature hippocampal neurons, exposure to the synthetic GR agonist dexamethasone (DEX) inhibited the BDNF-dependent dendrite outgrowth and synaptic formation. As a result, BDNF-induced synaptic proteins, such as NR2A, NR2B, GluR1, and synapsin I, were suppressed by DEX, and the inhibitory action of DEX influenced neuronal function even after the neurons had matured. Their subsequent study elucidated that GR directly interacts with TrkB receptor and promotes BDNF-triggered signaling for glutamate release via glutamate transporter (Numakawa et al. 2009). This raises the possibility that excessive glucocorticoid might decrease the TrkB-bound GR, which leads to decreased BDNF signaling (Figure 5). As the observed functional effects of glucocorticoid and GR were not accounted for by altered expression of BDNF, the findings show that stress-induced excess in glucocorticoid impacts not only on BDNF protein expression but also on function of the BDNF pathway.

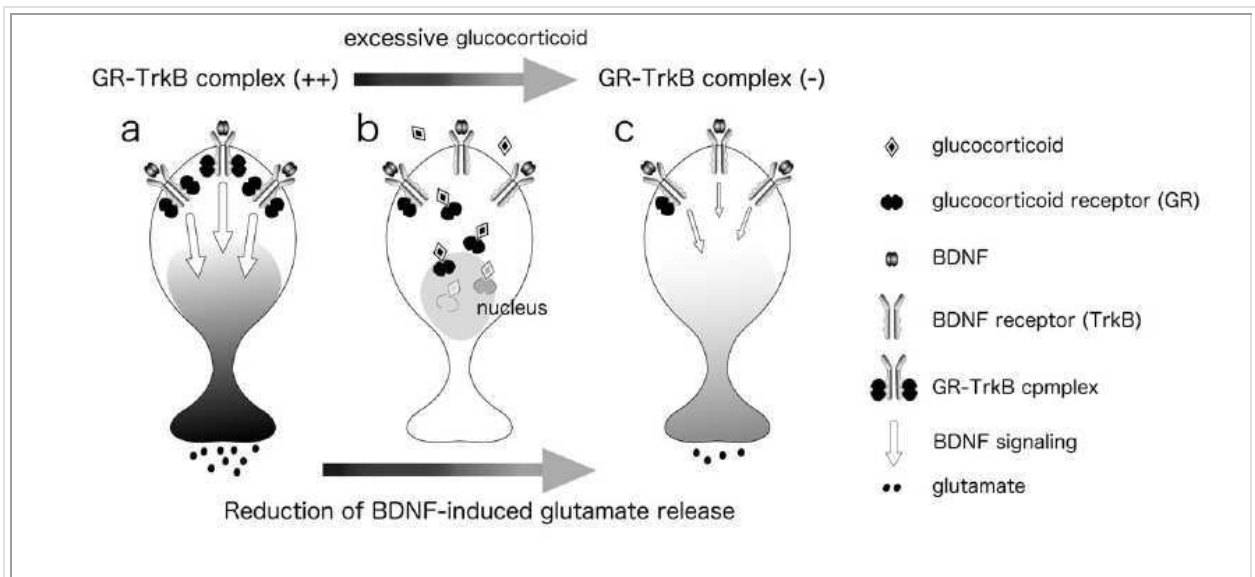


Figure 5: Excessive glucocorticoid might decrease the TrkB-bound glucocorticoid receptor (GR) and decrease brain-derived neurotrophic factor (BDNF) signaling and BDNF-induced glutamate release. (a) When GR-TrkB complex is rich, the BDNF-TrkB signaling for glutamate release is also rich. (b) Exposure to excessive glucocorticoid reduces TrkB-bound GR, which leads to (c) reduced BDNF-TrkB signaling and glutamate release (Kunugi et al. 2010).

From genes to plasticity and back: Current concepts in the pathophysiology of depression

I. Genetics of depression

Classical research on the genetic basis of depression consists of association studies of individual genes with the development of the disease. Epidemiological studies show that 40%-50% of risk for depression is genetic (Fava and Kendler 2000), which makes depression a highly heritable disorder. However, the research of specific, causative genes that underlie depression has not led to consistent results. In light of these problems, studies examining the role of gene-environment interactions on the emergence of particular symptoms or treatment responses have suggested the existence of 'susceptibility' genes rather than causative genes. This term refers to genes that render individuals relatively vulnerable or relatively invulnerable to the pathogenic effects of environmental stress and may thus indicate a higher risk (predisposition) for major depressive disorder (Kendler KS 1998, Aan Het Rot et al. 2009, Lekman et al. 2008). The depressogenic effect of stressful life events is substantially greater in individuals with high versus low genetic risk for major depression.

The most prominent genetic variants that have been linked to increased risk for major depression are related to the monoaminergic (5-HTT, 5-HT R, MAO-A, TPH2, COMT, DAT) and the neurotrophic system (BDNF, p75NTR), but genes related to other pathways have also been identified (see table below).

A relatively recent trend focuses on resilience to stress and "protection" genes. Resilience refers to adaptive coping strategies and capacity to perceive stressful events in a less threatening way, even when challenged by adversity several times. Cognitive reappraisal in order to re-evaluate or reframe adverse experiences in a more positive light and avoid their depressogenic effects is believed to constitute an important mechanism of resilience (Feder et al. 2009). "Susceptibility" and "protection" genes are two sides of the same coin that counts for genetic print x environmental influence and the development of depression.

Genes	Alteration	Role	References
5-HTTLPR (SLC6A4)	Short(S) Vs long (L) allele	Serotonin transporter S allele: higher risk for developing depression under stress conditions	Schinka JA, 2004, Mol Psy Belmaker, Agam 2008
5-HT 1A R	C(-1019)G (rs6295) SNP in the promoter	G allele leads to increased 5HT1A expression leading to reduction of 5-HT neurotransmission Associated to MDD, suicidality, trait anxiety	Lemonde et al. 2003, Strobel et al. 2003 Mann JJ, Currier DM, 2010
TPH	TPH1/2	Converts L-tryptophan to 5-HT TPH2 SNPs associated with major depression (less 5-HT)	Elder BL, Mosack V, 2011
MAO-A	VNTR polymorphism in the promoter MAOA-H vs MAOA-L allele	5-HT, NE degradation MAOA-H: highly active allele	Mann JJ, Currier DM, 2010
COMT	VAL allele	DA, NE degradation	MAssat I, 2005
DAT1 (SLC6A3)	Rs40184	DA transporter moderate the effects of maternal rejection in the onset of MDD	Haefel 2008
BDNF	V66M	Activity dependent secretion of BDNF Met allele: decreased BDNF trafficking	Egan et al. 2003 Lanni et al. 2009
p75NTR	S205L	involved in the pathogenesis of depressive disorder and suicidal behavior	Kunugi et al. 2004
CRHR1	Multiple individual SNPs	Gene x environment moderate the effect of child abuse on the risk for adult depressive symptoms	Bradley et al, 2008
IL-1β	Polymorphisms in the promoter : -511T allele vs -511C carriers	-511T allele trend of less severity of depressive symptoms and more favorable fluoxetine therapeutic response	Yu et al. 2003
TNF-α	-G308A	subjects with MDD had an increased frequency of the TNF2 (A) allele	Jun et al. 2003
NPY	SNP rs16147 in the promoter alters NPY expression	Lower haplotype-driven NPY expression, higher emotion-induced activation of the amygdala, related to trait anxiety and diminished resiliency	Zhou et al. 2008
SERPINE 1	Common SERPINE-1 polymorphisms	Role in MDD susceptibility and in the acute therapeutic response to selective serotonin reuptake inhibitors	Tsai et al. 2008

II. Epigenetic modifications

The term “epigenetics” refers to heritable modifications in gene expression (traits) that do not involve changes of the underlying DNA sequence. Environmental experiences can modify gene expression and thereby protein availability and function through several epigenetic modifications. These modifications (Figure 6) encompass covalent changes to DNA (for example, DNA methylation), post-translational modifications of histone N-terminal tails (for example, acetylation and methylation) and non-transcriptional gene-silencing mechanisms (for example, micro-RNAs) (Tsankova et al. 2007). Given that these changes can be long-lasting, epigenetics have been invoked to explain several aspects of depression, including high discordance rates between monozygotic twins, individual differences among inbred rodents, the chronic relapsing nature of the illness, and the strikingly greater prevalence of depression in women (Mill & Petronis 2007).

The field of depression research has focused on two main chromatin-modifying processes. The first is DNA methylation (of cytosine), which seems to be important in the influence of maternal behaviour on adult emotional processing. Adult offspring of rats born to mothers with low rates of maternal licking and grooming show increased anxiety and reduced expression of glucocorticoid receptors within the hippocampus compared with offspring of mothers with high rates of maternal behaviours. This reduced expression of glucocorticoid receptors is mediated by increased methylation of the glucocorticoid receptor gene promoter (effectively repressing gene expression). This long-lasting ‘molecular scar’ is established within the first week of life and is effectively reversed by cross-fostering (Szyf et al. 2007). Interestingly, this increase in methylation was also reversed by the infusion of trichostatin A, a histone deacetylase (HDAC) inhibitor (Weaver et al. 2004).

The second chromatin modification is histone acetylation. Histone acetylation is associated with transcriptional activation and decondensed chromatin and seems to be a key substrate for antidepressant action (Tsankova et al. 2004). Increased histone acetylation at the *Bdnf* promoter in the hippocampus was shown to be required for the ability of chronically administered imipramine to reverse certain deleterious effects of social defeat (Tsankova et al. 2006). Moreover, histone deacetylase (HDAC) inhibitors show antidepressant-like effects in the social-defeat assay and other behavioural assays (Tsankova et al. 2006, Schroeder et

al. 2007), and efforts are underway to develop more potent agents that are designed to target specific HDACs, such as HDAC5, a class II HDAC. The implications of these studies come with an important anatomical caveat: although inhibiting the actions of HDAC5 in the hippocampus seems to be therapeutically advantageous (Mayberg et al. 2005, Schroeder et al. 2007), mice that are globally deficient in HDAC5 are more vulnerable to social defeat (Renthal et al. 2007). Similarly, although imipramine increases HDAC5 expression in the hippocampus, it significantly reduces HDAC5 expression within the nucleus accumbens, further emphasizing the regional specificity of stress-related and antidepressant-related plasticity.

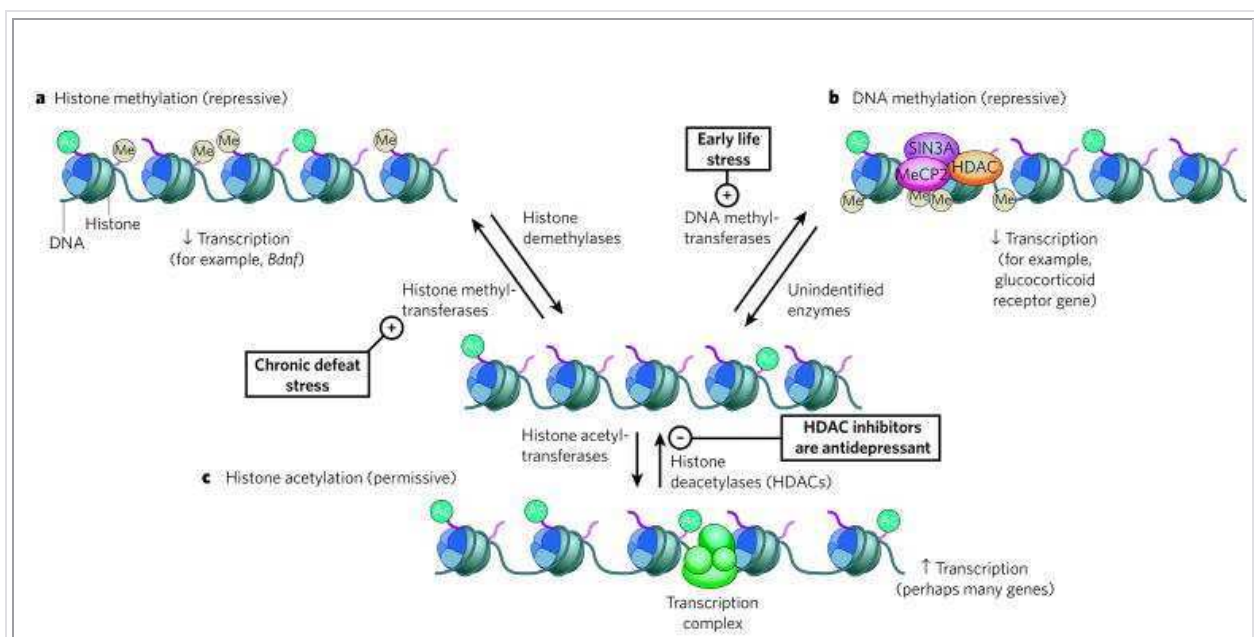


Figure 6: Epigenetic regulation in depression. The transcriptional potential of genes involved in neuroplastic responses to stress or antidepressant treatments can be regulated through chromatin-remodelling events catalysed by specific enzymes. **a**, The methylation of histones on specific lysine residues (for example, Lys 9 and Lys 27) is associated with condensed chromatin (heterochromatin) and is important in the repression of *Bdnf* expression in the hippocampus after social defeat. The pluses and minuses indicate activation or inhibition, respectively, of a particular process. **b**, By contrast, repression of other genes can occur through the methylation of cytosine within CpG islands of promoter regions, attracting proteins involved in transcriptional repression, such as SIN3A, MeCP2 (methyl-CpG binding protein 2) and histone deacetylases (HDACs). DNA methylation of the promoter of the glucocorticoid receptor gene occurs in rat pups born to mothers with inherently low levels of maternal behaviour. Although such methylation events have been reported to be reversible, the enzymes responsible for demethylating DNA have yet to be identified. **c**, Histone acetylation, catalysed by histone acetyltransferases, is associated with decondensed chromatin (euchromatin), increasing the activity of transcriptional complexes. HDAC inhibitors (which activate the expression of numerous genes that have not yet been identified with certainty) show antidepressant properties in several assays. Ac, acetyl; Me, methyl. (Krishnan and Nestler, 2008)

III. Resilience and synaptic plasticity: signal transduction pathways

Delayed therapeutic onset of antidepressant treatments is associated with long-term neuroplastic changes necessary to restore morphological and functional dysfunction of neural networks in the brain during depression. This is achieved by reestablishing proper patterns of gene expression and function to enhance cell growth and neuronal connectivity. Increases in the amount of synaptic monoamines induced by antidepressants activate membrane receptors and second messenger intracellular cascades that in longer timescale produce neuroplastic regulation. Four major signaling pathways have been intensively studied for their role in depression and in antidepressant action: the cyclic adenosine monophosphate (cAMP) and phosphoinositol second messenger systems, the glycogen synthase kinase-3 (GSK-3) signaling pathway, and the mitogen activated protein kinase (MAPK) pathway.

a) The cAMP second messenger system

Several 5-HT and adrenoceptor (AR) subtypes (G-protein-coupled receptors, GPCRs) are coupled to adenylyl cyclase (AC), the enzyme that catalyses the formation of cAMP from ATP. Stimulatory G_s stimulate while inhibitory G_{i/o} proteins inhibit AC. Once formed, cAMP activates its major target cAMP-dependent protein kinase A (PKA), which in turn phosphorylates numerous substrates to regulate gene transcription, neurotransmitter receptor efficacy, and neurotransmitter release (Brandon et al. 1997, Nguyen and Woo 2003). One major substrate of PKA in the CNS is Ser-133 of the cAMP regulatory element binding protein (CREB). Ser-133 phosphorylation activates CREB to promote the transcription of CRE-containing genes that regulate cell proliferation, development, plasticity, and survival. PKA activity and CREB phosphorylation have been reported to increase after chronic antidepressant treatment (Tardito et al. 2006, Thome et al., 2000). CREB is not only activated but also upregulated by chronic antidepressants treatment, in particular in the hippocampus (Nibuya et al. 1996).

Phosphodiesterases (PDEs) downregulate the cAMP system by degrading cAMP to AMP, and the cAMP system is tightly controlled by adjusting the equilibrium between cAMP production and degradation through the regulation of ACs and PDEs (Kamenetsky et al. 2006). One approach to enhance the cAMP pathway is by inhibition of phosphodiesterase (PDE). Indeed, PDE4A and PDE4B may be relevant targets for development of agents that possess antidepressant effects either as monotherapy or in combination with agents that increase intrasynaptic monoamine levels, due to the possible synergism of effects on the cAMP cascade. The idea that PDE inhibitors may have potential antidepressant activity was initially proposed by Wachtel in the early 1980s (Wachtel and Schneider, 1986). In the 1980s and early 1990s, a number of open-label and controlled clinical trials demonstrated that rolipram, a specific inhibitor of the high-affinity cAMP PDE4, may have antidepressant efficacy in depressed patients (reviewed in Manji et al, 2003). In addition, there is some evidence that rolipram may have a faster onset of response compared with standard antidepressants. Despite these data, the potential use of rolipram for depression was limited because of side effects such as nausea and emesis.

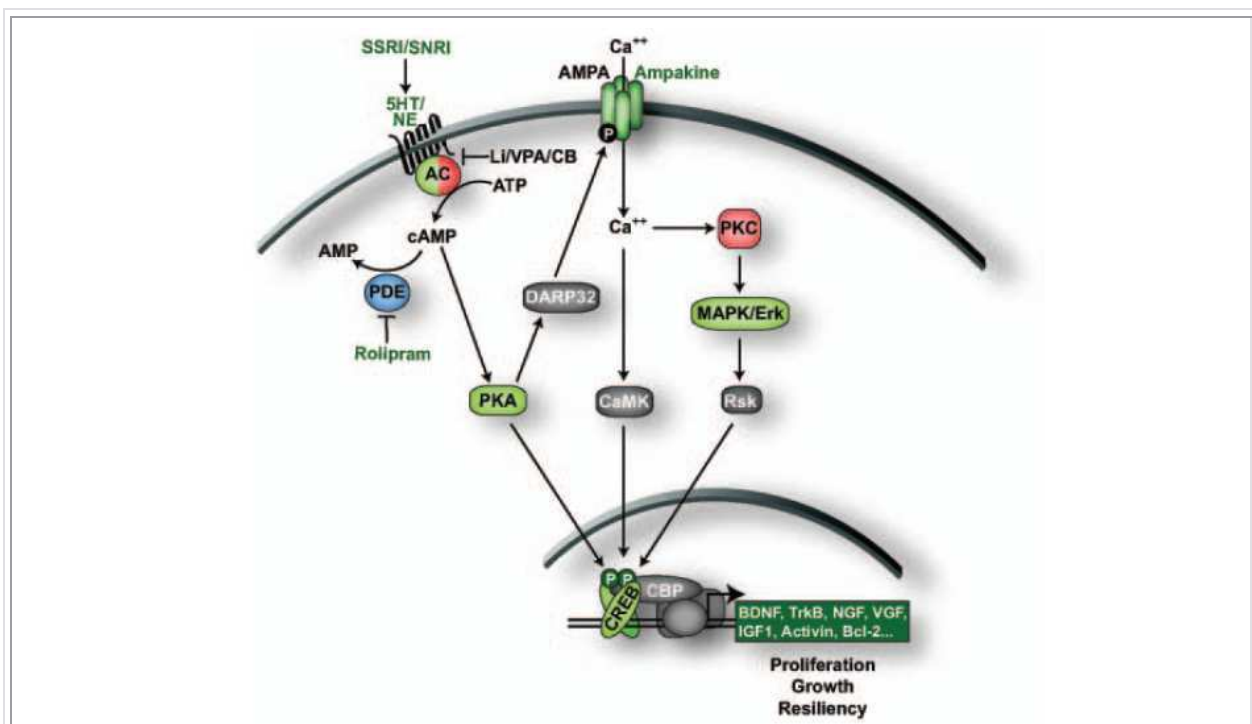


Figure 9: The cAMP second messenger system. Antidepressants upregulate 5-HT and NE signaling to activate the cAMP signaling cascade. cAMP production results in the activation of CREB-mediated gene transcription. PKA activates CREB both directly by phosphorylation and indirectly by triggering Ca²⁺ influx and the subsequent activation of CAMK and ERK. CREB triggers the expression of genes that promote cell proliferation, growth and resiliency, effects that could contribute to the ADT-like effects observed in behavioral models. (Tanis and Duman, 2007)

While monoamine signaling is directly coupled to CREB activation through PKA, antidepressants may also activate CREB via phosphorylation by Ca²⁺-calmodulin-regulated kinases (CAMKs) and/or the MAP kinase ERK. Both CAMKIV and ERK are activated in rat frontal cortex by antidepressants (Tiraboschi et al. 2004), possibly via the dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP32). DARPP32 is phosphorylated and activated by PKA, leading to blockade of protein phosphatase-1 (PP1). Indeed, activation of the 5-HT neurotransmitter system by SSRIs activates DARPP32, and results in increased phosphorylation of AMPA subunit GluR1, an event that enhances AMPA conductance (Alt et al. 2006, Svenningsson et al. 2002a, b). The antidepressant effects of AMPA potentiators may also be due to regulation of CREB-mediated gene transcription via activation of CAMKs (as previously mentioned).

b) The phosphoinositol cycle

Another second messenger pathway, mostly implicated in the action of mood stabilizers like lithium, is the phosphoinositol cycle: phospholipase C (PLC) after activation of GPCRs and Trks hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol tris-phosphate (IP₃) and diacylglycerol (DAG) (Figure 9) (Huang and Reichardt 2003, Rhee SG. 2001). IP₃ promotes the release of Ca²⁺ from intracellular stores, resulting in the activation of CAMK, and the subsequent phosphorylation of CREB. Ca²⁺ release, as well as DAG, activates multiple protein kinase C (PKC) isoforms which then translocate to their active sites where they associate with isoform-specific proteins such as receptors for activated C-kinase (RACKs). PKC isoforms phosphorylate a diverse set of substrates to regulate neurotransmission, neuronal excitability, development, and gene expression (Battaini F 2001, Keenan and Kelleher 1998).

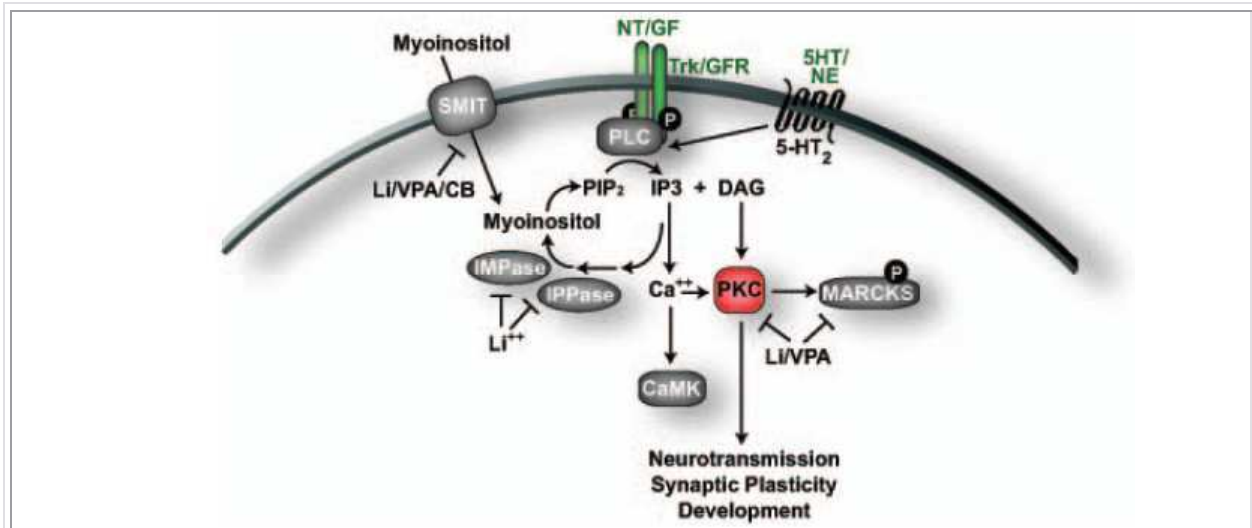


Figure 10: Phosphoinositol signaling. Following activation by growth factor and G-protein-coupled receptors, PLC catalyzes the production of IP3 and DAG to trigger release of Ca²⁺ from intracellular stores, and the activation of PKC to regulate neurotransmission, synaptic plasticity, and development. Mood stabilizers inhibit multiple aspects of this pathway, including influx of myo-inositol through SMIT, recycling of IP3 into PIP2, and the activity of PKC. (Tanis and Duman, 2007)

c) The GSK-3 signaling pathway

GSK-3 activity is linked to the activation of transcription factors that promote cell cycle arrest and apoptosis (Grimes and Jope 2001, Linseman et al.2004, Watcharasit et al.2002). Conversely, GSK-3 activity is directly or indirectly linked to the downregulation of b-catenin, Jun, CREB, heat shock factor-1 (HSF-1), and nuclear factors of activated T cell (NFAT), transcription factors that regulate cell proliferation, growth, resiliency, and immune responses (Grimes and Jope 2001). One of the best-characterized downstream targets of GSK-3 is b-catenin, which modulates cell adhesion in the cytoplasm and also translocates to the nucleus to activate transcription of T cell-specific transcription factor, lymphoid enhancer factor (TCF/LEF) target genes. Phosphorylation of b-catenin by GSK targets it for ubiquitin-dependent degradation, downregulating TCF/LEF gene transcription.

Numerous cellular stimuli converge to downregulate GSK-3, which is generally active under basal conditions (Figure 10). Wnt glycoproteins bind to Frizzled receptors, which leads to regulation of Disheveled and inhibition of GSK-3. Multiple kinases, including AKT, PKA, PKC, and RSK, phosphorylate GSK-3 to inhibit its kinase activity in response to cellular stimuli. Activation of neurotrophic factor, growth factor, and select GPCRs (such as 5-HT1A) recruits

and activates PI3K that in turn activates AKT that then phosphorylates and inactivates GSK-3. GSK-3 is reactivated following dephosphorylation by PP1 (Katso et al. 2001, Raymond et al. 2001, Bennechib et al. 2000). GSK-3 activity is downregulated by a wide variety of ADTs and mood stabilizers and antipsychotics. Increased serotonin activity mediated by antidepressants has been shown to increase GSK-3 inhibitory phosphorylation (Roh et al. 2005, Li et al. 2004). ECS increases AKT activity, GSK-3 phosphorylation, β -catenin levels and the expression of Wnt-2 (Madsen et al. 2003, Kang et al. 2004, Roh et al. 2003). Direct inhibition of GSK-3 activity elicits ADT and antimanic properties in animal models. Systemic administration of selective GSK-3 inhibitors or heterozygous deletion of GSK-3 produced ADT effects in the forced swim test (Kaidanovich-Beilin et al. 2004, Gould et al. 2004, O'Brien et al. 2004).

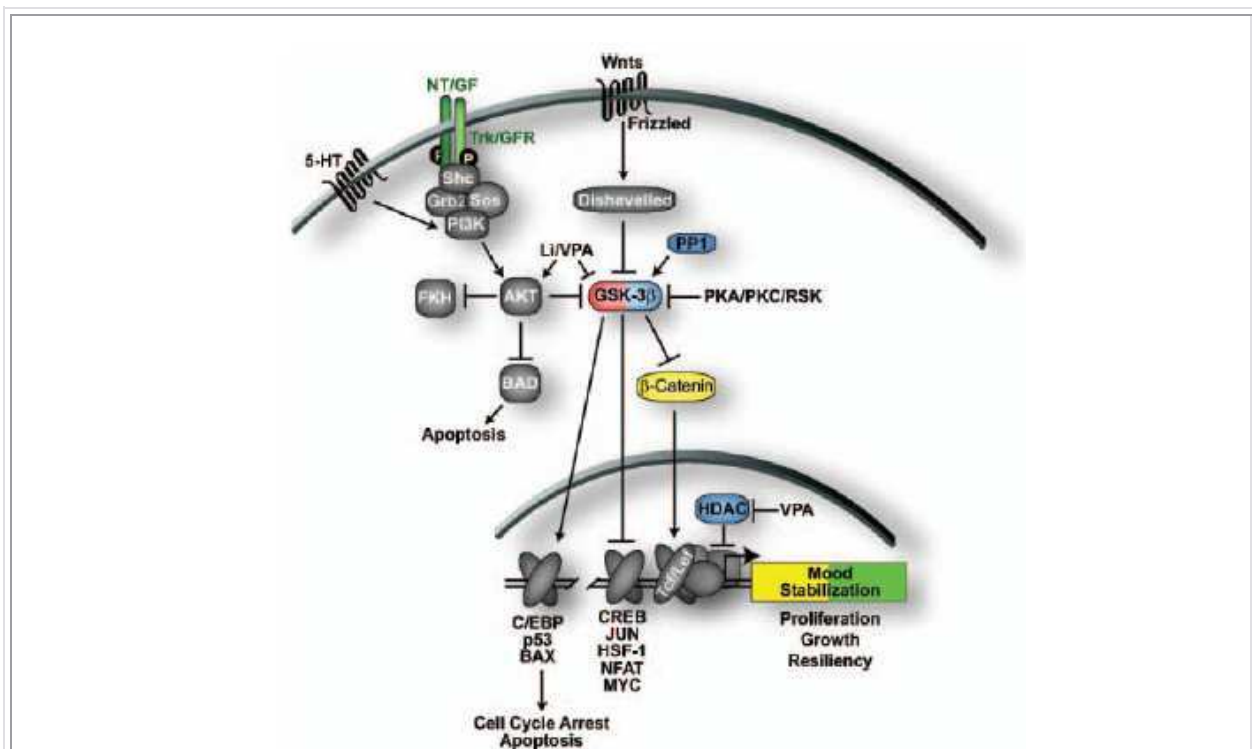


Figure 11: The GSK-3 signaling pathway. GSK-3 regulates multiple transcription factors to trigger cell cycle arrest and apoptosis, while inhibiting the transcription of genes that promote cell proliferation, growth and resiliency. Wnt glycoproteins inhibit GSK-3 activity through interactions with Dishevelled while receptor tyrosine kinases and select G-protein-coupled receptors activate PI3K, which activates AKT to phosphorylate and inhibit GSK-3. AKT also inhibits apoptosis through phosphorylation of Bad, a proapoptotic protein. (Tanis and Duman, 2007)

d) Mitogen-activated protein kinases (MAPKs)

Sequential activation of protein kinases (protein kinase cascades) is a common mechanism of signal transduction in many cellular processes (Campbell et al. 1995). During the past years several intracellular signaling cascades have been elucidated, including the central group of the mitogen-activated protein kinase (MAPK) signaling cascades (Seger et al. 1995, Chang and Karin 2001, Pearson et al. 2001, Johnson and Lapadat 2002). Each MAPK cascade is activated either by a small GTP-binding protein (smGP; Ras family protein) or by an adaptor protein, which transmits the signal either directly or through a mediator kinase (MAP4K) to the MAPK kinase kinase (MAP3K) level of the cascade (Figure 12). Subsequently, the signal is transmitted down the cascade by enzymes located at the following tiers, which are referred to as MAPK kinase (MAPKK), MAPK, and also MAPK-activated protein kinase (MAPKAPK). The core cascade is usually composed of MAP3K, MAPKK, and MAPK, whereas the components in the other tiers are not always involved. The existence of three or more tiers in each of the MAPK cascades is probably essential for signal amplification, specificity determination, and tight regulation of the transmitted signal. The four distinct MAPK cascades currently known are named according to the subgroup of their MAPK components: (1) extracellular signal regulated kinase1 and 2 (ERKs); (2) c-Jun N-terminal kinase (JNK), also known as stress activated protein kinase 1 (SAPK1); (3) p38MAPK, also known as SAPK2-4 or p38 α - δ ; and (4) ERK5, also known as Big MAPK (BMK).

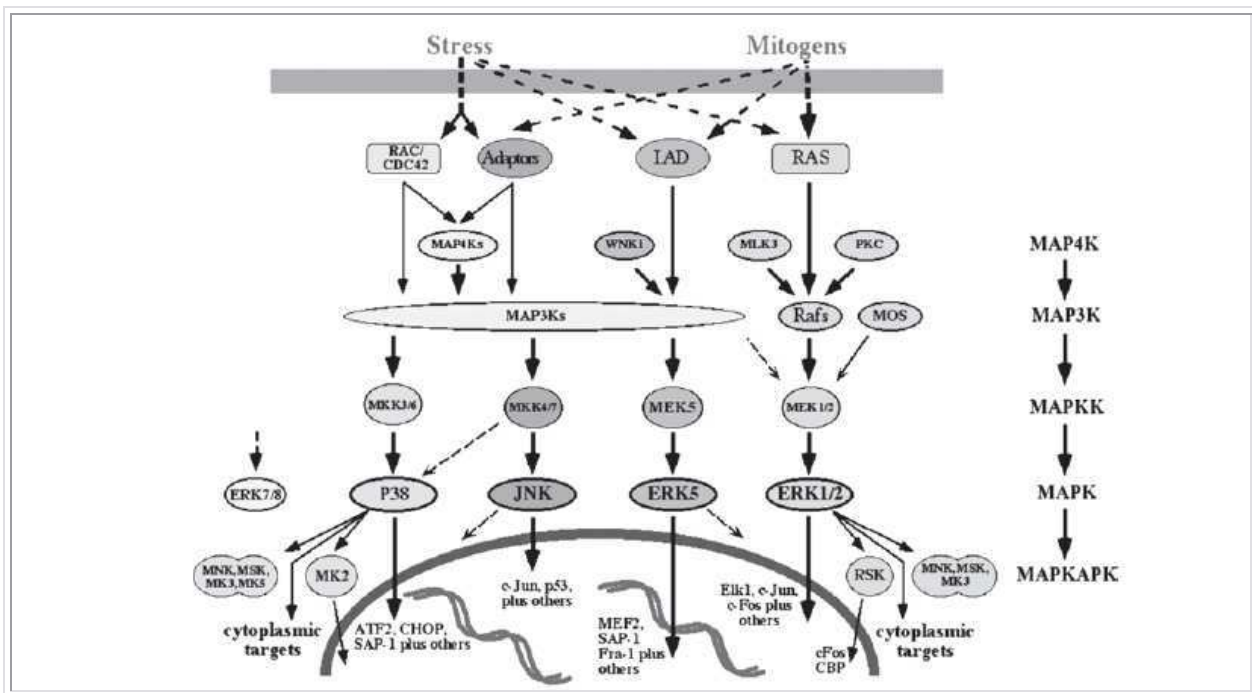


Figure 12: Schematic representation of the MAPK signaling cascades (Rubinfeld and Seger 2005).

ERK1/2 signaling cascade

ERKs were the first MAPKs to be described in mammals (Rossomando et al. 1989). Two isoforms, ERK1 and ERK2, also known as p44 and p42 respectively, are expressed in mammalian cells with 83% of sequence identity. The functions attributed to ERK1/2 at both the cellular and physiological levels are diverse (Lloyd 2006). ERK1/2 modulates cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, GAP junction formation, actin and microtubule networks, neurite extension and cell adhesion. Physiologically, ERK1/2 is required for immune system development, homeostasis and antigen activation, memory formation, heart development, and the response to many hormones, growth factors and insulin (Ramos JW 2008).

In neurons ERK1/2 is activated by a plethora of extracellular signals including neurotrophins, monoamines, glutamate, cytokines and CRF. The binding of neurotrophins (NT-3/4, NGF, BDNF) to receptor tyrosine kinase (Trk) induces dimerization and autophosphorylation of Trk at tyrosines (pTy). The autophosphorylation and activation of Trks leads to recruitment of adaptor proteins to activation site on the cell membrane. These proteins include Grb2, which in turn will bind son of sevenless, drosophila homolog 1 (SOS) that stimulates the exchange of guanine diphosphate (GDP) bound to Ras for GTP, and Ras then initiates a protein kinase cascade that includes, sequentially, Raf, MEK1/2 and ERK1/2 (Figure 13). ERK activity is terminated by the dephosphorylation of the Thr-Glu-Tyr motif by specific phosphatases like mitogen-activated protein kinase phosphatases (MKPs) and protein serine/threonine phosphatase 2A (PP2A) (Bhalla et al. 2002) (Figure 14).

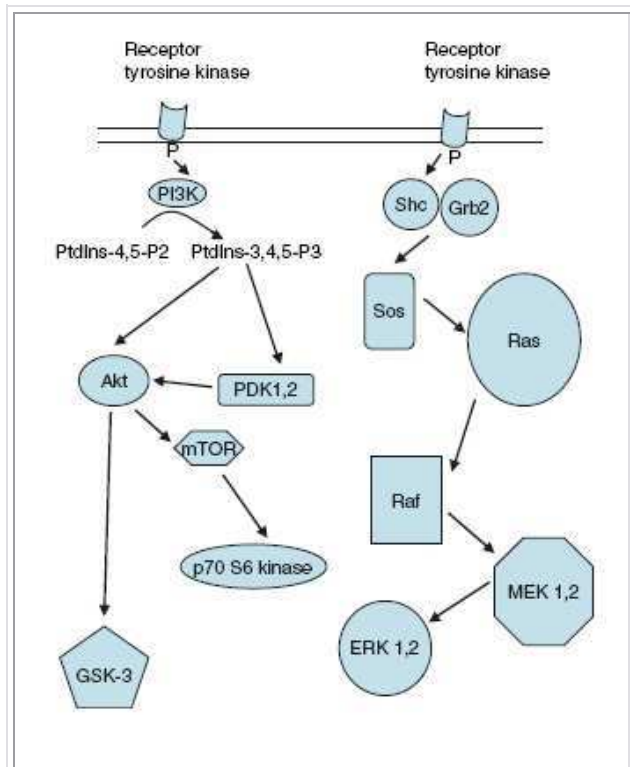


Figure 13: Pathways for activation of ERK and Akt by tyrosine kinase receptors. Receptor tyrosine kinases, activate ERK through a pathway utilizing Ras, Raf, and MAP kinase kinase (MEK). Activation of Akt proceeds through a pathway requiring PI3K and the phosphoinositide dependent protein kinases (PDK) 1 and 2. Subsequent activation of p70 S6 kinase can occur with activation of mTOR (Cowen DS 2007).

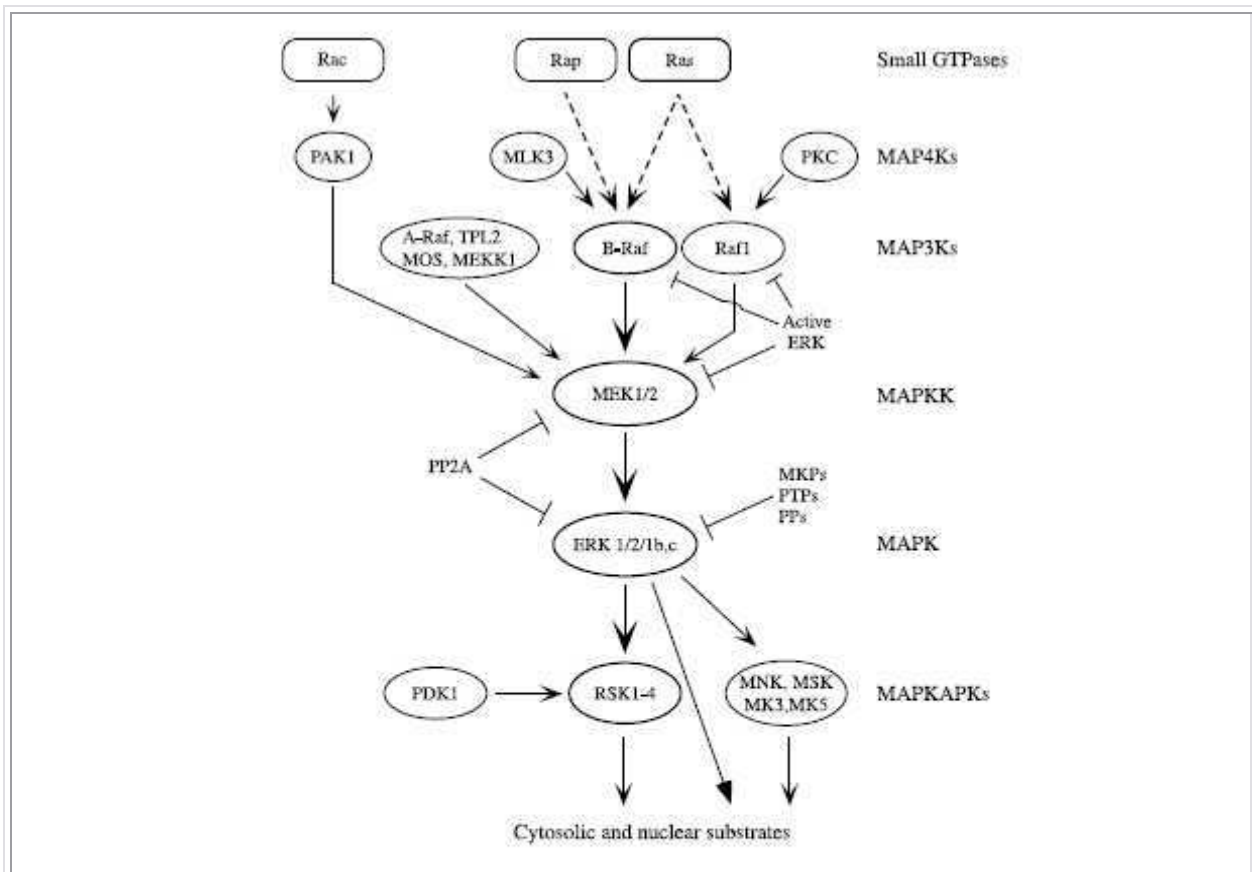


Figure 14: Schematic representation of the ERK signaling cascade. Activation and inactivation processes are indicated. Dashed lines indicate indirect activations, and dark arrow stand for the main pathway upon growth factor activation. The phosphorylation by PAK (on Ser298) increases MEK-ERK interaction, which facilitates the kinetics of ERK activation. It can be inhibited by a feedback phosphorylation by ERK (on Thr292). ERK also negatively regulates B-Raf and Raf1 by a feedback phosphorylation (Yoon and Seger 2006).

A significant role of ERK1/2 in the control of synaptic plasticity and memory in the adult brain has been established in behavioral and cellular studies (reviewed in Sweatt 2001, 2004, Thomas and Huganir 2004). ERK1/2 activated by glutamate receptor signals show a strong influence over gene expression and thereby exerts a transcription-dependent regulation of synaptic plasticity (long-term potentiation, LTP and long-term depression, LTD). The Ca²⁺-permeable ionotropic glutamate receptors, NMDA, AMPA and kainate receptors, activate ERK1/2 through a biochemical route involving the Ca²⁺-sensitive Ras-guanine nucleotide releasing factor, Ca²⁺/calmodulin-dependent protein kinase II, and phosphoinositide 3-kinase (Figure 15). The metabotropic glutamate receptor (mGluR), however, activates ERKs primarily through a Ca²⁺-insensitive pathway involving the transactivation of receptor tyrosine kinases and the synaptic adaptor protein Homer family (reviewed in Wang et al. 2007). Apart from transcriptional-dependent regulation, ERK1/2 cascade also mediates

synaptic AMPA-Rs delivery and removal during LTP and LTD respectively (Stornetta and Zhu 2011).

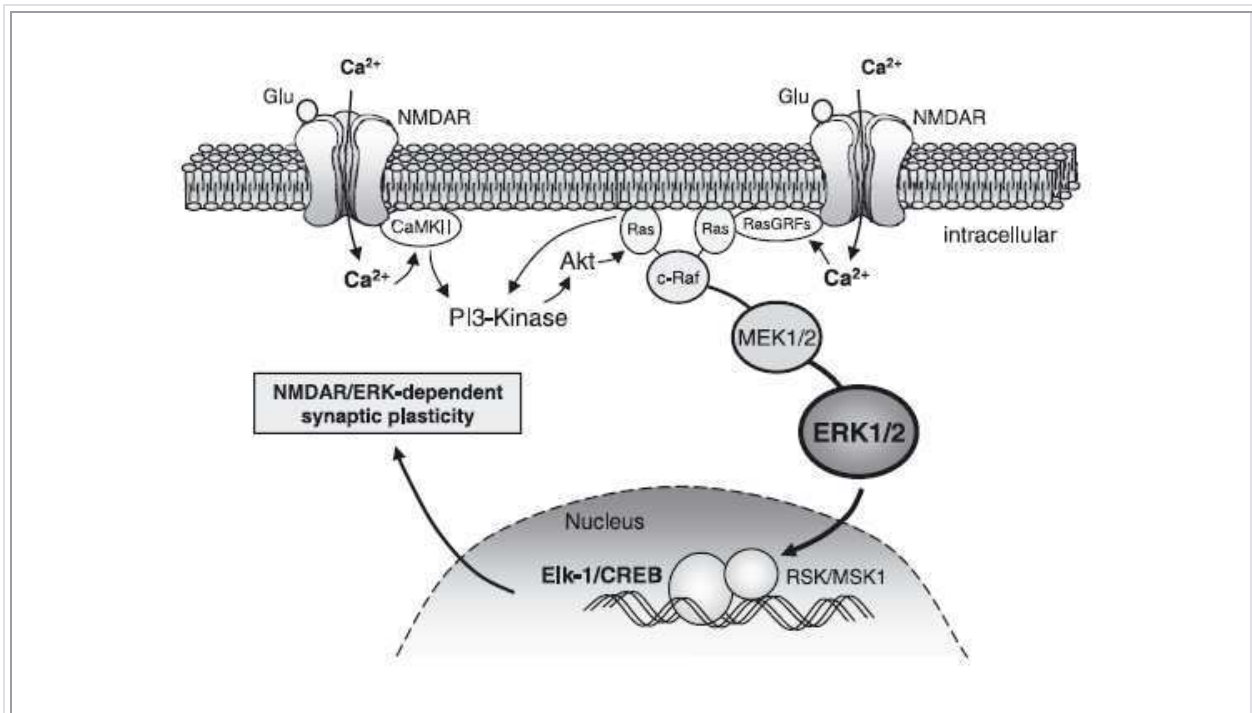


Figure 15: A schematic diagram illustrating signaling pathways from NMDA receptors (NMDAR) to Ras-ERK cascades in neurons. Glutamate (Glu) activation of NMDARs triggers Ca²⁺ influx, which activates CaMKII and PI3-kinase. Active PI3-kinase then activates Ras through its protein kinase activity involving Akt kinase. Alternatively, NMDARs activate Ras through a direct interaction with RasGRFs. Once activated, ERK translocates to the nucleus to activate the transcription factors Elk-1 and CREB through RSK and MSK1. The NMDAR/ERK-dependent gene expression then contributes to the development of various forms of synaptic plasticity. Abbreviations: CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; MEK1/2, mitogen-activated protein kinase kinase 1 and 2; MSK1, mitogen- and stress-activated protein kinase-1; PI3-kinase, phosphoinositide 3-kinase; RasGRF, Rasguanine nucleotide releasing factor; RSK, ribosomal S6 kinase (Wang et al.2007).

Increasing evidence also shows that glutamate receptor-dependent activation of the ERK1/2 pathway is critical for the development of striatal neuronal plasticity and is an important molecular mechanism for the long-lasting behavioural plasticity induced by drugs of abuse. Drugs of abuse like cocaine, D-amphetamine, morphine and nicotine activate ERK1/2 pathway through dopamine D1 and glutamate receptors in a subset of neurons in brain reward circuits (Girault et al. 2007). ERK1/2 drug-induced activation also interferes with drugs rewarding effects, craving and relapse, drug-associated memory consolidation and reconsolidation. ERK1/2 is also activated by non-addictive drugs, like antipsychotics and antidepressants, with distinct regional patterns in the mouse brain (Valjent et al. 2004). Addictive drugs strongly activate ERK1/2 in nucleus accumbens, lateral bed nucleus of the

stria terminalis, central amygdala and deep layers of prefrontal cortex, while antidepressants activate ERK1/2 in hippocampus and cerebral cortex.

Apart from dopamine, norepinephrine and serotonin have been shown to induce activation of the ERK1/2 intracellular pathway directly or indirectly through the glutamatergic and neurotrophic pathways (Cowen DS. 2007). It has been hypothesized, that the actions of 5-HT may be mediated indirectly through increased synthesis of peptide growth factors. However, there is increasing evidence that some subtypes of 5-HT receptors can directly couple to activation of the ERK and Akt pathways. Such coupling suggests a more direct potential role for 5-HT in mediating the long-term actions induced by antidepressants. Finally, CRF receptors have been proposed to act via the PLC-PKC and ERK-MAPK cascades, although their dominant mode of signaling is the Gs-coupled AC-PKA cascade (Hauger et al. 2009).

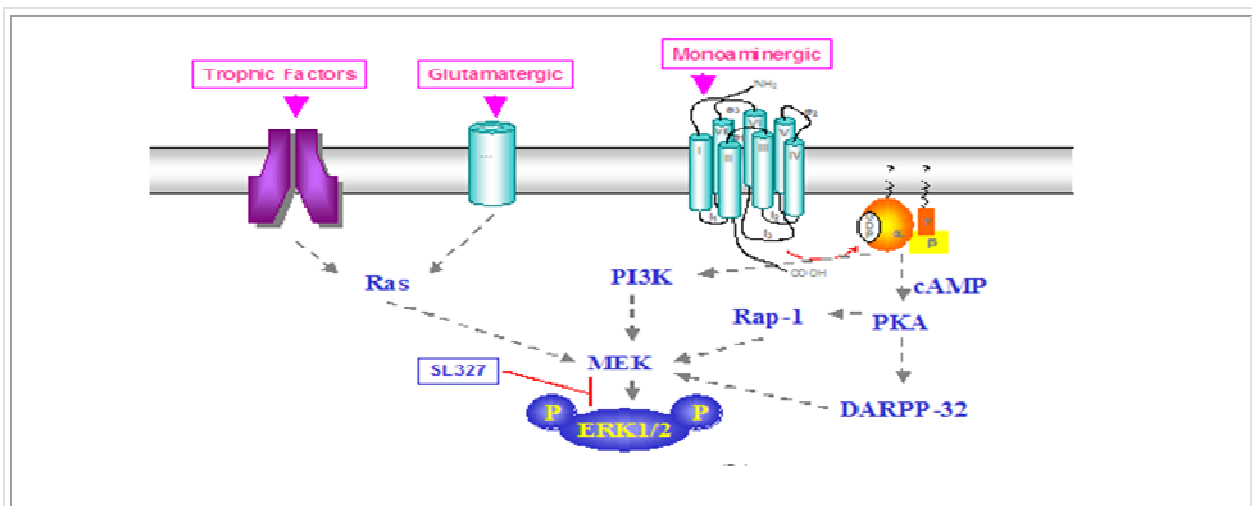


Figure 16: A simplified diagram illustrating signaling pathways relevant to depression and antidepressant action that activate ERK1/2 in neurons.

AIM OF THE STUDY

Multiple overlapping physiological systems are implicated in the pathophysiology of depressive disorders, as previously discussed. Scientific research, although advanced, has not yet completely elucidated the exact mechanisms underlying depression. Today, the delayed onset of action and the inefficiency of monoaminergic-based treatment to treat a significant proportion of patients highlight the need to develop new drugs with a more specific mode of action.

Plasticity-related mechanisms are currently considered to play a major role in both pathology of depression and antidepressant activity. We focused our attention in elements that govern cellular and synaptic plasticity, namely the AMPA receptor and the ERK signaling cascade. The work presented here is thus divided in two major parts.

The objective of the first part was to examine the potential antidepressant effects of AMPA R potentiation in a chronic animal model of depression.

The second part of this study is focused in ERK signaling and the role of molecular partners downstream of ERK (a) in the response to stress and (b) in depressive-like states.

Each topic will be discussed more in details at the beginning of the equivalent part.

MATERIALS AND METHODS

Behavioural tests

Animals

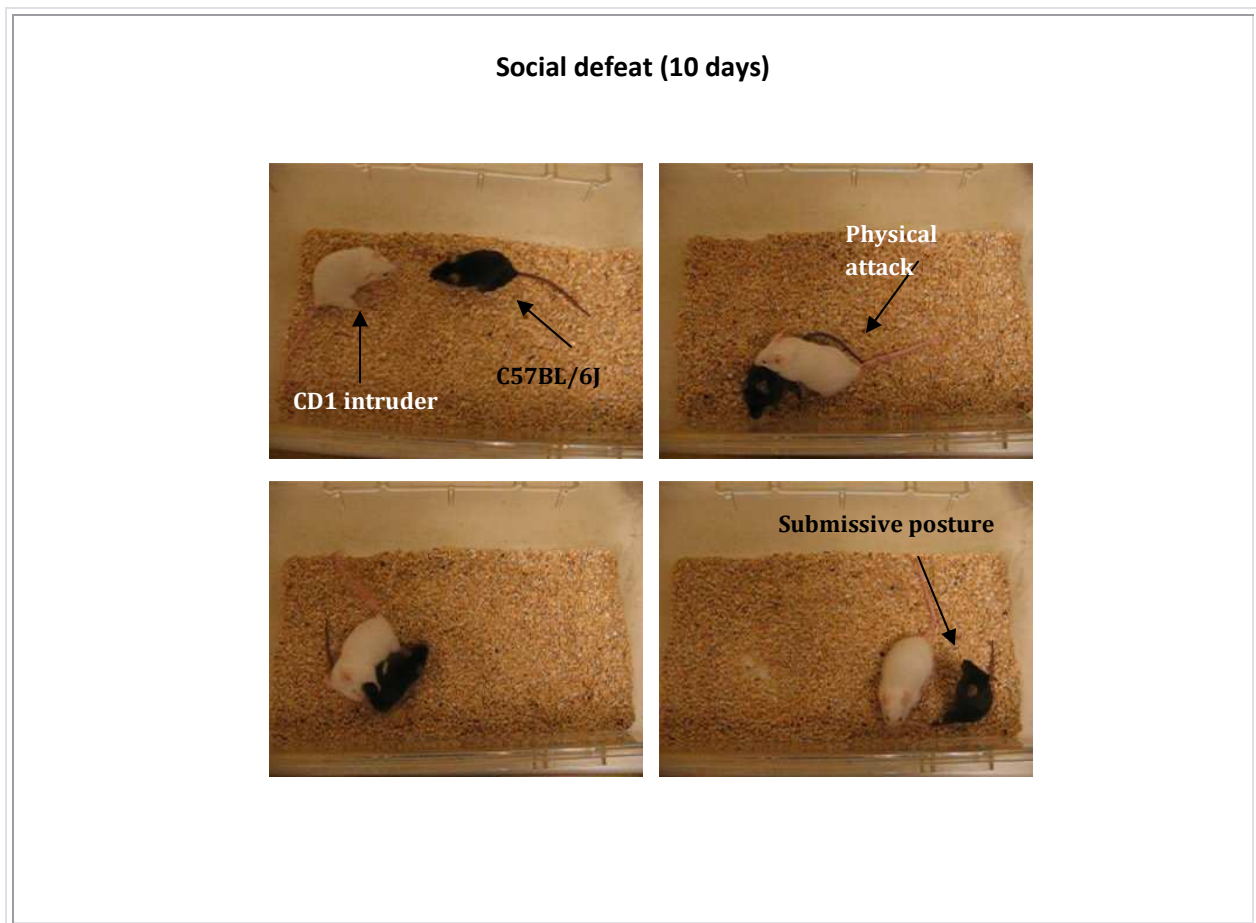
Adult male C57BL/6J or BALB/c mice (6 to 8 weeks old) were purchased from Charles River (France). Animals were housed five per cage (except for chronic stress protocols where mice were housed singly) under standard housing conditions with free access to food and water.

I. Models of depression

a) Social defeat stress

We used an adapted protocol from Berton et al. 2006 that we first validated in the laboratory. Experimental mice were submitted to social defeat stress for 10 consecutive days. Every day, each experimental mouse was introduced into the home cage of an unfamiliar resident and was physically defeated. Resident mice were CD1 breeders selected for their attack latencies reliably shorter than 30 sec upon 3 consecutive screening tests. After 1 min of physical attack (Figure 1), residents and intruders were maintained in sensory contact for 24 hr using a perforated plexiglass partition dividing the resident home cage in two halves. Every day experimental mice were exposed to a new resident home cage. Control animals were housed by pair, one on each side of a perforated plexiglass partition, and were handled daily.

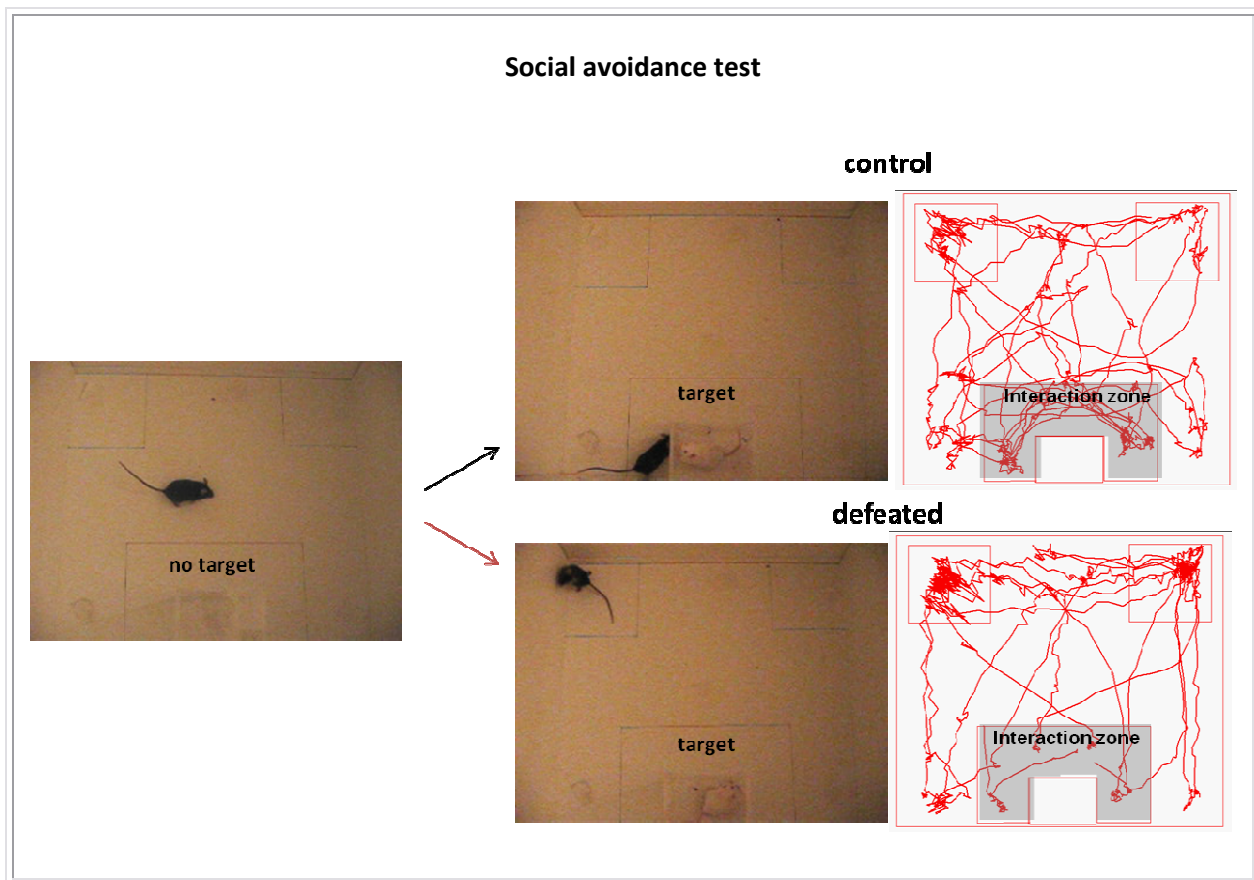
Figure 1



Social approach-avoidance test

A videotracking system was used to score approach-avoidance behaviors toward an unfamiliar social target. The arena was a white open field (42 x 42 cm). Each experimental mouse was introduced into the open field and its trajectory was tracked for two consecutive sessions of 2.5 min. During the first session (“no target”) the open field contained an empty perforated plexiglass cage (10 x 6.5 cm) located at one end of the field. During the second session (“target”), the conditions were identical except that a social target animal (an unfamiliar CD1 male mouse) had been introduced into the cage (Figure 2). Between the 2 sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately one min. The videotracking data from both the “no target” and “target” conditions were used to determine the time spent by the experimental mouse in the “interaction zone” (a 8 cm wide corridor surrounding the cage) and the “corners” of the open field opposite to the location of the cage.

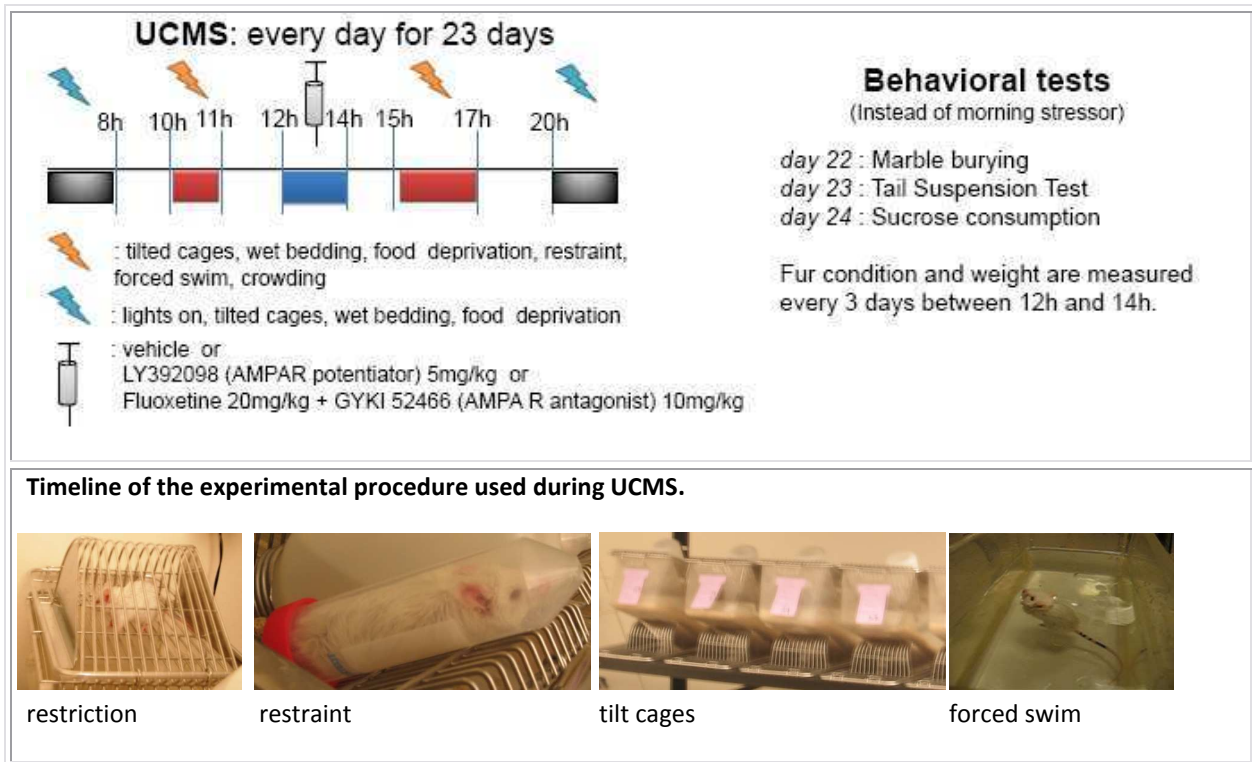
Figure 2



b) Unpredictable chronic mild stress

After a two week acclimation period BALB/c male mice (8 weeks old) were individually housed and subjected to a three week mild stress protocol. Stressors, typically wet bedding, tilted cages, lights on at night, crowding, restraint, food deprivation, forced swim, were applied twice a day for a two hour period and overnight in a randomized order during 21 days (Figure 3). Control (non-stressed) mice were held in a room next to that of the stressed mice. During this period the animal's weight and fur condition were measured every three days. At the end of the chronic stress protocol the emotional state of the animals was evaluated in the tail suspension (TST), the marble burying, and the sucrose preference tests.

Figure 3



II. Tests of high face validity

Sucrose consumption

For the sucrose preference test mice were first habituated to drink from two graduated pipettes one filled with water, and the other with sucrose solution for 3 d, the side of the sucrose pipette being alternated each day. On day 4 and after an overnight (15h) deprivation of water, the two pipettes were presented again; however, one was filled with water and the other with 4% sucrose (Figure 4).

Fur condition rating

The score of the animal's coat state was calculated as the sum of seven scores, one score for each of seven parts of the body (Surget et al. 2008) with some modifications. Namely, the state of each part was rated on a 3-point scale (3, very good; 2, fair; 1, poor).

Marble burying test

The marble burying test (Li et al. 2006) was performed in cages 10 x 15 x 20 cm filled up to the 7,5 cm mark with sawdust on top of which were placed 12 marbles evenly spaced in 4 rows and 3 columns (Figure 4). The number of marbles buried was counted for every minute up to the first 10 minutes and then for every 5 minutes up to 30 minutes. Buried marbles were defined as being buried by at least three quarters of bedding.

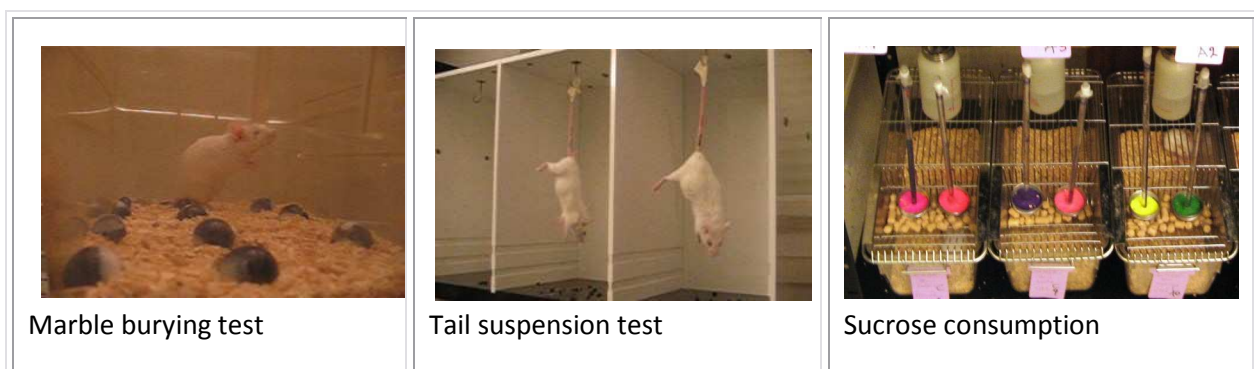
Locomotor activity

Horizontal activity (ambulations) was assessed in transparent activity cages (20x15x25 cm), with automatic monitoring of photocell beam breaks (Imetronic, France). The mice were placed into the recording chamber at 19:00 hours and locomotor activity was recorder for a 1-h period.

Elevated plus maze test

The elevated plus maze test was conducted in an apparatus consisting of a central platform (7x7 cm), two open and two closed arms (30x7 cm), located at a height of 55 cm above the floor, under a 50 lux illumination. Mice were placed in the central platform and time spent and entries in enclosed and open arms were measured in a single 5min session.

Figure 4



III. Tests of high predictive validity

Tail Suspension Test (TST)

Behavioral despair was investigated in the TST as previously described (Crozatier et al. 2007) using an automated TST apparatus (Bioseb, Chaville, France). Mice were individually suspended by their tail using a paper adhesive tape placed 1 cm from the tip of the tail and immobility (seconds) was automatically recorded during the 6-min test period (Figure 4).

Forced Swimming Test (FST)

Behavioral despair was also investigated in the FST (also called the Porsolt test) to measure the potential antidepressant effect of TAT-DEF-Elk1 peptide. The forced swimming test was conducted in clear plastic cylinders (diameter 20 cm; height 25 cm) filled with 6 cm of water (22–25°C) for 6 min. The duration of immobility was measured manually during the last 4 min of the 6-min trial. A mouse was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water.

Novelty Induced Hypophagia

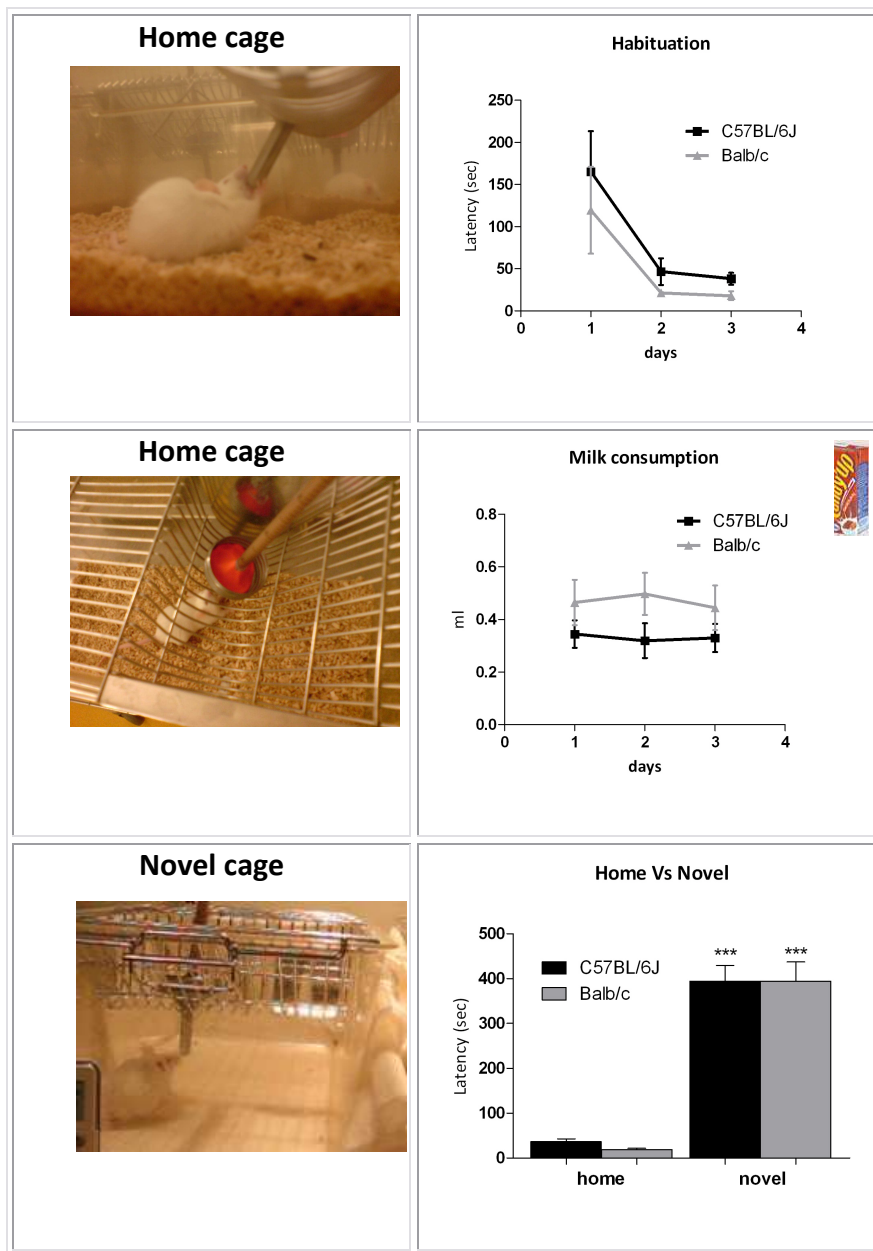
Novelty induced hypophagia is a test conceived to evaluate the anxiolytic effects of antidepressants and their onset of action with a strong predictive and construct validity. During this test animals have to make a choice between novelty stress and palatable food consumption. We used an adapted protocol from Dulawa and Hen 2005. Mice are trained to drink chocolate milk for 3-4 consecutive days. Mice are presented with diluted (1:3; milk: water) chocolate milk (Candy up) for 30min each day. Milk is presented in 5 ml serological pipettes adapted on water bottles' sippers with modeling clay. Pipettes are closed with parafilm and positioned through wire cage lids.

Home cage testing occurred during habituation when mice were briefly removed from their cages to position pipettes containing milk, and testing began when mice were returned to their cages. The latency to drink, and the volume consumed were recorded. Home cage testing occurred under dim lighting (approx. 50 lux). Novel cage testing consists of mice placed into new clean cages of bigger dimensions but without shavings, with pipettes containing the milk positioned. Novel cage testing occurred under bright lighting (approx.

1200 lux), with white paper placed under cages to enhance aversiveness and the latency to drink was measured during 10min.

I have used two mice strains during the validation of this protocol in the laboratory. Differences in habituation and milk consumption were observed as depicted in Figure 5.

Figure 5

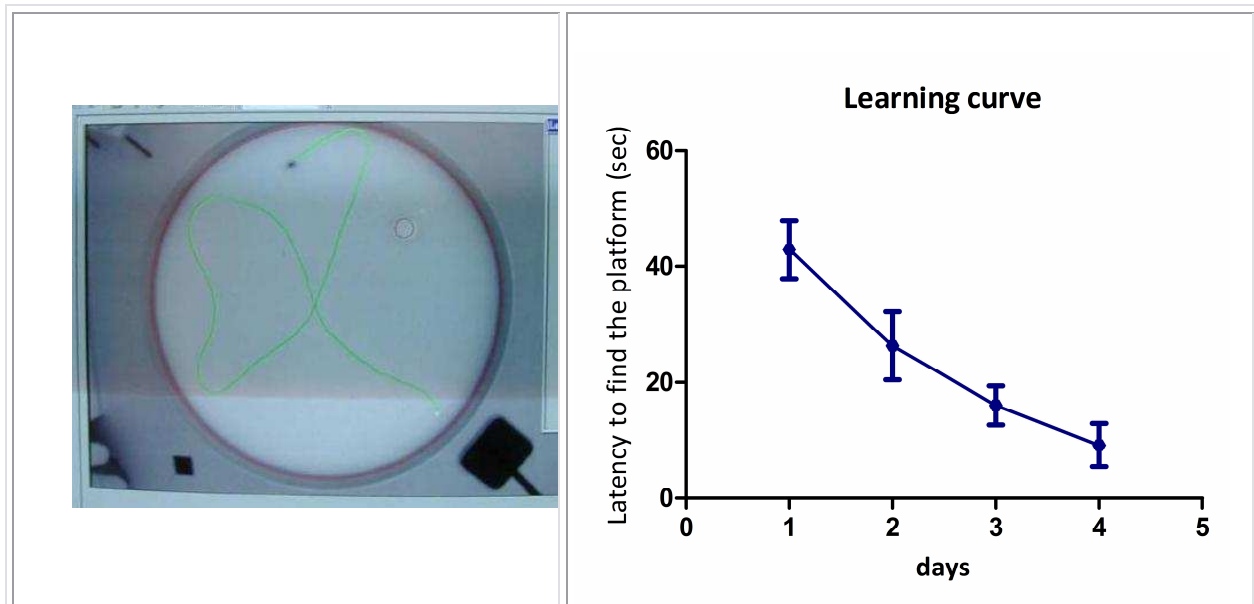


IV. Cognitive testing

Water maze

The Morris water maze is classically used to assess spatial cognitive performances in rodents (Morris, 1984). The experimental setup consists of a 1,5m diameter pool filled up to 20cm with opaque water (white color, Acusola OP 301 pacifier, Rohm Ihaas, France) and several extra maze visual cues. In the spatial version, mice are trained to swim to an escape platform placed at a constant location that could be located only by learning spatial relationships among distal extra maze cues. The platform is invisible as submerged in the opaque water. After several sessions (6sessions/day for 3days and then 3sessions/day for 4 days) of 90sec each animals learn to locate directly the platform (learning curve, last 4 days are depicted in Figure 6). During the probe trial, platform is completely removed from the pool and time spent in the four quadrants is automatically recorded by a video tracking system (View Point, France).

Figure 6



Latent learning

We used an adapted protocol from Noda et al. 2001 in an open field (30X50X15cm high) with an alcove (10X10X10 cm) in the middle of one of the long walls of the enclosure. A drinking tube, identical to those used in the home cages, was inserted into the center of the alcove ceiling with its tip 7 cm above the floor (Figure 7). The test consisted of two trials: a training trial (the 1st day) and a test trial (the 2nd day). In the training trial, mice were placed individually into one corner of the open field of the apparatus. Each mouse was allowed 5 min to explore the environment (open field + alcove). After exploration, mice were immediately returned to their home cages. Mice were then deprived of water for 15h (over-night) and tested the next day 24h after the training trial. In the test trial, mice were again individually placed in the test apparatus. The time taken to drink water from the drinking tube was manually recorded as the drinking latency. Non-trained mice (naive mice) were prepared for comparison with the trained mice (control mice) in terms of their ability to find the water source in the same environment.



Biochemical Approaches

I. Immunohistochemistry

Mice were anesthetized by sodium pentobarbital and perfused transcardially with 4% paraformaldehyde (PFA) in PBS solution, delivered with a peristaltic pump at 20 ml/min during 5 min. Brains were removed and post-fixed in the same fixative solution overnight. Sections (30 μ m) were cut with a vibratome (Leica) and then kept in a solution containing 30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, and 0.1% diethylpyrocarbonate (DEPC; Sigma Aldrich) at -20°C until they were processed for immunohistochemistry. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5 containing NaF 0.1 M) and incubated for 15 min in 0.2% Triton X-100 in TBS. After rinses, sections were saturated for 1 h with 3% BSA, 0.2% Triton in TBS. Sections were then rinsed in TBS and incubated overnight at 4°C with primary antibodies: phospho-p44/42 MAP Kinase (Thr202-Tyr204) and Phospho-MSK1 (Thr581) (1:500, Ozyme); p-Elk-1 (B-4), Egr-1 (588) and c-Fos (4) (1:200 Santa Cruz); overnight at 4°C in TBS. After rinses, sections were incubated for 2 h at RT with the following secondary antibodies: Cy3- conjugated anti-rabbit (1:500, GE Healthcare) or anti-mouse (Jackson ImmunoResearch 1:400). Nuclei were then counterstained with Hoechst (Invitrogen) and sections mounted under coverslips with Mowiol.

II. In situ hybridization

Mice were sacrificed and brains were rapidly frozen in isopentane at -30°C. In situ hybridization was assessed on 20 μ m-thick slices cut on a cryostat at -21°C after 1h post-fixation with 3,7 % formaldehyde in PBS, washed with PBS, rinsed with water, dehydrated in 50% and 70% ethanol and air-dried. All antisense oligonucleotide probes used were designed by Helios BioSciences (France), labeled with [35S]-dATP (PerkinElmer, France) using terminal deoxy-transferase enzyme (Promega). Sections were then covered with 140 μ l of a hybridization medium (Helios, Bioscience, France) containing 400000 dpm of the labeled oligonucleotide. Slides were incubated overnight at 42 °C, washed and exposed to a BASSR Fujifilm Imaging Plate for 5 days. The plates were scanned with a Fujifilm BioImaging

Analyzer BAS-5000 and analyzed with MCID Analysis Software. Optical density values were calibrated using autoradiographic [³⁵S] microscales 20 x 74 kBq, 20 x 2 nCi (Amersham, UK). Data are presented as mean ± SEM expressed as a percentage of vehicle values to allow direct visual comparisons of the effects of the drugs between regions.

III. Synaptosomal fraction preparation

After decapitation, brains were rapidly removed and the hippocampus and the prefrontal cortex were dissected. Tissues were homogenized in 500µL per 50mg tissue of a sucrose buffer (4mM Hepes/NaOH pH 7.4, 320mM sucrose), in a Dounce homogenizer (Pestle A). After centrifugation (1000g, 10min), the supernatant (S1) was kept and pellet was resuspended in the same volume and re-centrifuged (1000g, 10min). Supernatant (S1') was kept and added to S1 and centrifuged for 15min at 12500g. Pellet was resuspended in 5ml of sucrose buffer and centrifuged for 15min at 12500g. Pellet was kept as the synaptosomal fraction and conserved at -20°C until use.

IV. Cytoplasmic and nuclear fractions

We used an adapted protocol from Kitchener et al. 2004. Tissues were homogenized in 500 µL per 50 mg tissue of B1 buffer, in a Dounce homogenizer (Pestle A). After centrifugation (2000xg, 3xmin), the supernatant was kept as the cytoplasmic fraction, and the pellet was resuspended in B1 buffer, and passed 10 times in a 25-G needle. After centrifugation (2000g, 3xmin), the pellet was washed again in S1 buffer and then resuspended in 1.2 pellet volume of B2 buffer, kept under agitation for 1 h and centrifuged at 22 000 g for 30 min. The supernatant was kept as the nuclear fraction. 10% glycerol was added to the cytoplasmic fractions and the protein samples were kept frozen at -80 °C. All B1 and B2 buffers contained 5 µL/mL Protease inhibitor cocktail (# P8340 Sigma) and 0.5 µL/mL DTT 1 m. **B1:** 10mM Hepes, pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.1mM EGTA, pH 7, 5µl/ml protease inhibitors, 0.5µl/ml DTT 1M. **B2:** 10mM Hepes, pH 7.9, 400mM NaCl, 1.5mM MgCl₂, 0.1mM EGTA, pH 7, 5% glycerol, 5µl/ml protease inhibitors, 0.5µl/ml DTT 1M.

V. Western blot

Protein concentration was quantified using the Bradford method (Bio-Rad Protein Assay). 10µg or 40µg protein were heated at 70°C for 10min together with 5µl Nu PAGE LDS Sample Buffer 4X (Invitrogen) and 2µl DTT 500mM (reducing agent), in a total final volume 20µl. Protein migration was performed in NuPAGE Novex 10% Bis-Tris Midi Gel (Invitrogen), with NuPAGE MES SDS Running Buffer at 120V and then proteins were transferred on a nitrocellulose membrane (Invitrogen) in 1X Tris-Borate buffer (50mM Tris, 50mM Boric Acid) at 100V for 40min. Membranes were then blocked in a 5% dry milk-PBS-Tween 0.1% solution for 1h and primary antibody was added in 5% dry milk in PBS-Tween 0.1% and left overnight at 4°C under agitation. Primary antibodies: anti-GluR1, anti-GluR2/3, anti-NR2B (1:1000), anti-NR2A (1:5000)and Millipore), anti-NR1 (1:750 Santa Cruz); GR (M-20) (1 :1000 Santa Cruz) ; Anti-phospho-CREB (Ser133) and anti-CREB (1 :1000 Millipore) ; ERK 1/2 (MK1) (1 :1000 Santa Cruz) ; phospho-p44/42 MAP Kinase (ERK1/2) (Thr202-Tyr204) (1:1000 Ozyme); H2B (1:5000 Abcam); GAPDH (1:5000 Calbiochem). Secondary antibodies were added for 1h at room temperature in 5% dry milk in PBS-Tween 0.1%. Secondary antibodies were purchased by Tebu-bio SA: Anti-Rabbit IRDye800CW® Conjugated and anti-mouse IRDye700CW® Conjugated. These antibodies are conjugated with fluorochromes that emit in different wave lengths and are specifically designed for protein detection using the ODYSSEY Infrared Imaging system (LI-COR Biosciences). Membranes were then scanned and bands were quantified.

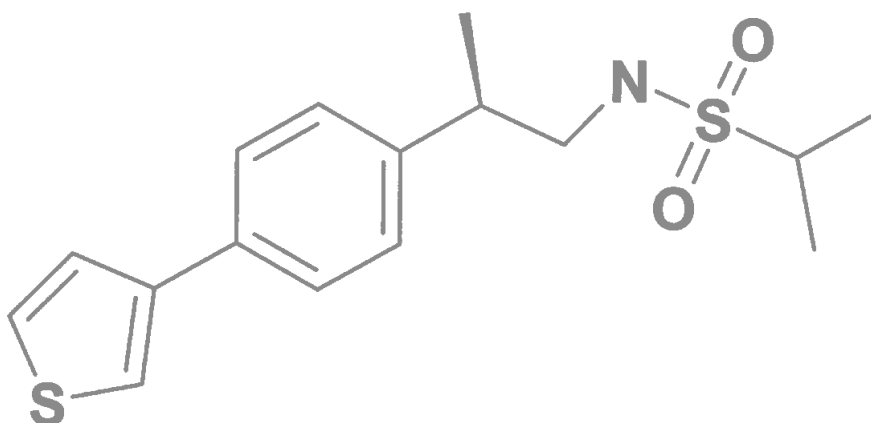
EXPERIMENTAL STUDIES – RESULTS

PART I

The effect of AMPA R potentiation in animal models of depression

LY 392098

N-2-(4-(3-thienyl)phenyl)propyl 2-propanesulfonamide



EXPERIMENTAL STUDIES – RESULTS

PART I. The effect of AMPA R potentiation in animal models of depression

INTODUCTORY REMARKS

Glutamatergic receptors as novel antidepressant targets

Glutamate exerts its physiological effects through two types of receptors: the metabotropic (G protein-coupled receptors, GPCR) glutamate receptors (mGluRs) and the ionotropic ligand-gated ion channels. The ionotropic receptors are separated into three subgroups based upon their pharmacology: AMPA, NMDA and kainate (KA) receptors. Several compounds that target glutamatergic receptors are currently studied in the context of depression.

i) Metabotropic glutamate receptors

Metabotropic glutamate receptors are divided into eight subtypes (mGluR1-8) that fall within three groups based upon homology and functional biology. Receptors of the group I (mGluR1 and mGluR5) are coupled to the activation of phospholipase C, and in general enhance glutamatergic signal. Receptors of the group II (mGluR2 and mGluR3) and group III (mGluR4, 6, 7 and 8) are Gi-coupled and negatively modulate excitatory neurotransmitter efflux and neuronal excitability. Group II mGluRs are mostly distributed at the preterminal portions of the axons, extrasynaptically, and may be activated by glutamate spillover from the same or a distant synapse. Group III mGluRs may act as inhibitory autoreceptors located in presynaptic active zones. In general, mGluR group I and II antagonism is consistent with an antidepressant action. Group I selective antagonism has shown antidepressant effects in the tail suspension and the forced swimming tests (Belozertseva et al. 2007). Similarly, repeated administration of the mGluR5 antagonist MPEP produced antidepressant-like effects in the rat olfactory bulbectomy model of depression (Palucha et al. 2005), and chronic administration of MPEP significantly increased the expression of hippocampal BDNF

(Legutko et al. 2006). mGluR2/3 antagonists exhibit dose-dependent antidepressant-like effects in behavioral animal models of depression (Chaki et al. 2004) and increase cell proliferation in the adult mouse hippocampus after subchronic administration (Yoshimizu and Chaki 2004). On the contrary, agonists (and not antagonists) of the group III have shown dose-dependent antidepressant-like effects in a forced swimming test (Palucha et al. 2004).

ii) NMDA receptors

In the past years, it has been demonstrated that antidepressant drugs produce time- and dose- dependent changes in the radioligand binding properties of the NMDA receptor (Paul et al. 1993, 1994, Nowak et al. 1993, 1998, Skolnick et al. 1996). Some antidepressants bind to NMDA receptors and inhibit the binding of NMDA receptor ligands *in vitro* and that antidepressants can modulate the release and/or the reuptake of glutamate. Acute, preclinical screening procedures (tail suspension test, forced swimming test) revealed antidepressant-like properties of NMDA antagonists since the 90s (Trullas and Skolnick 1990, Petrie et al. 2000) reinforcing the glutamate hypothesis of depression. Chronic treatment with NMDA receptor antagonists results in behavioral effects analogous to those produced by chronic antidepressant treatments in the chronic mild stress paradigm (Papp and Moryl 1994, Farley et al. 2012). These results were later confirmed in clinical studies, showing that cycloserine and amantadine, a partial NMDA agonist and a NMDA antagonist respectively, relieve depressive symptoms in humans (Crane GE 1959, Vale et al. 1971, Bode et al. 1997, Huber et al. 1999, Stryjer et al. 2003). Interest for NMDA antagonists in depression was renewed recently based on studies that have shown a rapid-onset of action of their antidepressant effects. Thus, a single subanaesthetic dose of ketamine (NMDA antagonist) resulted in a rapid antidepressant effect within hours of administration that was sustained for at least one week (Berman et al. 2000, Zarate et al. 2006).

Ketamine increases the presynaptic release of glutamate, and the net increase in extracellular glutamate levels preferentially favors the AMPA receptor over NMDA receptors, as ketamine blocks the latter. It has been proposed that the effects of ketamine are mediated by an enhanced AMPA receptor activation (Moghaddam et al. 1997, Machado-Vieira et al. 2009). It was recently shown that pre-treatment with the AMPA receptor antagonist NBQX attenuated not only the antidepressant-like effects induced by NMDA

receptor antagonists but also the regulation of hippocampal phosphorylated GluR1 AMPA receptors (Maeng and Zarate 2007). Taken together, the results suggest it is likely that NMDA receptor antagonists such as ketamine might exert their rapid antidepressant-like effects by enhancing AMPA receptors relative to NMDA receptors throughout critical neuronal circuits (Maeng and Zarate 2007, Maeng et al. 2008, Mathew et al. 2008).

Riluzole, a neuroprotective agent used in slowing the progression of amyotrophic lateral sclerosis, inhibits presynaptic glutamate release and increases glial cell glutamate uptake. Several studies in humans have revealed antidepressant-like properties (Coric et al. 2003, Zarate et al. 2004, 2005, Sanacora et al. 2004, 2008, Pittenger et al. 2008a) and in rats, riluzole increases glutamate clearance from the synaptic cleft concomitant with the amelioration of stress-induced depressive-like behavior when administered chronically (Banasr et al. 2010).

iii) AMPA receptors

An emerging set of data converges on the possibility that AMPA receptors play a key role in the treatment and possibly the etiology of mood disorders (Alt et al. 2006, Sen and Sanacora 2008, Witkin et al. 2007, Pittenger et al. 2008a,b, Krystal 2007, Maeng and Zarate 2007, Palucha and Pilc 2007, Pilc et al. 2008). Acute or repeated dosing with the SSRI fluoxetine changed the phosphorylation state of GluR1 subunit of AMPA receptors to one that favors increased channel conductance. Binding of serotonin to specific serotonin receptors changes the phosphorylation state of DARPP-32. Depending on its phosphorylation state DARPP-32 can act either as a phosphatase inhibitor or a kinase inhibitor and is thus in a position to regulate signal transduction cascades. Serotonin-mediated changes in the phosphorylation state of DARPP-32 induce GluR1 phosphorylation at Ser831 and Ser845 after acute and at Ser845 after chronic administration of fluoxetine. Furthermore, in DARPP-32 KO mice both GluR1 phosphorylation and the antidepressant potency of fluoxetine were significantly decreased (Svenningsson et al. 2002a,b). In the tail suspension test, antidepressant action of the SSRI fluoxetine but not that of the TCA imipramine was blocked by the AMPA antagonist (Li et al. 2001). Similar results were shown for the antidepressant effects of lithium (Gould et al. 2008).

Chronic and subchronic treatments with desipramine, paroxetine and antimanic agents with a predominantly antidepressant profile resulted in increased GluR1 and GluR2/3 levels in the membrane-enriched hippocampal extracts, with no significant change in the total extract, suggesting a trafficking of the AMPA receptor subunits from intracellular pools to synaptic sites in the hippocampus induced by antidepressants (Martinez-Turrillas et al. 2002, Du et al. 2007). Reported modifications in phosphorylating enzymes by chronic antidepressants could perhaps play a role in hippocampal membrane insertion of AMPA receptor subunits. The antidepressant-induced increase in the number of GluR1- and GluR2/3-containing AMPA receptors at the synapses may indicate an enhanced AMPA receptor-mediated synaptic transmission which could help to counteract the alterations in neuronal connectivity that appears to underlie the pathophysiology of mood disorders.

A recent study demonstrated that mice with deletion of the main AMPA receptor subunit (GluR1) represent a depression model with good face and construct validity, resembling behavioral and neurochemical features of depression in humans. These mice display increased learned helplessness, decreased serotonin and norepinephrine levels, and disturbed glutamate homeostasis along with increased glutamate levels and increased NMDA receptor expression (Chourbaji et al. 2008).

AMPA receptor has allosteric modulatory site(s) for which potent positive modulators (potentiators) have been designed. These compounds, also known as ampakines, do not activate AMPA receptors themselves but slow the rate of receptor desensitization and/or deactivation in the presence of an agonist and thereby increase ligand-gated current flow. Several positive modulators (piracetam, aniracetam, cyclothiazide, CX516, CX614, LY392098, LY404187, LY451395, LY503430, and S18986) of AMPA receptors have shown antidepressant-like effects in animal models of depression (Bai et al. 2001, Sanacora et al. 2008, Alt et al. 2006, O'Neill and Witkin 2007). In the forced swimming test, the antidepressant-like effects of LY392098 were blocked by AMPA receptor antagonist GYKI53655 (Li et al. 2001).

PART Ia: Antidepressant-like effects of an AMPA receptor potentiator under a chronic mild stress paradigm

Objective of the study

However no study has assessed the effects of AMPA potentiators in chronic models of depression. Based on the above, we assessed the possible antidepressant profile of the AMPA receptor potentiator LY392098 in the unpredictable chronic mild stress (UCMS). We also assessed the ability of the AMPA antagonist GYKI52466 to block the effects of fluoxetine in this model. Chronic mild stress models are considered of high face, construct and predictive validity. In these models prolonged exposure to uncontrollable and unpredictable stressors results in depressive like behaviors that can be prevented or reversed by chronic but not acute antidepressant treatment (Griebel et al. 2002, Mineur et al. 2006, Surget et al. 2008).

Antidepressant-like effects of an AMPA receptor potentiator under a chronic mild stress paradigm

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Abstract

Enhancement of AMPA receptor (AMPA) function has emerged as a novel strategy for treatment of depression. Nevertheless, studies on AMPAR function in chronic animal models used to predict antidepressant efficacy are surprisingly lacking. We investigated the role of AMPARs in antidepressant action in an unpredictable chronic mild stress (UCMS) model in BALB/c mice. After 3 wk of UCMS, BALB/c mice developed a number of depressive-like behaviours that were successfully prevented by fluoxetine (20 mg/kg) administration. The AMPAR potentiator LY392098 [N-2-(4-(3-thienyl)phenyl)propyl 2-propanesulfonamide] (5 mg/kg), when administered alone, functioned like classic antidepressants by reducing weight loss, fur deterioration and immobility in the tail suspension test. However, LY392098 did not restore sucrose preference and did not reduce anxiety (marble-burying) in stressed mice. In the same protocol, the AMPAR antagonist GYKI (10 mg/kg) reversed most, but not all, of the antidepressant-like actions of fluoxetine. Thus, the antidepressant-like effects of LY392098 were fully predicted by the AMPAR dependence of effects demonstrated for fluoxetine. Our results demonstrate that, in the UCMS paradigm, AMPAR activation exhibits antidepressant-like activity that relates preferentially to specific depressive-like responses and that those specific responses can be defined by their regulation by AMPAR modulation under conditions of stress.

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Key words: AMPA potentiator, chronic mild stress, glutamate, mood disorders, plasticity.

Introduction

AMPA receptor (AMPA) activation is currently considered as one of the most promising new approaches for new antidepressant therapies as the balance between glutamate and GABA in general is becoming increasingly relevant in the field of depression.

Direct evidence comes from preclinical models of antidepressant activity. Specifically, AMPAR

potentiators such as LY392098 are active in both the forced swim test (FST) and the tail suspension test (TST), two behavioural despair models that are used to screen and identify compounds with antidepressant activity (Bai *et al.* 2001; Li *et al.* 2001). There is also an increasing body of proof-of-concept evidence suggesting a role for AMPARs in depression and in the actions of antidepressant drugs. AMPAR potentiators result in neurobiological adaptations similar to those produced by antidepressants that are currently used in the clinical setting, including BDNF induction and increases in hippocampal progenitor cell proliferation (reviewed in Alt *et al.* 2006a; Bleakman *et al.* 2007; Skolnick, 2002). Data from studies using AMPA antagonists have further implicated a role for

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AMPA activation in mediating antidepressant-like actions of a number of either clinically used or experimental compounds. Thus, the AMPA antagonists NBQX and GYKI 52466 reduced the effects of lithium in the FST and/or TST (Gould *et al.* 2008) and GYKI 53655, another AMPA antagonist, prevented the effects of LY392098 in the FST and TST (Li *et al.* 2001). NBQX also prevented both antidepressant-like effects in the TST and serotonin increases in the medial prefrontal cortex induced by MGS0039 an mGluR_{2/3} receptor antagonist with antidepressant potential (Karasawa *et al.* 2005). NBQX also prevented antidepressant-like effects of the NMDA receptor antagonists ketamine, MK801 and Ro25-6981, an NR2B antagonist (Maeng *et al.* 2008). Given the clinical validation of ketamine (Zarate *et al.* 2006) and NR2B receptor blockade (CP101,606) (Preskorn *et al.* 2008) as effective antidepressants for treatment-resistant depression, identification of AMPAR potentiation as a potential mechanism for these effects (Maeng *et al.* 2008) is significant. Additional preclinical support comes from the finding that AMPAR subunit 1 (GluR-A) knockout mice show increased learned helplessness (Chourbaji *et al.* 2008).

However, only one study has previously evaluated this mechanism in antidepressant-detecting assays that require subchronic dosing (Knapp *et al.* 2002). Although models such as the FST and TST are widely used as tests of antidepressant efficacy, additional appreciation of the potential value of AMPAR potentiation in animal models of depression should be gained. Chronic mild stress models are considered of high face, construct and predictive validity. In these models prolonged exposure to uncontrollable and unpredictable stressors results in depressive-like behaviours that can be prevented or reversed by chronic but not acute antidepressant treatment (Griebel *et al.* 2002; Mineur *et al.* 2006; Surget *et al.* 2008).

In this paper, we provide the first evidence showing that an AMPAR potentiator can relieve some effects engendered by chronic mild stress. Specifically we examined (i) the possible antidepressant profile of the AMPAR potentiator LY392098 and (ii) the role of AMPAR activation in the antidepressant-like actions of the reference antidepressant fluoxetine.

Materials and methods

Animals

Male BALB/c mice (aged 6 wk; $n = 10$ per group) were purchased from Charles River (France). This strain

was chosen because BALB/c mice are particularly sensitive to stress, and respond in a reproducible manner to unpredictable chronic mild stress (UCMS) protocols. Experiments started after a 2-wk acclimation period; at the beginning of experiments mice were aged 8 wk. During the 2-wk acclimation period all mice were housed under standard conditions and had access to rodent pellet food and water available *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive (86/809/EEC) regarding the care and use of animals for experimental procedures and approved by the Comité d'éthique INSERM/UPMC/CNRS.

Drugs

GYKI 52466 was purchased from Tocris (USA). Fluoxetine and LY392098 [*N*-2-(4-(3-thienyl)phenyl)propyl 2-propanesulfonamide] were synthesized by Eli Lilly and Company (USA). GYKI 52466 [10 mg/kg in acidified saline; a dose comparable to that used by Gould *et al.* 2008 (5 mg/kg) and Le Merrer & Stephens, 2006 (5–10 mg/kg)], fluoxetine (20 mg/kg in 5% DMSO, 5% cremofor, 90% saline; Crozatier *et al.* 2007; Moutsimilli *et al.* 2005, 2008) and LY392098 (5 mg/kg in saline; Li *et al.* 2001, 2003) were injected intraperitoneally at a volume of 10 ml/kg. A number of studies from our group in rats and mice have shown that there are no behavioural effects upon intraperitoneal injection of 5% DMSO, 5% cremofor, 90% saline vehicle in TST, locomotion and anxiety measures in mice (Crozatier *et al.* 2007; Herzog *et al.* 2008, Tzavara *et al.* 2003, 2006). In addition, previous chronic administration studies from our group have shown that there is no difference in biochemical (Moutsimilli *et al.* 2005, 2008) and behavioural (not shown) outcomes upon repeated administration of different vehicles (5% DMSO, 5% cremofor, 90% saline *vs.* acidified saline *vs.* saline) in mice; therefore, vehicle values were pooled and vehicles were treated as a single experimental group in this study.

Chronic mild stress protocol and behavioural tests

After a 2-wk acclimation period mice were subjected to a 3-wk mild stress protocol. Mice subjected to the UCMS protocol were singly housed. Stressors were applied twice a day for a 3-h period and overnight in a randomized order; vehicle or drugs were injected daily between 12:00 and 14:00 hours (Fig. 1*a*). Stressors typically included wet bedding, tilted cages, forced swim stress (for 6 min), paired housing, food and water deprivation during confinement in

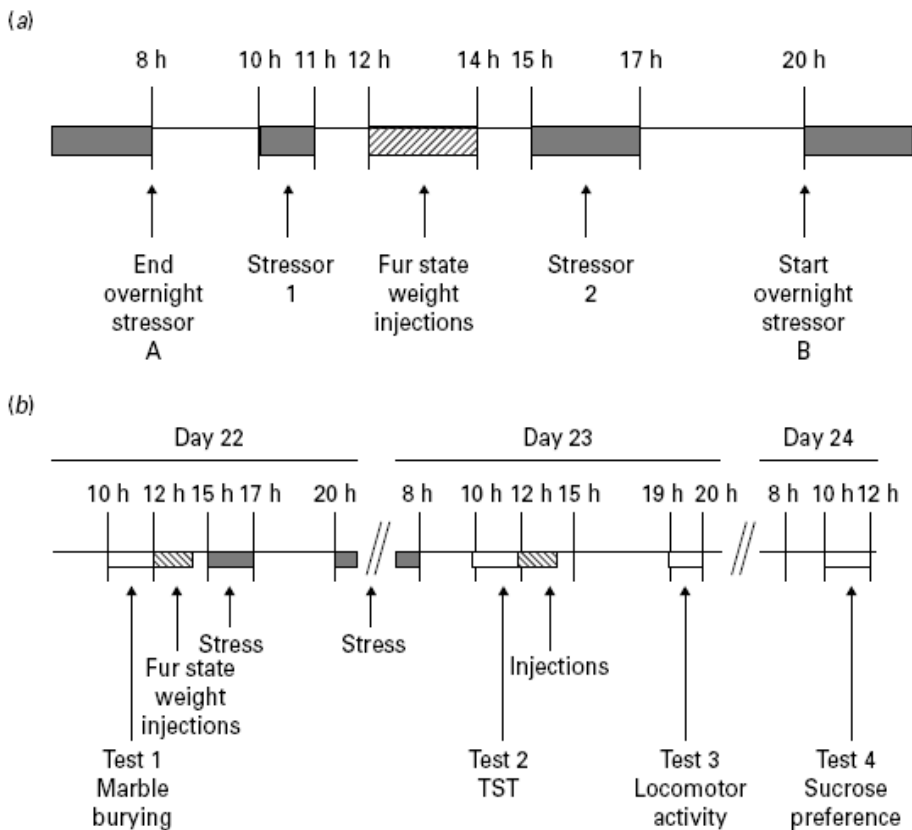


Fig. 1. Timeline of the experimental procedures. (a) The timeline of a typical unpredictable chronic mild stress (UCMS) day is depicted, showing the stressors applied, the interval between stressors, the time the mice were injected and the time for fur condition and weight measurements, when applicable. (b) The timeline of the behavioural testing at the end of the UCMS is depicted showing the order of the tests, interval between stressors and/or tests and stressors, and injection times, when applicable.

a 15 cm × 30 cm wired cage, crowding; overnight stressors included tilted cages, wet bedding, unpredictable illumination and reversed light–dark cycle. Control (non-stressed) mice were held in a room next to that of the stressed mice and were group-housed ($n = 4$ per cage).

During this period the animal's weight and fur condition were measured every 3 d. At the end of the chronic stress protocol the emotional state of the animals was evaluated in the marble-burying test, TST, locomotor activity and the sucrose preference tests; the tests were conducted throughout a 3-d period during which mice continued to receive injections and stressors (Fig. 1b).

Fur condition rating

The score of the animal's coat state was calculated as the sum of seven scores, one score for each of seven parts of the body (Surget *et al.* 2008) with some modifications. Namely, the state of each part was rated on a 3-point scale (3, very good; 2, fair; 1, poor).

TST

Behavioural despair was investigated in the TST as previously described (Crozatier *et al.* 2007) using an automated TST apparatus (Bioseb, France). Mice were individually suspended by their tail using a paper adhesive tape placed 1 cm from the tip of the tail and immobility (in seconds) was automatically recorded during the 6-min test period. In one TST experiment (Fig. 2a) one of the mice was observed climbing on its tail, this animal was excluded from the statistical analysis.

Marble-burying test

The marble-burying test (Li *et al.* 2006) was performed in cages 10 × 15 × 20 cm filled up to the 7.5 cm mark with sawdust on top of which were placed 12 marbles evenly spaced in four rows and 3 columns. The number of marbles buried was counted for every minute up to the first 10 min and then for every 5 min up to 30 min. Buried marbles were defined as being buried by at least three quarters of bedding.

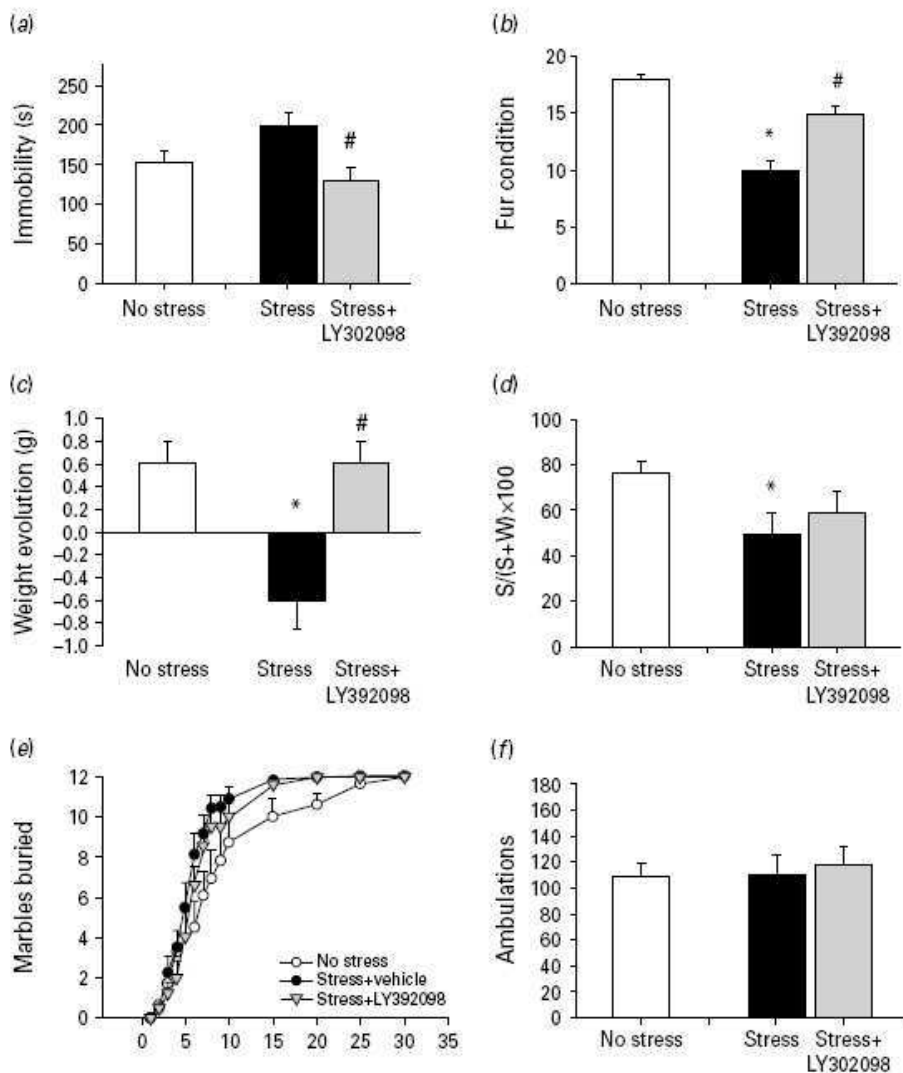


Fig. 2. Effects of the AMPA potentiator LY392098 in the unpredictable chronic mild stress test in the BALB/c mouse. (a) Antidepressant-like effects of the AMPA potentiator LY392098 in the tail suspension test. Stressed mice treated with LY392098 (□) exhibit reduced immobility compared to vehicle-treated (■) stressed controls ($^{\#}p < 0.05$, one-way ANOVA and Duncan's *post-hoc* test; $n = 9-10$ per group). (b) Antidepressant-like effects of the AMPA potentiator LY392098 in the fur condition. Stressed mice treated with LY392098 (□) exhibit reduced coat degradation compared to vehicle-treated (■) stressed controls ($^*p < 0.05$ compared to no-stress, $^{\#}p < 0.05$ compared to stress-vehicle, one-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (c) Antidepressant-like effects of the AMPA potentiator LY392098 on weight loss. Stressed mice treated with LY392098 (□) exhibit increased weight gain compared to vehicle-treated (■) stressed controls ($^*p < 0.05$ compared to no-stress, $^{\#}p < 0.05$ compared to stress-vehicle, one-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (d) The AMPA potentiator LY392098 did not restore sucrose preference. Stressed mice treated with LY392098 (□) did not differ in sucrose preference from vehicle-treated (■) stressed controls ($^*p < 0.05$ compared to no-stress, one-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (e) The AMPA potentiator LY392098 did not reduce anxiety in the marble-burying test. Stressed mice treated with LY392098 (□) did not differ in the number of marbles they buried from vehicle-treated (■) stressed controls (repeated-measures ANOVA, $n = 10$ per group). (f) No difference in locomotor activity between any of the groups.

Sucrose preference test

For the sucrose preference test mice were first habituated to drink from two graduated pipettes one filled with water, and the other with sucrose solution for 3 d, the side of the sucrose pipette being alternated each day. On day 4 the two pipettes were presented again;

however, one was filled with water and the other with 4% sucrose.

Locomotor activity

Horizontal activity (ambulations) was assessed in transparent activity cages ($20 \times 15 \times 25$ cm), with

automatic monitoring of photocell beam breaks (Imetronic, France). The mice were placed into the recording chamber at 19:00 hours and locomotor activity was recorded for a 1-h period.

Experiments

Expt 1

In this experiment we sought to assess the potential antidepressant profile of the AMPA potentiator LY392098, by examining its ability to reverse physical and behavioural alterations induced by chronic stress. The experimental groups compared were (i) control non-stressed, non-treated mice; (ii) stressed-mice treated with vehicle; (iii) stressed mice treated with LY392098 (5 mg/kg).

Physical (weight and fur condition) measurements and behavioural testing were conducted as above.

To ensure that our results are not due to a generalized action of LY392098, the possible effects of repeated treatment with the AMPA potentiator were assessed in non-stressed mice, in a second set of mice. These were control mice that were group-housed ($n=4$ per cage) and administered with vehicle or LY392098, daily between 12:00 and 14:00 hours for a 3-wk period. Physical (weight and fur condition) measurements and behavioural testing were conducted exactly as for stressed mice as described above and as depicted in Fig. 1b, with the difference that no stress was ever applied.

Expt 2

In this experiment we sought to assess the ability of the AMPAR antagonist GYKI52466 to reverse antidepressant effects of fluoxetine in the chronic mild stress protocol.

For this, during the 3-wk stress period mice were randomly assigned to one of the following treatment groups: (i) vehicle; (ii) GYKI52466 (10 mg/kg); (iii) fluoxetine (20 mg/kg); (iv) GYKI52466 (10 mg/kg) in combination with fluoxetine (20 mg/kg).

Physical (weight and fur condition) measurements and behavioural testing were conducted as above.

As for expt 1, in order to ensure that our results are not due to a generalized action of fluoxetine, GYKI52466 or their combination, the possible effects of repeated treatment with the above compounds were assessed in non-stressed mice, in a second set of mice. These were control mice group-housed ($n=4$ per cage) and were administered with vehicle, fluoxetine, GYKI52466 or their combination daily between 12:00 and 14:00 hours for a 3-wk period. Physical

(weight and fur condition) measurements and behavioural testing were conducted exactly as for stressed mice as described above and as depicted in Fig. 1b, with the difference that no stress was ever applied.

Statistics

Data are presented as mean \pm s.e. of $n=10$ mice per group.

Expt 1

Statistical analysis was performed by one-way ANOVA (three groups: non-stressed, stressed + vehicle, stressed + LY392098) for weight loss, fur condition, TST, locomotor activity and sucrose preference and by repeated-measures (time) one-way (three groups: non-stressed, stressed + vehicle, stressed + LY392098) ANOVA for marble-burying. When one-way ANOVA revealed significant group effects Duncan's *post-hoc* test was utilized to compare between the three groups.

To analyse the effects of repeated treatment with the AMPA potentiator in non-stressed mice we utilized *t* test comparisons between vehicle-treated and LY392098-treated mice.

Expt 2

Statistical analysis was performed by two-way [treatment 1 (vehicle or GYKI52466) \times treatment 2 (vehicle or fluoxetine)] ANOVA for weight loss, fur condition, TST, locomotor activity and sucrose preference and by repeated-measures (time) two-way (treatment 1 \times treatment 2) ANOVA for marble-burying. When two-way ANOVA revealed significant main or interaction effects Duncan's *post-hoc* test was utilized to compare between groups. Statistical analysis was done separately for stressed and non-stressed mice.

All statistical analyses were performed with Statistica software (SysStat, Germany).

Results

Expt 1

For immobility in the TST one-way ANOVA revealed a statistically significant difference between groups [non-stressed, stressed + vehicle, stressed + LY392098; $F(2,26)=3.66$, $p<0.05$]. Stressed mice treated with LY392098 displayed more attempts to escape in the TST compared to vehicle-treated stressed mice ($p=0.017$, Duncan's *post-hoc* test; Fig. 2a).

For fur condition, one-way ANOVA revealed a statistically significant difference between groups

[non-stressed, stressed + vehicle, stressed + LY392098; $F(2,27)=56$, $p<0.001$]. In stressed mice treated with vehicle, fur was markedly degraded compared to non-stressed mice ($p=0.00006$, Duncan's *post-hoc* test; Fig. 2b), whereas in LY392098-treated stressed mice fur degradation was prevented ($p=0.0015$, Duncan's *post-hoc* test; Fig. 2b).

For weight loss, one-way ANOVA revealed a statistically significant difference between groups [non-stressed, stressed + vehicle, stressed + LY392098; $F(2,27)=4.7$, $p<0.05$]. Stress resulted in weight loss in vehicle-treated mice ($p=0.0093$, Duncan's *post-hoc* test; Fig. 2c), whereas weight evolution for LY392098-treated stressed mice did not differ from that of non-stressed mice ($p=0.54$, Duncan's *post-hoc* test; Fig. 2c).

For sucrose preference, one-way ANOVA revealed a statistically significant difference between groups [non-stressed, stressed + vehicle, stressed + LY392098; $F(2,27)=3.5$, $p<0.05$]. Stress resulted in a marked loss of sucrose preference in vehicle-treated mice ($p=0.023$, Duncan's *post-hoc* test; Fig. 2d). Loss of sucrose preference was attenuated in LY392098-exposed mice but this effect did not reach statistical significance ($p=0.45$, Duncan's *post-hoc* test; Fig. 2d).

For marble-burying, repeated-measures (time) one-way ANOVA revealed a significant interaction for time \times group [$F(26, 351)=1.74$, $p<0.05$]. Duncan's *post-hoc* test showed that stressed + vehicle and stressed + LY392098 mice buried more marbles than non-stressed mice ($p=0.014$ and $p=0.011$ for stressed + vehicle and stressed + LY392098, respectively, *vs.* non-stressed for the last time-point); there was no difference between stressed + vehicle and stressed + LY392098 mice ($p=1$ for the last time-point; Fig. 2e).

For locomotor activity one-way ANOVA revealed no statistically significant difference across the groups tested (Fig. 2f). This suggests that the reduction of immobility in the TST seen with repeated LY392098 in stressed mice is not due to a non-specific generalized effect on locomotor activity.

To ensure that our results were not due to a generalized action of LY392098 that was not specific to the control of stress-induced behavioural signs, the possible effects of repeated treatment with the AMPA potentiator were assessed in non-stressed mice. There was no effect of repeated LY392098 on any of the parameters tests in non-stressed mice (Fig. 3).

Expt 2

For immobility in the TST, two-way ANOVA revealed a statistically significant interaction for treatment 1

(AMPA antagonist or vehicle) \times treatment 2 (fluoxetine or vehicle) [$F(1,36)=5.96$, $p<0.05$]. Duncan's *post-hoc* test revealed that fluoxetine prevents stress-induced increases in immobility in the TST ($p=0.038$ for vehicle + vehicle *vs.* vehicle + fluoxetine); this antidepressant-like effect is blocked by co-administration of GYKI52466 ($p=0.044$ for vehicle + fluoxetine *vs.* GYKI52466 + fluoxetine; $p=1$ for vehicle + vehicle *vs.* GYKI52466 + fluoxetine). GYKI52466 administered alone did not affect stress-induced increases in immobility in the TST ($p=0.23$ for GYKI52466 + vehicle *vs.* vehicle + vehicle) (Fig. 4a).

For fur condition, two-way ANOVA revealed a statistically significant interaction for treatment 1 \times treatment 2 [$F(1, 36)=7.19$, $p<0.05$]. Duncan's *post-hoc* test showed that fluoxetine prevents stress-induced fur degradation ($p=0.0008$ for vehicle + fluoxetine *vs.* vehicle + vehicle); this antidepressant-like effect is blocked by co-administration of GYKI52466 ($p=0.0015$ for vehicle + fluoxetine *vs.* GYKI52466 + fluoxetine). In contrast, GYKI52466 administered alone has no effect on this behavioural parameter ($p=0.79$ for GYKI52466 + vehicle *vs.* vehicle + vehicle) (Fig. 4b).

For weight-loss, two-way ANOVA revealed a statistically significant interaction for treatment 1 \times treatment 2 [$F(1, 36)=5.9$, $p<0.05$]. Duncan's *post-hoc* test showed that fluoxetine prevents stress induced reduction in body weight ($p=0.008$ for vehicle + fluoxetine *vs.* vehicle + vehicle); this antidepressant-like effect is blocked by co-administration of GYKI52466 ($p=0.005$ for vehicle + fluoxetine *vs.* GYKI52466 + fluoxetine). GYKI52466 administered alone has no effect ($p=0.79$ for GYKI52466 + vehicle *vs.* vehicle + vehicle) (Fig. 4c).

For sucrose preference, two-way ANOVA did not show a statistically significant interaction for treatment 1 \times treatment 2, but revealed a significant main effect of fluoxetine [$F(1, 36)=8$, $p<0.01$]. Fluoxetine prevents stress-induced loss of sucrose preference ($p=0.044$, Duncan's *post-hoc* test). However, fluoxetine + GYKI52466-treated mice do not differ from fluoxetine + vehicle-treated mice ($p=0.65$) (Fig. 4d).

For marble-burying, repeated-measures (time) two-way ANOVA revealed a significant interaction for time \times treatment 1 \times treatment 2 [$F(13, 468)=3.577$, $p<0.001$]. Duncan's *post-hoc* test showed that fluoxetine-treated mice buried less marbles than saline-treated mice ($p=0.032$ for vehicle + fluoxetine *vs.* vehicle + vehicle for the last time-point); this effect was accentuated in mice treated with fluoxetine + GYKI52466 ($p=0.0073$ for GYKI52466 + fluoxetine *vs.* vehicle + fluoxetine for the last time-point).

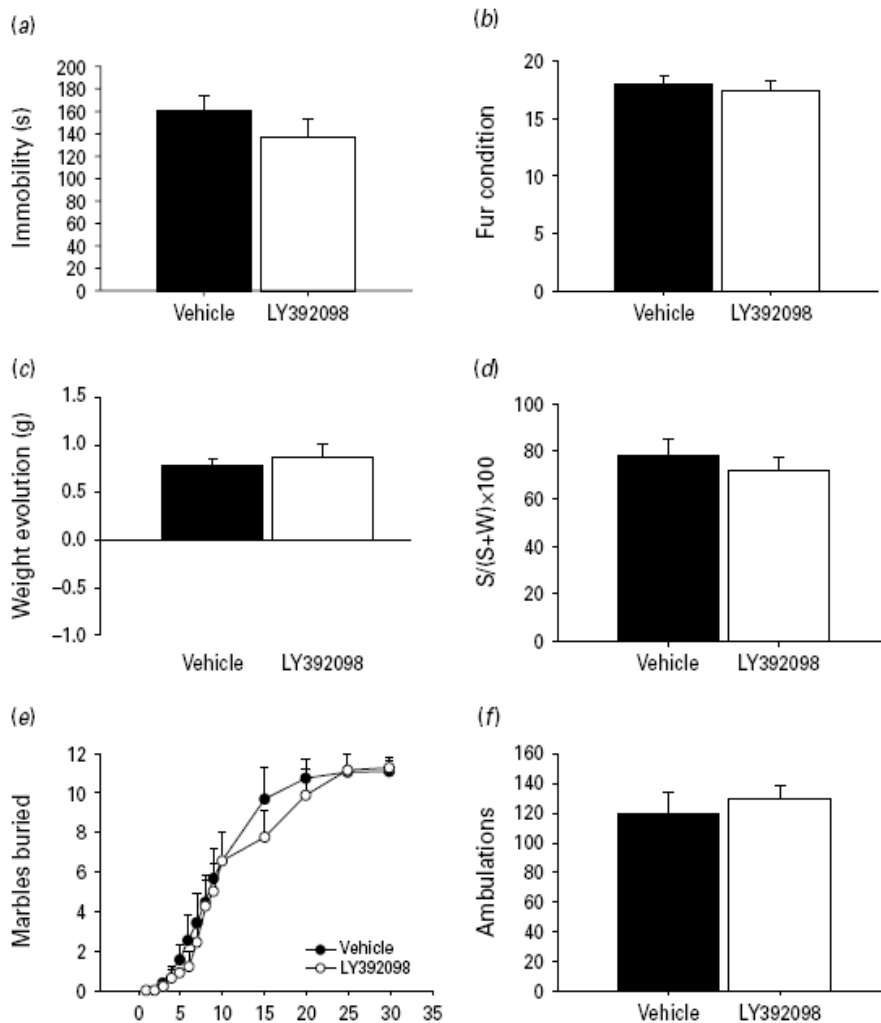


Fig. 3. Effects of repeated administration of the AMPA potentiator LY392098 in the non-stressed BALB/c mouse. (a) Immobility in the tail suspension test; (b) fur condition; (c) weight evolution; (d) sucrose preference; (e) marble-burying test; (f) locomotor activity. LY392098 did not affect any of these parameters ($n = 10$ per group).

GYKI52466 alone had no effect ($p = 0.94$ for GYKI52466 + vehicle *vs.* vehicle + vehicle for the last time-point) (Fig. 4e).

For locomotor activity, two-way ANOVA revealed no statistically significant difference among the groups tested (Fig. 4f). This suggests that the effects on immobility in the TST seen with fluoxetine, GYKI52466, or their combination in stressed mice, are not due to non-specific generalized effects on locomotor activity.

To ensure that our results are not due to a generalized action of fluoxetine, GYKI52466 or their combination, the possible effects of repeated treatment with the above compounds were assessed in non-stressed mice. None of the treatments affected fur condition, weight evolution, sucrose preference, or locomotor activity in non-stressed mice.

For the TST, two-way ANOVA revealed a statistically significant effect of treatment 2 (fluoxetine *vs.*

vehicle) [$F(1,36) = 10.6$, $p < 0.01$]; but not for interaction between treatment 1 (AMPA antagonist or vehicle) \times treatment 2 (fluoxetine or vehicle). Duncan's *post-hoc* test showed that fluoxetine reduces immobility in the TST ($p = 0.0043$ for vehicle + fluoxetine *vs.* vehicle + vehicle); this effect is only partially affected by co-administration of GYKI52466 ($p = 0.32$ for GYKI52466 + fluoxetine *vs.* vehicle + fluoxetine and $p = 0.039$ for GYKI52466 + fluoxetine *vs.* GYKI52466 + vehicle). GYKI52466 administered alone did not affect immobility in the TST ($p = 0.83$ for GYKI52466 + vehicle *vs.* vehicle + vehicle) (Fig. 5a).

For marble-burying, repeated-measures (time) two-way ANOVA revealed a significant interaction for treatment 2 (fluoxetine *vs.* vehicle) [$F(13, 468) = 5.4$, $p < 0.001$]. Duncan's *post-hoc* test showed that fluoxetine-treated non-stressed mice buried less marbles than saline-treated mice ($p = 0.033$ for vehicle + fluoxetine *vs.* vehicle + vehicle for the last time-point);

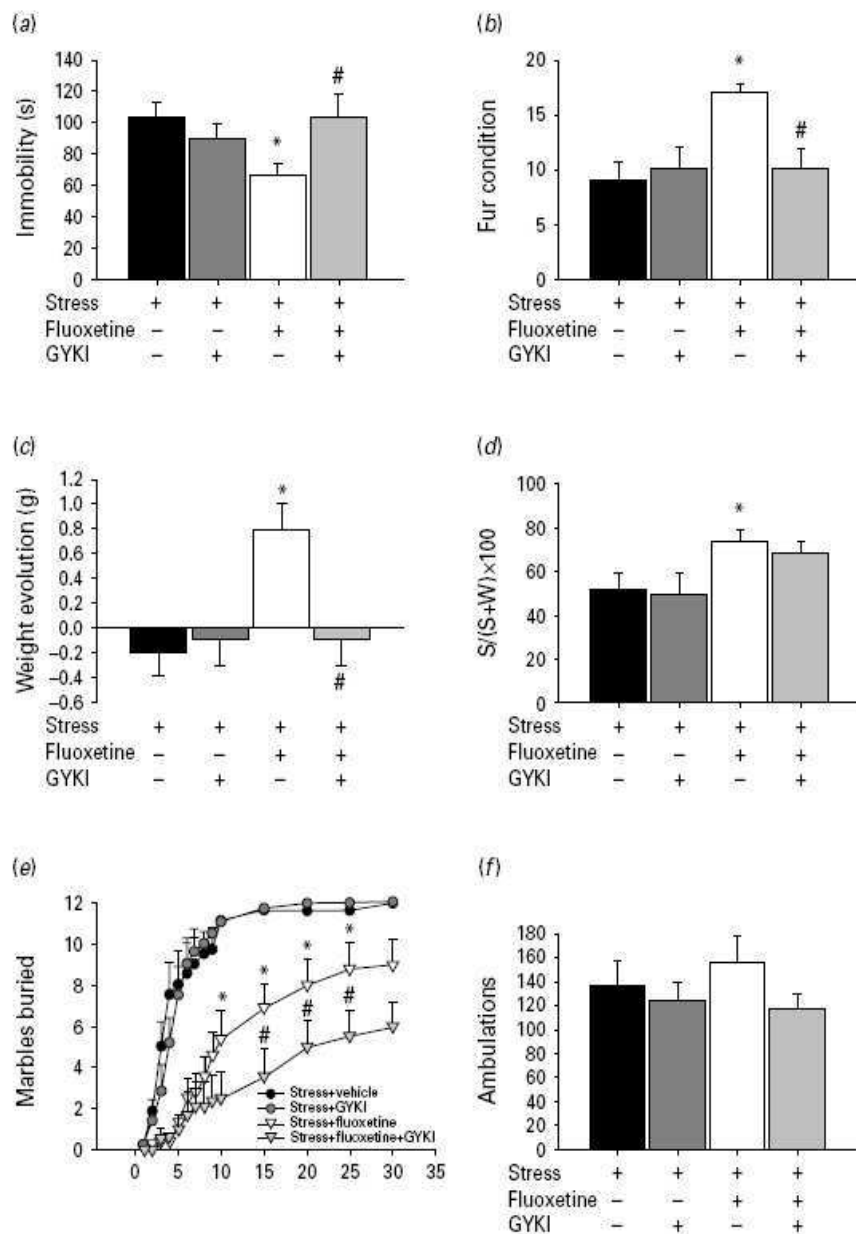


Fig. 4. Effects of the AMPA antagonist GYKI52466 on the antidepressant effects of fluoxetine in the unpredictable chronic mild stress in the Balb/C mouse. (a) The AMPA antagonist GYKI52466 blocks the antidepressant effects of fluoxetine in the TST. Stressed mice treated with fluoxetine + GYKI did not differ from vehicle-treated stressed mice and displayed increased immobility compared to mice treated with fluoxetine alone (* $p < 0.05$ compared to stress, # $p < 0.05$ compared to stress-fluoxetine, two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (b) The AMPA antagonist GYKI52466 blocks the antidepressant effects of fluoxetine in the fur condition. Stressed mice treated with fluoxetine + GYKI did not differ from vehicle-treated stressed mice and displayed greater coat deterioration compared to mice treated with fluoxetine alone (* $p < 0.05$ compared to stress, # $p < 0.05$ compared to stress-fluoxetine, two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (c) The AMPA antagonist GYKI52466 blocks the antidepressant effects of fluoxetine in weight loss. Stressed mice treated with fluoxetine + GYKI did not differ from vehicle-treated stressed mice and displayed reduced weight gain compared to mice treated with fluoxetine alone (* $p < 0.05$ compared to stress, # $p < 0.05$ compared to stress-fluoxetine, two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (d) The AMPA antagonist GYKI52466 does not block the antidepressant effects of fluoxetine in sucrose preference. Stressed mice treated with fluoxetine + GYKI did not differ from fluoxetine-treated stressed mice (* $p < 0.05$ compared to stress, two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (e) The AMPA antagonist GYKI52466 potentiates the anxiolytic effects of fluoxetine in the marble-burying test. Stressed mice treated with fluoxetine + GYKI buried even fewer marbles than fluoxetine-treated stressed mice (* $p < 0.05$ compared to stress, # $p < 0.05$ compared to stress-fluoxetine, repeated-measures two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (f) No difference in locomotor activity between any of the groups.

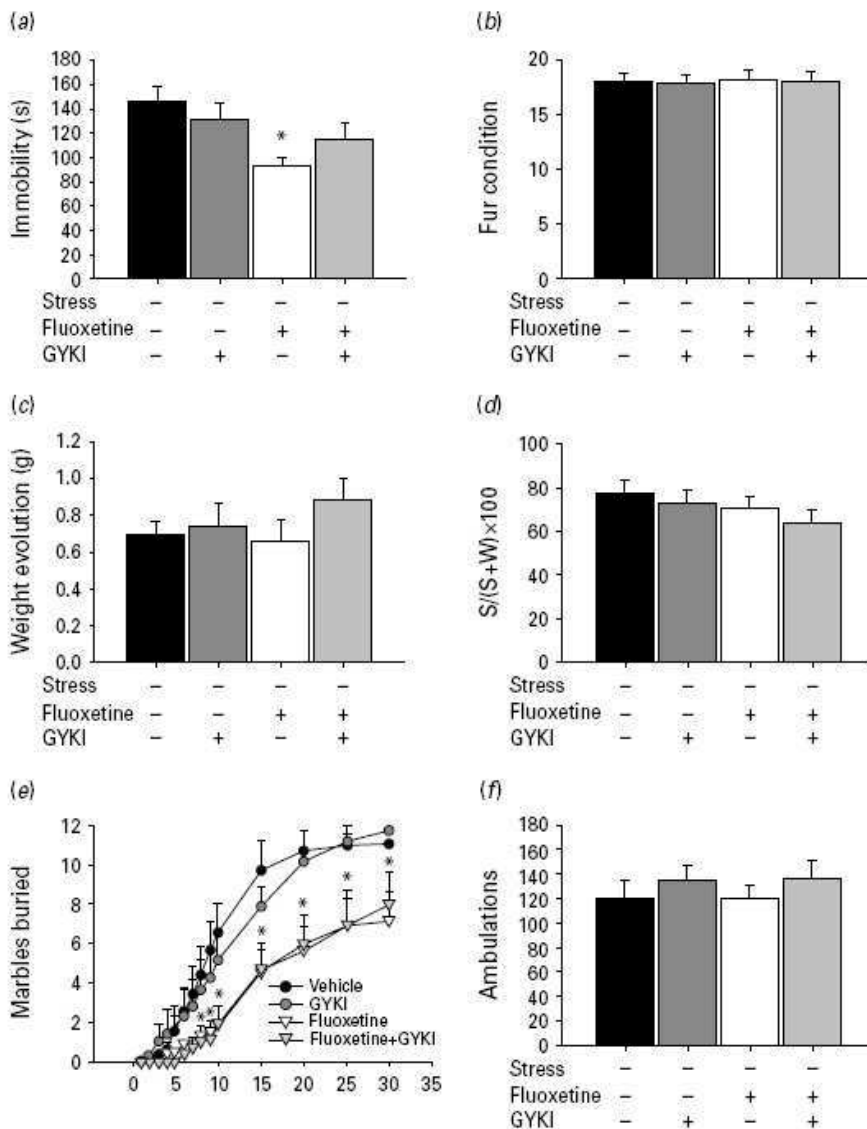


Fig.5. Effects of repeated administration of the AMPA antagonist GYKI52466, fluoxetine, or their combination in the non-stressed BALB/c mouse. (a) Immobility in the tail suspension test. Fluoxetine reduces immobility ($*p < 0.05$ compared to vehicle-treated, two-way ANOVA, Duncan's *post-hoc* test; $n = 10$ per group). (b) Fur condition. (c) Weight evolution. (d) Sucrose preference. No effect of any of the treatments ($n = 10$ per group). (e) Marble-burying test. Mice treated with fluoxetine or fluoxetine+GYKI buried less marbles than vehicle-treated mice ($*p < 0.05$ compared to vehicle, repeated-measures two-way ANOVA and Duncan's *post-hoc* test; $n = 10$ per group). (f) Locomotor activity. No effect of any of the treatments ($n = 10$ per group).

this effect was maintained in mice treated with fluoxetine+GYKI52466 ($p = 0.83$ for GYKI52466 + fluoxetine *vs.* vehicle+fluoxetine and $p = 0.033$ for GYKI52466 + fluoxetine *vs.* GYKI52466 + vehicle for the last time-point). GYKI52466 alone had no effect ($p = 0.83$ for GYKI52466 + vehicle *vs.* vehicle+vehicle for the last time-point) (Fig. 5e).

Discussion

This study assessed the role of AMPAR function in a chronic model relevant to depression and

antidepressant reversal under conditions of chronic mild stress. We used a chronic mild stress protocol, in which BALB/c mice were subjected to a sequence of unpredictable stressors for 3 wk. In this study, after 3 wk of unpredictable stress we observed marked trends for increased helplessness and increased anxiety-like signs, as well as anhedonia in BALB/c mice. We also observed a progressive physical degradation, manifested as a marked deterioration of the state of the coat of the stressed animals that is attributed to reduced grooming. Growth, measured as body weight gain, was reduced in stressed BALB/c mice

compared to non-stressed controls. These behavioural changes resulting from stress are comparable to those reported in the literature (Griebel *et al.* 2002; Mineur *et al.* 2006; Surget *et al.* 2008). Effects of a positive allosteric modulator of AMPARs was then compared to that of fluoxetine for its ability to prevent stress-induced behavioural deterioration. Further, an AMPAR antagonist was used to prevent effects of the SSRI antidepressant fluoxetine to ascertain which behavioural changes induced by chronic mild stress were responsive to AMPA-mediated changes.

The AMPAR potentiator LY392098 was administered daily throughout the 3-wk period in mice submitted to stress. The dose of LY392098 (5 mg/kg) was selected to be in the range of doses effective in acute tests of antidepressant activity (Li *et al.* 2001, 2003). LY392098 administration resulted in a less pronounced depressive-like phenotype in the mice when impacted by chronic stress than vehicle-treated mice. It should be noted that repeated LY392098 administration did not elicit any effect in non-stressed BALB/c mice, suggesting that the effects observed in stressed mice are specific to stress-induced behavioural alterations. Namely, depressive-like signs were markedly attenuated in stressed BALB/c mice receiving LY392098 compared to vehicle-receiving BALB/c controls. AMPAR potentiator-treated mice did not lose as much weight as vehicle controls, their coat was healthier and their attempts to escape in the TST were enhanced. These antidepressant-like effects were also not due to a non-specific effect of LY392098 on general activity, since LY392098 did not affect locomotion as measured in an actimeter. Thus, the effects of LY392098 were similar to those seen with different classes of compounds with antidepressant activity. Indeed, clinically useful antidepressants such as fluoxetine and imipramine, as well as putative antidepressant compounds with novel mechanisms of action (e.g. CRF1 antagonist SSR125543, or the vasopressin antagonist SSR149415) (Griebel *et al.* 2002; Surget *et al.* 2008) prevent stress-induced depressive-like behaviours in chronic stress models. However, LY392098 did not reduce anxiety-like behaviours as measured by the latency to bury marbles in stressed BALB/c mice. This is unsurprising since AMPAR antagonists were shown to display anxiolytic potential in rodents (Alt *et al.* 2006*b*). The fact that the AMPA/kainate blockade mediates anxiolytic-like effects whereas AMPA potentiation induces antidepressant-like effects, illustrates the idea that different molecular strategies might be needed for treating distinct symptom clusters of depressive disorders. LY392098, at the dose used, did not restore sucrose preference to a

statistically significant level in stressed BALB/c mice. The role of AMPAR in perception of positive emotional valence and reward is unclear, and AMPA effects appear to be dependent on the region and the GluR subunit studied (Todtenkopf *et al.* 2006). Further experiments with different behavioural paradigms are needed to study the effects of AMPA potentiators in hedonic homeostasis and its alterations in depressive states.

In a parallel experiment the AMPA antagonist GYKI52466 was used to investigate whether AMPAR activation mediates antidepressant-like effects of fluoxetine in chronic mild stress. Fluoxetine, administered at 20 mg/kg throughout the chronic stress, prevented or alleviated the expression of depressive-like physical signs and behaviours. Similar results have been reported in the literature, as discussed above (Griebel *et al.* 2002; Mineur *et al.* 2006; Surget *et al.* 2008). Co-administration of the AMPAR antagonist GYKI52466, at 10 mg/kg, reversed most, but not all, of the antidepressant actions of fluoxetine, whereas GYKI52466 alone had no effect in any of the physical or behavioural changes induced by chronic mild stress in BALB/c mice. In particular, GYKI52466 prevented the effects of fluoxetine in the TST, in fur deterioration and in weight loss. In contrast, GYKI52466 did not alter the effects of fluoxetine on sucrose preference and even potentiated the effects of fluoxetine in the marble-burying test. Chronic antidepressant administration was shown to modulate neuronal glutamatergic protein expression (Moutsimilli *et al.* 2005; Tordera *et al.* 2005) and several lines of evidence have suggested a role of AMPAR in mediating the effects of some classes of classical antidepressants. Fluoxetine has been found to alter AMPAR phosphorylation in a manner that is expected to increase AMPAR signalling (Svenningsson *et al.* 2002), and similar effects were seen with serotonergic compounds that may have antidepressant activity (Svenningsson *et al.* 2007*a*), while the effects of tianeptine in the TST are not seen in phosphomutant GluR1 mice (Svenningsson *et al.* 2007*b*). Increased expression of GluR1 has also been correlated with enhanced activity of antidepressants (Crozatier *et al.* 2007) in the same manner that LY392098 at low doses was shown to enhance antidepressant potency (Li *et al.* 2003). Furthermore, chronic administration of antidepressants was shown to increase GluR1 mRNA expression (Svenningsson *et al.* 2002), as well as AMPAR expression and synaptic targeting (Du *et al.* 2007; Martínez-Turrillas *et al.* 2007; Tan *et al.* 2006). Results of the present study are consistent with these biochemical findings since AMPAR activation was necessary for the full expression of

the antidepressant-like effects of fluoxetine on a host of parameters in mice exposed to UCMS.

Although based upon limited parametric analysis (e.g. one dose of fluoxetine), the data comparing the behaviours/signs for which subchronic fluoxetine demonstrated AMPAR-dependent modifications to those behaviours/signs affected by LY392098 reveals critical new information on the AMPAR hypothesis of mood disorders (Alt *et al.* 2006a). AMPAR dependence on the effects of fluoxetine were demonstrated in the TST, fur deterioration, and body weight measures. Similarly, LY392098 was effective in preventing the effects of stress on these measures. Conversely, fluoxetine did not show AMPAR-dependent effects in the marble-burying or sucrose preference assays, the two assays for which LY392098 did not significantly modify stress-induced behavioural changes. These data provide at least two important conclusions. (1) Subchronic fluoxetine-induced antidepressant-like effects under these conditions are dependent upon AMPARs for some, but not all, behavioural signs and (2) the antidepressant-like efficacy of the AMPAR potentiator LY392098 is consistent with this mechanism of action; activity against behaviours driven by AMPAR modulation and lack of efficacy against those in which AMPAR modulation is less relevant.

Overall the data presented here show that the AMPA potentiator LY392098 exhibits antidepressant-like activity in key stress-induced behavioural alterations in the chronic mild stress protocol in mice. However, it should be noted that depression is not a homogenous disease but rather embraces a constellation of symptoms and diagnostic criteria. It has been proposed that different symptom clusters may distinguish between different types of depression. Thus, the prevalence of anhedonia and irritability in contrast to the prevalence of fatigue and impulsivity (reviewed in Gold & Chrousos, 2002) might be relevant to the need for different treatment options for different aspects/subtypes of mood disorders. A lack of effect on a standard measure of hedonic response in rodents, confirmed by the lack of effect of the AMPAR antagonist on the effects of fluoxetine on anti-anhedonic activity (sucrose consumption), suggests a partial antidepressant-like activity that is also supported by a lack of non-specific motor effects in stressed as well as control animals. AMPAR-mediated antidepressant effects preferentially prevent specific depressive-like responses, such as immobility in the TST, coat degradation, and weight loss, that may reflect a motivational component of emotionality. It is suggested that enhancement of AMPAR function could constitute

a targeted psychotherapeutic approach for relevant subtypes of depression.

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Statement of Interest

J.M.W. is an employee of Eli Lilly and Company. E.T.T. is a former employee of Eli Lilly and Company.

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PART Ib: Early onset of action of AMPAR potentiation and concomitant BDNF increased expression

In PART Ia we have shown that a chronic administration of the AMPA R potentiator LY392098 was shown to inverse stress-induced fur deterioration, weight loss and increased resignation and an AMPAR antagonist prevented fluoxetine beneficial effects on the same parameters. In those experiments we have used fluoxetine as a positive control. All drugs were administered for 21 days based on the state of the art protocols with reference antidepressants. Daily observations gave rise to the idea that LY392098 treatment could exhibit its effects earlier than a 3-week period of time. Moreover, as discussed, elements in the literature have shown that AMPAR phosphorylation and increased membrane AMPAR insertion follow treatment with reference antidepressants (Svenningsson et al. 2002a,b, 2007, Martinez-Turillas et al. 2002), suggesting AMPA receptor activation as a downstream target mediating antidepressant action.

Hypothesis:

Our hypothesis is that targeting directly AMPARs could therefore precipitate antidepressant efficacy. To assess this question we used the model of novelty-induced hypophagia (NIH). This model based on anxiety, is widely used to screen onset of antidepressant action/anxiolytic effects of an antidepressant treatment. During this test, animals have to face the stress of a novel environment (mild stressor) in order to access a highly palatable drink. The animal experiences conflict between the desire to approach and drink, and the anxiety-induced avoidance of the novel environment. Anxiolytics administered acutely reduce the latency to consume in the novel environment, as expected. Antidepressants also reduce latency to consume only after a chronic administration but not after an acute injection, providing a test to directly study onset of antidepressant action.

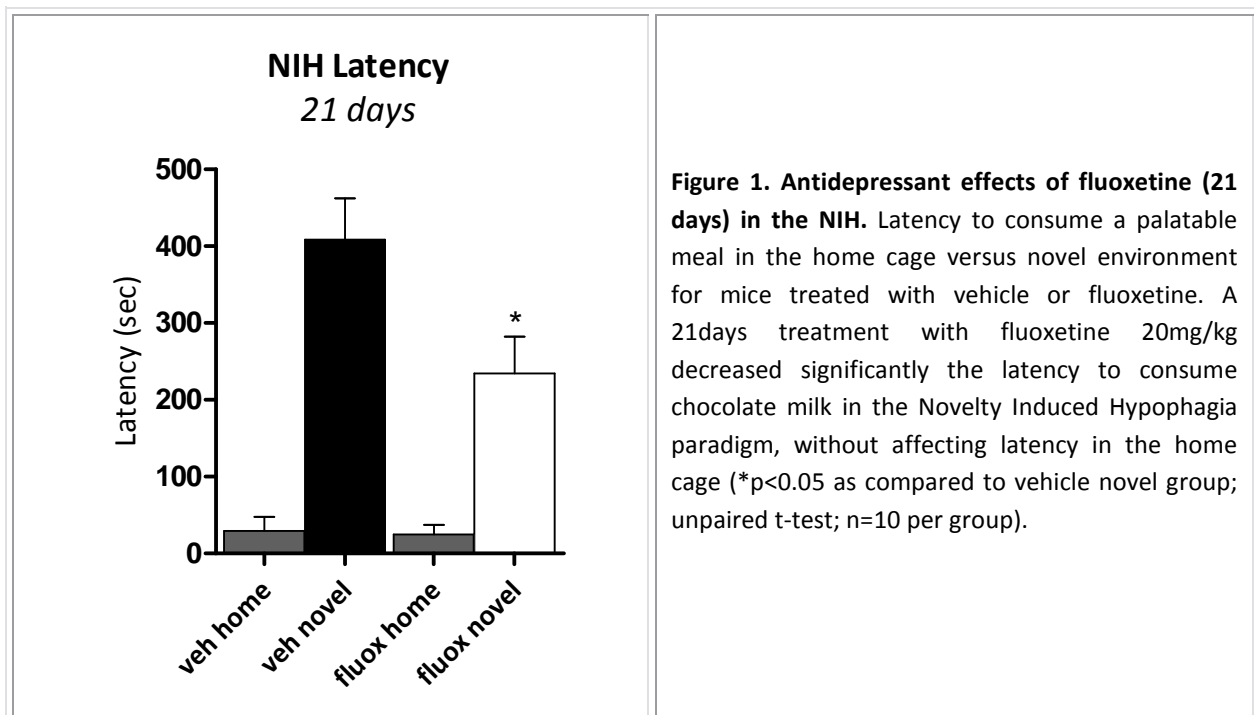
Objective of the study

Based on the above, we used the novelty-induced hypophagia paradigm to evaluate the delay of onset of action of the AMPA receptor potentiator LY392098. Behavioral outcome was associated with BDNF expression in the hippocampus of Balb/c adult mice.

Results

In order to evaluate the onset of antidepressant action, we used the novelty-induced hypophagia test. Chronic treatment (21 days) was first assessed to ensure that our experimental setup is sensitive to fluoxetine chronic effects as described in the literature (Dulawa and Hen, 2005).

Chronic (21days) treatment with fluoxetine 20mg/kg was administered i.p. and animals were tested on the day 22. Mice were habituated to consume chocolate milk in their home cage and latency to drink was measured in a novel cage the day of the test (Figure 1). Fluoxetine treatment significantly decreased the latency to consume chocolate milk in the novel cage as compared to vehicle-treated mice, without affecting latency to consume in the home cage.



Subsequently to determine the time-point of onset of action of the AMPA potentiator LY392098 we investigated whether a short period of LY392098 administration could exhibit antidepressant effects. We tested 5 days of treatment with LY392098 5mg/kg i.p. and animals were tested on the day 6. Novelty-induced increase of latency to consume in the novel cage was significantly reduced by LY392098 (Figure 2). To ensure that alterations in the latency to consume reflect an antidepressant effect and are not due to changes in appetite we also measured total milk consumption in the home cage. Consumption did not differ significantly, showing that treatment effects are not due to changes in intake (Figure 3).

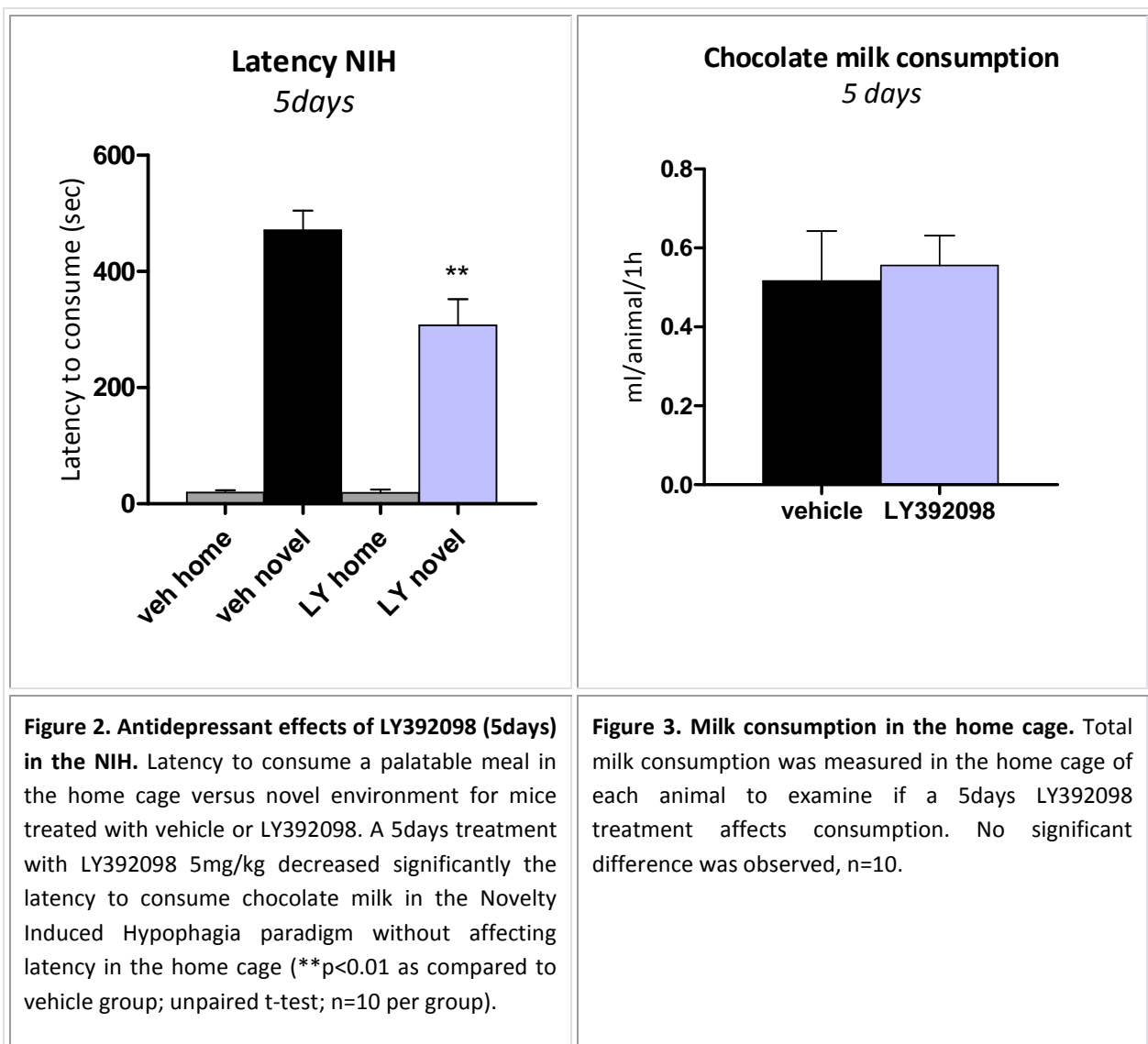


Figure 2. Antidepressant effects of LY392098 (5days) in the NIH. Latency to consume a palatable meal in the home cage versus novel environment for mice treated with vehicle or LY392098. A 5days treatment with LY392098 5mg/kg decreased significantly the latency to consume chocolate milk in the Novelty Induced Hypophagia paradigm without affecting latency in the home cage (**p<0.01 as compared to vehicle group; unpaired t-test; n=10 per group).

Figure 3. Milk consumption in the home cage. Total milk consumption was measured in the home cage of each animal to examine if a 5days LY392098 treatment affects consumption. No significant difference was observed, n=10.

BDNF expression has been repeatedly shown to correlate with antidepressant action. We assessed BDNF mRNA expression in animals treated with LY392098 5mg/kg for 5 consecutive

days. Having demonstrated an early onset of action of LY392098, we then examined if this effect correlates with changes in BDNF expression in hippocampus. Treated animals were sacrificed 24h after the last injection and brains were rapidly frozen. *In situ* hybridization for BDNF mRNA was assessed on hippocampal slices. LY392098 treatment was found to significantly increase BDNF expression in CA1 and CA3 regions of hippocampus (Figure 4).

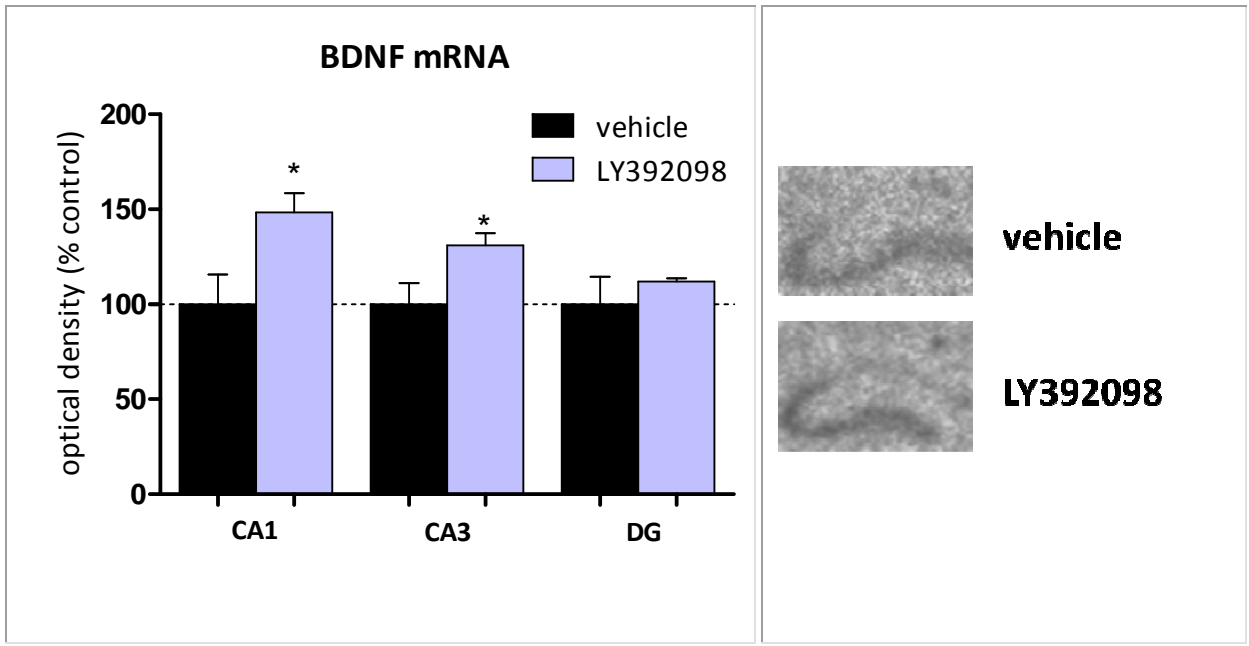


Figure 4. BDNF expression in hippocampus after 5days LY392098 5mg/kg. LY392098 significantly increased BDNF expression in CA1 and CA3 but not DG in hippocampus (unpaired t-test to compare vehicle vs LY392098 in CA1, CA3 or DG;*p<0.05, n=5).

Discussion

Chronic administration of the AMPA-R potentiator LY392098 was found to inverse stress-induced effects in the chronic mild stress (UCMS) paradigm. We evaluated the onset of this antidepressant action using the novelty-induced hypophagia (NIH) model, a model sensitive to chronic antidepressant treatment or acute anxiolytic effects. A subchronic treatment of LY392098 (5 days) decreased latency to consume in the NIH. Although, this may be attributed to an anxiolytic effect, it is more believed to be a hint of antidepressant action as an acute administration of desipramine in CREB^Δ mutant mice reduces latency to consume in the NIH but has no effect on the elevated plus-maze (Gur et al. 2007). This effect can be attributed to an enhancement of the motivational aspect that helps animals overcome the mild anxiogenic environment and access the palatable drink. In our previous work, we found no anxiolytic effects of a chronic LY392098 administration after a chronic stress protocol (Farley et al. 2010). Thus, our findings possibly reflect an antidepressant effect on motivation consistent to the one seen in UCMS. It should be noted that a 5day treatment with LY392098 did not alter milk consumption in the home cage, so effects in the NIH are not due to changes in appetite and milk intake. Effects of LY392098 in NIH reflect a very early onset of action. A subchronic dosing of LY392098 has already been reported to exhibit rapid onset antidepressant-like effects in a social interaction test that measures submissive behavior in rats (Knapp et al. 2002).

Hippocampal atrophy and decreased BDNF expression have been related to stress-induced pathophysiological alterations at the origin of depression in animal models and humans, while increased expression of BDNF in hippocampus has been repeatedly shown to correlate with and to mediate antidepressant action (Bleakman et al. 2007). We examined whether the short subchronic treatment (5days) with LY392098 could also potentiate BDNF expression in hippocampus. After 5 days of repeated LY392098 administration, BDNF mRNA was increased in the CA1 and CA3 area of hippocampus but not in the DG of subchronically LY-treated mice.

It is well known that AMPA R potentiators increase BDNF expression both *in vitro* and *in vivo* (Zafra et al. 1990, Hayashi et al. 1999, Lauterborn et al. 2000, Altar C.A. 1999, Alt et al. 2006). *In vivo* the AMPA R potentiator-induced increase in BDNF occurs after 21 days of treatment.

Chronic administration of the AMPA receptor potentiator LY451646 has also been reported to increase progenitor cell proliferation in adult rat hippocampus (Bai et al. 2003). All known effective antidepressant therapies have been shown to enhance BDNF expression in hippocampus. Traditional antidepressants are thought to induce BDNF expression by activating the transcription factor Ca²⁺/cAMP response element-binding protein (CREB) through protein kinase A (PKA) and Ca²⁺/calmodulin-dependent protein kinase (CaM kinase) signal transduction pathways (reviewed in Duman et al. 1997, Alt et al. 2005). AMPA receptors have also been linked to BDNF induction through a Ca²⁺-independent mitogen-activated protein kinase (MAPK) pathway (Hayashi et al. 1999, Legutko et al. 2001).

The present finding that LY392098 increases BDNF expression after 5 days is in line with our behavioral observation in NIH. Antidepressant treatment has been shown to increase BDNF expression after a chronic administration, a lapse of time that correlates with onset of action. In a recent study, concomitant α_2 -adrenoceptor blockade accelerates behavioral effects of chronic antidepressant treatment as measured in the novelty suppressed feeding test in rats, a similar test to NIH, normally sensitive only to longer periods (3 weeks) of antidepressant treatment (Yanpallewar et al. 2010). In the same study, amelioration of the behavioral outcome was associated with a rapid increase (7 days of treatment) of BDNF expression and hippocampal neurogenesis.

The BDNF gene has a rather complex transcriptional regulation with eight promoters generating multiple distinct transcripts that all lead to the same protein. Thus, upregulation of the mature BDNF protein can be mediated through several different ways, depending on the stimulus and the region studied. Differential region-specific regulation of exon-containing BDNF isoforms has been reported in the literature following diverse antidepressant treatment (Dias et al. 2003, Dwivedi et al. 2006). It would be interesting to further examine which BDNF mRNA isoforms are regulated by the AMPA R potentiator LY392098 in each hippocampal area.

Delayed onset of action constitutes a major problem of all current antidepressant treatment. Amelioration of mood is only observed several weeks after the beginning of treatment, a lapse of time related to an increased risk of suicide temptatives. Moreover, all patients do not respond equally to different antidepressant treatments. In order to determine the adequate medication for each patient, a period of several weeks (~4 weeks) is needed

before switching to another class of antidepressants in case of non-response. Taken together, these elements point out the imperative of developing new drugs with earlier onset of action.

In summary these experiments show for the first time that AMPA-R potentiators could constitute a novel class of antidepressant treatment with a faster onset of action. Furthermore, AMPAkinases could be used as an adjunct to classical antidepressants to accelerate their actions or as a first line medication, followed by a classical antidepressant treatment.

EXPERIMENTAL STUDIES-RESULTS

PART II

The effect of selective inhibition of ERK/Elk1 signaling in animal models of depression



PART II. The effect of selective inhibition of ERK/Elk1 signaling in animal models of depression

INTRODUCTORY REMARKS

I. ERK and depression

Little is known on the exact role of ERK1/2 pathway in the pathophysiology of depression and in the antidepressant action. Recent studies have tried to decipher ERK1/2 implication in depressive-like states, but results are so far controversial. Acute stress induces activation of MAPKs in cortex, hippocampus, and striatum and of MAPKs to a smaller extent (Shen et al. 2004). Inhibition of ERK1/2 activation in the models of TST and FST has shown both antidepressant and pro-depressant actions. Systemic administration of U327, a MEK inhibitor that crosses the blood–brain barrier (BBB), reduced immobility and increased swimming time in the forced swimming test. Systemic administration of its parent compound U0126, which cannot penetrate the BBB, had no effect in the same tests indicating that the antidepressant-like effects of U327 are due to inhibition of MEK activation in the CNS (Einat et al. 2003). Consistent with this, Galeotti and Ghelardini (2011) have shown that acute stress (tail suspension test, TST, and forced swimming test, FST) activate ERK1/2 in hippocampus and that icv inhibition of MEK using U0126 had antidepressant-like effects in the same tests.

On the other hand, in a study of Duman et al. (2007) acute administration of PD184161 (MEK inhibitor) produced depressive-like responses in learned helplessness, FST, and TST. The behavioral effects of desipramine and sertraline in the FST were significantly reduced by coadministration of PD184161 indicating that the actions of these two major classes of antidepressants, 5-HT and norepinephrine (NE) selective reuptake inhibitors are mediated, at least in part, by activation of the MAPK signaling pathway. In the TST, the MEK inhibitor significantly blocked the effect of desipramine, but did not significantly alter the antidepressant actions of three SSRIs.

In chronic experiments, Qi et al. (2006) found that chronic forced swimming resulted in a depressive-like behavior with a concomitant decrease of phosphorylated ERK1/2 (pERK1/2) in hippocampus and prefrontal cortex. The same group showed later that local sub-chronic (7d) inhibition of ERK1/2 activation in dorsal hippocampus (dHP) resulted in anhedonia and anxiety-like behavior, and in median prefrontal cortex (mPFC) induced anhedonia and locomotor impairment in rats. The phosphorylation of the cyclic AMP-responsive-element-binding protein (CREB) was decreased following the ERK pathway inhibition in both regions (Qi et al. 2009). However, Li et al. 2009 found decreased JNK and P-CREB activity in hippocampus of chronically stressed rats but no effect of chronic unpredictable stress on ERK1/2 activation.

In a recent study of Duric et al. (2010), a major negative regulator of the ERK cascade, the phosphatase MKP-1, was assessed after a chronic stress model in rats. Chronic unpredictable stress (CUS) significantly upregulated MKP-1 in DG, CA1 and CA3 regions of hippocampus, but not in cortex, showing a decrease in ERK1/2 activation selectively in hippocampus induced by stress. Antidepressant treatment with fluoxetine completely inverted these effects in DG and partially in CA1. Selective upregulation of MKP-1 in DG produced profound depressive-like responses similar to the effect of CUS (decreased sucrose preference, increased escape failures in the active avoidance test, increased latency to feed in the novelty-suppressed feeding test and increased immobility in the forced swim test). Experiments on MKP-1^{-/-} mice demonstrated that Mkp-1^{-/-} mice are normal in the absence of stress and are resistant to CUS-induced behavioral deficits (anhedonia and anxiety). Analysis of pERK1/2 (decreased) and total ERK1/2 (no effect) in the hippocampus of stressed WT or Mkp-1^{-/-} mice (no effect on pERK1/2) further confirmed the hypothesis that decreased ERK signaling through stress-induced MKP-1 upregulation contributes to the expression of depressive symptoms.

In humans, a whole-genome expression analysis of postmortem hippocampal tissues from individuals with depression and healthy controls that were matched for age, gender, tissue pH and postmortem interval revealed MKP-1 as significantly increased in both the dentate gyrus and CA1 of MDD subjects (Duric et al. 2010). MEK2 was significantly downregulated in the CA1 of subjects with MDD, whereas expression of ERK2 was decreased in the dentate gyrus. Inhibition of ERK signaling (that is, decreased levels of pERK), has been previously

demonstrated in postmortem hippocampus of depressed individuals who committed suicide (Dwivedi et al. 2001, 2006a, Hsiung et al. 2003). Downstream targets of ERK (MSK1, CREB1, CREBL, BDNF, VGF, and VEGFA) were also downregulated.

II. ERK and antidepressant action

Studies on the effects of antidepressants on the ERK pathway have so far yielded inconsistent results. Chronic treatment of rats with venlafaxine (SNRI) did not induce significant changes on immunostaining of phosphorylated RSK (a downstream target of ERK) in the frontal cortex and hippocampus (Khawaja et al. 2004). Chronic treatment of rats with fluoxetine (SSRI) and reboxetine (NRI) significantly reduced levels of phosphorylated ERK1, but not those of phosphorylated ERK2 in nuclear fraction of the hippocampal tissue (Tiraboschi et al. 2004). The same treatments did not significantly alter levels of pERK1/2 in prefrontal cortex. Chronic treatment of rats with desipramine (TCA) significantly elevated levels of pERK2 in nuclear fraction of the hippocampal tissue and decreased levels of pERK1 in nuclear fraction of the prefrontal cortical tissue (Tiraboschi et al. 2004).

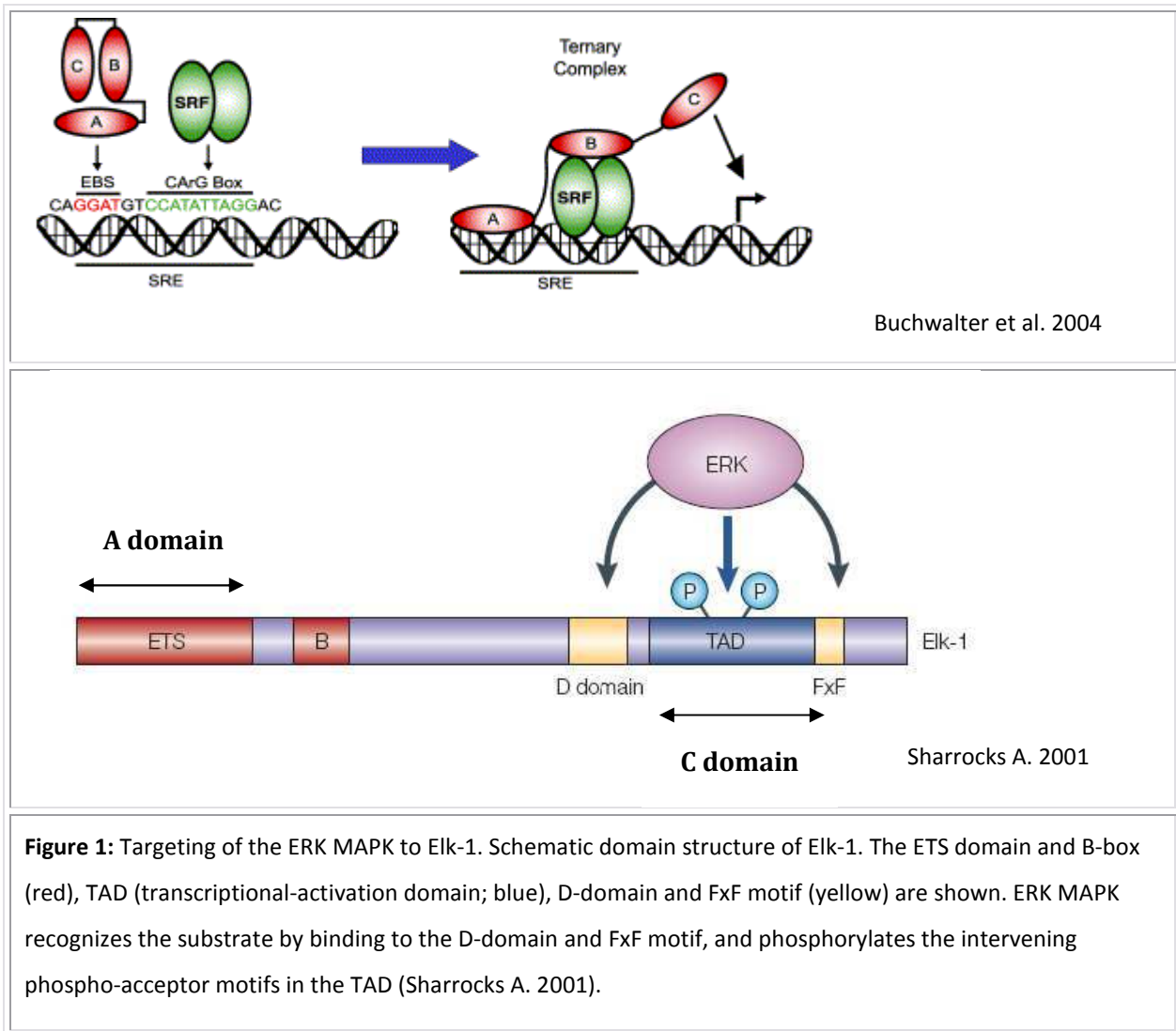
Another study found that chronic treatment of rats with fluoxetine, but not imipramine (TCA), significantly reduced levels of pERK1 and pERK2 in nuclear fractions of the hippocampal and frontal cortical tissues and levels of pERK1 in hippocampal synaptosomes (Fumagalli et al. 2005). However, Qi et al. (2008) showed that chronic treatment with fluoxetine significantly increases pERK2 in hippocampus of naïve rats and inverses stress-induced decrease of pERK2 in hippocampus in the model of chronic forced swim stress.

Up to now, manipulation of ERK1/2 signaling during a stress response or an antidepressant treatment has shown both beneficial and hazardous effects. All the above show that ERK1/2 pathway is crucial in mechanisms underlying depressive states and antidepressant action, but further evidence is necessary to decipher the exact role of ERKs in mood modulation. Studying downstream targets of ERK1/2 may accord important information on the implication of the ERK cascade in depression and antidepressant action.

III. Downstream modules of ERK signaling: Elk-1 Vs MSK-1

Cumulative data have revealed that MAPK signaling cascades activate transcription factors in order to transduce extracellular signals from ligand-activated cell surface receptors to the nucleus and regulate gene expression. ERK1/2 pathway has been shown to activate both Elk-1 and CREB transcription factors in a concomitant way (Vanhoutte et al. 1999). Evidence from studies using *MSK1*^{-/-} or *MSK2*^{-/-} or double-knockout mice and pharmacological inhibitors have established that MSK mediates ERK-induced phosphorylation of CREB at Ser133 (Wiggin et al. 2002) and thereby stimulates transcription of CREB-target genes. CREB has been implicated in depressive states and antidepressant action, through the regulation of transcription of the BDNF gene (as already discussed).

ERKs phosphorylate Elk-1 in several sites thereby increasing its transcriptional activity (Gille et al. 1992, Cruzalegui et al. 1999). Elk-1 is a member of the ternary complex factors (TCFs) subfamily of the ETS-domain transcription factors, which contains a C-terminal transcriptional activation domain with multiple MAPK core consensus phosphorylation sites (Buchwalter et al. 2004). Indeed, stimulation of cells with growth factors and other ligands induces phosphorylation of 6–9 distinct sites on Elk1, all of which seem to be simultaneously phosphorylated. The phosphorylation enhances DNA binding of Elk1 through the A domain, may participate in recruitment of coactivators such as CBP, p300, Srb or SRF through the B domain, and eventually, dramatically increases the transcriptional activity of Elk1 (Figure 1). The phosphorylated sites on Elk1 that have been identified are Thr353, Thr363, Thr368, Ser383, Ser389, Thr417, and probably Ser324, Thr336 and Ser422 (Cruzalegui et al. 1999). Out of these sites, it was shown that phosphorylation of Ser383 alone is crucial for the activity of Elk1. Phosphorylation of Ser389 seems to play some role in Elk1 activity as well, while the role of phosphorylation of the other residues is not fully understood (Buchwalter et al. 2004). Interestingly, extracellular signal-dependent phosphorylation and activation of Elk1 can also be mediated by other MAPKs, including JNK and p38MAPK (Whitmarsh et al. 1997), but the phosphorylation by these other MAPKs usually occurs under fewer circumstances and in a slower kinetics.



The rapid and efficient phosphorylation of Elk1 by ERKs is enabled by a direct interaction between the two proteins. This interaction is mediated by the two classical docking domains, which are present in Elk1, and their counterparts in ERKs. Thus, one of the sites is the D-domain which contains the DEJL (docking site for ERK and JNK LXL) consensus sequence that permits the binding to ERK, JNK, and p38MAPK families. The other docking site is the hydrophobic FXFP or DEF (docking site for ERK, FxF) (Jacobs et al. 1999), which interacts with the hydrophobic interaction domain of ERKs (Figure 1). Interestingly, the binding through each of the docking domains is essential for the phosphorylation of a distinct subset of sites. The binding through the DEF motif, specific for the ERK1/2 pathway, is both necessary and sufficient to direct the phosphorylation of Ser383 and its nearby residues, whereas the interaction through the D domain of Elk1 is required to induce the phosphorylation of other Ser/Thr residues, such as Ser363 (Fantz et al. 2001). This well-regulated phosphorylation of

Elk1 makes the transcription factor a very good model substrate for ERKs, as well as for phosphorylation-dependent regulation of transcriptional activation. Interestingly, it has been shown that blunted zif268 or MSK1 signalling differentially affects (abolishes or exacerbates) performance in the cocaine-place-preference paradigm, suggesting that functional responses to ERK activation are conditioned by subsequently occurring molecular events (Brami-Cherrier et al. 2005).

HYPOTHESIS:

I. Selective inhibition of the ERK/Elk1 signaling module as a novel antidepressant strategy

Despite the compelling evidence presented above, the implication of ERK in depression and antidepressant drug action remains largely unknown. A number of high profile studies have aimed to study the role of ERK in depression. Although this work was done in laboratories that are leaders in the field, the results have been inconclusive and rather contradictory (Duman et al. 2007, Einat et al. 2003, Tronson et al. 2008) as discussed above.

It is noteworthy that the above studies have used inhibitors of MEK, which block ERK activation and non-selectively inhibit all downstream targets of ERK.

We content that this lack of selectivity has been a major limitation: The numerous substrates of ERK include nuclear transcription factors, cytoskeletal proteins, signalling proteins and receptors and it is unlikely that all these substrates serve the same functions. There are, therefore, branch points in the pathway that generate diversity and specificity of ERK signalling, depending on the contingencies of a given cellular system and prior information stored in it. This is achieved by the recruitment of distinct molecular partners as discussed above for Elk1 and MSK1 signaling.

Our hypothesis is that a selective inhibition of the ERK/p-Elk1 signaling module, rather than non-selective modulation of ERK, could constitute a novel antidepressant strategy.

Indeed, CREB signaling is implicated in the antidepressant action of all actual antidepressant treatments as well as in BDNF regulation. Inhibiting CREB activation from ERK would be rather pro-depressant and would block the antidepressant effect (Duman et al. 2007). Indeed, ERK inhibition has been shown to have pro-depressant effects (Qi et al. 2009). However, ERK inhibition has also shown antidepressant effects implying that downstream modules of ERK could also contribute to the antidepressant action.

On the other hand, calcineurin has been shown to be the major phosphatase for Elk-1 dephosphorylation both *in vitro* and *in vivo*, and plays a critical role in Elk-1 regulation (Sugimoto et al. 1997). Chronic treatment with the antidepressant fluoxetine increases

calcineurin expression in the hippocampus, as previously shown in the laboratory (Crozatier et al. 2007). Moreover, chronic stress decreases calcineurin in rat hippocampus and calcineurin inhibition induces depressive-like behaviors (Zhu et al. 2011). These data provide evidence that increased phosphorylation of Elk-1 is implicated in depressive-like states and that dephosphorylation of Elk-1 mediates antidepressant action.

II. Innovative tools to selectively inhibit protein/protein interactions

We hypothesized that MSK1/CREB pathway constitutes an antidepressant pathway downstream ERK, while Elk-1 pathway is rather pro-depressant. In that case, direct and specific inhibition of Elk-1 phosphorylation from ERK would have antidepressant-like effects and could constitute a potent antidepressant target.

How can we selectively inhibit phosphorylation of Elk-1 from ERK without altering phosphorylation patterns of other molecular partners or ERK itself?

The phosphorylation and activation of ERK1/2 substrates is regulated by protein-protein interactions through ERK1/2 docking sites. Active ERK1/2 localize to effectors containing DEF or D-domain (docking domain) motifs. The DEF domain docking site is responsible for recruitment of active ERK1/2 to Elk-1 and IEG (immediate early gene) targets during sustained signaling. The D domain is required for activation of the ERK1/2 effector kinases MSK-1 and RSK, and may also be important for ERK1/2 inactivation by phosphatases. Signaling to either docking site is functionally separated (Dimitri et al. 2005). Physically, docking sites on ERK are situated in diametrical positions as shown in Figure 2. This permits the separate manipulation of each site, using specifically designed peptides that mimic the docking domains of the substrates, without interfering in phosphorylation patterns of other substrates.

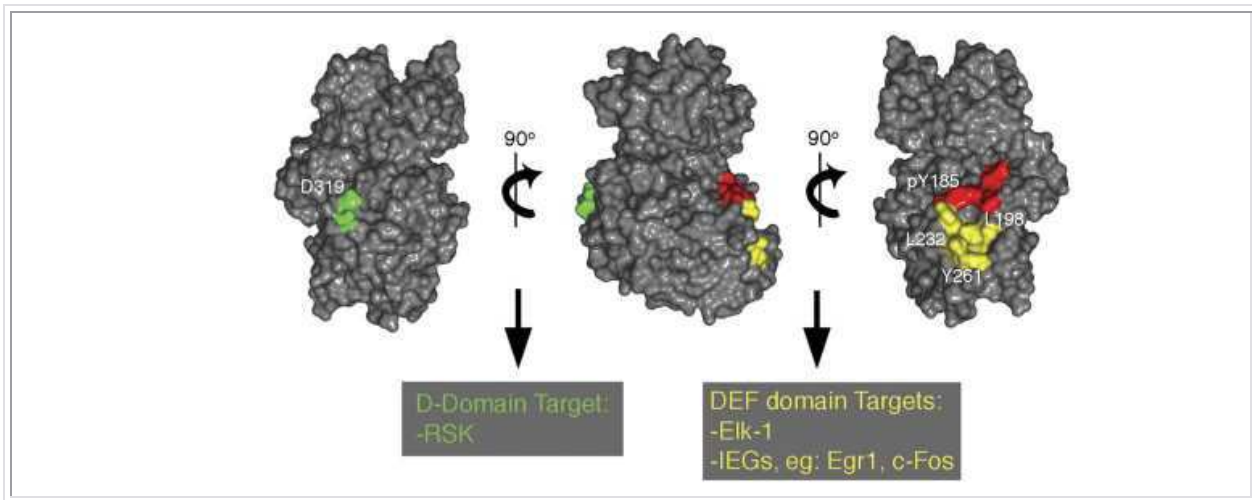


Figure 2: Docking sites on ERK1/2 (Dimitri et al. 2005).

A peptide that mimics Elk-1 docking domain was designed and validated in vitro by the group of J.Caboche (Lavour et al. 2007, Bernard et al. 2011). This peptide carries a membrane penetrating sequence (TAT) from the HIV virus on its N terminus, the sequence FXFP for the docking site DEF and some amino acids of Elk-1 on its C terminus. Administration of this peptide in vivo would be expected to specifically block ERK1/2-Elk-1 interaction, inhibiting activation of Elk-1 from ERK1/2 (Figure 3).

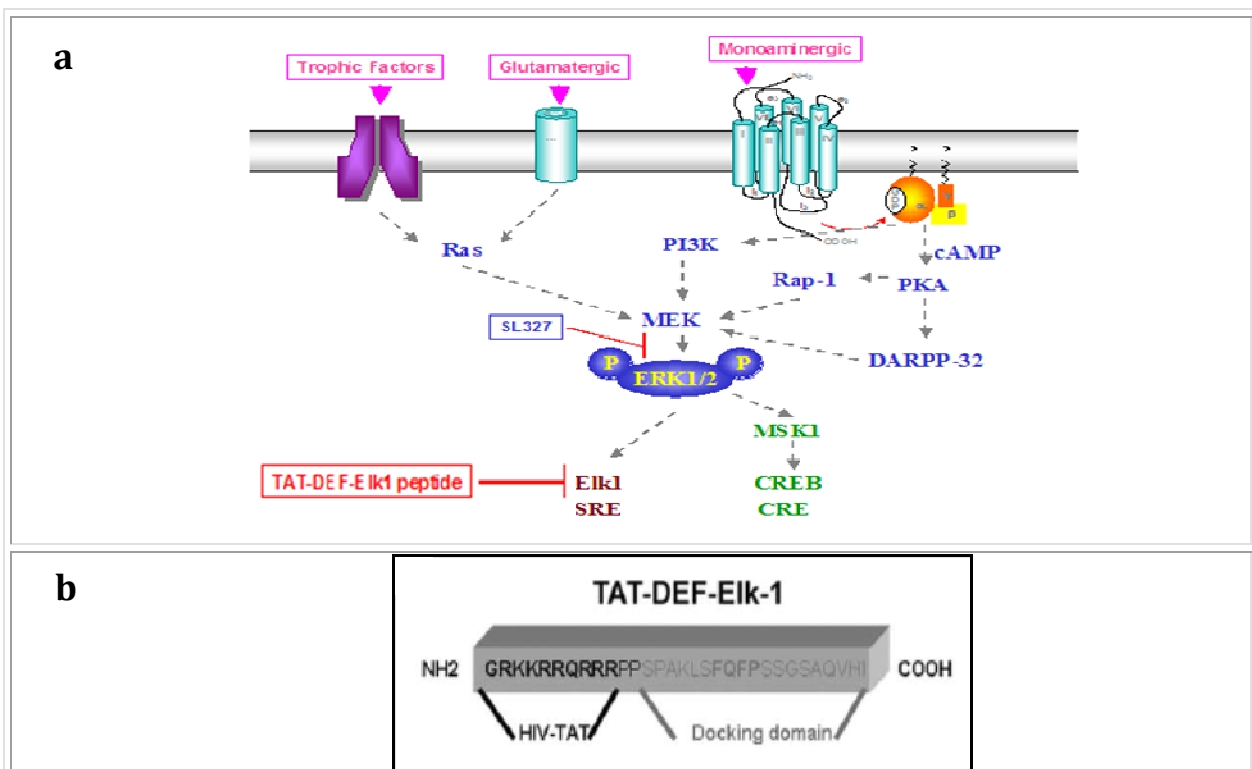


Figure 3: (a) Schematic illustration of ERK1/2 signaling cascade in neurons. Inhibition of ERK phosphorylation by the MEK inhibitor SL327 is depicted, as well as inhibition of Elk1 phosphorylation by ERK in the presence of the TAT-DEF-Elk1 peptide. (b) Design of the sequence of the TAT-DEF-Elk1 peptide.

PART IIa: Selective inhibition of ERK/Elk1 signaling modulates response to stress

Hypothesis

Acute stress has been shown to rapidly increase phosphorylation of ERK1/2 in prefrontal cortex and hippocampus (Meller et al. 2003, Shen et al. 2004, Yang et al. 2004). Activation of ERK mainly activates its downstream targets to regulate gene expression. The presence of the TAT-DEF-Elk1 peptide in the cytoplasm would block ERK/Elk1 protein-protein interaction, thus inhibiting activation of Elk1 and Elk1-mediated gene expression induced by stress. Systemic administration of the TAT-DEF-Elk1 peptide is expected to cross the brain-blood barrier (BBB) and penetrate the cell membrane as it carries a TAT sequence.

Objective of the study

Based on the above, we assessed *in vivo* acute administration of the TAT-DEF-Elk1 peptide to examine whether specific inhibition of ERK/Elk1 signaling can have an antidepressant-like action. We administered the TAT-DEF-Elk1 peptide systemically to validate its specificity *in vivo* in brain regions related to depression. Activation of different modules of the ERK cascade and Elk1-regulated gene expression were measured by fluorescent immunohistochemistry.

In a behavioral level, TAT-DEF-Elk1 effects were evaluated in the tail suspension and forced swimming tests (TST and FST respectively). These tests are widely used to screen antidepressant action as reference antidepressants decrease immobility after an acute administration.

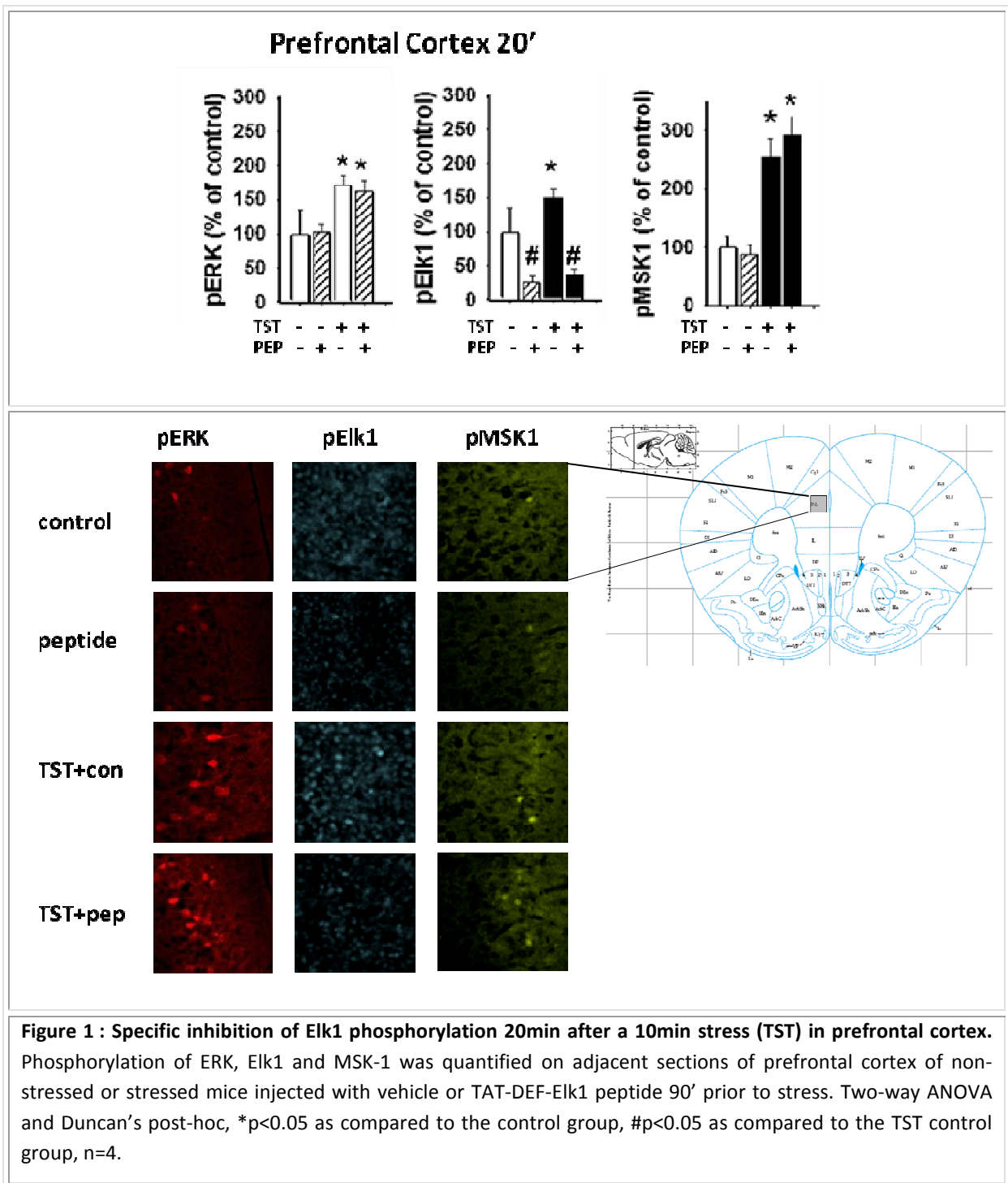
Results

I. *In vivo* validation of the specificity of the TAT-DEF-Elk-1 peptide

In order to confirm phosphorylation of Elk1 by ERK after an acute stress *in vivo* and evaluate whether the TAT-DEF-Elk1 peptide specifically blocks ERK/Elk1 signaling, we assessed levels of phosphorylation of ERK, Elk1 and MSK-1 by immunohistochemistry. Adult (8 weeks) male C57BL/6J mice were subjected to a tail suspension stress during 10 min and transcardially perfused 20, 60 and 120 min after the beginning of the stress. Phosphorylation of ERK1/2, MSK-1 and Elk-1 were examined at 20 min and the induction of the immediate early genes zif268 and c-fos at 60 and 120 min after stress in prefrontal cortex, ventral striatum and hippocampus (CA1). TAT-DEF-Elk1 peptide was administered at a dose of 2mg/kg i.p. 90min before stress.

TAT-DEF-Elk1 peptide blocks pElk1 but not pERK or pMSK1 after stress

Stress significantly increased phosphorylation of ERK, Elk1 and MSK1 in all regions studied (Figures 1, 2, 3). TAT-DEF-Elk1 peptide administration specifically inhibited stress-induced phosphorylation of Elk1 in the prefrontal cortex and hippocampus without altering pERK1/2 or pMSK1 amounts. Administration of TAT-DEF-Elk1 showed no effect on phosphorylation of ERK, Elk1 or MSK-1 in ventral striatum (Figure 2).



Nucleus Accumbens 20'

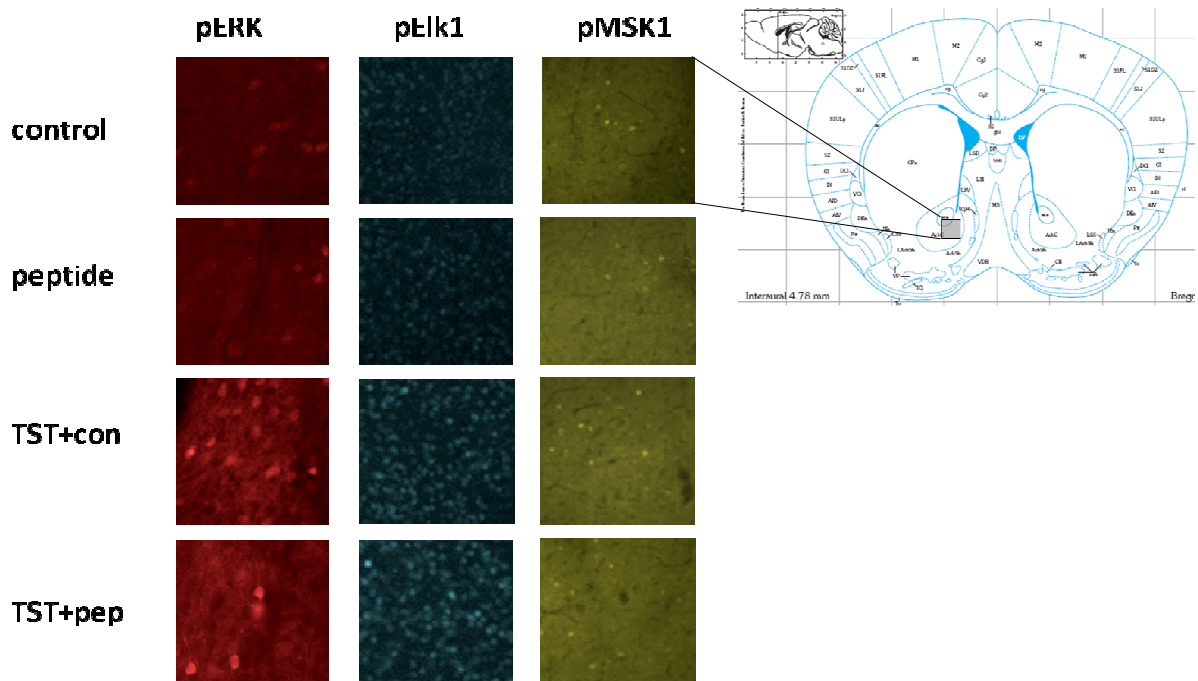
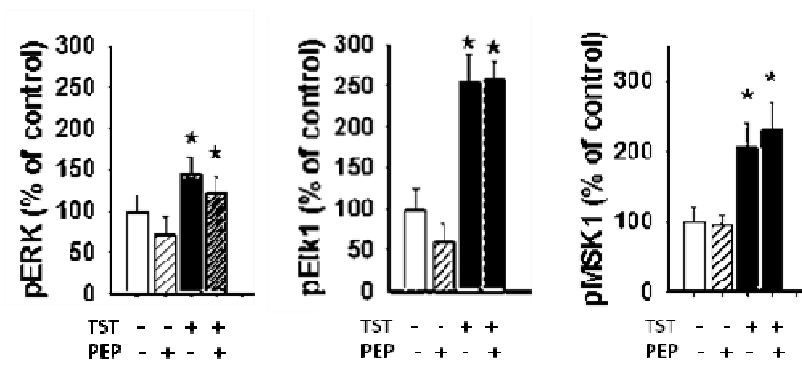


Figure 2: Specific inhibition of Elk1 phosphorylation 20min after a 10min stress (TST) in ventral striatum. Phosphorylation of ERK, Elk1 and MSK-1 was quantified on adjacent sections of striatum of non-stressed or stressed mice injected with vehicle or TAT-DEF-Elk1 peptide 90' prior to stress. Two-way ANOVA and Duncan's post-hoc, * $p < 0.05$ as compared to the control group, $n = 4$.

Hippocampus – CA1 20'

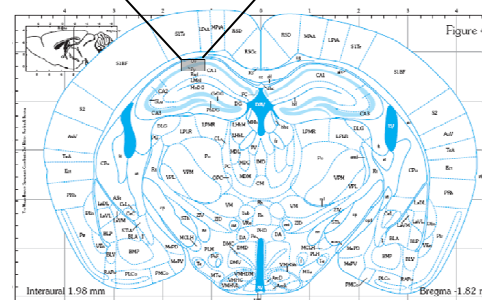
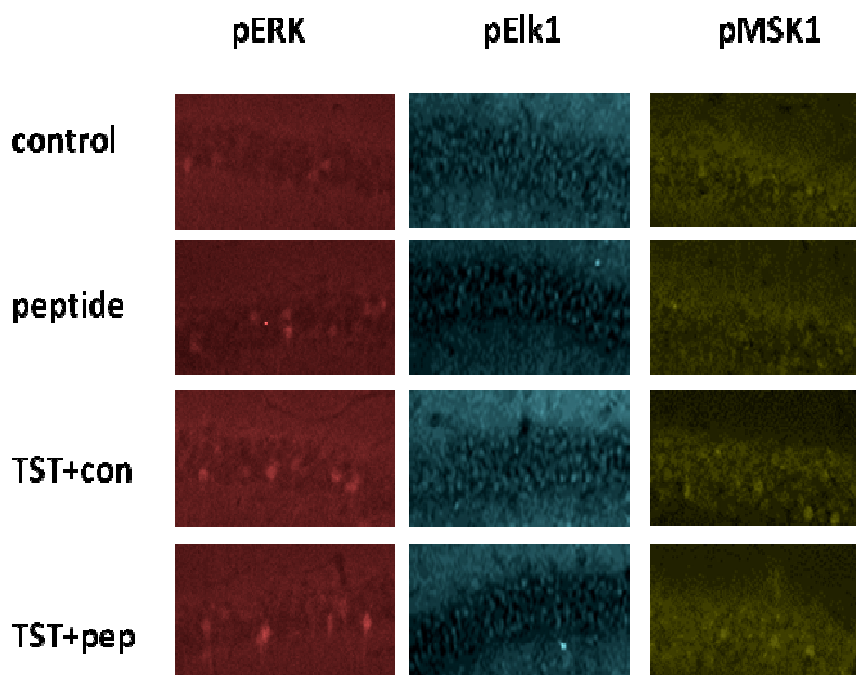
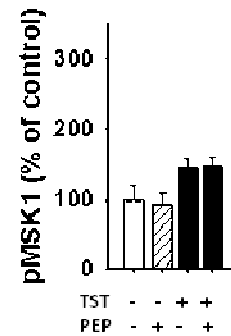
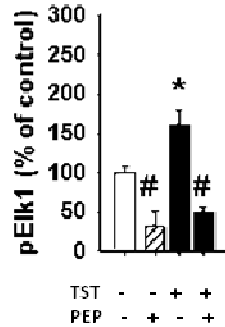
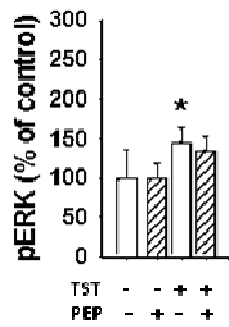


Figure 3: Specific inhibition of Elk1 phosphorylation 20min after a stress (TST) in hippocampus. Phosphorylation of ERK, Elk1 and MSK-1 was quantified on adjacent sections of hippocampus of non-stressed or stressed mice injected with vehicle or TAT-DEF-Elk1 peptide 90' prior to stress. Two-way ANOVA and Duncan's post-hoc, * $p < 0.05$ as compared to the control group, # $p < 0.05$ as compared to the TST control group, $n = 4$.

Immediate early genes (IEGs) expression after stress and TAT-DEF-Elk1 peptide

Stress had no effect on zif268 induction when measured 60min after the beginning of stress in prefrontal cortex or ventral striatum (Figures 4, 5). To the contrary, expression of zif268 was significantly increased 60min after stress in the CA1 area of hippocampus (Figure 6). Administration of the TAT-DEF-Elk1 peptide decreased zif268 levels in prefrontal cortex and ventral striatum, both in basal and after stress conditions. In hippocampus, administration of the TAT-DEF-Elk1 peptide had no effect alone, but showed a tendency to decrease stress-induced increase of zif268 levels.

Zif268 was also measured at 120min after stress (Figures 4, 5, 6). Stress induced a significant increase in zif268 expression in prefrontal cortex and to a smaller extent in hippocampus, whether no effect was observed in ventral striatum 120 min after stress. Administration of the TAT-DEF-Elk1 peptide abolished stress-induced increase of zif268 expression in prefrontal cortex and significantly decreased basal levels. No effect was observed in ventral striatum. In hippocampus basal levels were intact while after stress TAT-DEF-Elk1 significantly decreased zif268 expression.

The expression of c-fos was also measured 60 and 120 min after stress (Figures 4, 5, 6). Stress significantly increased c-fos expression in all regions studied at both time points. This effect was greater in hippocampus. Administration of the TAT-DEF-Elk1 peptide had no effect on c-fos induction 60min after stress, while it abolished stress effects in all regions 120min after stress. No significant effect was found in basal levels.

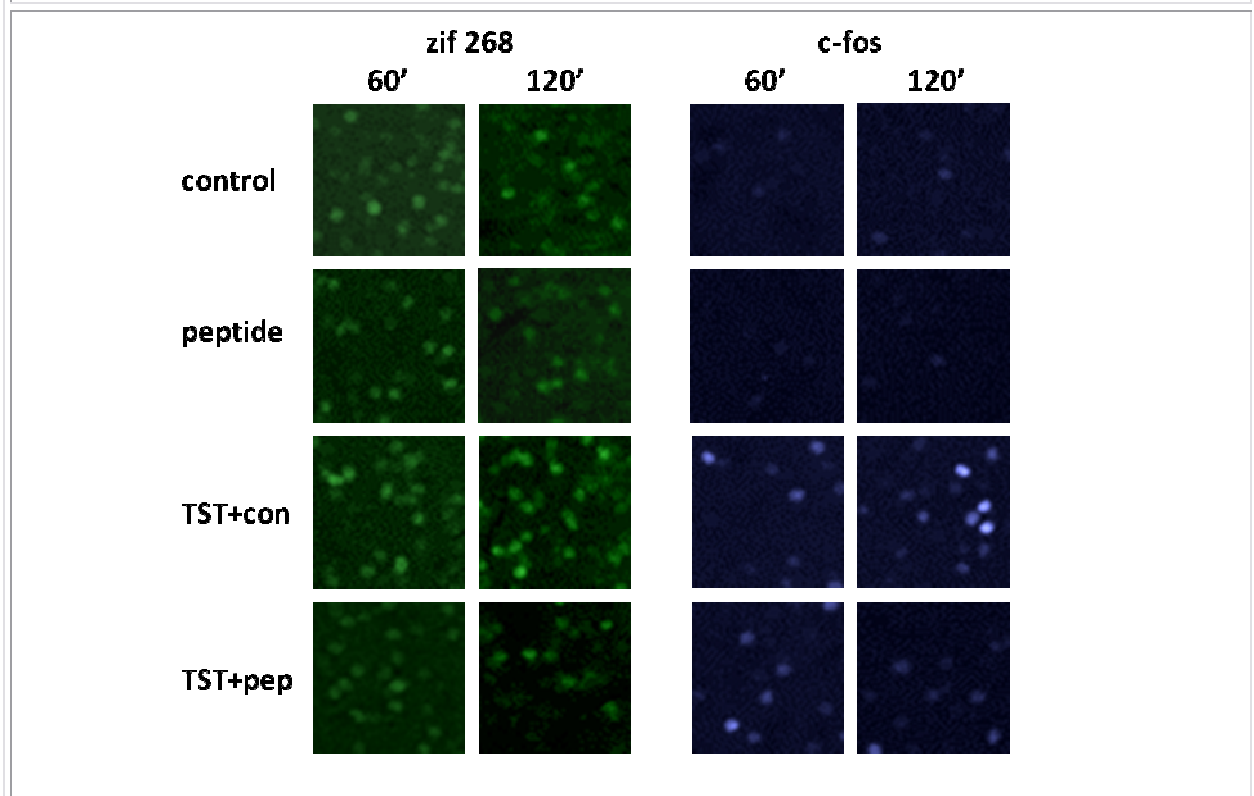
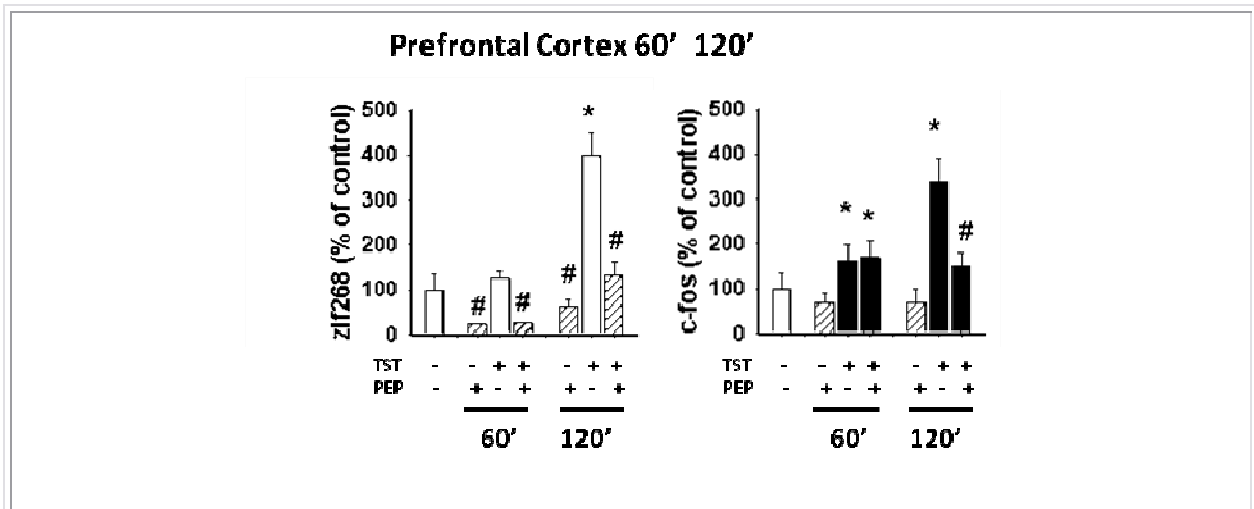


Figure 4: Expression of zif268 and c-fos 60 and 120min after a 10min stress (TST) in prefrontal cortex. Zif268 and c-fos expression was quantified on adjacent sections of prefrontal cortex of non-stressed or stressed mice injected with vehicle or TAT-DEF-Elk1 peptide 90' prior to stress. Two-way ANOVA and Duncan's post-hoc, * $p < 0.05$ as compared to the control group, # $p < 0.05$ as compared to the TST control group, $n = 4$.

Nucleus Accumbens 60' 120'

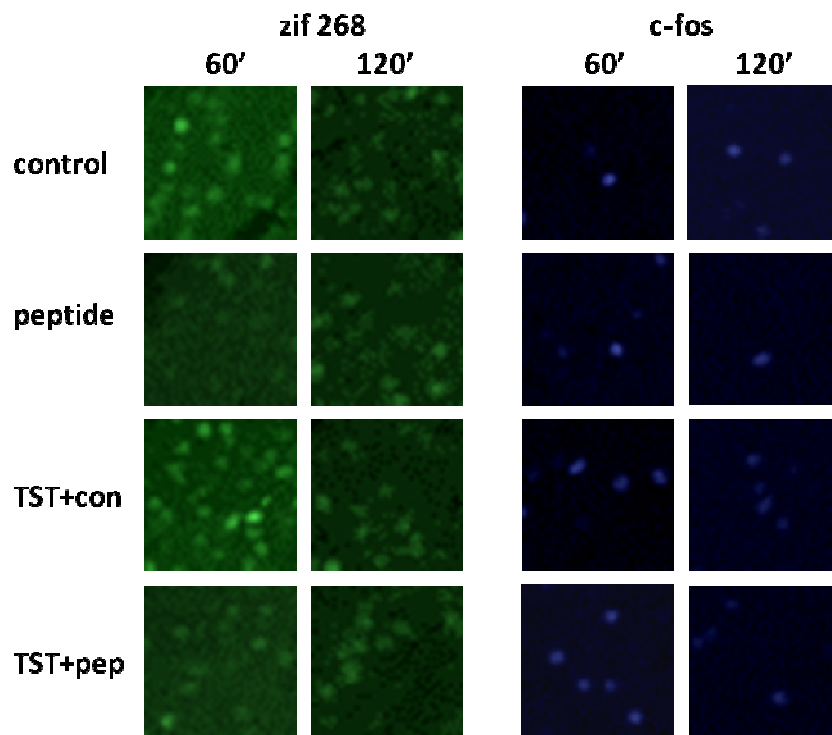
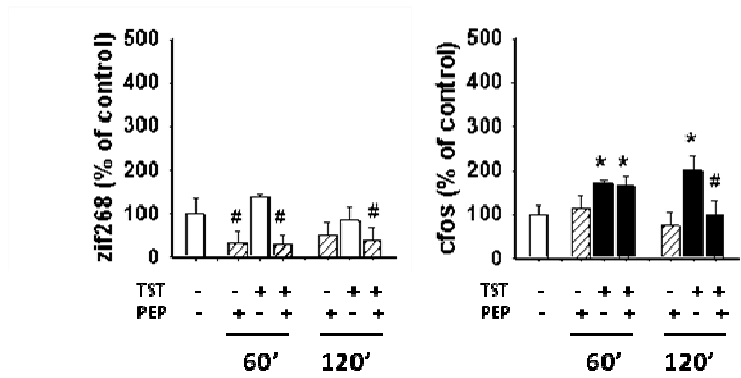


Figure 5: Expression of zif268 and c-fos 60 and 120min after a 10min stress (TST) in ventral striatum. Zif268 and c-fos expression was quantified on adjacent sections of striatum of non-stressed or stressed mice injected with vehicle or TAT-DEF-Elk1 peptide 90' prior to stress. Two-way ANOVA and Duncan's post-hoc, *p<0.05 as compared to the control group, #p<0.05 as compared to the TST control group, n=4.

Hippocampus - CA1 60' 120'

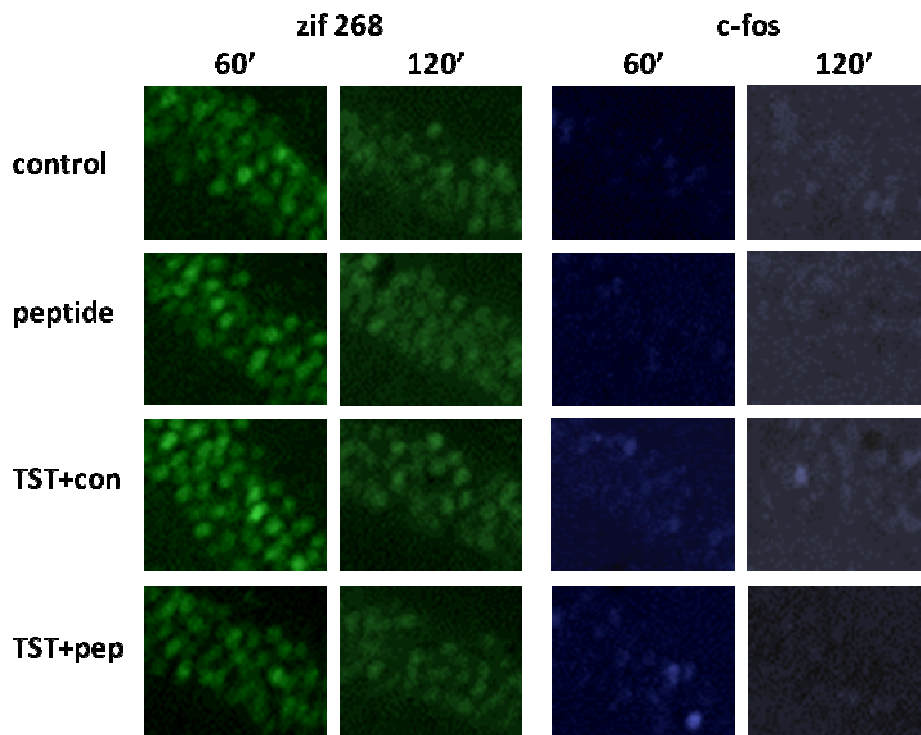
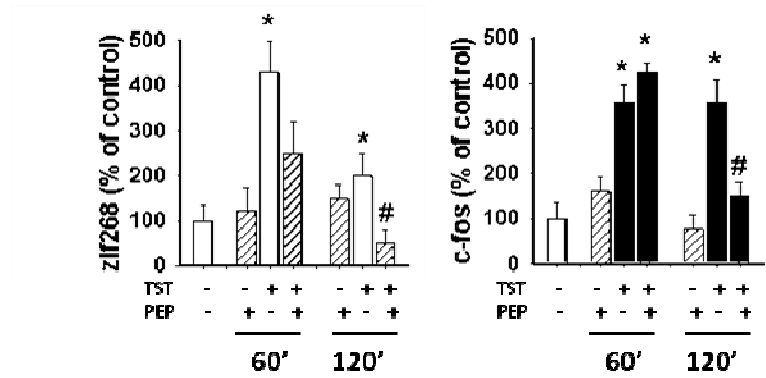
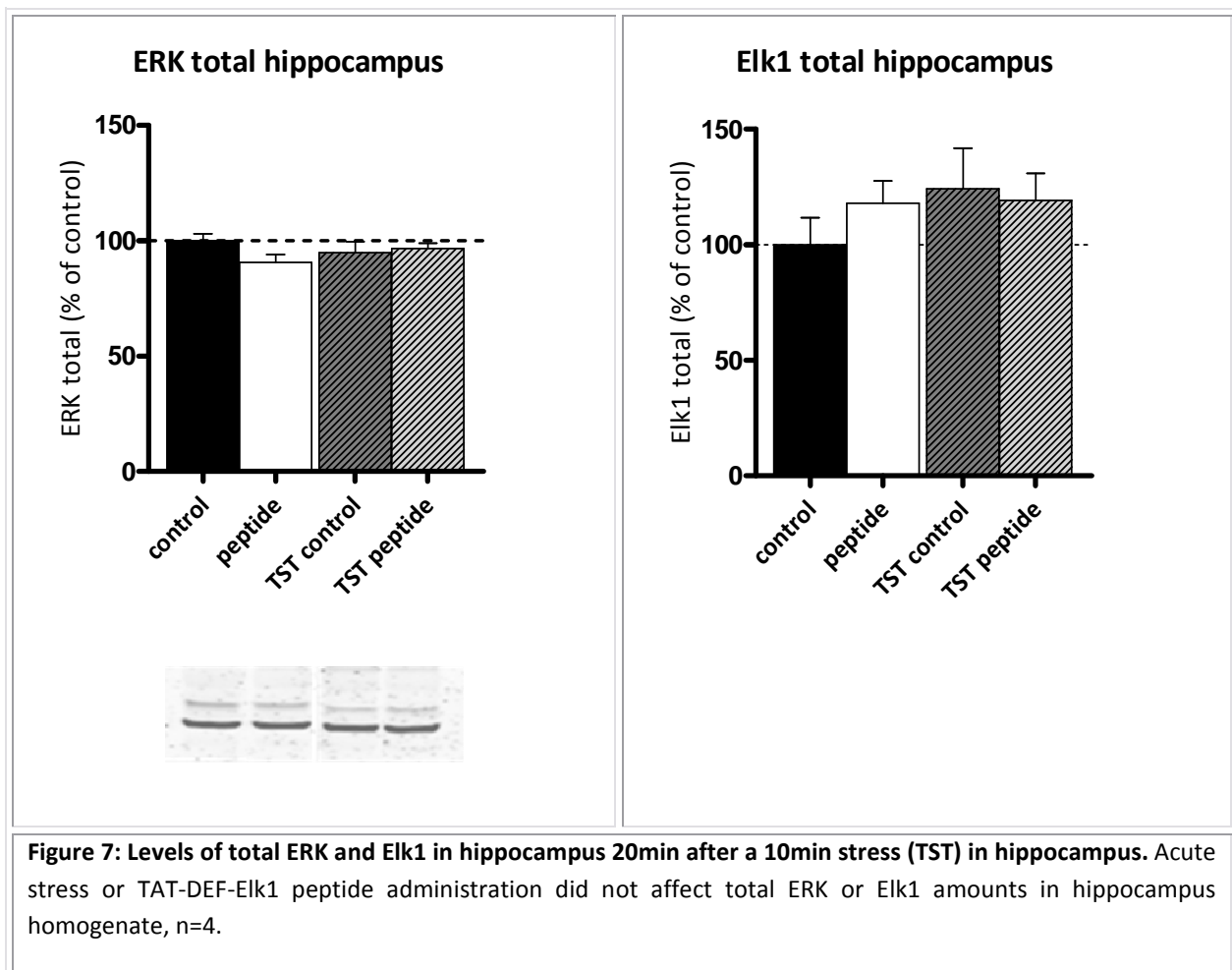


Figure 6: Expression of zif268 and c-fos 60 and 120min after a 10min stress (TST) in hippocampus. Zif268 and c-fos expression was quantified on adjacent sections of hippocampus of non-stressed or stressed mice injected with vehicle or TAT-DEF-Elk1 peptide 90' prior to stress. Two-way ANOVA and Duncan's post-hoc, * $p < 0.05$ as compared to the control group, # $p < 0.05$ as compared to the TST control group, $n = 4$.

No changes in total ERK or Elk1 protein expression

Alterations in phosphorylated amounts of proteins may result from alterations in total amounts of the same proteins in their non-phosphorylated form. To examine if the observed changes in pERK and pElk-1 amounts were indeed due to alterations of the activation state we measured total protein levels of ERK and Elk1 in hippocampal tissue by Western blotting. TAT-DEF-Elk1 peptide was injected i.p. at 2mg/kg 90min prior to a 10min tail suspension stress, as for the immunohistochemistry experiments, and animals were sacrificed 20min after stress. No significant effects were found in ERK or Elk1 total protein levels after stress and/or TAT-DEF-Elk1 peptide administration (Figure 7). All measures were normalized with GAPDH as a cytosolic marker and expressed as percentage of the control.



No changes in CREB signaling

We also measured phosphorylated and total amounts of CREB by Western blotting in hippocampal nuclear fraction of the same experimental conditions. The transcription factor CREB is activated by multiple intracellular signaling cascades. ERK/MSK-1 signaling is one of them. Increased phosphorylation of CREB has been related to antidepressant action and to BDNF expression, as mentioned before. No significant effect of stress and/or treatment was found on pCREB or CREB levels in hippocampus (Figure 8). Measures are normalized with H2B as a nuclear marker and expressed as percentage of the control.

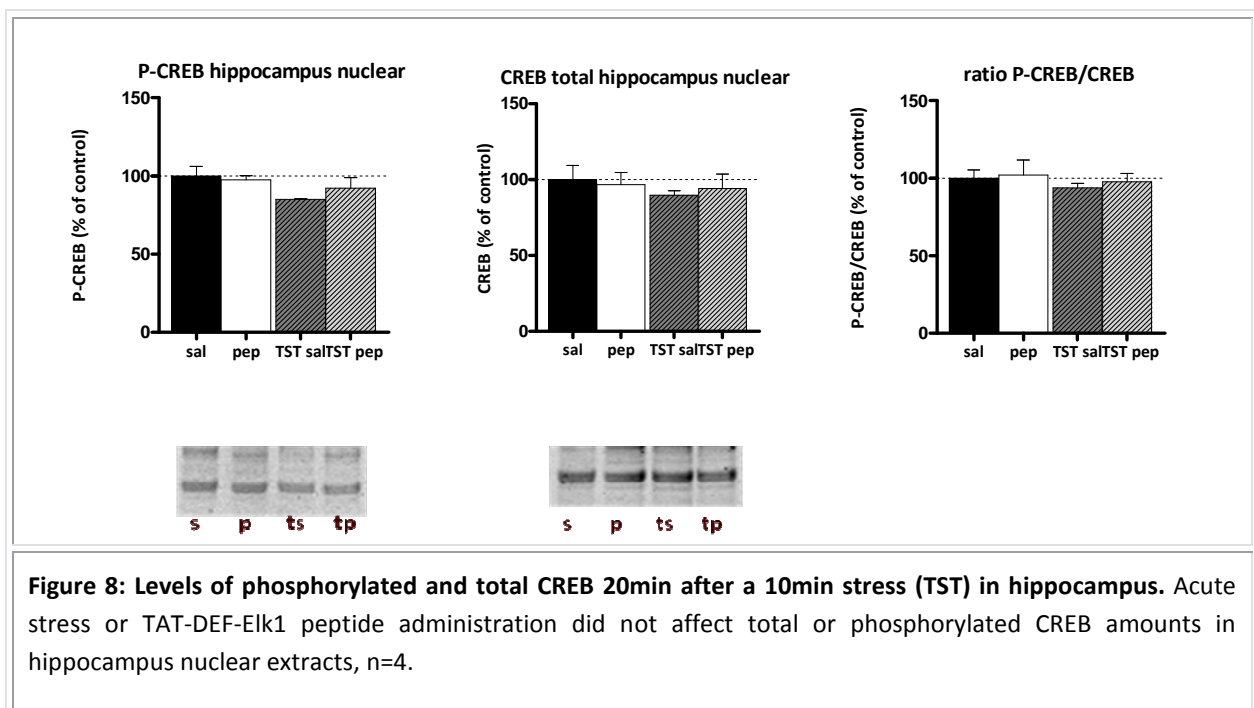
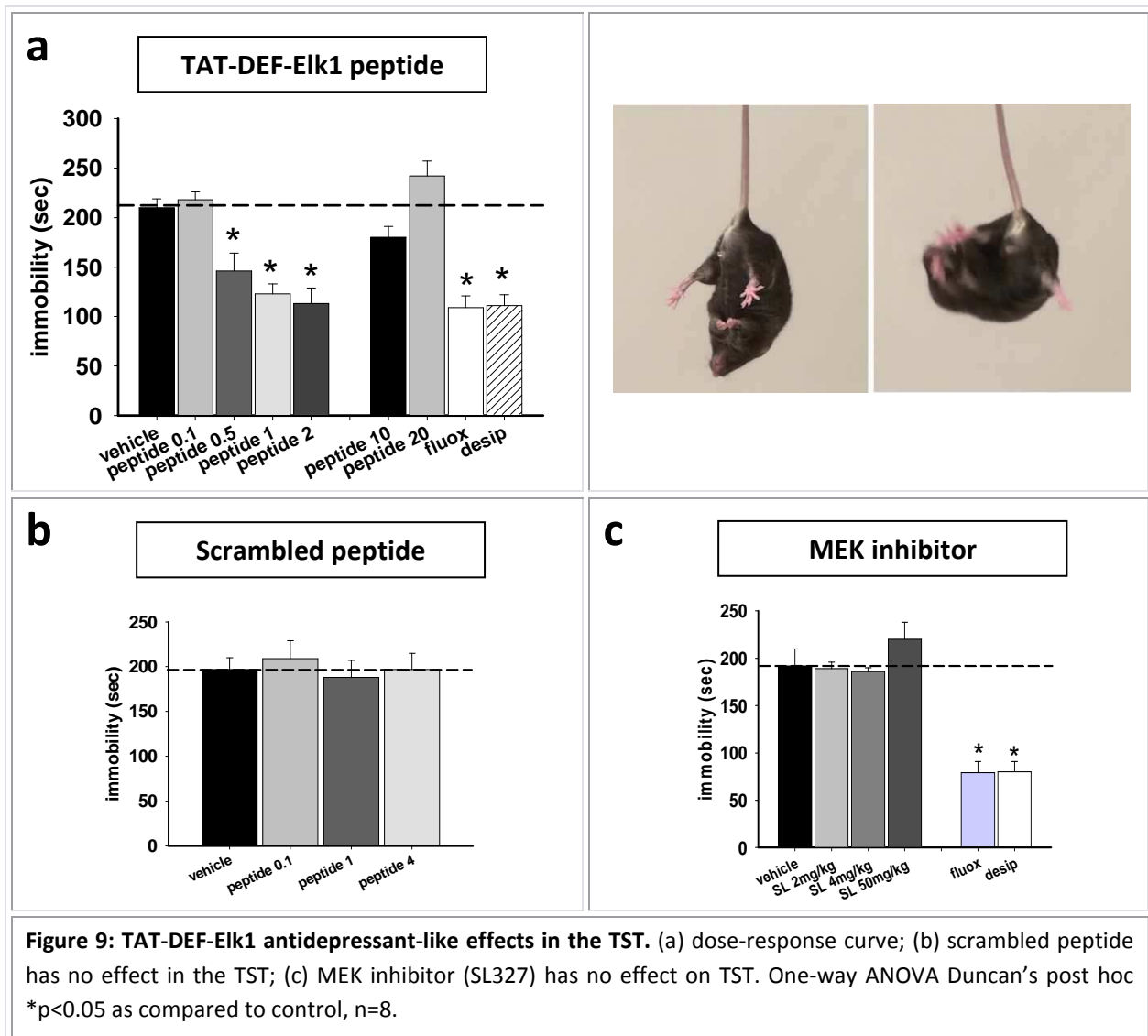


Figure 8: Levels of phosphorylated and total CREB 20min after a 10min stress (TST) in hippocampus. Acute stress or TAT-DEF-EIk1 peptide administration did not affect total or phosphorylated CREB amounts in hippocampus nuclear extracts, n=4.

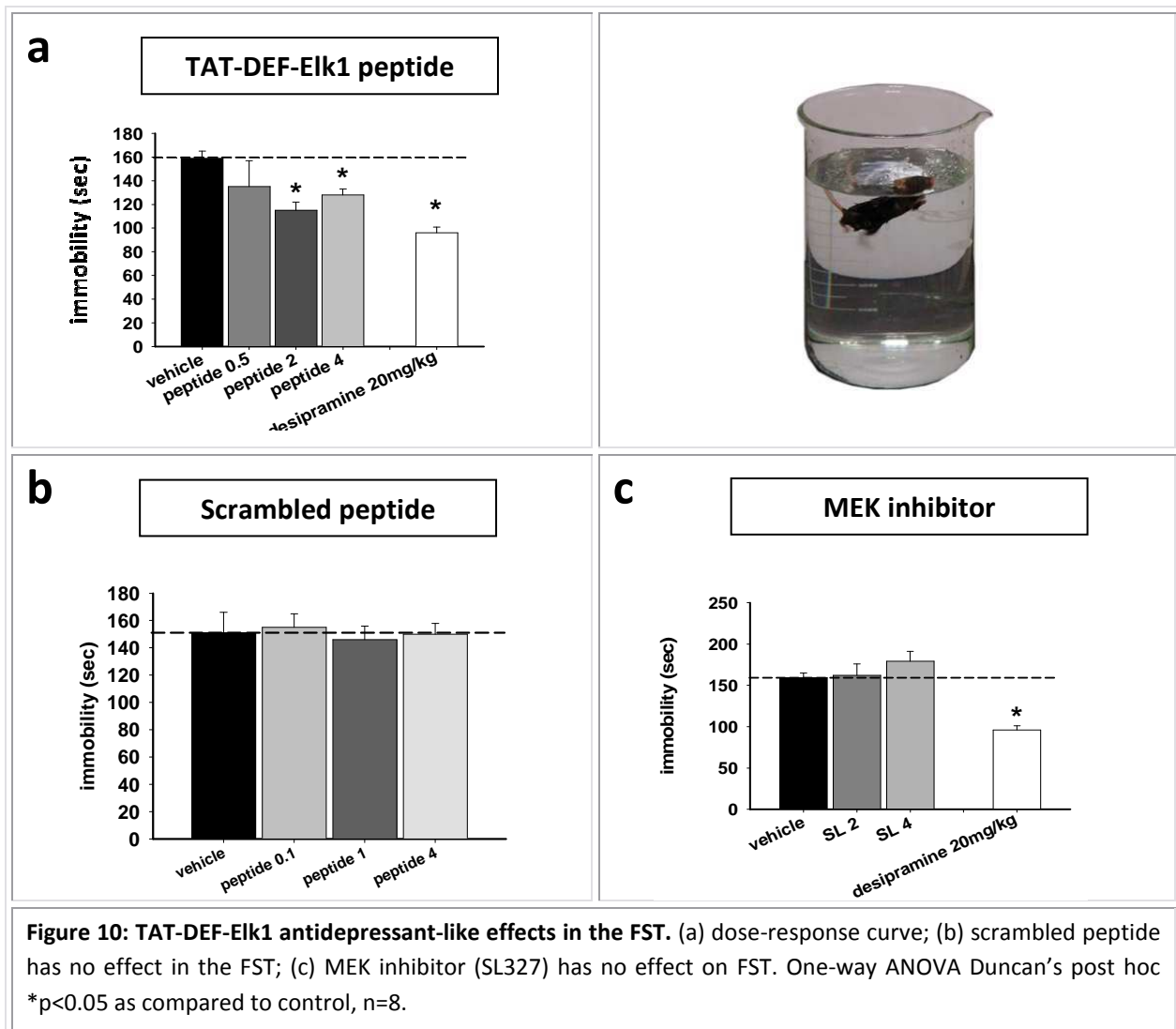
II. Antidepressant-like effects of TAT-DEF-Elk1 peptide

To examine if the TAT-DEF-Elk1 peptide has an antidepressant potential we used the tail suspension test (TST), a test widely used for screening antidepressant action. TAT-DEF-Elk1 peptide was administered at several doses, 90min prior to test and the reference antidepressants fluoxetine (20mg/kg) and desipramine (15mg/kg) were used as positive controls.

Low doses (0.5, 1 and 2mg/kg) significantly reduced immobility on the TST. Higher doses (10 and 20mg/kg) had no effect on immobility (Figure 9a). To verify the specificity of the peptide on behavior, we used a scrambled peptide as a control, which is a peptide of the same length and molecular mass but with a different amino-acid sequence. Scrambled peptide showed no significant effect on immobility time when administered at the same (effective doses) of the TAT-DEF-Elk1 peptide 90min prior to test (Figure 9b). We also tested the MEK inhibitor SL327 to examine whether an inhibition of the phosphorylation of ERK may have the same effect as the specific inhibition of pElk1 on TST. SL327 showed no significant effect at doses tested (Figure 9c).

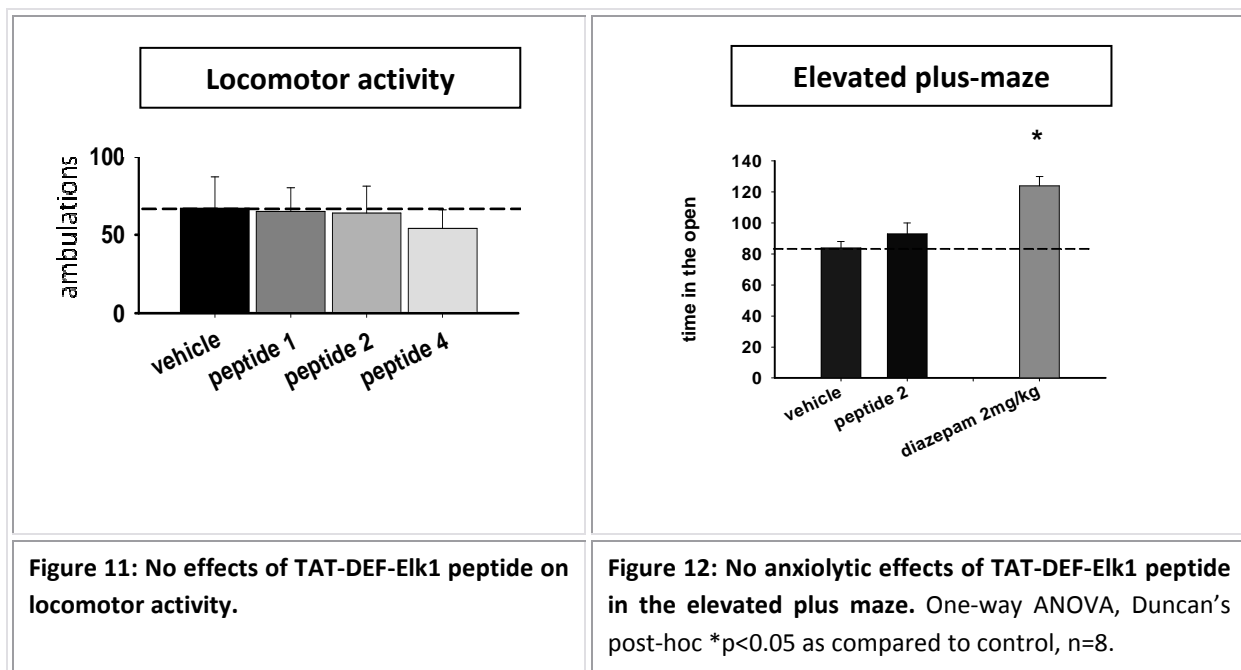


To further examine the antidepressant effect of the TAT-DEF-Elk1 peptide, we also used the forced swimming test (FST). The FST, also known as Porsolt test, is a test equivalent to the TST, used to screen antidepressant action. We administered the TAT-DEF-Elk1 peptide at 0.5, 2 and 4mg/kg 90min prior to test. We used desipramine (20mg/kg) as reference drug. TAT-DEF-Elk1 peptide significantly decreased immobility time in the FST at doses 2 and 4mg/kg (Figure 10a). Scrambled peptide and the MEK inhibitor SL327 were also tested in FST. No significant effect was observed upon administration of either of these compounds (Figure 10b, c).



Locomotion was measured to ensure that the effects observed in these two tests were not due to an overall alteration of locomotor activity but rather reflected an antidepressant-like effect. Administration of the TAT-DEF-Elk1 peptide at 1, 2, 4mg/kg 90min before had no effect on locomotor activity (Figure 11).

We tested the more effective dose of TAT-DEF-Elk1 peptide (2mg/kg) in the elevated plus maze test, to examine whether it has anxiolytic effects. We used the anxiolytic diazepam (2mg/kg) as a reference drug. No effect of the TAT-DEF-Elk1 peptide was observed in this test as expressed by the time passed in the open arms of the elevated plus-maze (Figure 12).



III. AMPA inhibition blocks TAT-DEF-Elk1 effects on TST

Substantial evidence implicates AMPA receptors in the antidepressant action, as already discussed. Several antidepressant compounds have been shown to modulate AMPA receptors in the synapse and AMPA receptors positive modulators exhibit antidepressant-like effects, as shown previously. Moreover, AMPA GluR1 subunit phosphorylation has been shown to mediate fluoxetine's antidepressant effect. To test if the effects of TAT-DEF-Elk1 peptide on TST are AMPA-mediated we administered the AMPA R antagonist GYKI52466 (10mg/kg) 10min before the peptide and we measured immobility time in the TST. TAT-DEF-Elk1 was administered i.p. 90min before testing at 2mg/kg. AMPA antagonist completely blocked the antidepressant effect of the peptide without affecting immobility time when administered alone (Figure 13).

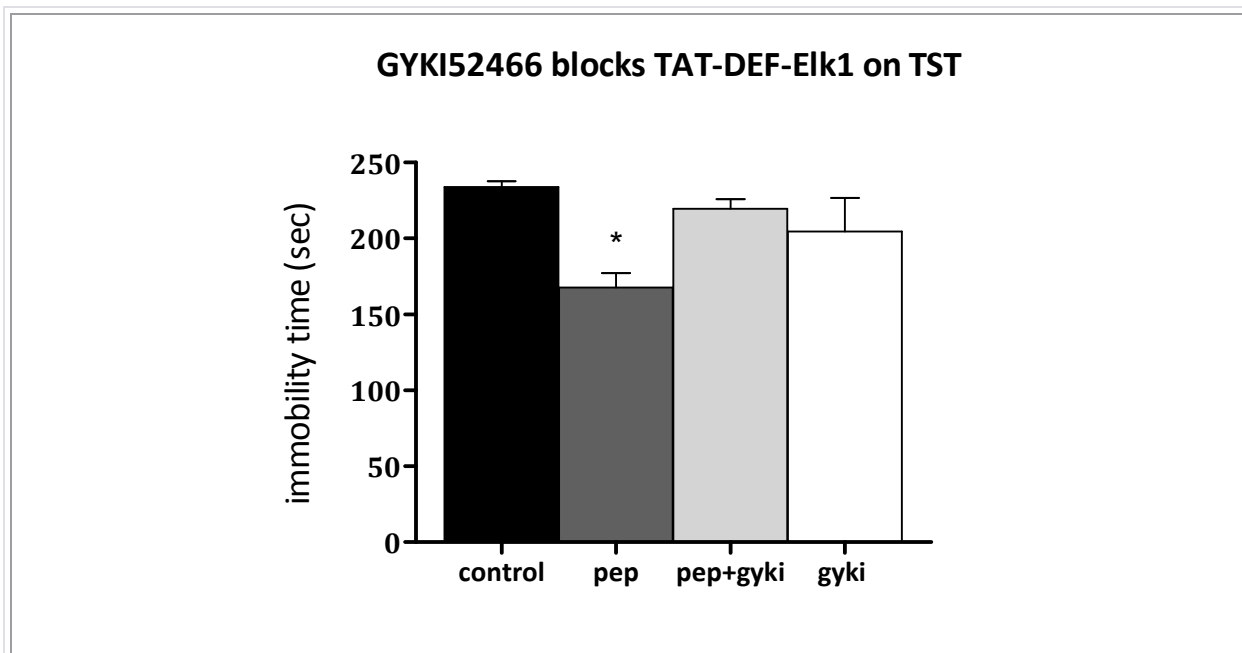
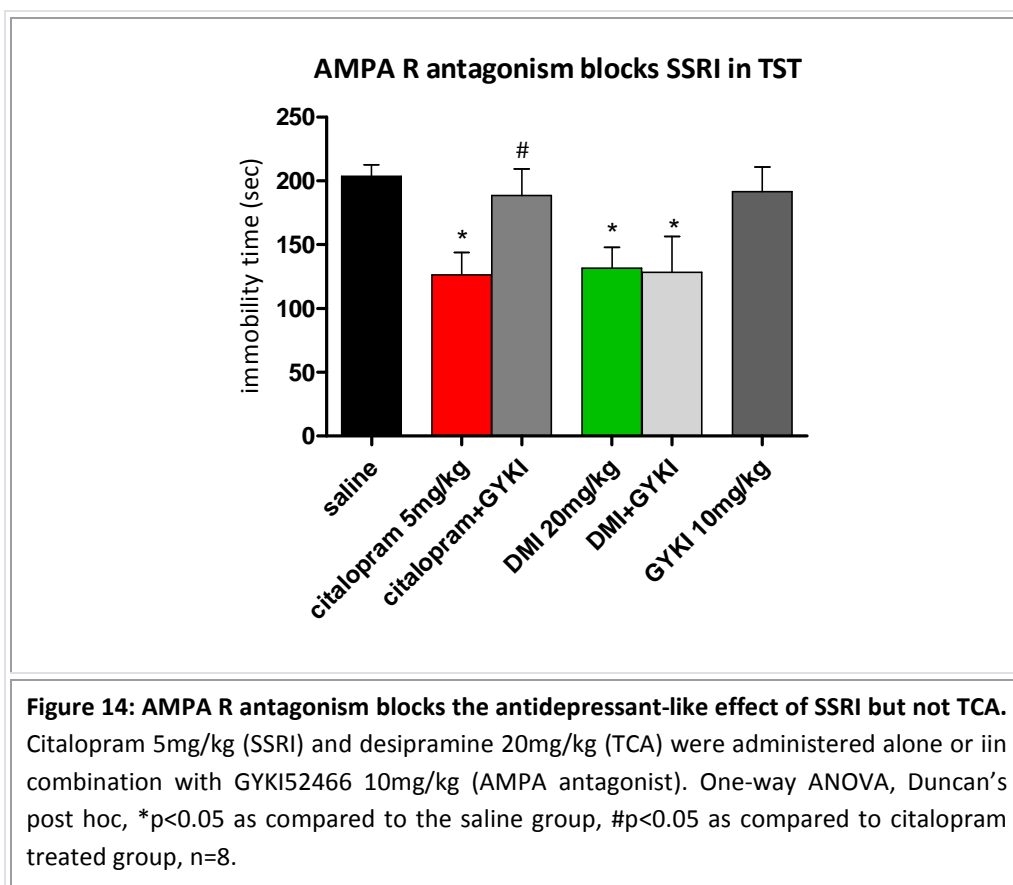


Figure 13: AMPAR antagonist blocks TAT-DEF-Elk1 effects on TST. Immobility time during TST of control mice, TAT-DEF-Elk1 treated mice, AMPA antagonist GYKI52466 and TAT-DEF-Elk1 treated mice and GYKI52466 treated mice. AMPA antagonist blocks the TAT-DEF-Elk1 antidepressant-like effect in TST without affecting behavior when administered alone. One-way ANOVA, Duncan's post-hoc * $p < 0.05$ as compared to control, $n = 8$.

Subsequently, we administered the AMPA antagonist GYKI 52466 with the SSRI citalopram and with the tricyclic (TCA) desipramine to test if AMPA antagonism can block the effect of these compounds on TST. Citalopram 5mg/kg significantly decreased immobility in TST and this was blocked by prior administration of GYKI52466 10mg/kg. Desipramine 20mg/kg significantly decreased immobility in the TST and prior administration of GYKI 52466 did not affect this effect (Figure 14). GYKI 52466 did not affect immobility when administered alone.



From the above, AMPA R mediates the effect of TAT-DEF-Elk1 peptide as well as SSRIs on TST. Desipramine effect on TST was not blocked by the AMPA antagonist, implying that this desipramine decreases immobility time via a different pathway. These results gave rise to the question of possible synergy between TAT-DEF-Elk1 and these compounds. In order to evaluate if the TAT-DEF-Elk1 peptide can have a synergistic effect with SSRIs or TCAs, we administered ineffective doses of fluoxetine (5mg/kg) and desipramine (5mg/kg) and measured immobility time in the TST. We used effective doses of the same compounds as positive controls. TAT-DEF-Elk1 peptide was used at 0.5mg/kg. The TAT-DEF-Elk1 peptide 0.5mg/kg had no effect on immobility when administered alone and neither did desipramine at 5mg/kg. Co-administration of the two significantly decreased immobility time, to the same extent as an effective dose (15mg/kg) of desipramine (Figure 15a). Fluoxetine showed no effect on immobility when administered at 5mg/kg and neither did co-administration of fluoxetine (5mg/kg) with the TAT-DEF-Elk1 peptide 0.5mg/kg. Fluoxetine 10mg/kg significantly decreased immobility in TST (Figure 15b).

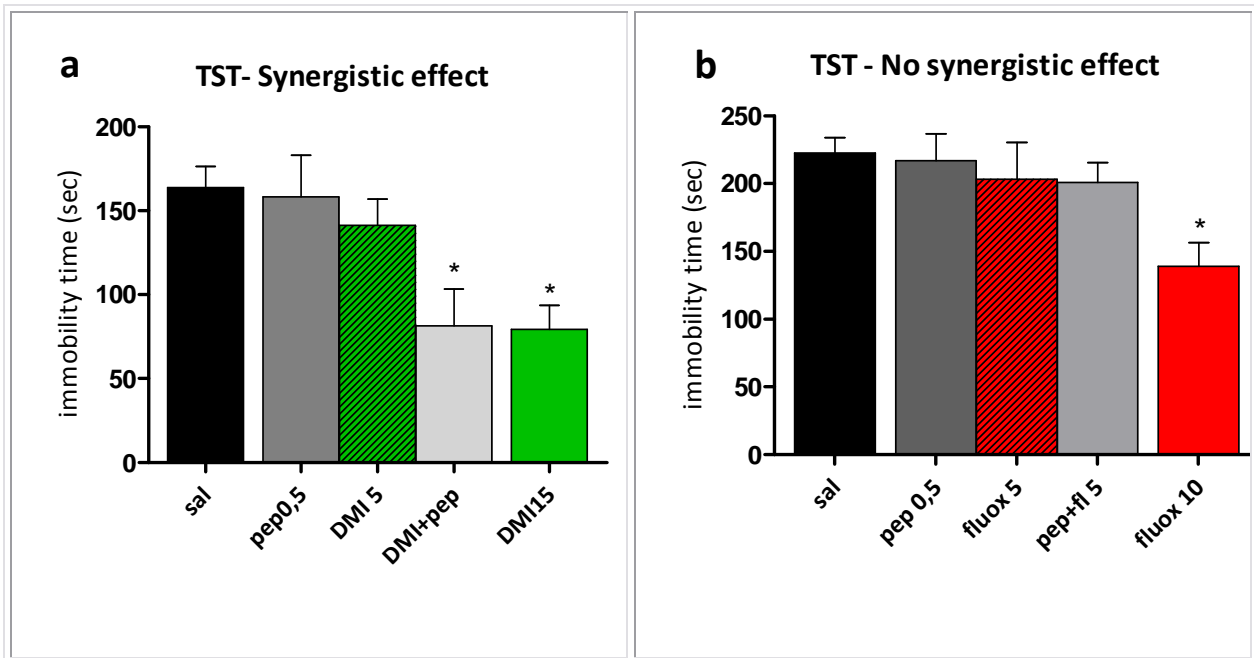
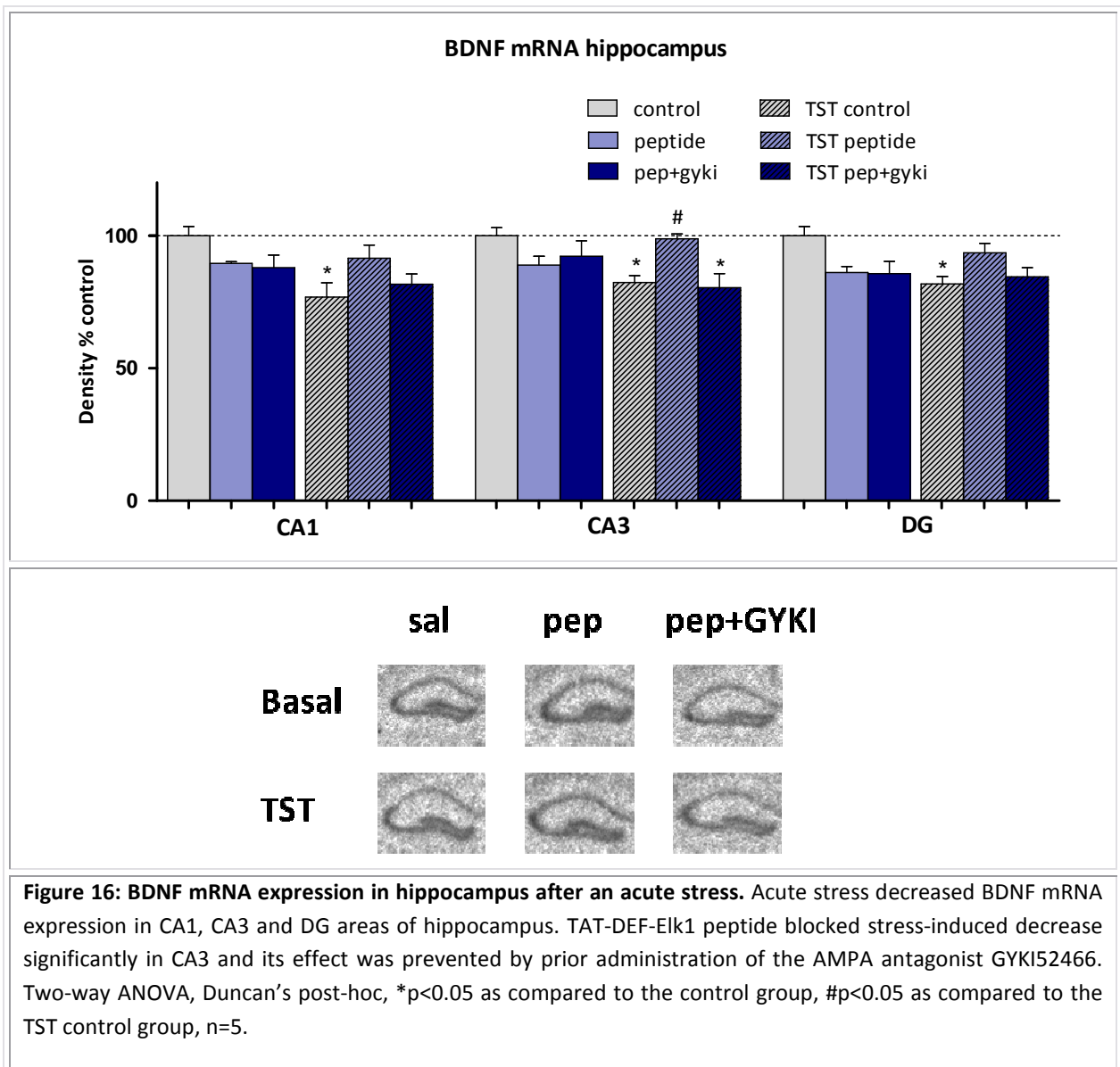


Figure 15: TAT-DEF-Elk1 peptide has synergistic effects with desipramine (TCA) but not with fluoxetine (SSRI) in the tail suspension test. (a) TAT-DEF-Elk1 peptide 0.5mg/kg has no effect in TST, desipramine 5mg/kg has no effect in TST, co-administration of the two significantly decreases immobility similar to an effective dose 15mg/kg of desipramine. (b) TAT-DEF-Elk1 peptide 0.5mg/kg has no effect in TST, fluoxetine 5mg/kg has no effect in TST, co-administration of the two has no effect either. Fluoxetine 10mg/kg significantly decreases immobility in TST. One-way ANOVA, Duncan's post hoc, * $p < 0.05$ as compared to the saline group, $n = 8$.

IV. Effects of TAT-DEF-Elk1 peptide on biomarkers expression related to antidepressant action

Studies of the role of intracellular signal transduction and regulation of gene expression in impaired neuroplasticity in depression have led to the neurotrophic hypothesis of depression, with brain-derived neurotrophic factor (BDNF) as an important mediator of neuronal plasticity and a potential target for antidepressant drug development. Stress has been shown to decrease BDNF expression in the hippocampus, which may contribute to the neuronal atrophy and sometimes neural cell loss in key limbic regions in the brain seen in patients with depression. Conversely, the efficacy of some classes of antidepressants is proposed to involve increased expression of BDNF.

In rodents, both acute and chronic stressors have been shown to reduce BDNF mRNA in hippocampus. We examined the effects of a 10min TST on BDNF mRNA expression in hippocampus of male adult C57BL/6J mice. A prior treatment of the peptide alone or the peptide + GYKI was also assessed. Mice were injected 2mg/kg of TAT-DEF-Elk1 peptide 90min prior to TST. When AMPA antagonist (GYKI52466, 10mg/kg) was used, it was injected 10min prior to the peptide administration. Acute stress (TST) was applied for 10min and mice were sacrificed 20min after the end of stress. Brains were rapidly frozen in isopentane at -30°C for in situ hybridization. BDNF expression was significantly decreased by stress in all areas of hippocampus. TAT-DEF-Elk1 administration blocked stress effects on BDNF expression and antagonism of AMPA receptors completely inverted its effects in CA3 (Figure 16).



GluR1 mRNA expression was also measured. GluR1 AMPA subunit has been implicated to the antidepressant action and stress has been shown to modulate GluR1 mRNA expression. Acute stress significantly decreased GluR1 expression in CA1 region of hippocampus and TAT-DEF-Elk1 peptide inverted this effect. This effect was also AMPA dependent as prior inhibition of AMPA receptors blocked the action of the peptide (Figure 17).

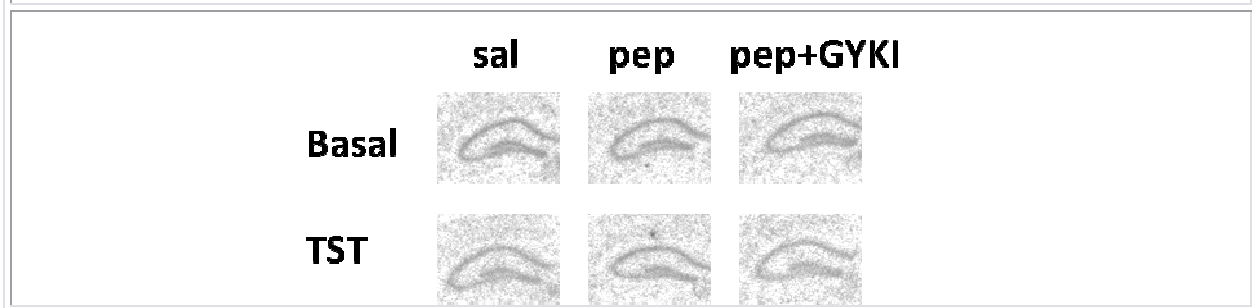
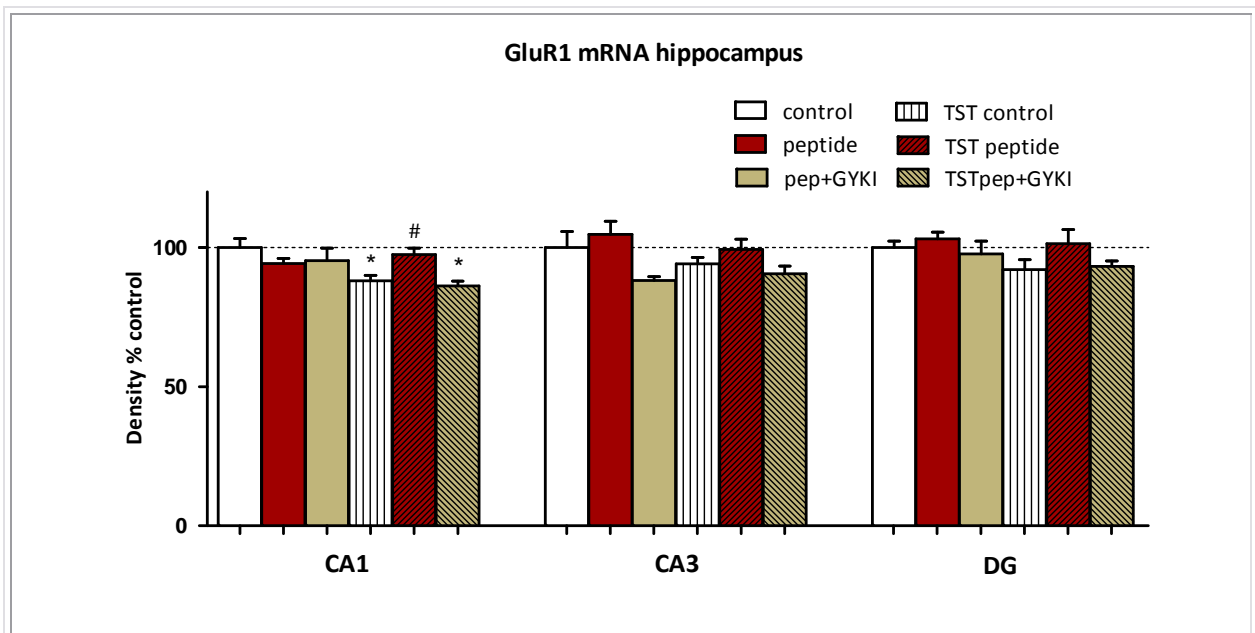


Figure 17: GluR1 mRNA expression in hippocampus after an acute stress. Acute stress significantly decreased GluR1 mRNA expression in CA1 area of hippocampus. TAT-DEF-Elk1 peptide blocked stress-induced decrease in CA1 and its effect was prevented by prior administration of the AMPA antagonist GYKI52466. Two-way ANOVA, Duncan's post-hoc, * $p < 0.05$ as compared to the control group, # $p < 0.05$ as compared to the TST control group, $n = 5$.

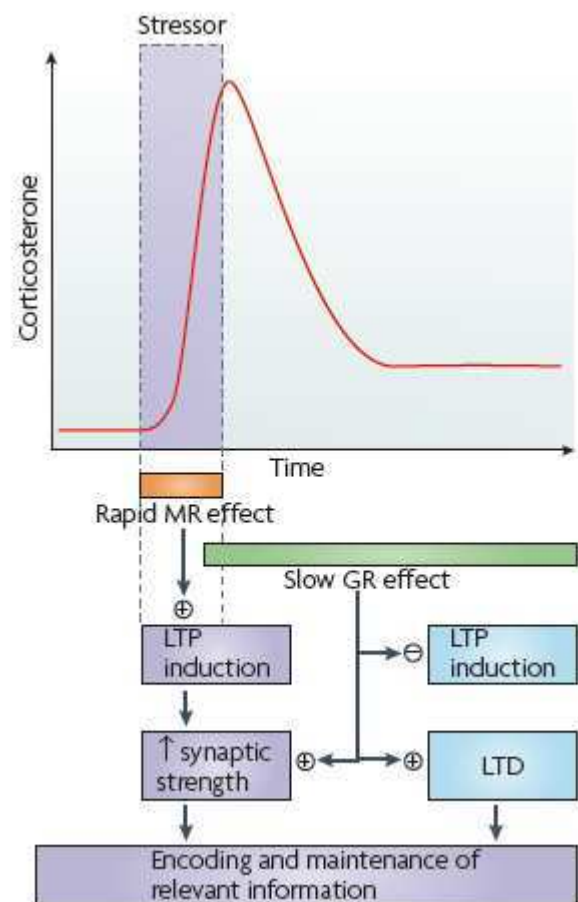
PART IIb: TAT-DEF-Elk1 peptide prevents stress-induced alterations in plasticity markers and behavior

HYPOTHESIS

Enhancement and impairment of hippocampal synaptic plasticity and cognitive performances following stress have both been described in the literature. A unifying model in the literature, firstly proposed by Diamond et al. (2007) and further developed by Krugers et al. (2010) explains the impact of an acute stress on memory formation and hippocampal plasticity. In summary, the onset of stress activates the hippocampus, thereby producing a rapid and dramatic increase in levels of intracellular calcium. The increased calcium serves as the trigger stimulus to briefly produce an enhancement, followed by impairment, of the induction of endogenous synaptic plasticity in the hippocampus.

This enhancement of plasticity, facilitation of LTP induction, is believed to strengthen stress-related memory formation (Krugers et al. 2010). Substantial evidence in the literature has shown that the fast enhancing effects of

stress are mediated by norepinephrine and corticosteroids within minutes after stress exposure. During the initial phase corticosteroid hormone levels start to rise. Via mineralocorticoid receptors (MR) activation, these hormones rapidly and reversibly (via non-genomic actions) increase miniature excitatory postsynaptic currents (mEPSCs) frequency presumably reflecting increased presynaptic release of glutamate (Karst et al. 2005,



Olijslagers et al. 2008) and increase lateral diffusion of predominantly GluR2 containing AMPA receptors (Groc et al. 2008).

The slow developing effects of glucocorticosteroid exposure include protein synthesis (genomic effects) and result in an increase in membrane expression and synaptic insertion of GluR2-containing AMPARs in the hippocampus (Groc et al. 2008). After the initial phase, norepinephrine levels return to baseline but plasma corticosterone levels still arise and via slow genomic action can persistently affect neuronal function. Via glucocorticoid receptors (GR) these hormones slowly increase synaptic incorporation, lateral diffusion and mobility of GluR2 containing AMPARs (Groc et al. 2008, Martin et al. 2009) along with slowly increasing AMPA receptor synaptic transmission (Karst and Joels 2005, Martin et al. 2009).

A critical role of the ERK1/2 cascade in neuronal plasticity has been well established in the past years (Impey et al. 1999, Lisman et al. 2002, Sweatt JD 2001, 2004). Phosphorylation of ERK1/2 is crucial for plasticity maintenance as inhibition of ERK1/2 has been shown to abolish both long-term potentiation (LTP) and long-term depression (LTD) induction in hippocampus (Thiels et al. 2002, Yang et al. 2004). Apart from exerting a transcription-dependent regulation of synaptic plasticity, ERK1/2 cascade also mediates synaptic AMPA-Rs delivery and removal during LTP and LTD respectively (Seger et al. 1995, Stornetta and Zhu 2011).

Elk1 implication in the control of synaptic plasticity, as a downstream target of ERK1/2, has been also demonstrated; Davis et al. (2000) first suggested that Elk-1, in addition to CREB, form an important link in the ERK1/2 pathway to transduce signals from the cell surface to the nucleus to activate the genetic machinery necessary for the maintenance of synaptic plasticity in the dentate gyrus of hippocampus. Few years later, Thiels et al. (2002) demonstrated that ERK activation during LTD results in increased phosphorylation of Elk-1 but not of the transcription factor cAMP response element-binding (CREB) protein. Their findings indicate that the ERK cascade transduces signals from the synapse to the nucleus during LTD in hippocampal area CA1 in vivo, as it does during long-term potentiation (LTP) in area CA1, but that the pattern of coupling of the ERK cascade to transcriptional regulators differs between the two forms of synaptic plasticity. The findings of Yang et al. 2004 propose a decisive role of ERK1/2 in the stress-induced alterations in the inducibility of LTP and

subsequent LTD in the CA1 region of the hippocampus. Increased phosphorylation of Elk1 and upregulated expression of its downstream targets (c-fos, junB, egr1) has also been reported after LTD (Lindecke et al. 2006).

Egr-1 (early growth response-1, also called *Zenk*, *Tis8*, *Zif/268*, *NGFI-A* or *Krox24*) is an immediate-early gene with serum-response-elements (SRE) in its promoter, regulated downstream of the ERK1/2 MAPK signaling pathway via the transcriptional factor Elk-1 (Sgambato et al. 1998a, b). Egr-1 has been shown to mediate GR fast and slow responses through a MAPK-independent and MAPK-dependent mechanisms, respectively (Revest et al. 2005). Corticosterone administration in hippocampus enhances aversive memory as measured in the fear conditioning test, via a MAPK/Egr-1 dependent mechanism.

Moreover, acute stress or administration of glucocorticoids has been shown to disrupt the retrieval of hippocampal-dependent spatial memory via a NR2B-dependent facilitation of long-term depression (LTD) induction (de Quervain et al. 1998, Wong et al. 2007). The above show that stress causes impairment of cognitive function such as memory formation as well as memory retrieval through plasticity based mechanisms that implicate the ERK/MAPK signaling pathway.

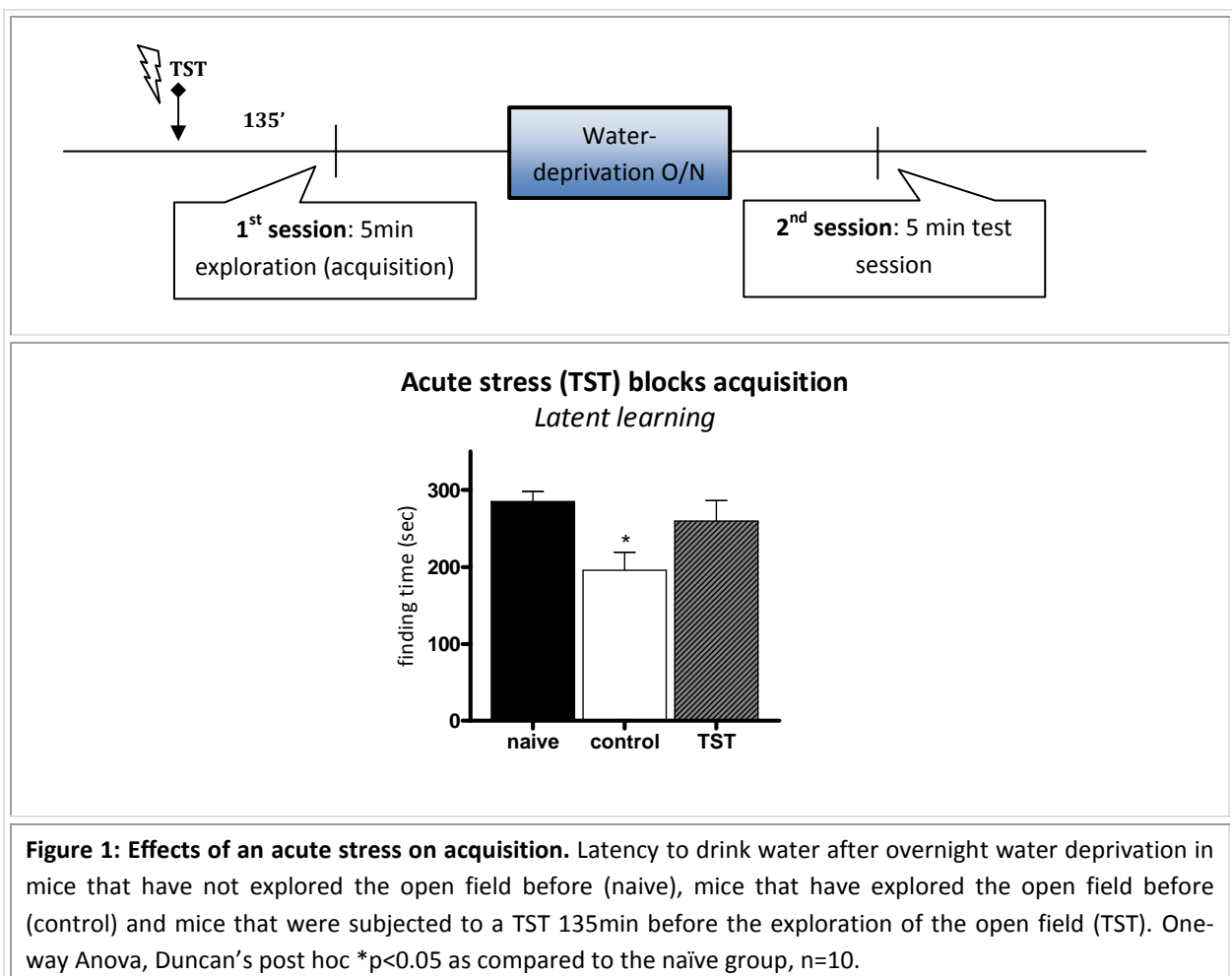
Objective of the study

We assessed the effects of a direct, specific inhibition of ERK/Elk-1 signaling *in vivo* on memory related behavioral and biochemical changes induced by an acute stress.

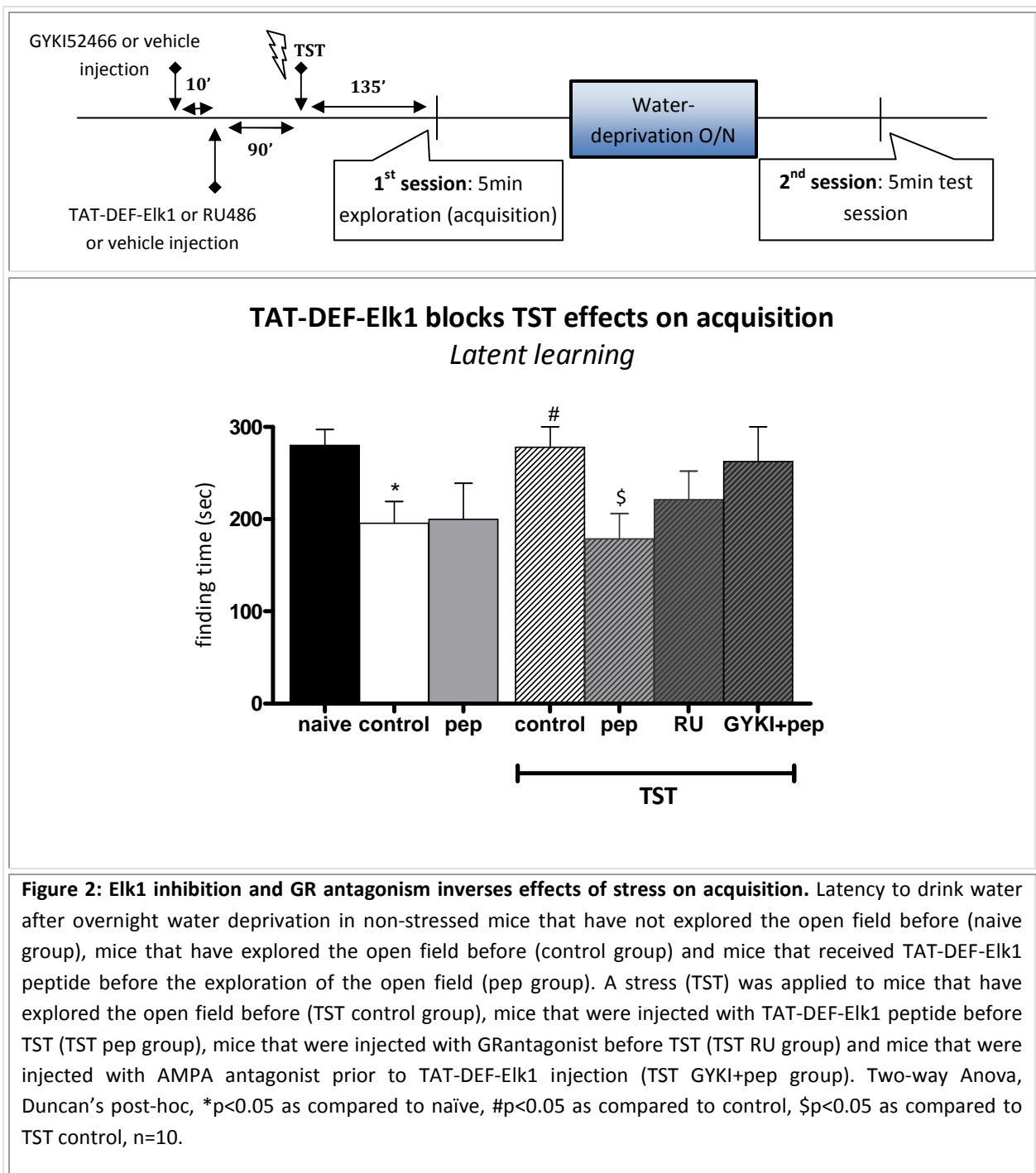
Results

I. *In vivo* inhibition of Elk-1 activation blocks stress-induced deficits in the latent learning

We tested the effects of an acute stress on memory encoding (acquisition) using the test of latent learning. During this test, adult (8 weeks) male C57BL/6J mice were placed in an open field with a drinking bottle fixed on the top of an alcove, free to explore (see materials and methods). One group of mice was subjected to a 10min tail suspension test (TST) 135min before the first session (acquisition). Mice were then water-deprived over-night and the following day finding time was manually recorded during the test session. To validate memory acquisition animals of the control group are compared to a naïve group, that is water-deprived animals that have not been exposed to the open field during the exploration session. During the test session, control animals found the drinking bottle significantly faster than naïve animals but a TST before acquisition blocked this effect as shown in Figure 1.



To examine if stress effects on acquisition are GR and/or Elk1 dependent we administered systemically the GR antagonist mifepristone (RU486 60mg/kg) and TAT-DEF-Elk1 peptide 2mg/kg both 90min prior to stress. Stress-induced inhibition of acquisition was significantly blocked by the peptide and partially by the GR antagonist. Furthermore, to test if the effect of the peptide is AMPA mediated, we administered the AMPA antagonist GYKI52466 (10mg/kg) 10min prior to TAT-DEF-Elk1. AMPA antagonism blocked the effects of the peptide as shown in Figure 2.

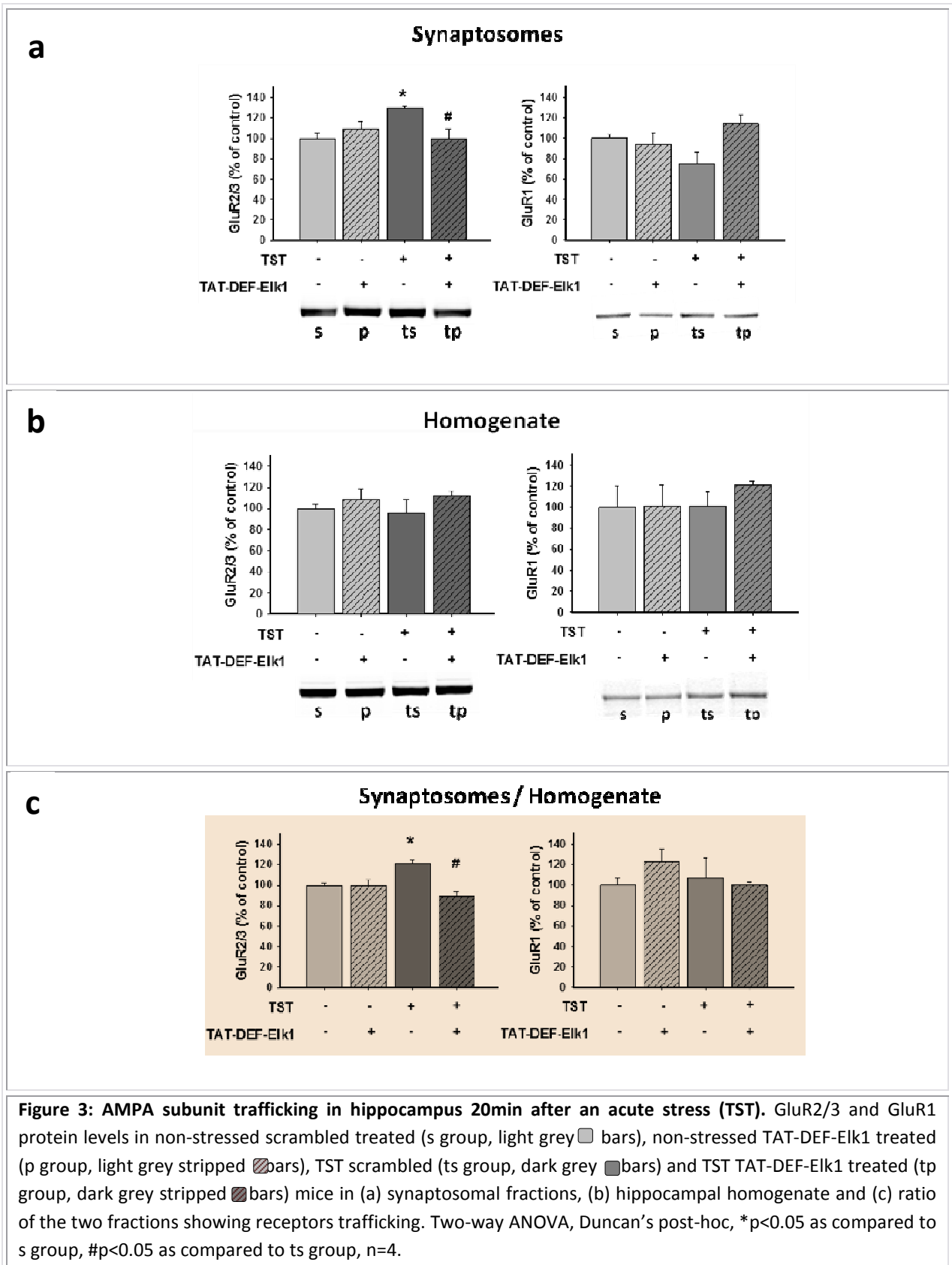


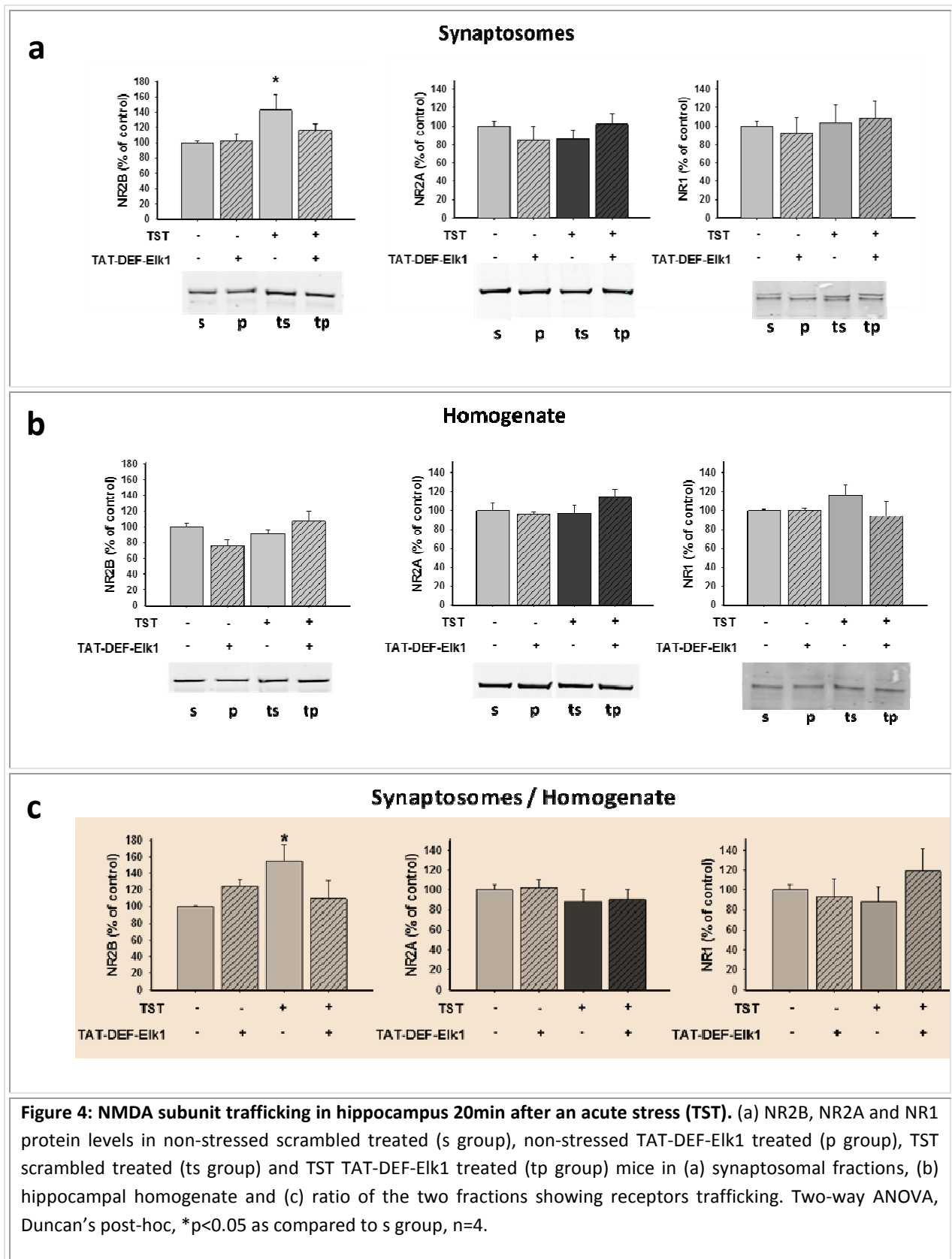
II. AMPA/NMDA synaptic movement during stress

Up to now, our results show that specific inhibition of ERK/Elk1 signaling during an acute stress inhibits the effects of stress on behavior (TST, FST, latent learning) and on biochemical changes such as BDNF and GluR1 expression. AMPA antagonism blocked the effects of the TAT-DEF-Elk1 peptide in all tests implying that AMPA receptors play a major role in the action of the peptide. We used the same model of acute stress (10min tail suspension stress) and administration of the peptide alone or before stress to study synaptic expression of AMPA receptor subunits under these conditions. Synaptic and total levels of GluR2/3 and GluR1 subunits were quantified by Western blotting 20min after stress in hippocampus homogenate and synaptosomal fractions of adult male C57BL/6J mice. TAT-DEF-Elk1 was administered at 2mg/kg i.p. 90min prior to stress. Stress significantly increased GluR2/3 synaptic expression without altering total amount of GluR2/3 in the homogenate. This effect was abolished in the presence of the peptide that showed no effect when administered alone (Figure 3). GluR1 amounts were not significantly changed in either of conditions.

NMDA receptors synaptic and total levels were also quantified in the same experimental conditions. Among NR2B, NR2A and NR1 subunits only NR2B synaptic expression was altered by stress as shown in Figure 4. Stress significantly increased NR2B synaptic amounts without altering total levels and this effect was prevented by prior administration of the TAT-DEF-Elk1 peptide. Administration of the peptide alone showed no effects on either NMDA subunit total or synaptic amounts.

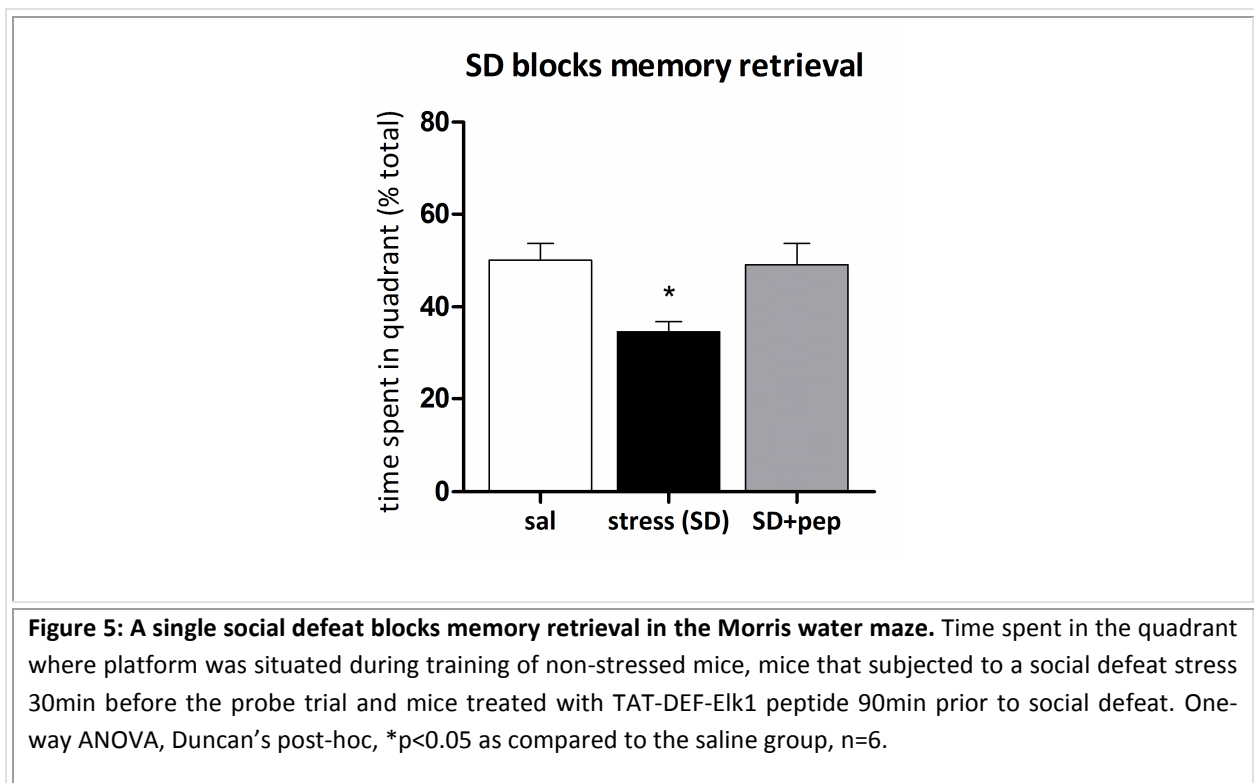
All measures were normalized with GAPDH and expressed as percentage of the control. As a control mice were injected with the scrambled peptide at the same dose and time as for the TAT-DEF-Elk1 peptide.





III. *In vivo* inhibition of Elk-1 activation blocks stress-impaired memory retrieval in the Morris water maze

Stress-induced alterations of the NR2B subunit in the synapse have been related to enhanced LTD in hippocampus in rats exposed to an acute stressor. This LTD was correlated with impaired memory retrieval in the quadrant test of the Morris water maze task (Wong et al. 2007). TAT-DEF-Elk1 peptide was earlier shown to inhibit stress-induced increase of NR2B synaptic expression. We thus tested the effects of the TAT-DEF-Elk1 peptide in an adapted experimental disposition on mice. Adult (8 weeks) male C57BL/6J mice were trained to find a hidden platform in the Morris water maze during 8 days (see Materials and Methods for details). Memory retrieval was assessed in the water maze quadrant test. During the quadrant test the platform is removed and the time passed in the quadrant where platform was placed is recorded. Animals were subjected to an acute social defeat stress 30min before the test. A single social defeat session significantly blocked memory retrieval. Prior administration (90min) of the TAT-DEF-Elk1 peptide (2mg/kg, i.p.) blocked stress-induced amnesia (Figure 5).



PART IIc: Antidepressant-like properties of TAT-DEF-Elk1 peptide in chronic models of depression

Hypothesis

The first parts of this work (PART IIa, b) revealed an important role for Elk-1 activation in response to stress. Acute selective inhibition of Elk1 activation from ERK, using the TAT-DEF-Elk1 peptide, showed antidepressant activity *in vivo* in two behavioral models of despair (TST and FST) and inversed acute stress effects on memory. Biochemical changes following acute stress were examined in a transcriptional and functional level and several stress-induced modifications were reversed by prior inhibition of Elk1 activation in brain regions related to depression. It is crucial to examine the TAT-DEF-Elk1 peptide effects after a chronic administration period and if a chronic treatment can reverse stress-induced alterations in chronic models of depression, more relevant to the etiopathophysiology of the disease. Several chronic stress models have been developed to study depressive-like states. They are considered of high face, construct and predictive validities. In these models, prolonged exposure to uncontrollable and/or unpredictable stressors results in depressive like behaviors that can be prevented or reversed by chronic but not acute antidepressant treatment.

Objective of the study

We have used two chronic animal models of depression to evaluate the antidepressant potential of the TAT-DEF-Elk1 peptide: the chronic mild stress paradigm and the social defeat stress. Behavioral output was associated with stress-induced transcriptional and functional modifications.

Results

I. TAT-DEF-Elk-1 chronic treatment

We first assessed chronic treatment of the TAT-DEF-Elk1 peptide to examine the effects of a long term inhibition of basal activation of Elk-1 on behavior and certain biochemical markers. Adult male C57Bl/J6 mice received a 21days treatment systemically of the TAT-DEF-Elk1 peptide at 1mg/kg. At the end of the treatment, mice were evaluated for their resignation state (TST), their fur condition, anhedonia (sucrose preference), anxiety (marble burying) and locomotor activity. Administration of the TAT-DEF-Elk1 peptide significantly decreased immobility time in TST as compared to vehicle treated mice (Figure 1a). Treatment did not affect fur condition, sucrose preference, anxiety or locomotion (Figure 1b, c, d and 2).

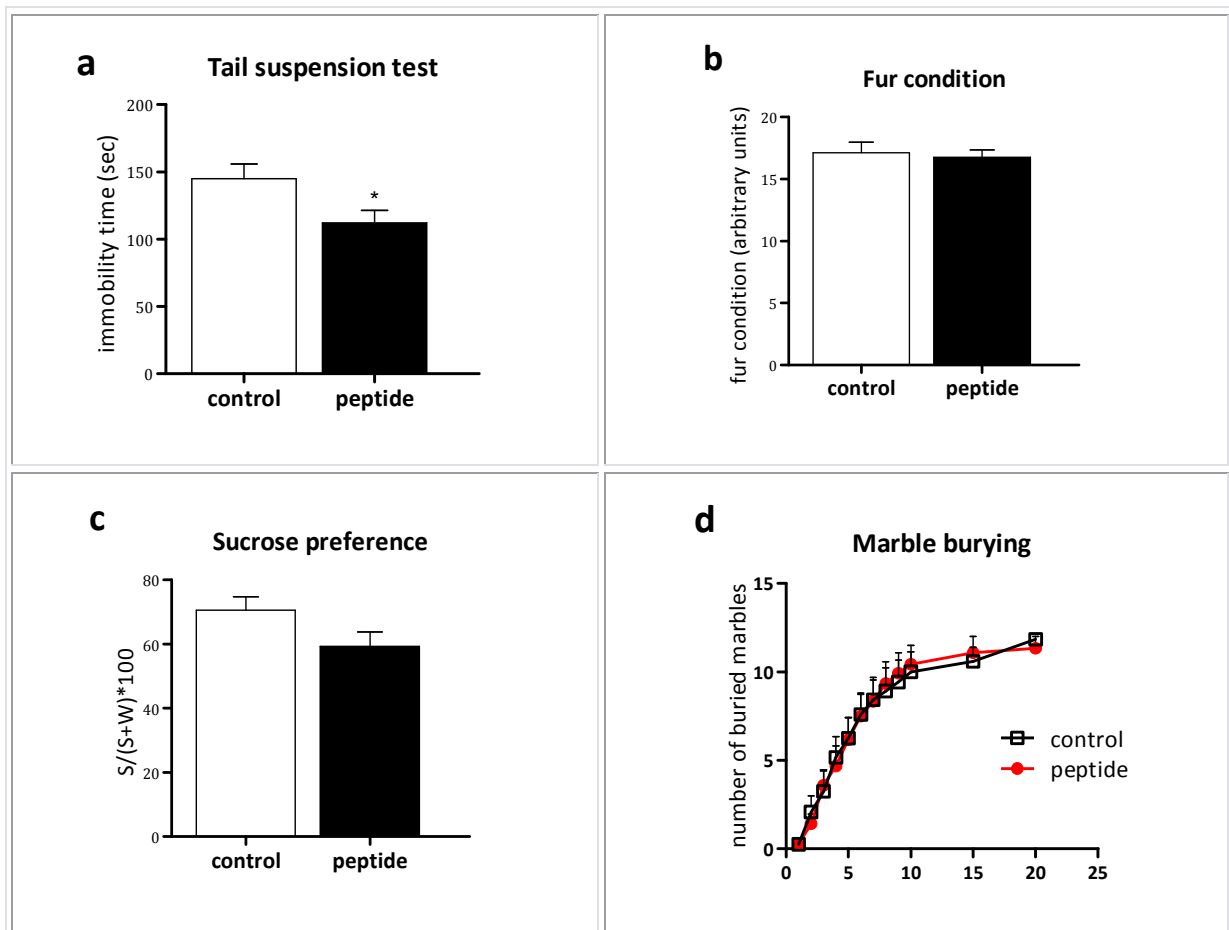


Figure 1: Effects of chronic administration of TAT-DEF-Elk1 peptide on different behavioral dimensions. (a) 21 days of treatment with the TAT-DEF-Elk1 peptide, 1mg/kg i.p., significantly decreased the immobility time in the tail suspension test. Chronic treatment with the peptide did not affect (b) self care as an evaluation of the fur condition (c) anhedonia as measured in the sucrose preference test (S=sucrose, W=water consumption) or (d) anxiety as measured in the marble burying test. T-test * $p < 0.05$ as compared to control, $n = 10$.

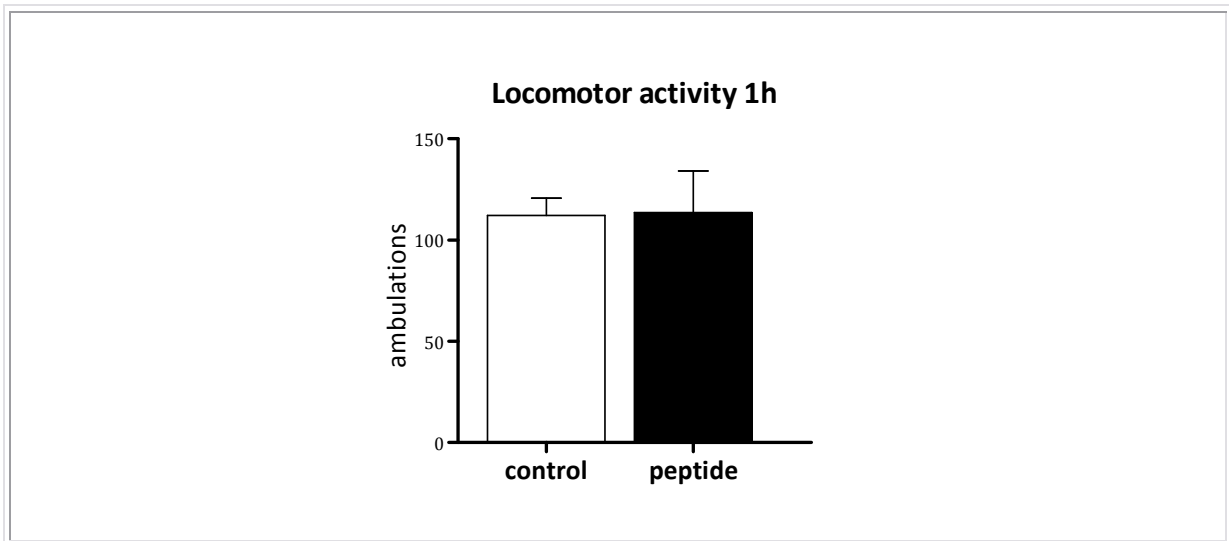


Figure 2: No effect of chronic administration of TAT-DEF-Elk1 peptide on locomotor activity.

ERK cascade after TAT-DEF-Elk1 chronic treatment

Total amounts of key proteins of the ERK cascade were quantified in hippocampal tissue by Western blot. There was no significant effect found on ERK, Elk1 or CREB total amounts after chronic administration of the peptide (Figure 3).

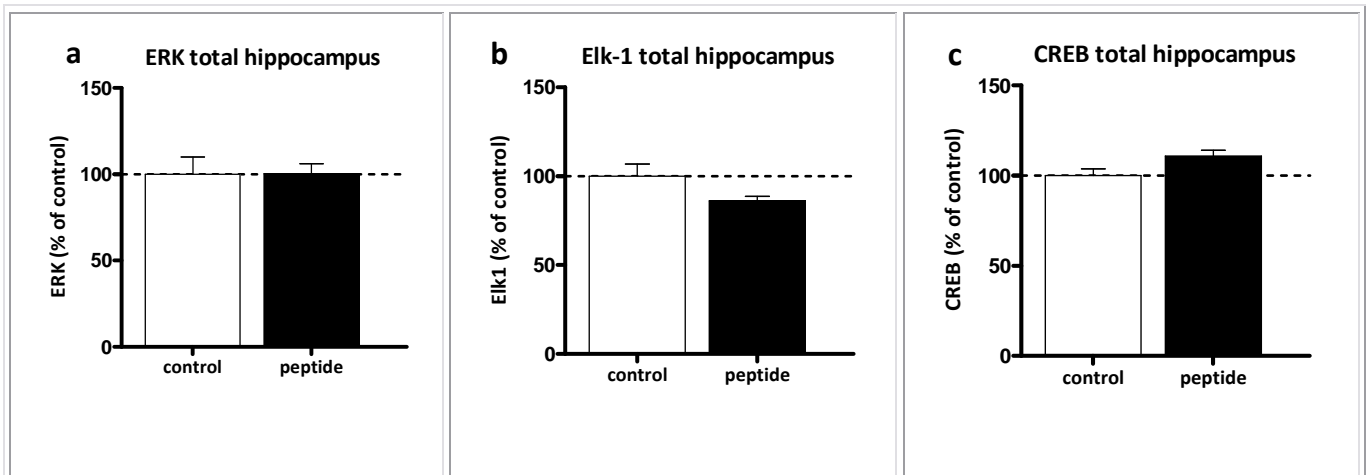
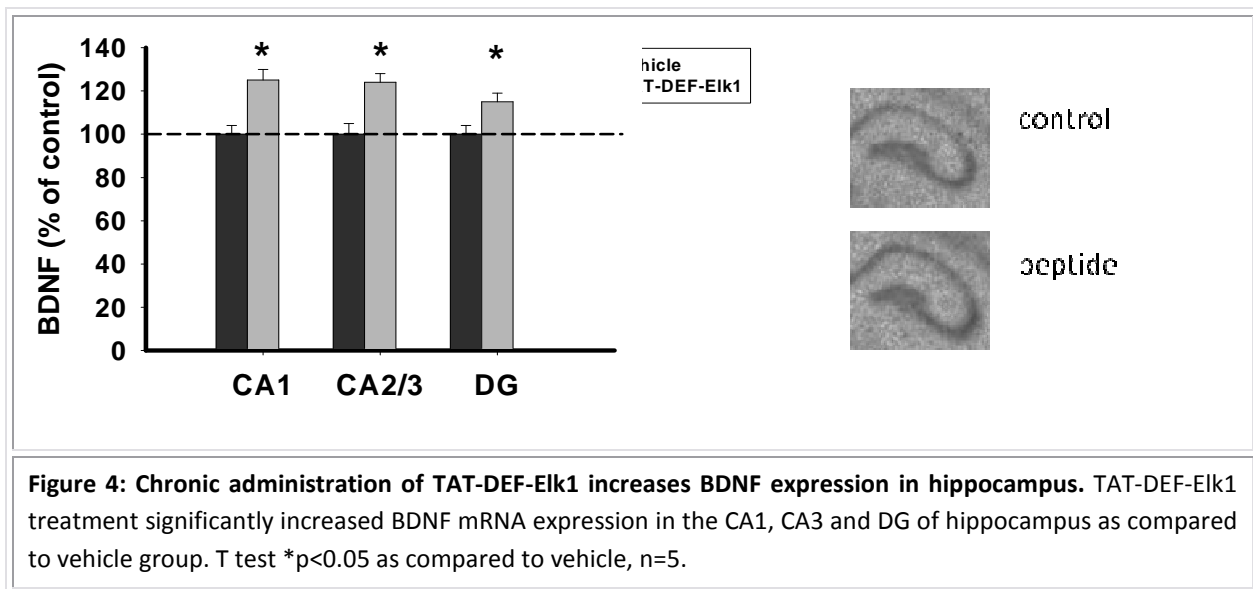


Figure 3: Chronic administration of TAT-DEF-Elk1 on total levels of ERK, Elk1 or CREB in hippocampus. Total levels of (a) ERK, (b) Elk1 and (c) CREB were not significantly affected by a 21days treatment with the TAT-DEF-Elk1 peptide 1mg/kg (black bars) as compared to control group (white bars), n=4.

BDNF expression

In parallel, BDNF mRNA expression was measured in hippocampus of chronically treated mice with TAT-DEF-Elk1 peptide 1mg/kg by in situ hybridization. Treatment significantly increased BDNF expression in all regions studied (Figure 4).



II. Unpredictable chronic mild stress: behavioral and biochemical evaluation

To examine whether a chronic administration of the peptide can prevent the depressive-like phenotype in mice submitted to a chronic stress, we first used the paradigm of unpredictable chronic stress. Adult male C57Bl/J6 mice were subjected to a 3-week chronic mild stress protocol as previously described (Farley et al. 2010). Mice submitted to multiple stressors and received vehicle, TAT-DEF-Elk1 (1mg/kg) or fluoxetine (20mg/kg) injections daily, i.p. during 21 days as shown in the Figure 5. At the end of the stress procedure, their emotional state was evaluated in the TST, fur condition, sucrose consumption and marble burying tests (Figure 6).

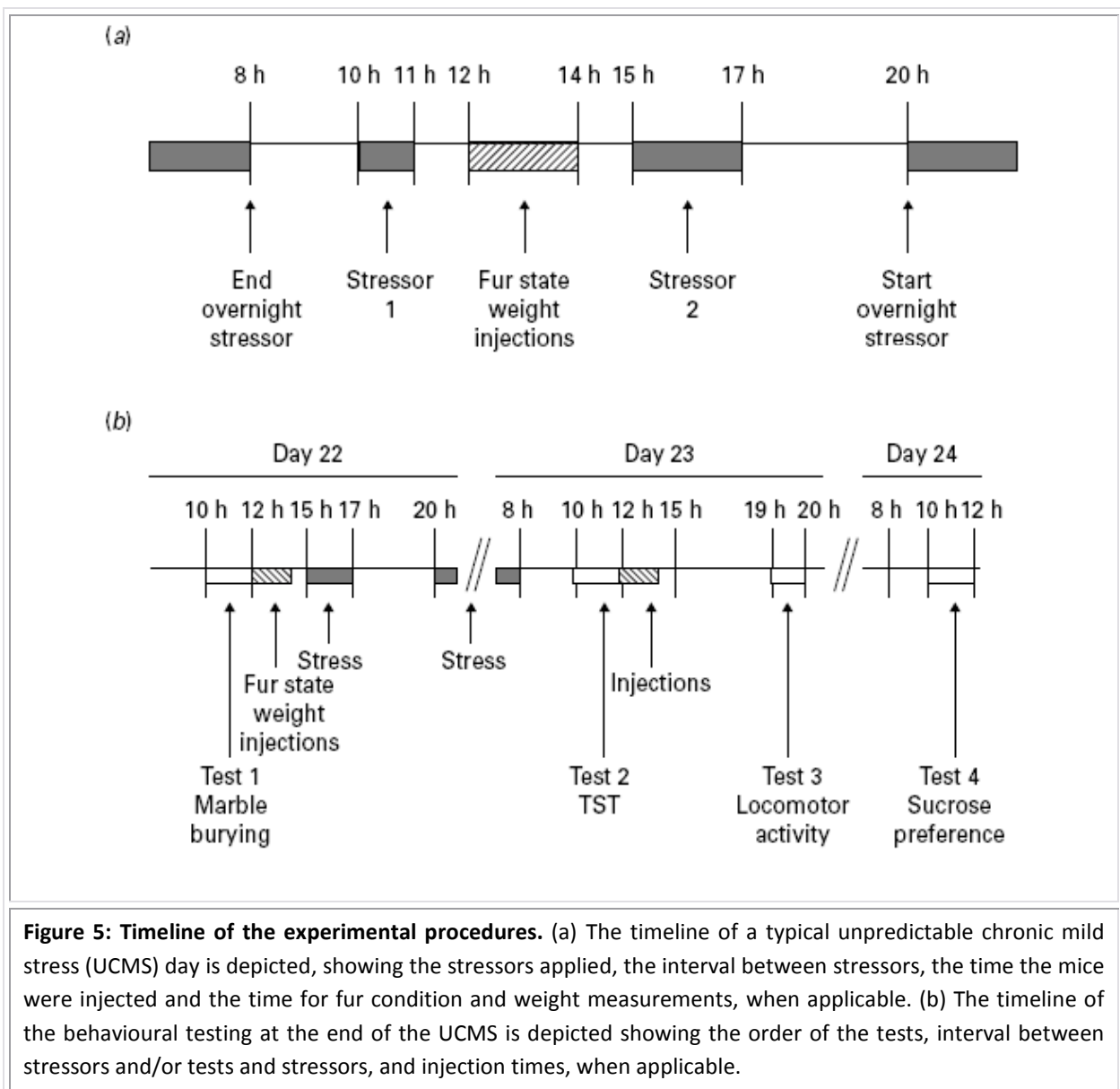
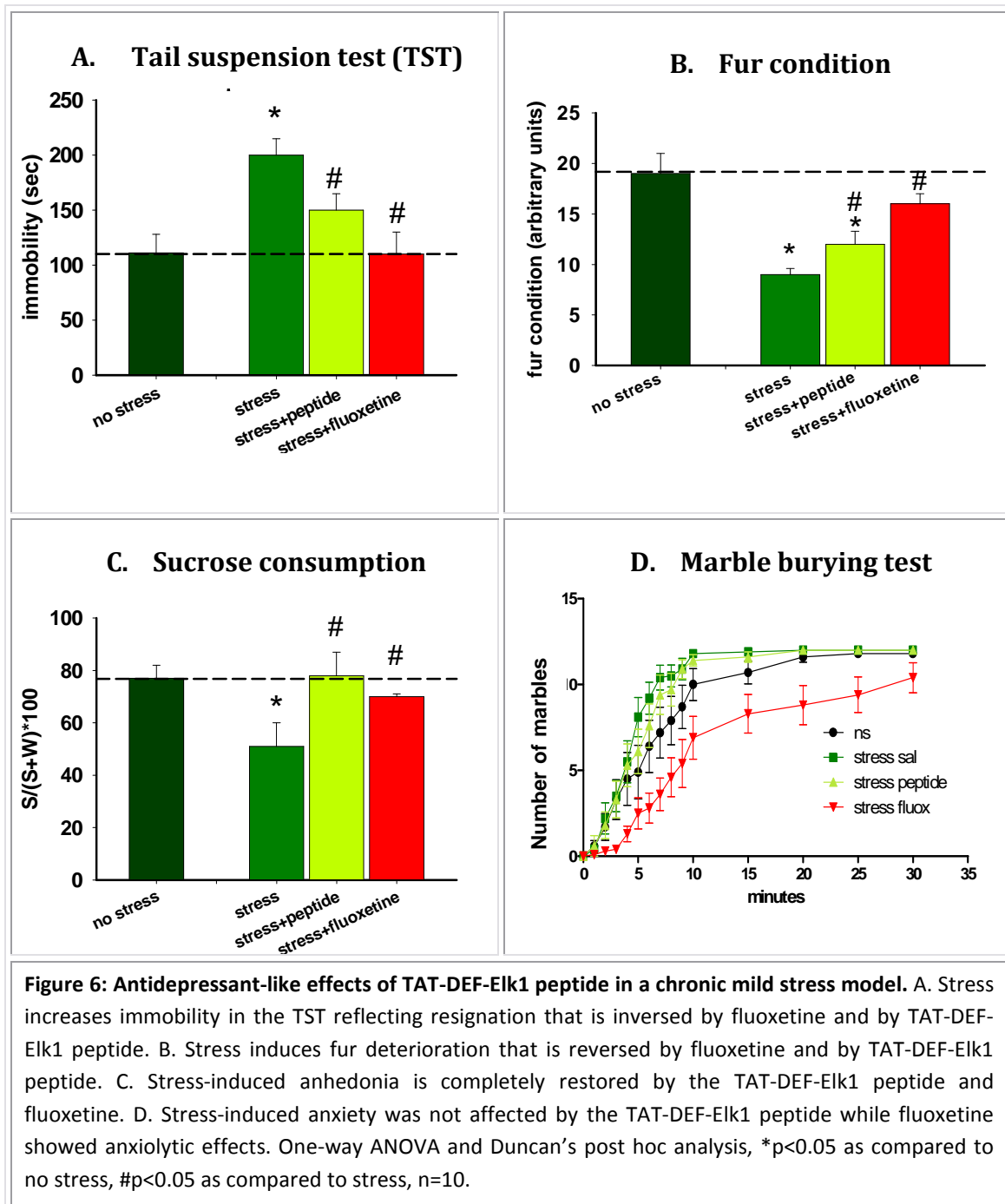
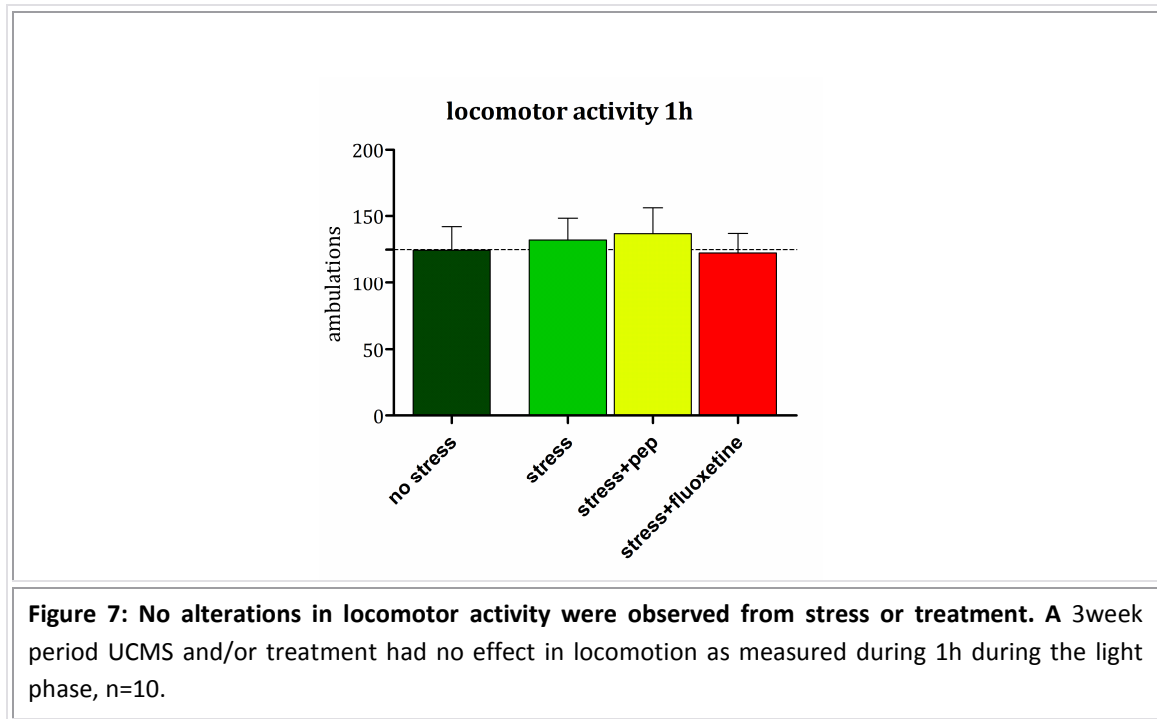


Figure 5: Timeline of the experimental procedures. (a) The timeline of a typical unpredictable chronic mild stress (UCMS) day is depicted, showing the stressors applied, the interval between stressors, the time the mice were injected and the time for fur condition and weight measurements, when applicable. (b) The timeline of the behavioural testing at the end of the UCMS is depicted showing the order of the tests, interval between stressors and/or tests and stressors, and injection times, when applicable.



Stressed mice exhibited increased immobility in the TST compared to non-stressed control group, reflecting a higher resignation induced by stress. Fluoxetine and TAT-DEF-Elk1 inverted increased immobility time (Figure 6A). Fur condition is deteriorated by UCMS and fluoxetine and TAT-DEF-Elk1 peptide significantly reversed this effect (Figure 6B). Stress-induced anhedonia as depicted by the sucrose preference test is restored by fluoxetine and

by TAT-DEF-Elk1 peptide (Figure 6C). Marble burying test was used to evaluate anxiety levels after a chronic mild stress protocol. Stressed mice show a tendency to more anxious state with no significant difference. Fluoxetine has an anxiolytic effect in this paradigm, while TAT-DEF-Elk1 had no effect (Figure 6D). Locomotion was not affected in either of these conditions (Figure 7).



BDNF and GR expression after UCMS

In the end of the UCMS procedure mice were decapitated and brains were quickly frozen in isopentane -30°C . Slices of $20\mu\text{m}$ were cut in cryostat and BDNF and GR mRNA level was assessed by in situ hybridization. Chronic stress has been shown to decrease BDNF and GR expression in hippocampus, an effect that can be reversed by chronic antidepressant treatment. In our experimental setting, UCMS significantly decreased BDNF expression in CA3 area of hippocampus and GR expression in dentate gyrus (DG). Administration of the TAT-DEF-Elk1 peptide completely restored BDNF mRNA levels (Figure 8) but did not alter GR mRNA levels (Figure 9).

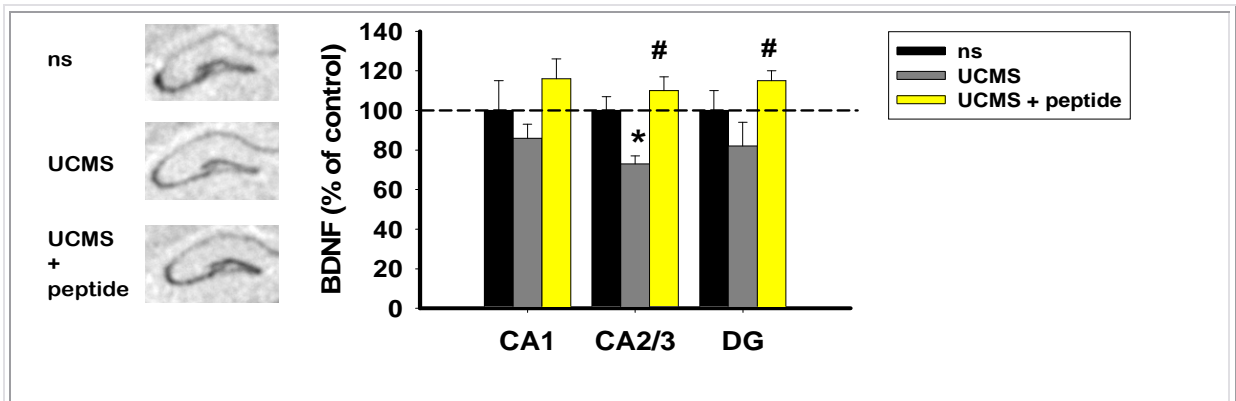


Figure 8: BDNF mRNA expression decreases after a UCMS in hippocampus. Significant decrease was observed in CA2/3 area of hippocampus that was completely abolished by the TAT-DEF-Elk1 peptide. A tendency to decrease was remarked in the other hippocampal regions. One-way ANOVA, Duncan's post hoc, * $p < 0.05$ as compared to non-stressed group, # $p < 0.05$ as compared to UCMS group, $n = 5$.

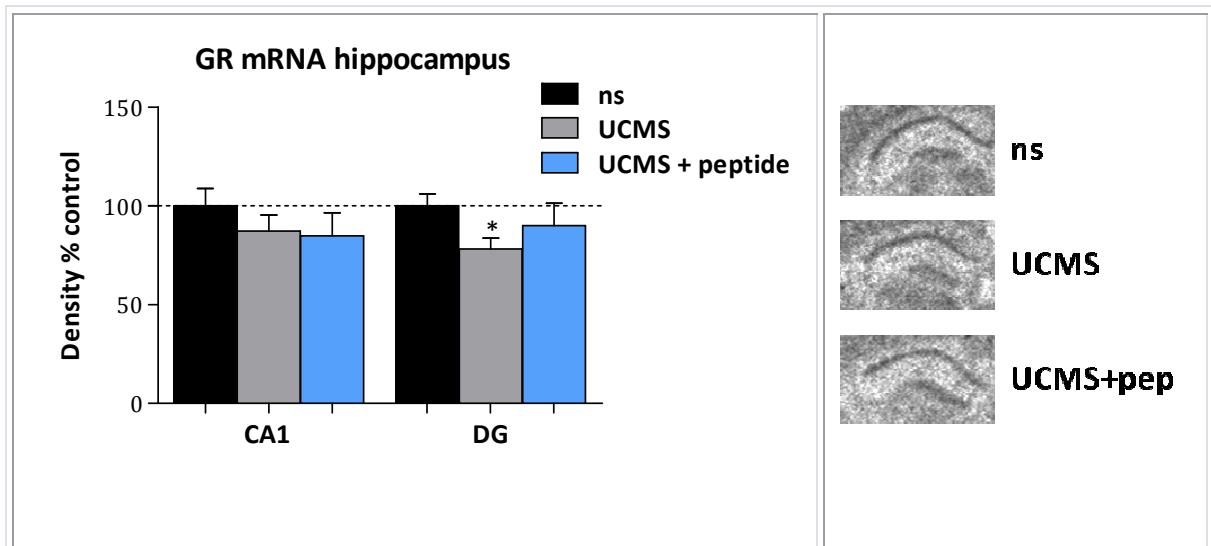


Figure 9: GR mRNA expression decreases after a UCMS in hippocampus. Significant decrease was observed in DG area of hippocampus that was not inverted by TAT-DEF-Elk1 peptide. One-way ANOVA, Duncan's post hoc, * $p < 0.05$ as compared to non-stressed group, $n = 5$.

III. Social defeat stress: behavioral and biochemical evaluation

Chronic administration of the TAT-DEF-Elk1 peptide reversed chronic stress effects in the UCMS paradigm. We then assessed another model of chronic stress, the social defeat stress, used to modelize social isolation and phobia.

Adult male C57Bl/6J mice were submitted to 10 consecutive days of social defeat stress (see Materials and Methods for details). In the end of the stress procedure, stressed and non-stressed mice were evaluated for social interaction in the social avoidance test (Figure 10). During the first session of this test mice are introduced in an open field (depicted below) with an empty plexiglass cage (no target) on one side and time spent in the interaction zone is automatically recorded. During the second session an aggressive mouse is inserted in the cage (target) and mice are re-introduced in the open field and time spent in the interaction zone is again recorded automatically. Non-defeated mice spend more time in the interaction zone when there is another mouse in the cage. Defeated mice spend the same time in the interaction zone during the two sessions showing a social avoidance behavior (Figure 10a). In Figure 10b the same results are expressed as a ratio of the interaction time during the target session on the time spent in the interaction zone during the first no target session.

Social avoidance behavior is maintained up to four weeks after the end of stress and can be reversed by chronic but not acute antidepressant treatment (Berton et al. 2006). As TAT-DEF-Elk1 peptide showed antidepressant efficacy in the UCMS protocol, we assumed that it could also reverse social avoidance after a chronic dosing. Adult male C57BL/6J mice were submitted to a 10day social defeat stress and their social interaction was evaluated in the social avoidance test as shown earlier. Mice were then treated with TAT-DEF-Elk1 peptide 1mg/kg or vehicle for 21 days and their social behavior as well as anxiety state was evaluated. TAT-DEF-Elk1 completely abolished stress-induced social avoidance after 3weeks of treatment and alleviated anxiety as measured in the elevated plus maze (Figure 11a, b).

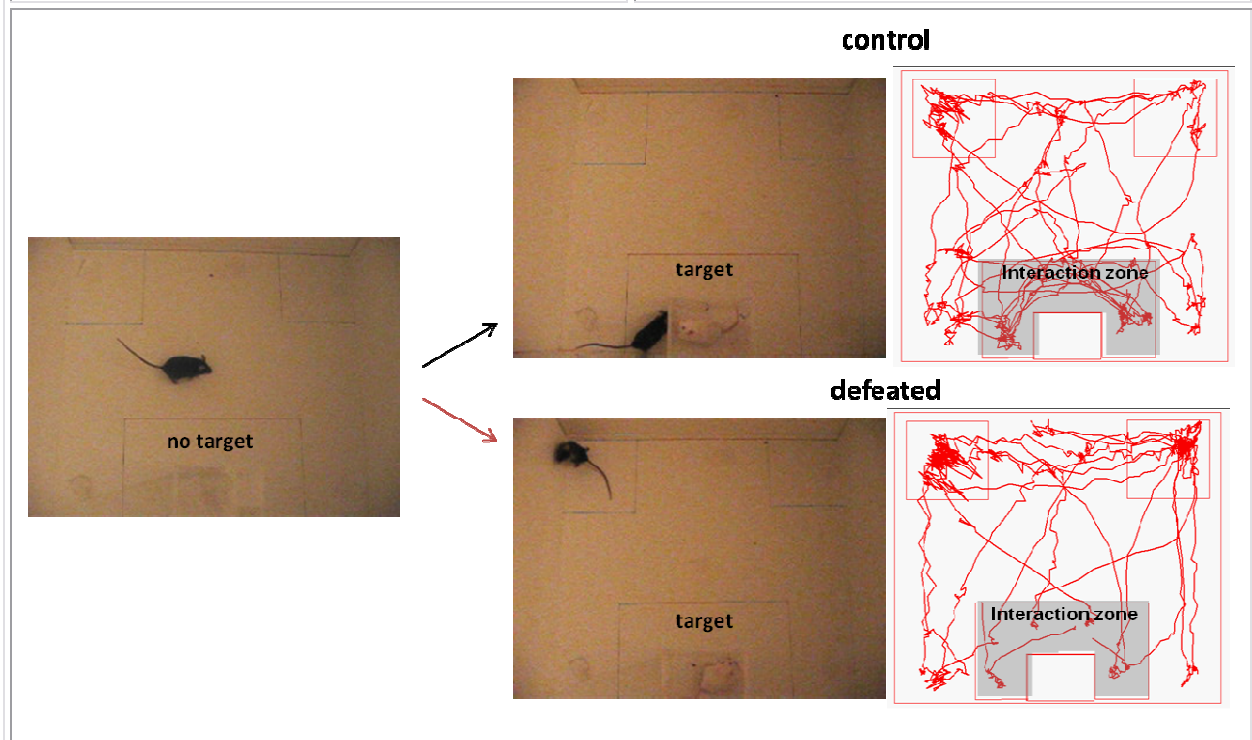
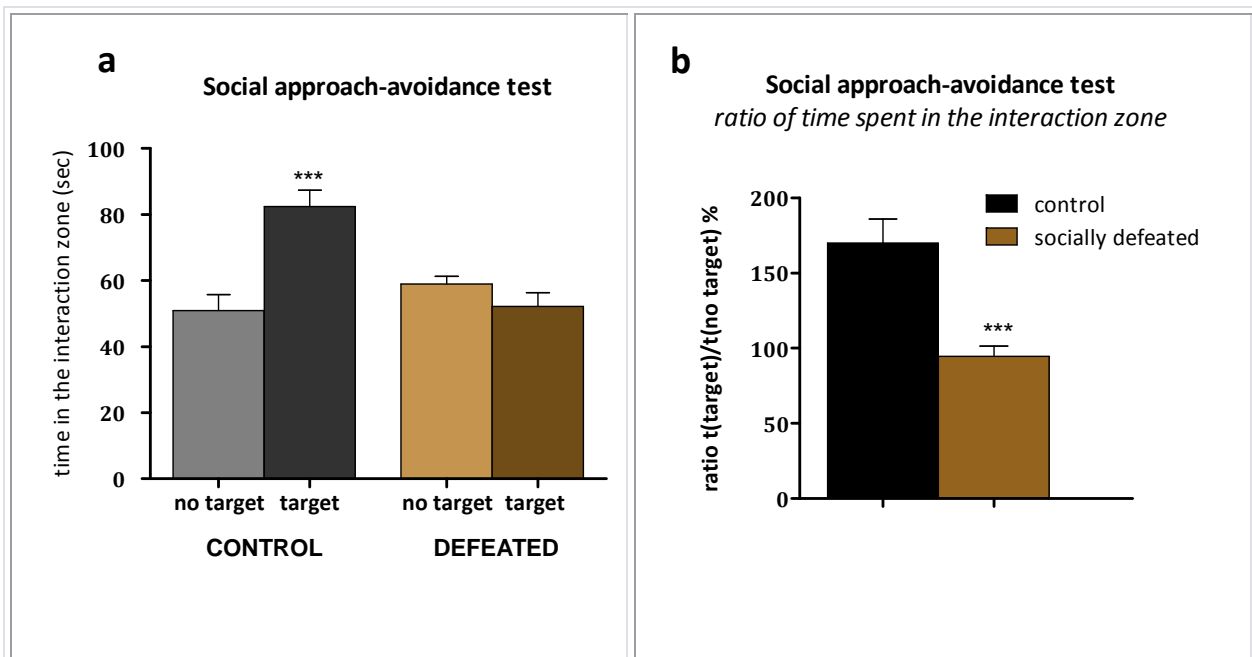


Figure 10: Social interaction test after 10 days of social defeat stress. (a) Time spent in the interaction zone during the first session (no target) and the second session (target) of non-defeated and defeated mice. Two-way ANOVA repeated measures $***p < 0.001$ as compared to no target control group. (b) Ratio of time spent in the interaction zone in the presence of an aggressor (target) on the time spent in the absence of an aggressor (no target) for non-defeated and defeated mice. Student t test $***p < 0.001$ as compared to control, $n = 15-25$.

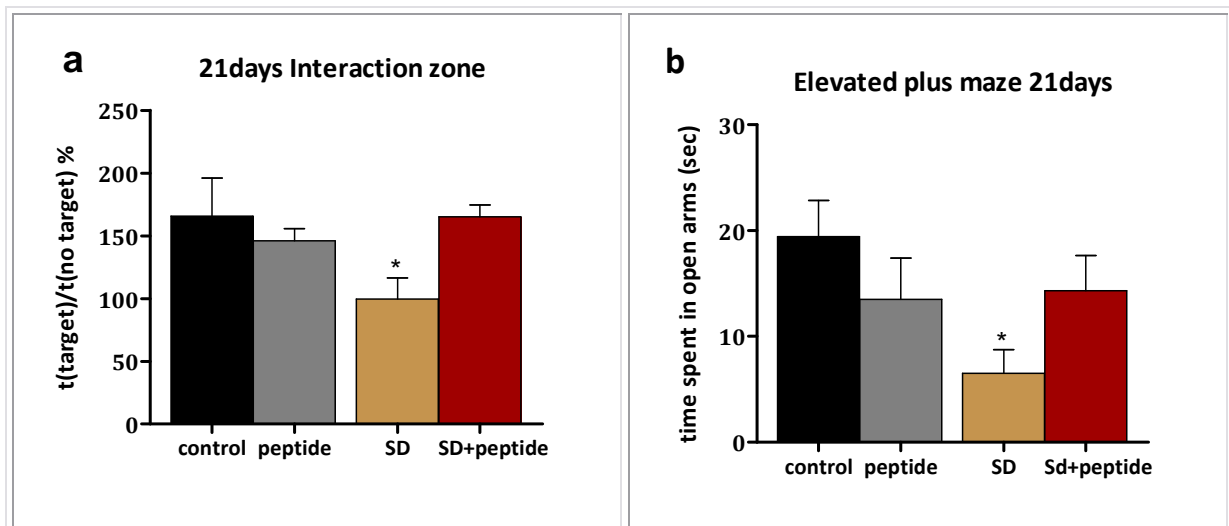
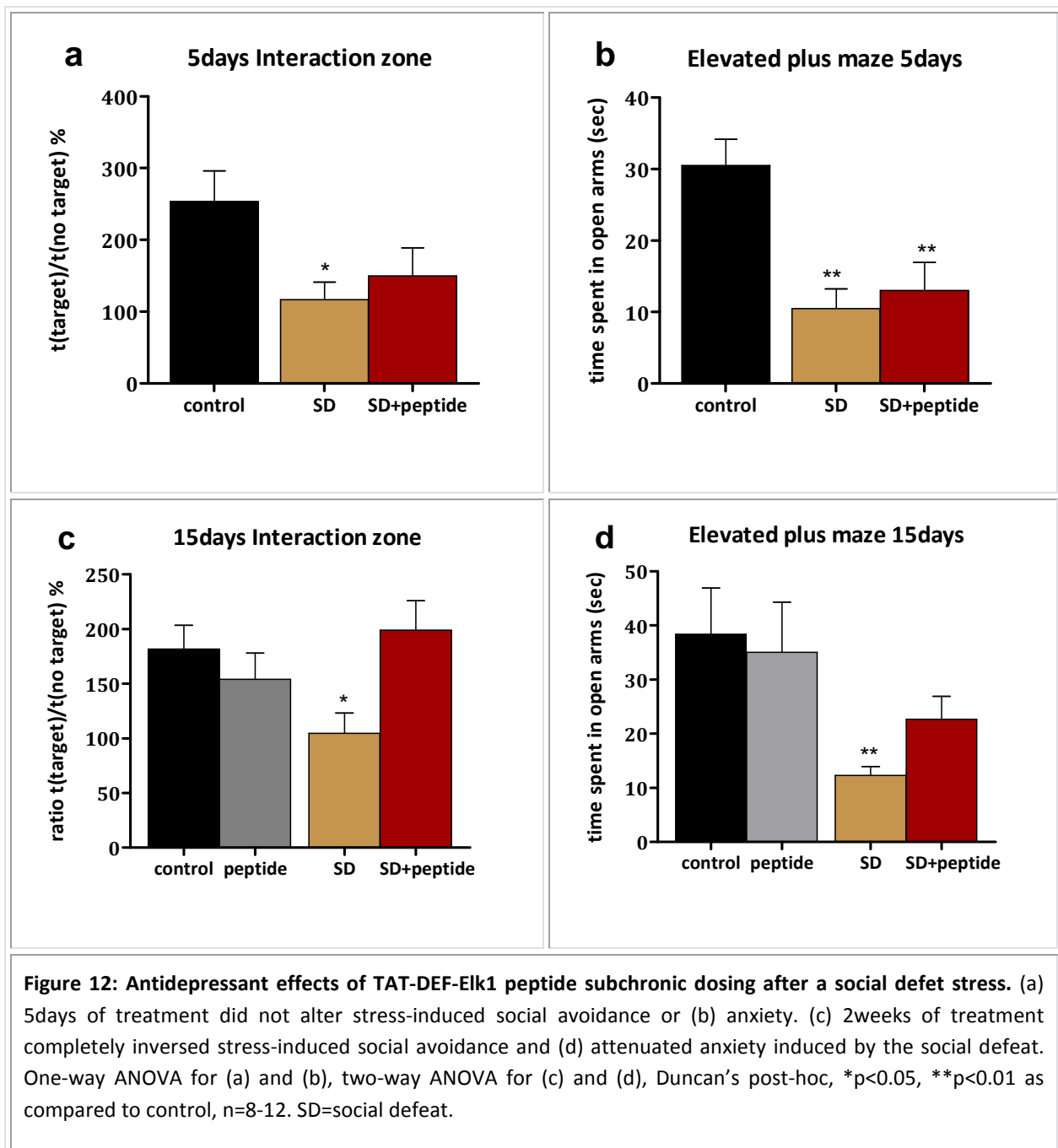


Figure 11: Antidepressant effects of a chronic treatment with the TAT-DEF-Elk1 peptide after a social defeat stress. (a) 3 weeks after the end of stress, socially defeated mice still present a social avoidance behavior that is completely reversed by a chronic treatment with TAT-DEF-Elk1 peptide. (b) Increased anxiety is also observed 3 weeks after the end of stress and was partially inverted by TAT-DEF-Elk1. Chronic treatment with the peptide alone had no effect in this tests. Two-way ANOVA, Duncan's post-hoc, * $p < 0.05$ as compared to control group, $n = 8-12$. SD = social defeat.

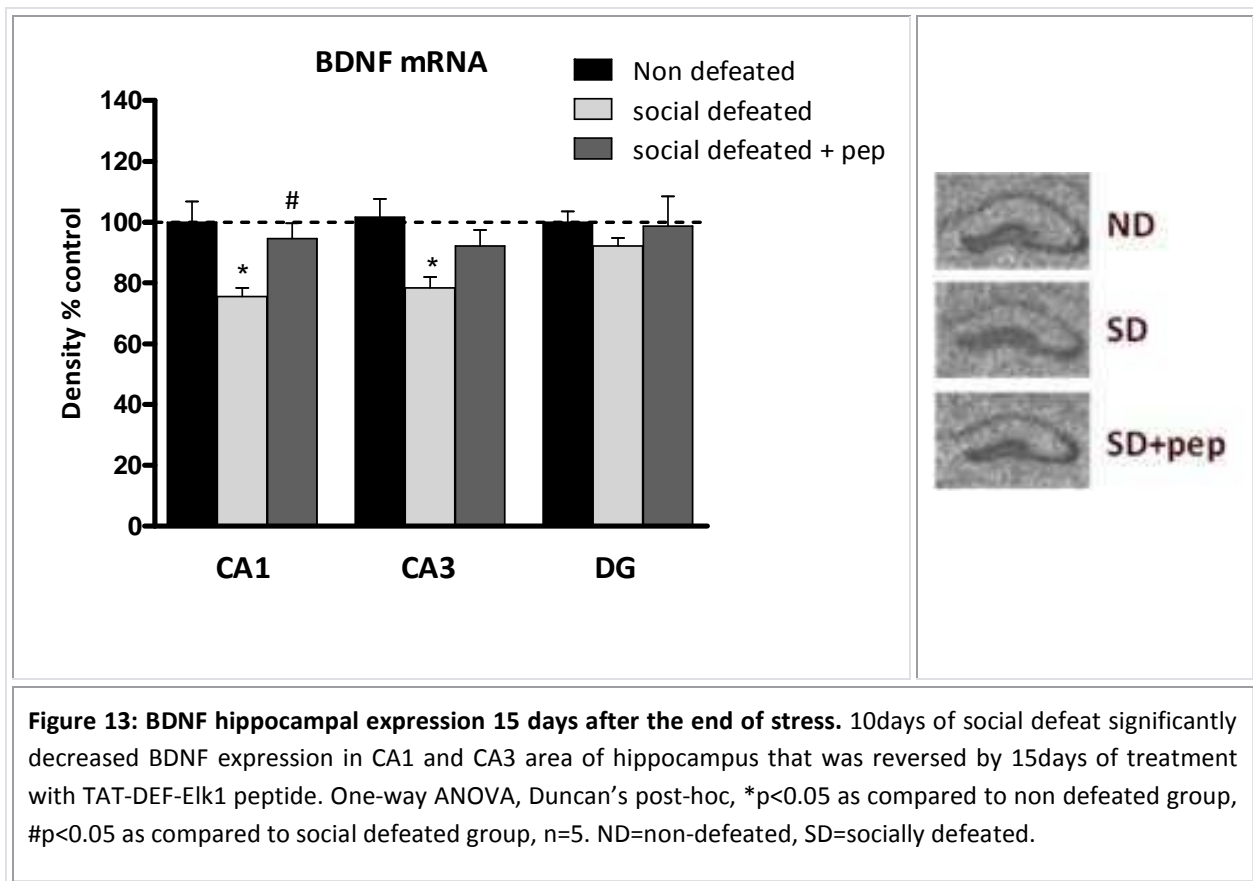
In order to evaluate the onset of action of the peptide, we performed two other sets of experiment with subchronic dosing of the peptide after 10days of social defeat. At the end of the stress procedure mice were treated either for 5days either for 15days with TAT-DEF-Elk1 peptide at 1mg/kg. The emotional state of mice was evaluated in the social avoidance test and in the elevated plus maze. A short period of treatment (5days) showed no significant effect on behavior in either test (Figure 12a, b). To the contrary, 15days of treatment completely inverted social avoidance and moderated anxiety in defeated mice as shown in the Figure 12c and d, respectively.

In an attempt to correlate behavior to stress-induced biochemical changes we used this behavioral setting (10days social defeat + 15days TAT-DEF-Elk1 treatment) to study alterations in hippocampus of non-stressed and stressed animals treated with TAT-DEF-Elk1 peptide or vehicle.



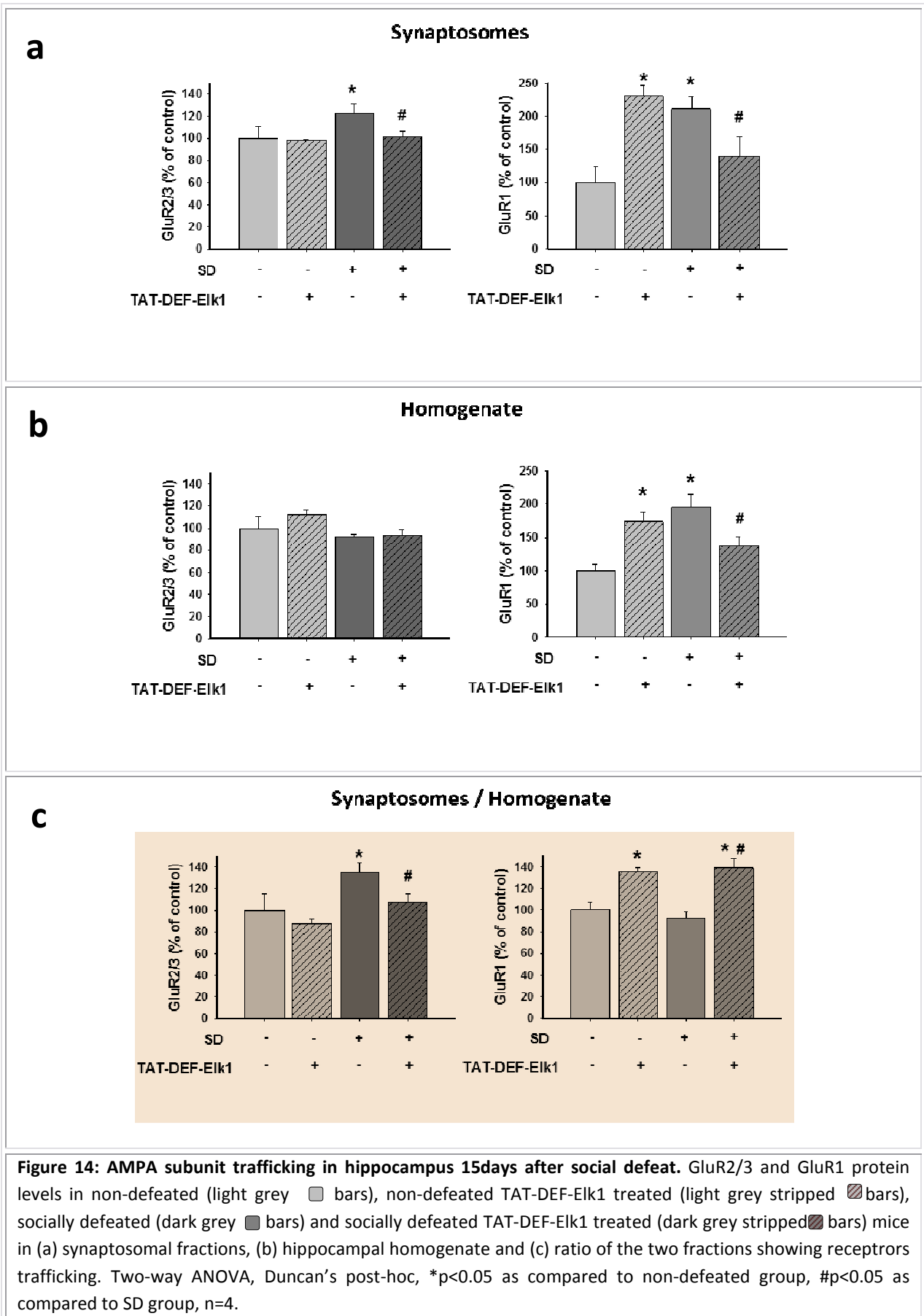
Social defeat stress induces biochemical modifications that correlate with behavior

A 10day social defeat stress significantly decreased BDNF mRNA expression in CA1 and CA3 areas of hippocampus as it was measured 15days after the end of the stress by in situ hybridization. A 15day TAT-DEF-Elk1 treatment (1mg/kg) completely inverted this effect as shown in Figure 13.



Acute administration of the TAT-DEF-Elk1 peptide was found to reverse stress-induced modifications in AMPA and NMDA trafficking in hippocampus (Results-Part IIb). Here, we studied the effects of a chronic stress (10days of social defeat) followed by 15days of vehicle or TAT-DEF-Elk1 1mg/kg treatment on glutamatergic modulation. Stress was found to significantly increase GluR2/3-containing AMPA receptors in the synaptic cleft and this effect was reversed by the peptide administration. GluR1-containing AMPA receptors were found increased by stress in both synaptic and homogenate fractions indicating a general increase of GluR1 expression. Interestingly, TAT-DEF-Elk1 peptide subchronic administration increased only the synaptic expression of GluR1-containing AMPA receptors both in non-defeated and defeated mice (Figure 14).

AMPA R trafficking



NMDA R trafficking

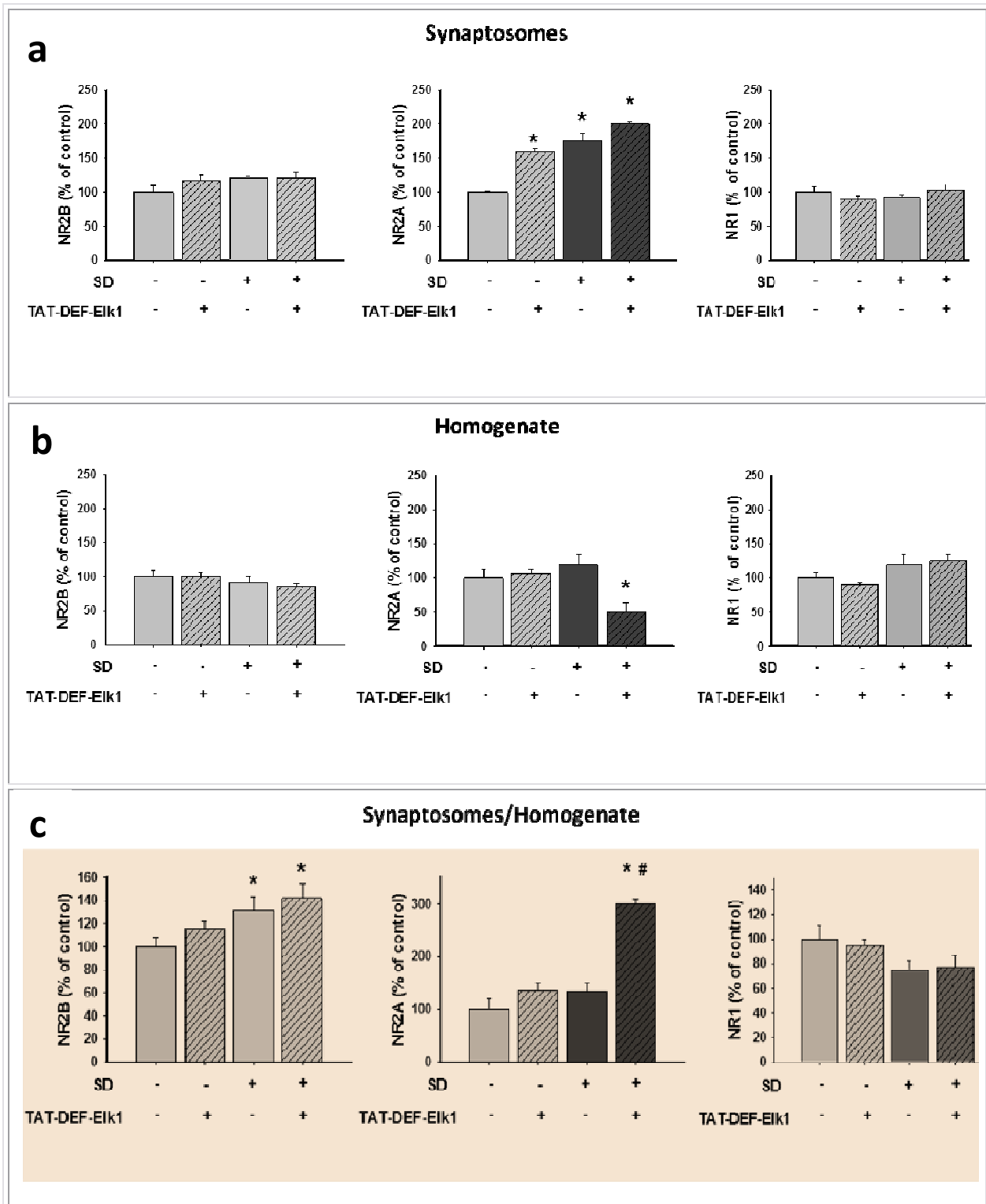


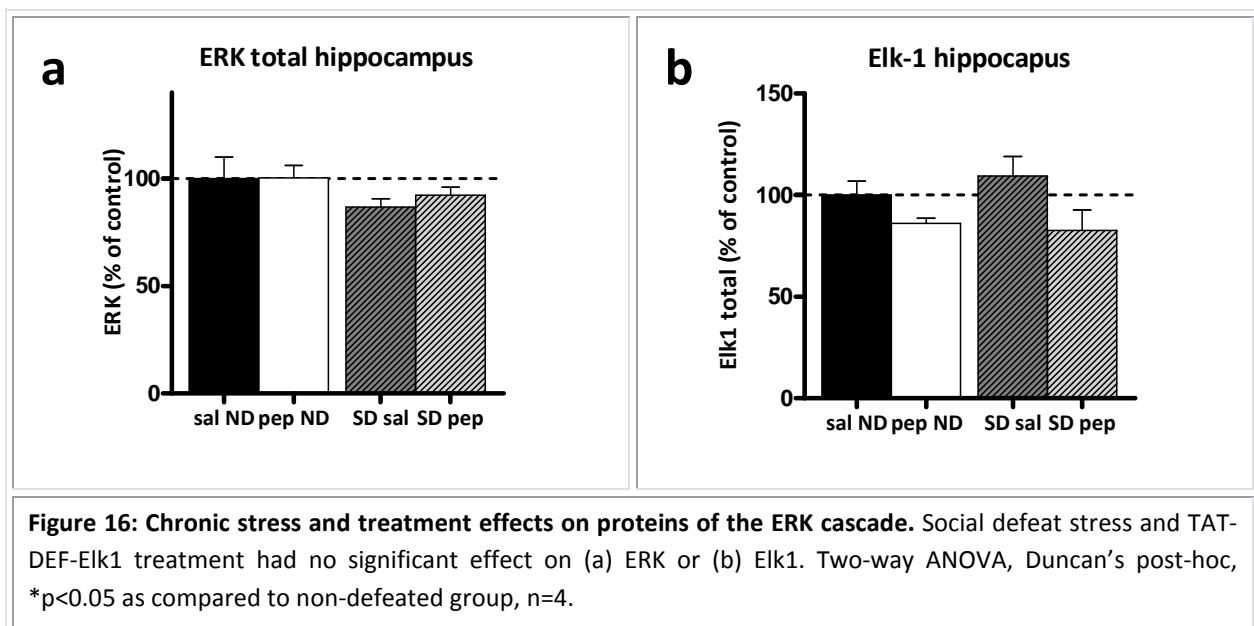
Figure 15: NMDA subunit trafficking in hippocampus 15days after social defeat.

(a) NR2B, NR2A and NR1 protein levels in non-defeated, non-defeated TAT-DEF-Eik1 treated, socially defeated and socially defeated TAT-DEF-Eik1 treated mice in (a) synaptosomal fractions, (b) hippocampal homogenate and (c) ratio of the two fractions showing receptors trafficking. Two-way ANOVA, Duncan's post-hoc, * $p < 0.05$ as compared to non-defeated group, # $p < 0.05$ as compared to socially defeated group, $n = 4$.

When to NMDA receptors, stress was found to significantly increase the synaptic expression only of the NR2B subunit and TAT-DEF-Elk1 treatment did not affect this increase. NR2A subunits were found upregulated in the synaptosomal fraction of TAT-DEF-Elk1 treated mice and stressed vehicle and TAT-DEF-Elk1 treated mice. Ratio of synaptosomal to homogenate fractions showed significant increase only in the stressed TAT-DEF-Elk1 treated mice. NR1 subunits were not affected in either condition (Figure 15).

ERK cascade

Protein levels of the ERK cascade were also studied by Western blot in hippocampal extracts to examine stress effects after 10days of social defeat followed by 15days of TAT-DEF-Elk1 treatment (1mg/kg). No significant effect was found on ERK, Elk-1 or CREB total amounts in hippocampus of non-defeated or defeated, vehicle- or TAT-DEF-Elk1-treated mice (Figure 16a, b, 17a). Calcineurin, the major phosphatase of Elk-1, was found significantly decreased by chronic social defeat stress and administration of TAT-DEF-Elk1 peptide alleviated this effect (Figure 17b).



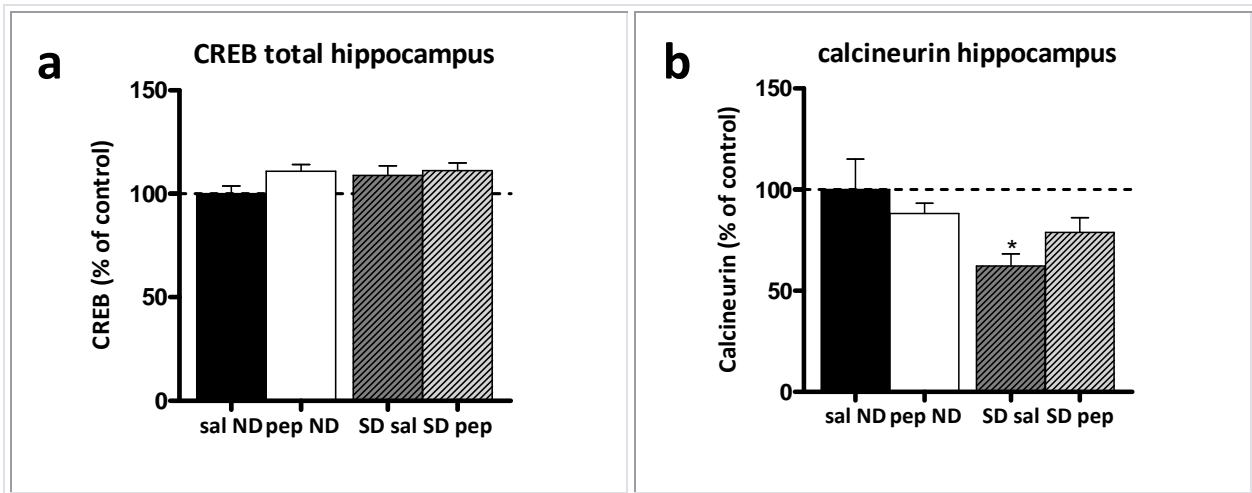


Figure 17: Chronic stress and treatment effects on proteins of the ERK cascade. Social defeat stress and TAT-DEF-Elk1 treatment had no significant effect on (a) CREB total levels in hippocampus. (b) Calcineurin levels were significantly decreased after a 10day social defeat stress and TAT-DEF-Elk1 treatment partially inverted this effect. Two-way ANOVA, Duncan's post-hoc, * $p < 0.05$ as compared to non-defeated group, $n = 4$.

MKP-1, also named DUSP-1 for dual-specific phosphatase, is known to regulate the activity of the ERK signaling cascade by dephosphorylation of ERK1/2. Upregulation of MKP-1 has been recently implicated in the pathophysiology of depression in humans and depressive-like states in mice (Duric et al. 2010). We assessed MKP-1 mRNA expression in hippocampus of vehicle- or TAT-DEF-Elk1 socially defeated mice and compared it to non defeat control mice. MKP-1 expression was found significantly increased in the CA3 region of hippocampus and with a tendency to increase in the other areas of hippocampus (Figure 18). TAT-DEF-Elk1 treatment restored MKP-1 mRNA levels in hippocampus.

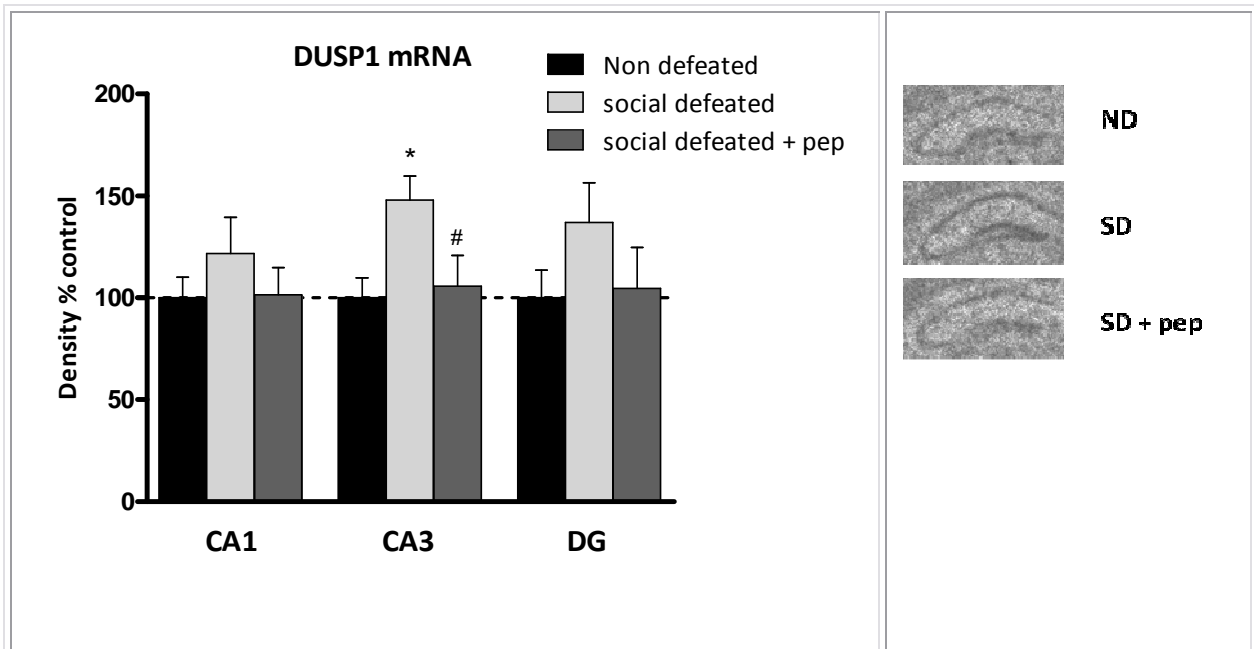


Figure 18: MKP-1 (DUSP1) mRNA expression in hippocampus of socially defeated mice, 15days after the end of stress. Social defeat significantly increased mRNA expression of the phosphatase MKP-1 in CA3 area of hippocampus. One-way ANOVA, Duncan's post hoc, * $p < 0.05$ as compared to the non-defeated group, # $p < 0.05$ as compared to social defeat group, $n = 5$.

PART IId: Delay of onset of antidepressant action of the TAT-DEF-Elk1 peptide

Hypothesis

In Part IIc of this work, antidepressant effects of chronic administration of the TAT-DEF-Elk1 peptide were demonstrated in two chronic models of depression: the UCMS and the social defeat stress. In both models, animals were submitted in repeated stressors for a long period of time and were evaluated in the end for behavioral outputs related to depressive states. TAT-DEF-Elk1 peptide attenuated stress-induced behavior in both models after a chronic (21days) treatment and in social defeat after a shorter (15 days) treatment. This indicates that a TAT-DEF-Elk1 treatment has an earlier onset of action than classical antidepressant treatments.

In Part IIb of this work, we found that an acute administration of the TAT-DEF-Elk1 peptide modulates response to stress through a glutamatergic component, similar to SSRIs but not tricyclics. AMPA R phosphorylation has been shown to mediate antidepressant effects of the reference antidepressant fluoxetine but not desipramine. Furthermore, TAT-DEF-Elk1 peptide showed synergistic effects with desipramine but not fluoxetine.

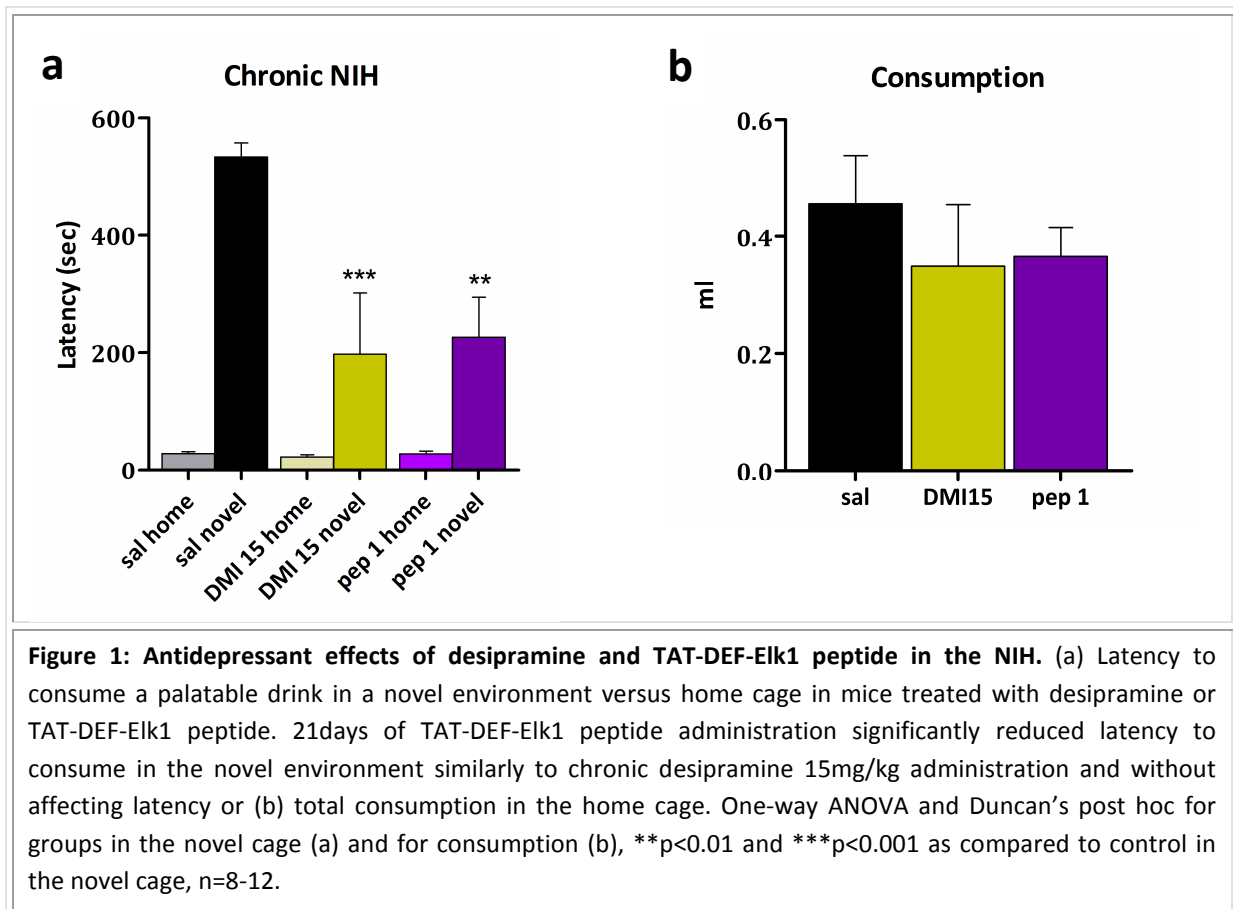
Objective of the study

We used the Novelty Induced Hypophagia (NIH) paradigm and subchronic periods of TAT-DEF-Elk1 peptide administration to estimate the exact delay of onset of action. We also evaluated if combinational treatment of the peptide with desipramine could show a synergistic effect on the delay of onset of action.

Results

I. Effects of chronic TAT-DEF-Elk1 administration in the NIH paradigm

We first assessed 21days treatment of the TAT-DEF-Elk1 peptide (1mg/kg) to validate its antidepressant action in these experimental conditions. We used desipramine 15mg/kg as reference antidepressant. Animals were habituated to consume chocolate milk in their home cage before the test. During habituation, latency to drink and consumption were measured to examine if treatment affects habituation or milk intake. Chronic administration of TAT-DEF-Elk1 or desipramine did not affect either of these two parameters. Latency to drink in the novel cage was significantly decreased by both treatments and to the same extent as compared to saline treated animals (Figure 1).



II. Effects of subchronic TAT-DEF-Elk1 administration in the NIH paradigm

In order to evaluate if the TAT-DEF-Elk1 peptide has an earlier onset of action we used two administration periods: 5 and 15days. TAT-DEF-Elk1 peptide was earlier shown to act synergistically with desipramine in the TST.

Subsequently, we also administered the two compounds concomitantly to examine if their synergistic effect can further decrease the delay of onset of antidepressant action. Adult male C57BL/6J mice were injected daily for 5 or 15 days with TAT-DEF-Elk1 1mg/kg, with desipramine 10mg/kg or with a combination of the two treatments and their latency to consume chocolate milk was measure in a novel cage 24h after the last injection.

A 5day treatment showed no effect in latency to consume in the novel cage of either treatment (Figure 2a). Latency to consume and total consumption in the home cage were not altered either (Figure 2a, b). Desipramine and TAT-DEF-Elk1 peptide had no effect when administered alone for 15days, while co-administration of the two significantly decreased latency to consume in the novel cage (Figure 2c) without altering latency to consume or total consumption in the home cage (Figure 2c, d).

Peptide 1mg/kg, DMI 10mg/kg

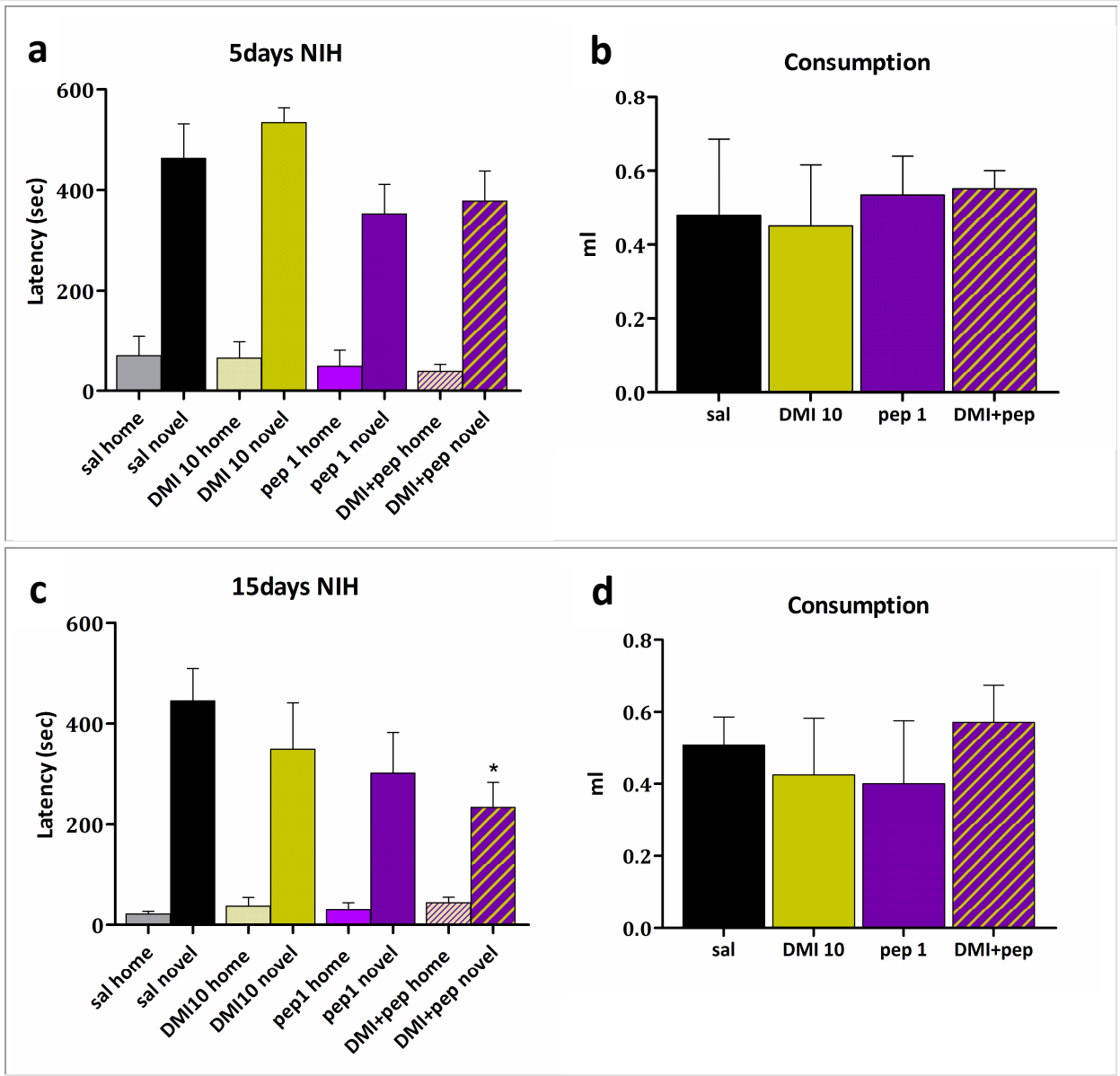


Figure 2: Early onset of action of co-administration of desipramine and TAT-DEF-Elk1 peptide. Latency to consume a palatable drink in a novel environment versus home cage in mice treated with desipramine 10mg/kg and/or TAT-DEF-Elk1 1mg/kg peptide. (a) 5days of TAT-DEF-Elk1 peptide administration alone or in combination with desipramine and (b) total consumption in the home cage. (c) 15days of TAT-DEF-Elk1 peptide administration alone or in combination with desipramine and (d) total consumption in the home cage. One-way ANOVA and Duncan’s post hoc for groups in the novel cage (a) and for consumption (b), *p<0.05 as compared to saline in the novel cage, n=8-12.

Peptide 2mg/kg, DMI 15mg/kg

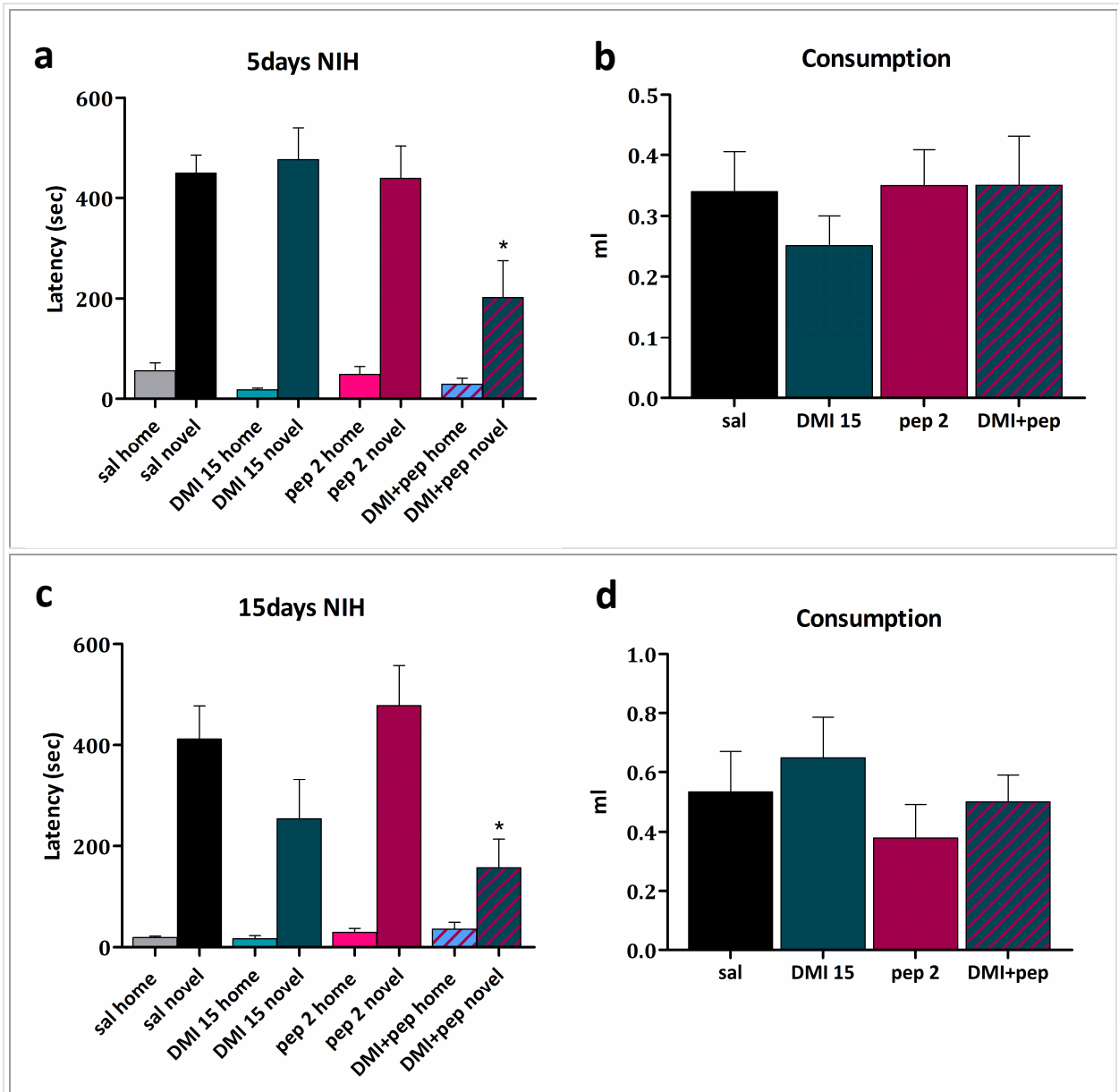


Figure 3: Very early onset of action of co-administration of desipramine and TAT-DEF-Elk1 peptide. Latency to consume a palatable drink in a novel environment versus home cage in mice treated with desipramine 15mg/kg and/or TAT-DEF-Elk1 2mg/kg peptide. (a) 5days of TAT-DEF-Elk1 peptide alone or in combination with desipramine and (b) total consumption in the home cage. (c) 15days of TAT-DEF-Elk1 peptide administration alone or in combination with desipramine and (d) total consumption in the home cage. One-way ANOVA and Duncan's post hoc for groups in the novel cage (a, c) and for consumption (b, d), * $p < 0.05$ as compared to control in the novel cage, $n = 8-12$.

Having shown a synergistic action after 15days of treatment with relatively low doses of desipramine and TAT-DEF-Elk1 peptide, we then increased the doses mutually to evaluate if this could further decrease the delay of onset. Subsequently, we assessed 5 and 15 days of treatment with TAT-DEF-Elk1 2mg/kg and desipramine 15mg/kg separately or in combination. Co-administration of the two compounds significantly decreased latency to consume after both 5 and 15days of administration. Desipramine or TAT-DEF-Elk1 peptide treatments alone showed no significant effect and total consumption was not affected by either treatment (Figure 3).

III. BDNF mRNA expression

Increased expression of BDNF mRNA in hippocampus is believed to mediate antidepressant action. We measured BDNF expression in mice that received a combinational treatment of desipramine (10mg/kg) and peptide (1mg/kg) for 15days. This treatment showed antidepressant action on the NIH to the same extent as chronic (21days) desipramine treatment. BDNF expression was significantly increased in CA1 and CA3 areas of hippocampus, similarly to chronic desipramine treatment as shown in Figure 4.

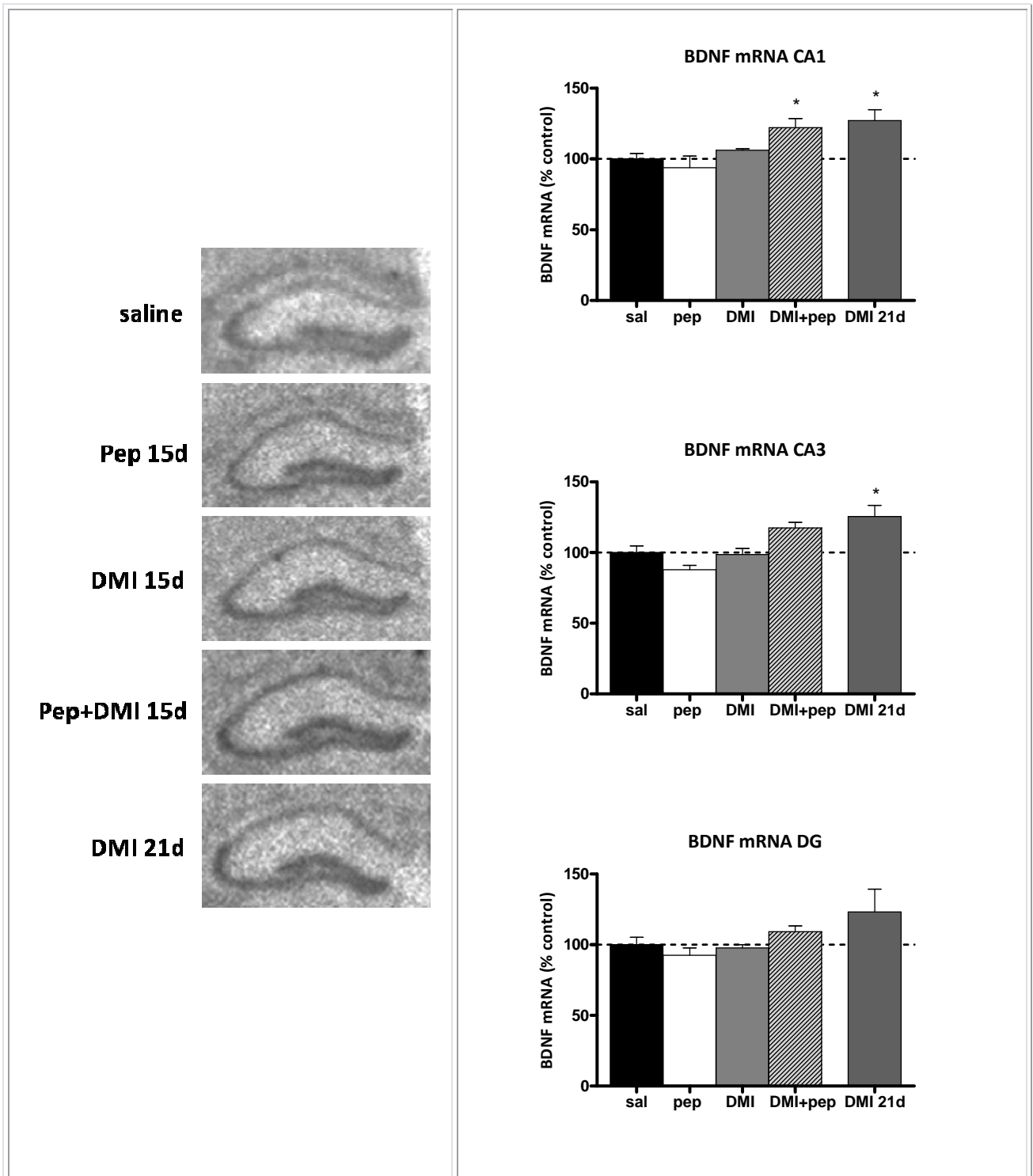
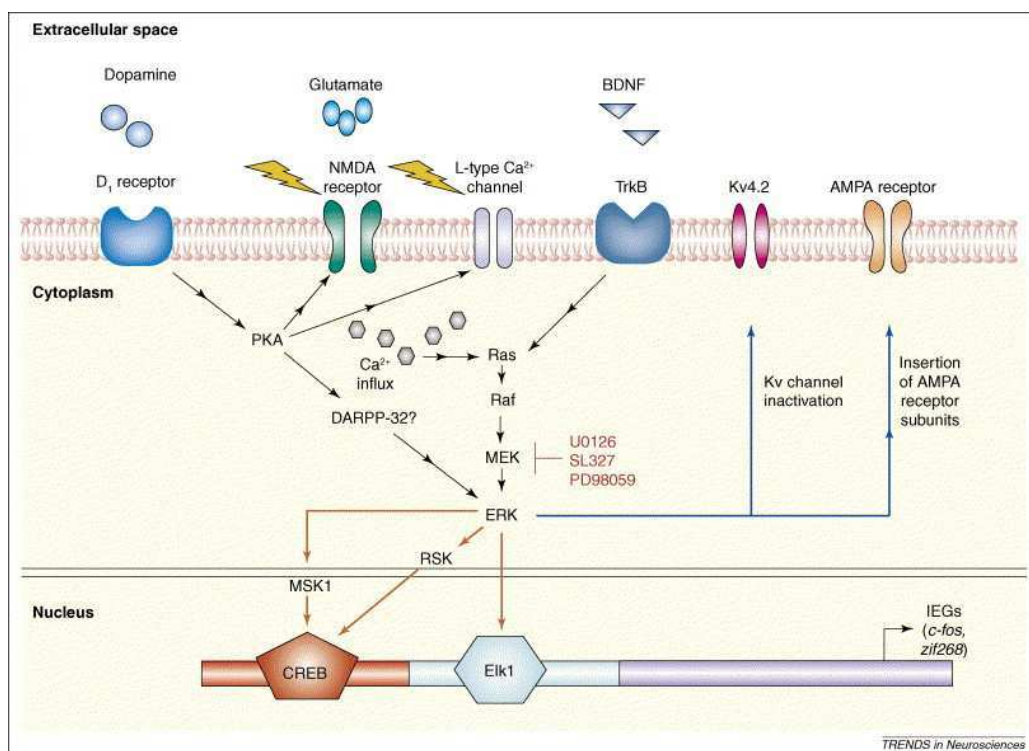


Figure 4: Increased BDNF expression in hippocampus after co-administration of desipramine and TAT-DEF-Elk1. A subchronic treatment (15days) with TAT-DEF-Elk1 peptide 1mg/kg (white bars) or desipramine 10mg/kg (grey bars) did not increase BDNF expression in hippocampus as compared to saline treated mice (black bars). A chronic treatment with desipramine (dark grey bars) 10mg/kg significantly increased BDNF mRNA expression in CA1 and CA3 area of hippocampus. Subchronic dosing of TAT-DEF-Elk1 peptide 1mg/kg combined to desipramine 10mg/kg significantly increased BDNF mRNA expression in CA1. One-way ANOVA, Dunnett's post hoc, * $p < 0.05$ as compared to saline group, $n = 5$.

DISCUSSION

The mitogen-activated protein kinase (MAPK) signaling pathways constitute a highly conserved mechanism of eukaryotic cells that control fundamental cellular functions such as proliferation, differentiation, development, cell survival and apoptosis. Among the MAPKs, the extracellular-regulated kinase ERK/MAPK pathway is considered today a major integrator of membrane and cytoplasmic signals that are further transduced to nucleus to modulate gene expression and cellular response. In neurons, this is achieved through specific upstream and downstream cascades, regulated among others by multiple feedback loops.

Mainly implicated as a central regulator of behavior and CREB activity, as well as a mediator of the antidepressant effects of neurotrophic and growth factors, ERK constitutes today a major candidate mediating etiology and therapeutics of mood disorders. Regulation of gene expression is now thought to underlie mechanisms that lead to pathology and are implicated in treatment of depression. ERK pathway, like other signal transduction cascades, forms a highly complex network that is activated by several stimuli and controls transcription factors activity. A very simplified schematic representation of the ERK cascade is depicted below.



The phosphorylation and activation of ERK1/2 substrates is regulated by protein-protein interactions. Evolutionary conserved docking sites on ERK1/2 offer the possibility to specifically block a protein-protein interaction of a subset of substrates.

“In theory, it should be possible to block specific docking sites on the kinases and hence specifically inhibit phosphorylation of a subset of substrates by a particular class of MAP kinase. As the MAP kinase pathways play important roles in many aberrant biological processes, such specific therapeutic agents would be of immense value” (Sharrocks et al. 2000).

In our approach, we used a membrane penetrating peptide that carries the HIV-TAT sequence on its N terminus and mimics the docking domain of Elk-1 for ERK (DEF domain), the TAT-DEF-Elk1 peptide. Once in the cytoplasm, TAT-DEF-Elk1 binds on the equivalent site on ERK1/2 inhibiting the interaction of the two proteins and thus the activation of Elk1 from ERK1/2. The specificity of this peptide has been previously validated *in vitro* and *in vivo* (Lavour et al. 2007, Besnard et al. 2011). Administration of this peptide *in vivo* revealed a considerable amount of data that suggests an antidepressant action of selective inhibition of ERK/Elk1 signaling in mice.

Elk1: A novel antidepressant target?

I. Antidepressant-like properties in animal models of depression

a) Acute

As a first evidence for antidepressant action, TAT-DEF-Elk1 peptide systemic administration decreased immobility time in the tail suspension and the forced swimming tests, similarly to reference antidepressants. These are two acute tests widely used in the literature for screening antidepressant action (Bai et al. 2001, Li et al. 2001).

Lack of effect after administration of the scrambled peptide (a peptide that consists of the same amino acids like the TAT-DEF-Elk1, but placed in a different sequence) and the MEK inhibitor SL327 on TST and FST indicates that the antidepressant-like activity observed after

administration of the TAT-DEF-Elk1 peptide is attributed to the selective inhibition of ERK/Elk1 signaling during an acute stress.

Indeed, systemic injection of TAT-DEF-Elk1 peptide *in vivo* specifically blocked phosphorylation of Elk1 without altering phosphorylation patterns of the other substrate of ERK1/2, namely MSK-1, or activation of ERK1/2 itself after an acute stress in prefrontal cortex and hippocampus.

Activation of ERK cascade from stress		pERK		pElk1		pMSK1	
		no stress	stress	no stress	stress	no stress	stress
PFC	Control	→	↑	→	↑	→	↑
	TAT-DEF-Elk1	→	↑	↓	↓	→	↑
CA1	Control	→	↑	→	↑	→	↑
	TAT-DEF-Elk1	→	↑	↓	↓	→	↑

b) Chronic

Acute identification of antidepressant activity does not necessarily imply that chronic administration would also exhibit antidepressant action able to reverse depressive-like states induced by chronic stress procedures. For that purpose, we assessed chronic stress models followed by chronic treatment of the TAT-DEF-Elk1 peptide. Chronic animal models of depression are considered of high construct, face and predictive validities despite their limitations of partial representation of human symptomatology. Advantageously, multiple models are developed today that study certain clusters of symptoms related to different etiology. Here, we used two different chronic models, namely the unpredictable chronic mild stress and the social defeat stress.

Unpredictable chronic mild stress is mainly validated for its ability to induce anhedonia, a core symptom occurring in all subtypes of depressive disorders. Moreover, conventional antidepressants have been shown to restore anhedonia after a chronic treatment (Willner P. 2005). In this study, anhedonia was completely prevented in chronically stressed mice that

received a chronic TAT-DEF-Elk1 treatment, as measured in the sucrose preference test. Interestingly, TAT-DEF-Elk1 treatment also inversed stress-induced resignation and self-care as measured in the TST and the fur condition state, respectively. It is worth noting that fluoxetine effects on the same measures were comparable to these of the peptide. Results are summarized in the table below.

UCMS and chronic TAT-DEF-Elk1 treatment effects on behavior				
Behavior measured (test)	Behavioral output			
	Stress/drug	vehicle	TAT-DEF-Elk1	fluoxetine
Resignation (TST)	No UCMS	→	↓	↓
	UCMS	↑	↓	↓
Anhedonia (sucrose preference)	No UCMS	→	→	→
	UCMS	↑	↓	↓
Self care (fur condition)	No UCMS	→	→	→
	UCMS	↓	↑	↑
Anxiety (elevated plus maze)	No UCMS	→	→	↓
	UCMS	↑	↑	↓
Locomotion	No UCMS	→	→	→
	UCMS	→	→	→

Social defeat stress is a model of social stress that leads to a remarkable decrease of social behavior. Mice are introduced every day into the cage of a dominant resident mouse of a different strain and are allowed to interact directly. Daily aggressions result in a pronounced avoidance of social interactions that lasts up to 4 weeks after the end of the stress (Berton et al. 2006). Social defeat has been proposed as a model of depression, post-traumatic stress disorder, physical abuse and anxiety. The fact that reference antidepressants have been shown to reduce signs of defeat after a chronic administration, validates this model as responsive to antidepressant action.

Chronic treatment (21days) of the TAT-DEF-Elk1 peptide completely inversed social phobia in defeated mice as measured in the social interaction/avoidance test and alleviated anxiety as

measured in the elevated plus maze. Subchronic treatment (15days) demonstrated the same efficacy in these tests.

Social defeat (SD) and chronic TAT-DEF-Elk1 treatment effects on behavior				
Behavior measured (test)	Stress/drug	vehicle	TAT-DEF-Elk1 (15d)	TAT-DEF-Elk1 (21d)
Social interaction	No SD	→	→	→
	SD	↓	↑	↑
Anxiety (elevated plus maze)	No SD	→	→	→
	SD	↑	↓	↓

II. Antidepressant biomarkers

a) BDNF

The “neurotrophic” hypothesis of depression posit a role for decreased trophic support in the hippocampal damage observed in depression, and a contribution of antidepressant induced trophic factor expression to their therapeutic action. Here, we studied BDNF expression in hippocampus as a major biomarker of depression and antidepressant activity. Both after acute and chronic stress, BDNF mRNA expression was found significantly decreased in hippocampus, predominantly in the CA3 area. We assessed BDNF expression after TAT-DEF-Elk1 treatment in each behavioral setting to examine if the observed antidepressant action correlates with reversal of reduced BDNF expression after stress. Both after acute and chronic stress, TAT-DEF-Elk1 treatment completely blocked the effect of stress on BDNF expression suggesting that stress modulates BDNF gene expression via a mechanism that implicates phosphorylation of Elk-1 from ERK.

BDNF mRNA has been consistently reported to decrease after a single or repeated stressor as well as corticosterone treatment in all areas of hippocampus (Smith et al. 1995, Schaaf et al. 1998, Duman and Monteggia 2006, Murakami et al. 2005). The mechanisms underlying the downregulation of BDNF expression from stress have been little investigated in the literature and have yet to be completely characterized. Epigenetic modifications have been

proposed to mediate alterations of BDNF mRNA expression after an acute stress (Fuchikami et al. 2009), a social defeat stress (Tsankova et al. 2006) and antidepressant treatment (Tsankova et al. 2004, 2006). Specifically, decreased histone acetylation (H3) that leads to a more closed formation of the chromatin has been implicated in acute stress effects on BDNF expression. Social defeat stress induced robust increases of repressive histone (H3) methylation at certain BDNF promoters. Electroconvulsive seizures and antidepressant treatment have been shown to increase H3 acetylation promoting opening of the chromatin. This enhances BDNF mRNA expression reversing stress-induced downregulation.

Treatment with the TAT-DEF-Elk1 peptide restored BDNF mRNA levels after stress and also upregulated BDNF levels after chronic administration in non-stressed mice. It would be interesting to further investigate histone modifications after chronic stress and chronic TAT-DEF-Elk1 treatment. In addition to its C-terminal transcriptional activation domain, Elk-1 contains an N-terminal transcriptional repression domain that can recruit the mSin3A-histone deacetylase 1 corepressor complex (Yang et al. 2001). An attractive hypothesis would be that inhibition of Elk1 from ERK during stress inhibits also histone deacetylase complex (HDAC) activity on BDNF promoters.

BDNF mRNA expression in hippocampus				
Treatment	No stress	TST	Social Defeat	UCMS
Vehicle	→	↓	↓	↓
TAT-DEF-Elk1 acute	→	↑	-	-
TAT-DEF-Elk1 15d	→	-	↑	-
TAT-DEF-Elk1 21d	↑	-	-	↑
Desipramine 15d	→	-	-	-
Desipramine 21d	↑	-	-	-
Desipramine + TAT-DEF-Elk1 15d	↑	-	-	-

b) GluR1 subunit of AMPA R

It is worth noting here changes found in GluR1 subunit expression after acute and chronic stress. Antidepressant efficacy has been related to increased synaptic expression of the GluR1 subunit of the AMPA receptors, as previously discussed (Svenningsson et al. 2002a, b, 2007, Martinez-turillas 2002, 2005, Du et al. 2007, Crozatier et al. 2007). Acute stress significantly reduced GluR1 expression in the CA1 area of hippocampus. This decrease was blocked by the TAT-DEF-Elk1 peptide through an AMPA R mediated mechanism, as AMPA antagonist inhibited TAT-DEF-Elk1 effect. GluR1 mRNA expression has been shown to decrease in CA1 after acute and chronic restraint stress (Rosa et al. 2002, Yaka et al. 2007). Short- and long-term administration of antidepressant agents have been shown to upregulate GluR1 synaptic expression in hippocampus (Gould et al. 2008). Consistent with this, GluR1 subunit was found to be increased in synaptosomal hippocampal fractions of mice chronically treated with TAT-DEF-Elk1 peptide, providing further evidence for antidepressant activity.

Early onset of antidepressant action

The delayed onset of action of antidepressants is a major drawback for both treatment and compliance. Chronic selective inhibition of ERK/Elk1 signaling demonstrated antidepressant effects in two chronic models of depression as discussed above. Subchronic treatment also showed antidepressant effects in the social defeat. In order to evaluate the exact onset of antidepressant activity of the TAT-DEF-Elk1 peptide we used the novelty induced hypophagia (NIH) paradigm. During this test, animals have to face the stress of a novel environment (mild stressor) in order to access a highly palatable drink. The animal experiences conflict between the desire to approach and drink, and the anxiety-induced avoidance of the novel environment. Anxiolytics administered acutely reduce the latency to consume in the novel environment, as expected. Antidepressants also reduce latency to consume only after a chronic but not acute administration, providing a test to directly study onset of antidepressant action.

Administration of the TAT-DEF-Elk1 peptide in combination with the tricyclic antidepressant desipramine showed synergistic effects in the TST. Thus, we assessed subchronic treatment

of each drug and co-administration of the two compounds to examine the hypothesis that a combinational treatment may accelerate the onset of antidepressant action. Combination of desipramine with the TAT-DEF-Elk1 peptide showed an antidepressant effect after 15 days of treatment. Interestingly, higher doses of both treatments further decreased the onset of antidepressant action at a 5-day delay. A chronic (21 days) treatment with TAT-DEF-Elk1 peptide decreased latency to consumption to the same extent as chronic desipramine in the NIH, consistent with reports in the literature (Dulawa and Hen, 2005).

It is noteworthy that the neurotrophic changes exhibit a slow onset, and parallel the time course required for the behavioral benefits of sustained antidepressant administration (Sahay and Hen 2007, Schmidt and Duman 2007). Combinational effective treatment of the TAT-DEF-Elk1 peptide and the tricyclic antidepressant desipramine also correlated with increased BDNF expression in CA1 and CA3 hippocampal areas to the same extent as chronic desipramine treatment. It would be interesting to further investigate which BDNF mRNA isoforms are upregulated from each treatment and if combination of the two amplifies upregulation of the same isoforms or if it accumulates upregulation of different ones. As it has been already described in the literature diverse classes of antidepressants may differentially recruit signaling pathways and transcription factors in a region-specific manner to regulate multiple BDNF transcripts (Dias et al. 2003). Moreover, chronic antidepressant treatment has been shown to inverse corticosterone-mediated downregulation of BDNF total mRNA in hippocampus; this is accomplished through different mechanisms as corticosterone has been reported to downregulate BDNF mRNA isoforms II and IV, while desipramine to upregulate isoforms I and III (Dwivedi et al. 2006). Chronic antidepressant treatments have been reported to regulate the transcription factors c-fos and zif-268 (Bjartmar et al. 2000, Morinobu et al. 1997) for which putative binding sites have been reported in the BDNF gene (Nakayama et al. 1994) suggesting that they may contribute to the regulation of BDNF expression. A more recent meta-analysis of BDNF gene expression and conserved over-represented TFBSs (transcription factors binding sites) in human, mouse and rat revealed the KROX family TFBSs to be abundant in the promoters of BDNF and BDNF-correlated genes in the CNS (Aid-Pavlidis et al. 2009). KROX family of transcription factors includes krox24, also called zif268 or egr-1.

Elk-1 implication in stress response: behavioral and plasticity-related changes

I. Stress-induced impaired cognitive function

Alterations in hippocampal function and impaired cognitive performances following stress have been constantly described in the literature (Lupien and McEwen 1997, Sandi et al. 2007, Krugers et al. 2010, Riedemann et al. 2010). The paradigm of latent learning is a test based on attention and information processing (Noda et al. 2001). We used the water-finding task, considered as a latent learning paradigm, to examine the effects of an acute stressor on memory related to spatial and attentional construction. In this test, during the acquisition (encoding) session mice are free to explore an apparatus that consists of an open field and an alcove to one of its sides. A drinking bottle filled with water is positioned on the top of the alcove, accessible to the animal. The next day, after an overnight water deprivation, mice are introduced again to the open field and latency to drink is recorded. Animals that have explored the apparatus before should exhibit shorter finding time (latency to drink). A stress (10min TST) 2h before the first exposition to the open field increased latency to find water in water-deprived C57BL/6J adult male mice, showing memory impairment. Time of stress exposure was chosen based on studies in the literature that showed that a stress before acquisition has no effect when delivered shortly before, but blocks acquisition when delivered 3hours before (Baker and Kim 2002). Prior administration of mifepristone, a glucocorticoid receptor (GR) antagonist, partially blocked the effects of stress, showing that stress effects are partially GR mediated. Prior administration of the TAT-DEF-Elk1 peptide completely blocked the effects of stress via an AMPA-mediated mechanism; administration of the AMPA antagonist GYKI52466 10min before the peptide blocked its effect. These findings suggest that Elk1 mediates stress effects on memory formation through an AMPA-component regulation.

In another behavioral set up, memory retrieval was tested in mice that were submitted to an acute stressor. Mice were trained during several sessions to find a hidden platform in the hippocampus-dependent spatial version of Morris water maze. After the training period,

mice were tested for memory retrieval in the quadrant test. Exposure to an aggressive mouse (single session of social defeat) 30min before the test significantly impaired memory retrieval. Consistent findings in the literature have shown that prior exposition to a single stressor inhibits memory retrieval in the water maze in rats only when occurring 30min before the test, a time point that corresponds to elevated levels of circulating corticosterone (Wong et al. 2007, de Quervain et al. 2009). The type of stressor used in rats 30min before the task is most commonly predator exposure or elevated platform (Czakoff et al. 2010). Although fewer studies have been conducted in mice, stressors like species-relevant inescapable stress or psychosocial stress have been mostly used to impair memory retrieval (El Hage et al. 2004). We chose a single social defeat session as an equivalent stressor. Social defeat blocked memory retrieval, and prior administration of the TAT-DEF-Elk1 peptide reversed stress-induced impairment.

Taken together, these results show that Elk1 phosphorylation from ERK mediates stress effects on cognitive function. We chose these paradigms to separately study two different memory procedures: memory encoding and memory retrieval during stress. Cognitive impairment has been shown to occur when stress or corticosterone treatment is delivered before the acquisition (impair in encoding), after acquisition (impair in consolidation) or before the retrieval process (impair in retrieval) as summarized in Lupien and McEwen (1997). The exact time point and nature of stress applied seem to play a major role in determining the behavioral output and the magnitude of impairment. The nature of the task examined is also an important parameter.

Synaptic plasticity changes are currently believed to underlie these procedures. Stress is proposed to facilitate LTP induction in hippocampus shortly after exposure and to further induce LTD impairing cognitive function. Enhanced LTP is believed to increase the threshold for further neuronal potentiation. This means that encoding new information is blocked unless stronger potentiation is provoked. Molecular basis of these procedures involve AMPA and NMDA trafficking into and out of the synaptic cleft. GluR2-containing AMPA receptors levels have been shown to increase and mediate LTP early after stress, while NR2B-containing NMDA receptors promote LTD induction responsible for stress induced amnesia. Using the model of TST as an acute stressor we subsequently studied AMPA and NMDA receptors movement after stress.

II. AMPA and NMDA receptors synaptic movement during stress

a) Acute

As mentioned earlier, corticosteroids alter AMPA R trafficking in the synaptic cleft, increasing GluR2-containing receptors by lateral movement and expression (Groc et al. 2008), an effect related to stress-induced modifications in the synaptic plasticity in hippocampus. Thus, we assessed AMPA receptor synaptic expression after a TST and pre-treatment with the TAT-DEF-Elk1 peptide. Adult male C57BL/6J mice were injected i.p. with vehicle or TAT-DEF-Elk1 peptide 90min prior to a 10min TST. AMPA R subunits were analyzed by western blotting. Stress was found to increase GluR2 levels in the synaptic cleft without altering total amounts of GluR2. Prior administration of the TAT-DEF-Elk1 peptide blocked GluR2 increase after stress without affecting AMPA R trafficking when administered alone (no stress). GluR1 subunit did not change in either of cases. This indicates that stress-induced GluR2 synaptic increase is mediated by the ERK/Elk1 pathway and correlates with impaired memory encoding as shown in the water-finding latent learning.

NMDA subunits, NR2A, NR2B and NR1 were also quantified. Stress was found to increase NR2B containing receptors in the synaptic cleft without altering the total amount. Prior administration of the TAT-DEF-Elk1 peptide inversed stress-induced NR2B synaptic increase. Impairment of memory retrieval in the water maze paradigm has been related to NR2B mediated LTD induction in hippocampus in rats (Wong et al. 2007). Long-term depression (LTD) has been previously shown to depend on NR2B activity (Liu et al. 2004, Yang et al. 2005). Administration of the TAT-DEF-Elk1 peptide in the same model of stress-induced amnesia blocked stress effects, consistent with blockage of NR2B synaptic increase. In collaboration with T.P. Wong, we studied the effects of the TAT-DEF-Elk1 peptide on hippocampal slices and the induction of LTD after corticosterone exposure. Interestingly, corticosterone application in the bath induced LTD that was blocked in the presence of the peptide (data not shown), further validating the role of Elk1 in glucocorticoid-mediated LTD.

Although limited information to indisputably correlate trafficking of glutamatergic receptors during stress with behavior (time after stress, stressor type), these results provide evidence for an important role of Elk1 activation mediating these procedures.

b) Chronic

Glutamatergic receptors, AMPA and NMDA trafficking was also assessed in hippocampal extracts of defeated and non defeated mice. GluR2/3 subunit was found increased by chronic social defeat stress and normalized by the peptide treatment. When to NMDA receptors subunits, NR2B was found increased by social defeat stress consistent with findings in literature (Yu et al. 2011). This increase was not normalized by the TAT-DEF-Elk1 treatment. It would be interesting to investigate cognitive functions in chronically stressed and treated mice.

The Elk-1 network in depression and antidepressant action

Elk-1 is a transcription factor that regulates expression of several clusters of genes. As previously discussed, immediate early genes (IEGs) expression such as *egr-1* (*zif268*) and *c-fos* are controlled by Elk-1. In a recent study of Boros et al. (2009) Elk-1 target genes were analyzed using a repressive form of Elk-1, the Elk-En, in cells. Several clusters of genes were identified that were regulated by Elk-1 alone or with other transcription factors (i.e. SRF or other ETS-domain transcription factors). The cluster of *Egr-1* genes was down-regulated in presence of induction of Elk-En expression without stimulation or after stimulation with the early growth factor (EGF) (MAPK dependent). The cluster of *FOS* genes on the other hand, was down-regulated after stimulation with EGF but induction of Elk-En without stimulation did not affect *fos* genes expression.

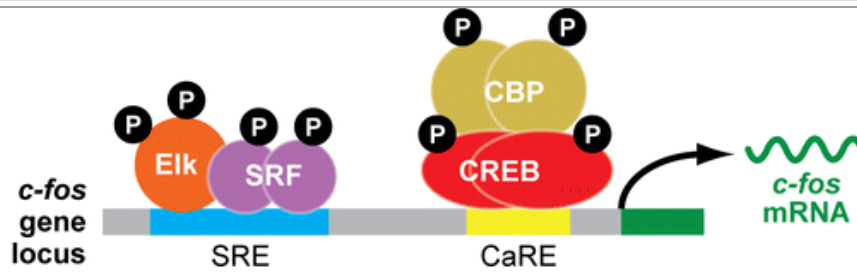
Consistently, our results showed that acute administration of the TAT-DEF-Elk1 peptide *in vivo* decreased basal levels of *egr-1* expression without altering *c-fos* basal expression in the

brain. Upon stimulation (acute stress), both genes were downregulated within a delay of two hours. Administration of the TAT-DEF-Elk1 peptide specifically blocked pElk1 without altering pERK or pMSK1 in prefrontal cortex and hippocampus. Expression of IEGs zif268 and c-fos was also measured 1 and 2h after stress. Stress increased expression of both genes in prefrontal cortex and hippocampus at both time points studied. In nucleus accumbens, zif268 was not affected 1h after stress. A summary of TAT-DEF-Elk1 peptide effects on stress-induced increase on pElk1 and IEGs expression is shown in the table below (n.s. = non-stressed, TST= tail suspension test).

Effects of the TAT-DEF-Elk1 peptide on stress-induced Elk1 signaling											
Protein		pElk1		Zif268				c-fos			
Time after stress		20'		60'		120'		60'		120'	
region	treatment	n.s.	TST	n.s.	TST	n.s.	TST	n.s.	TST	n.s.	TST
PFC	control	→	↑	→	→	→	↑	→	↑	→	↑
	peptide	↓	↓	↓	↓	↓	↓	→	↑	→	↓
NAc	control	→	↑	→	→	→	→	→	↑	→	↑
	peptide	→	↑	→	→	→	→	→	↑	→	↓
CA1	control	→	↑	→	↑	→	↑	→	↑	→	↑
	peptide	↓	↓	↓	↓	→	↓	→	↑	→	↓

From the above, inhibition of Elk1 activation prior to an acute stress blocks stress effects on Elk1 and its downstream targets, mainly zif268, 60 and 120min after stress. Stress increases on c-fos levels are present up to 2h later and prior administration of the TAT-DEF-Elk1 peptide does not affect this increase in a first place (60min) but completely abolishes expression 120min after stress. The expression of c-fos is regulated both from Elk1 and CREB transcription factors as shown in the figure below.

Regulation of the expression of c-fos gene from Elk1 and CREB



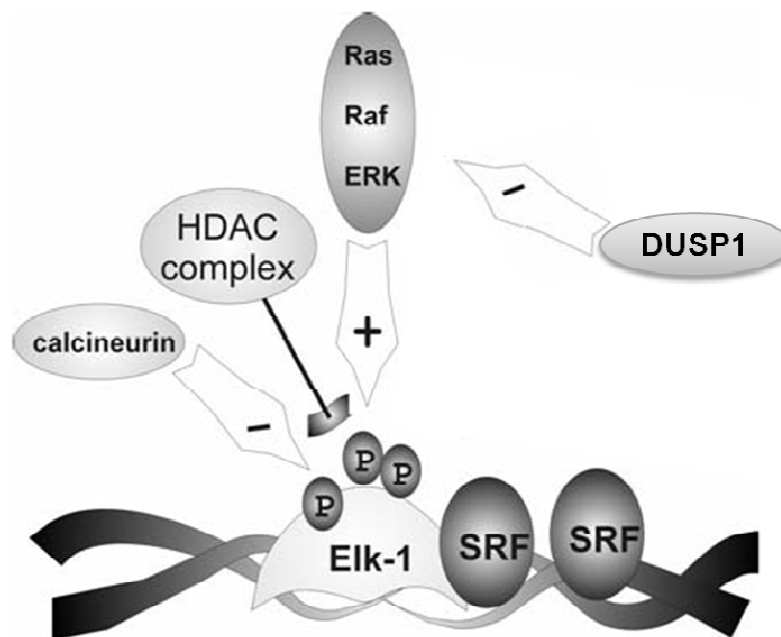
AR Flavell SW, Greenberg ME. 2008.
Annu. Rev. Neurosci. 31:563–90.

White and Sharrocks proposed recently that Elk-1 regulation on gene expression may achieve a second wave of gene expression in a temporally controlled manner, through rapid regulation of “immediate-early” induction of genes that themselves encode for another transcription factor (White and Sharrocks 2010). This shows that Elk-1 may affect gene expression in multiple ways and that the final output is a result of several parameters.

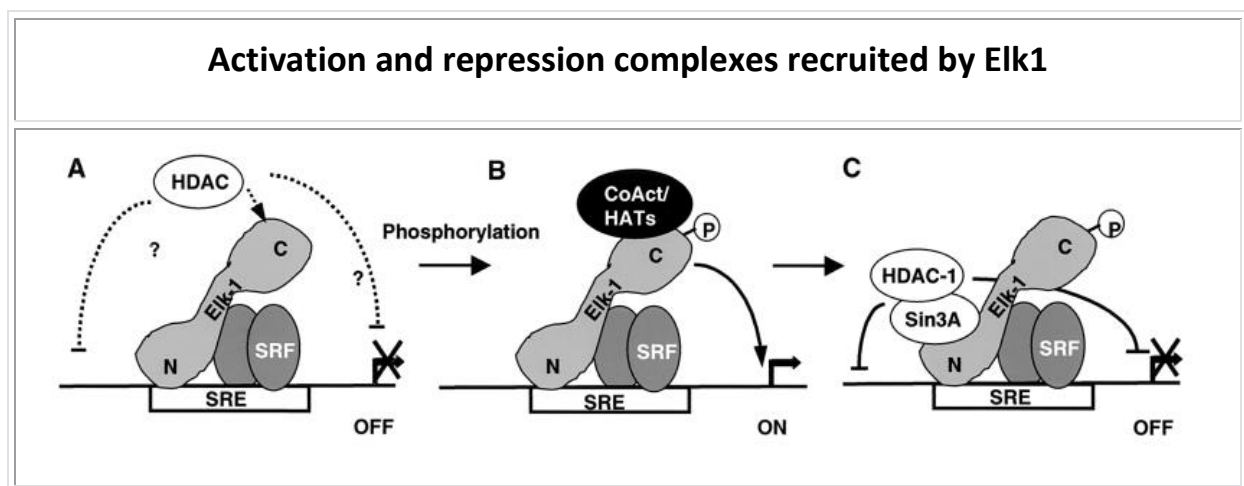
ERK cascade once activated is rapidly regulated by phosphatases as de-activators that act to prevent sustained phosphorylation and maintain phosphorylation at basal levels. The mitogen-activated phosphatase 1, MKP-1, also called dual-specificity phosphatase (DUSP1), is considered as an immediate early gene and is rapidly upregulated, among others, by GR receptors (Kassel et al. 2001). ERK phosphorylation basal levels are found decreased in depressed patients and in animals after a chronic stress. We classically correlate this to a depressive state. Is this decrease responsible for the behavior studied or does it mostly reflect a disturbance (pathological state) of a highly regulated system? And more importantly: does this modification mean that ERK is unable to respond “correctly” to stimuli? Logically, if pERK is downregulated, downstream targets like Elk-1 and MSK-1/CREB should also be downregulated. Decreased MSK-1/CREB signaling has been well characterized in depression and is believed to count for decreased BDNF expression, as CREB is a transcription factor for the promoter of BDNF gene. Conversely, inhibition of Elk1 phosphorylation from ERK showed antidepressant effects in all animal models studied, validating our initial hypothesis that Elk-1 signaling is mostly prodepressant.

Importantly, social defeat stress significantly decreased calcineurin (protein phosphatase 2B, PP2B) levels in hippocampus of vehicle-treated defeated mice. Calcineurin has been shown to specifically dephosphorylate Elk1 at phosphoserine 383, a site whose phosphorylation by MAP kinases makes a critical contribution to the enhanced transcriptional activity of Elk1 (Sugimoto et al. 1997, Tian and Karin 1999). Stress-induced decreased amounts of calcineurin imply a high phosphorylation state of Elk1 or a prolonged activation of Elk1 after a stimulus. This might lead to a maladaptive cellular response occurring in a depressive-like state. Consistent recent findings in the literature revealed a reduction of calcineurin in rat hippocampus after chronic stress and demonstrated that calcineurin inhibition induces depressive-like behaviors (Zhu et al. 2011). Moreover, chronic antidepressant treatment was previously found to increase calcineurin expression in the hippocampus (Crozatier et al. 2007). Convergenly, chronic inhibition of Elk1 phosphorylation from ERK using the TAT-DEF-Elk1 prevents the depressive behavior induced by a chronic stress, as shown in this work.

Regulation of the ERK/Elk1 cascade by phosphatases



Interesting findings of Sharrocks team suggest that Elk-1 undergoes temporal activator-repressor switching and contributes to both the activation and repression of target genes following activation of the ERK pathway *in vivo* (Yang et al. 2001). They proposed that after activation from ERK, both co-activator and the histone deacetylase (mSin3A-HDAC) co-repressor complexes are recruited, with co-repressor recruitment incurring a temporal delay. Removal of the co-activators can lead to a shutdown of the promoter by the co-repressor complex. Thus, Elk-1 plays a pivotal role in both the upregulation of immediate-early gene transcription and their subsequent rapid shutdown, controlling the precise “amount” of gene expression. The equilibrium between activation and repression via Elk-1 is altered following activation of the ERK1/2 signaling cascade (Yang et al. 2001). We propose that sustained activation of ERK by repeated stressors might disrupt this equilibrium, mediating persistent changes of chronic stress.

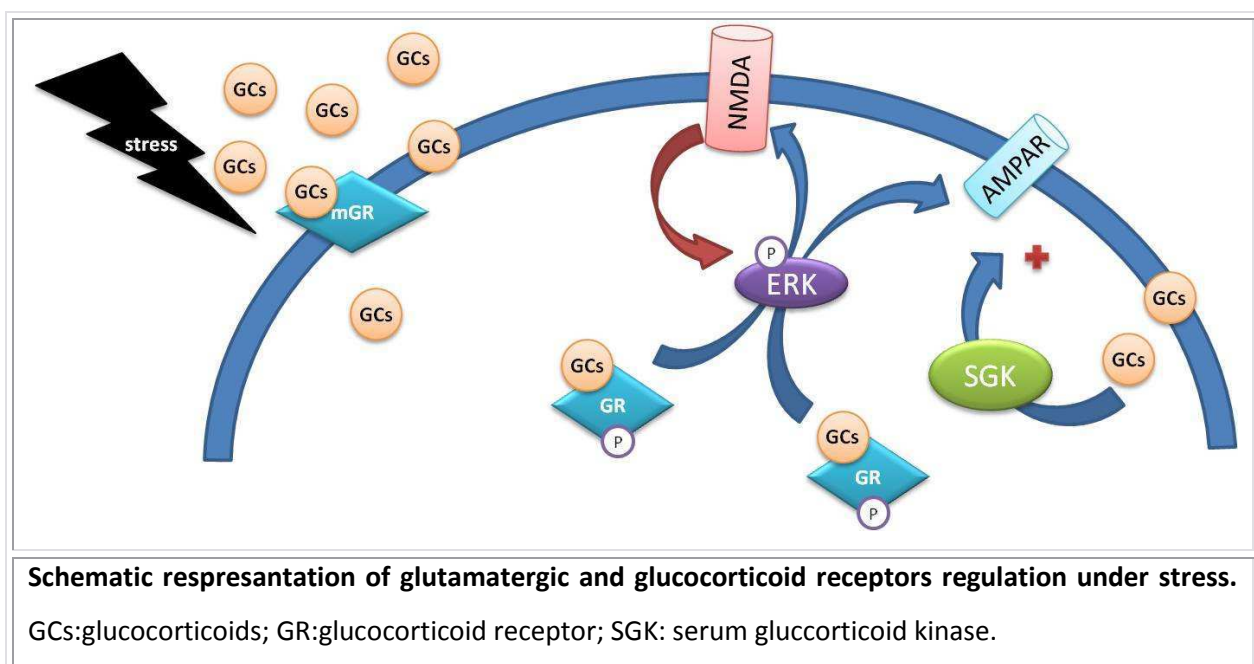


TAT-DEF-Elk1 peptide binds on activated ERK decreasing the available amount of pERK for the activation of Elk1. Acute administration of the TAT-DEF-Elk1 peptide showed antidepressant effects in low doses but showed no effect in higher doses. This indicates that optimal levels of pElk1 are necessary for antidepressant action/adaptive response. Chronic administration of TAT-DEF-Elk1 peptide under stress conditions presumably stabilizes levels of activated Elk1 from overactivated ERK, thus blocking a part of signal integration that leads to pathology.

PERSPECTIVES

Elk1: Linking glucocorticoid receptors (GR) and glutamate signaling in response to stress?

Few minutes after a stressor, plasma corticosterone levels arise and both via fast and slow action can persistently affect neuronal function. Electrophysiology studies have shown that corticosteroids affect AMPA and NMDA dependent changes in synaptic plasticity. Via glucocorticoid receptors (GR) these hormones slowly increase synaptic incorporation, lateral diffusion and mobility of GluR2 containing AMPARs (Groc et al. 2008, Martin et al. 2009) along with slowly increasing AMPA receptor synaptic transmission (Karst and Joels 2005, Martin et al. 2009), as previously discussed. ERK/MAPK pathway has been shown to mediate AMPA receptors delivery and removal during plasticity changes. Activation of Elk1 from ERK has been first demonstrated upon NMDA activation (Xia et al. 1996). In this work, we demonstrated a prominent role of Elk1 in AMPA/NMDA movement during stress. Hypothetically, Elk1 mediates GR-glutamatergic interaction during stress. From data in the literature, we know that GR modulates receptors movement via ERK phosphorylation (Stornetta and Zhu 2011) or serum glucocorticoids kinase SGK (Liu et al. 2010). Both kinases have been shown to activate Elk1.



We content that at the cellular level the ERK cascade might be positioned at the interface of regulatory processes that mediate cross-talk between NMDA-glutamate and glucocorticoid signalling.

It should be emphasized here that NMDA activation increases ERK/Elk-1 phosphorylation and activation (Vanhoutte et al. 1999); phosphorylated Elk-1 in turn increases zif268 expression and zif268-dependent transmission of genes, GR being one of those.

Recent studies revealed a direct link between GR activity and behavioral effects of glucocorticoids and induction of the expression of the immediate early gene zif268, also called Egr-1. Revest et al. (2005) showed that GR mediated activation of the ERK1/2 signaling pathway that culminates in Egr-1 expression is important in stress-related memories and critical for the glucocorticoid-induced enhancement of the fear conditioning. Few years later, Sarrazin et al. (2009) created a conditional transgenic mouse line that expresses a nuclear constitutively active form of the GR (Δ GR) prevalently in glutamatergic neurons of the dentate gyrus (DG) of the hippocampus. Δ GR lacks the hormone-binding and AF2 transcriptional activation domains and is able to activate GR-mediated transcription in the absence of glucocorticoids. DG-*Eno2*- Δ GR/EGFP bigenic mice displayed enhanced stress-related behaviors and in particular higher anxiety- and depression-related behaviors in response to unavoidable aversive situations. The behavioral phenotype in DG-*Eno2*- Δ GR/EGFP was associated with an up-regulation of the MAPK cascade and the downstream-regulated Egr-1 protein. As discussed earlier, egr-1 expression is mainly regulated by the transcription factor Elk-1.

Furthermore, recent studies have demonstrated an antidepressant-like effect of mifepristone in rats in the forced swim test (Wulsin et al. 2010), while GR antagonism seems to block activation of the ERK cascade in this model (Gutiérrez-Mecinas et al. 2011).

Throughout this thesis we have used a TST challenge as an acute stressor. We have shown that it precipitates depressive-like behaviors and cognitive impairment, in a temporal window that is coincident with ERK/Elk1 phosphorylation and activation.

Is Elk1 activation in our model GR dependent?

To address this issue we assessed the effect of a GR antagonist, mifepristone (RU486) on ERK, MSK-1 and Elk1 activation after an acute stress (10min TST) in hippocampus and prefrontal cortex. Phosphorylation of ERK, Elk1 and MSK-1 was measured by fluorescent immunohistochemistry on adjacent sections of each structure of non-stressed vehicle treated mice or stressed RU486 treated mice. Activation of the ERK cascade (phosphorylation of ERK, Elk1 and MSK-1) was found upregulated by stress in prefrontal cortex and hippocampus, consistent with previously findings. GR antagonism significantly blocked pERK and pElk1 in prefrontal cortex and pElk1 in CA1 area of hippocampus as shown in Figure 1.

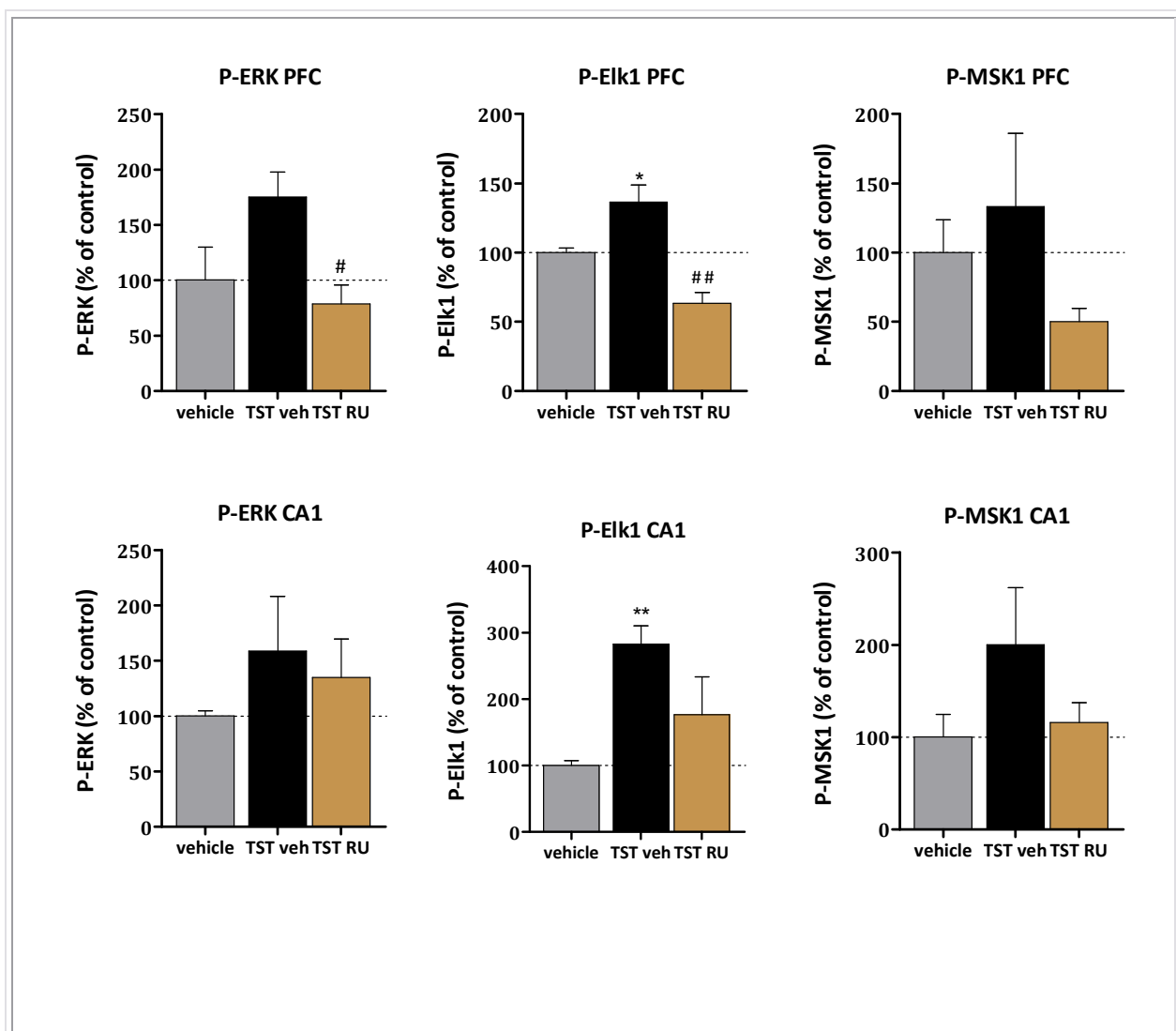
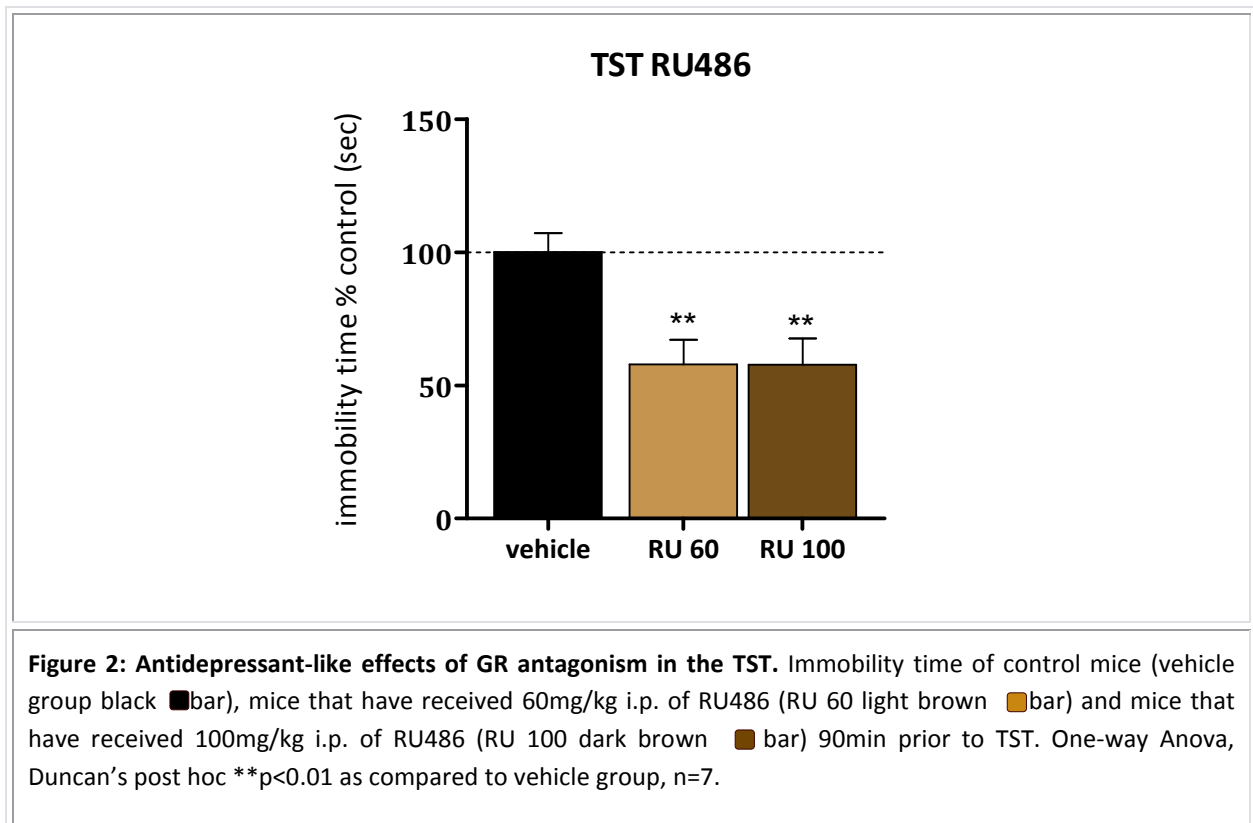


Figure 1: GR interferes with pERK - Elk-1 - MSK1 activation in prefrontal cortex and hippocampus after an acute stress. Phosphorylation of ERK, Elk1 and MSK-1 in prefrontal cortex and hippocampus after a 10min TST in animals treated with RU486 60mg/kg or vehicle 90min prior to stress. One-way ANOVA, Duncan's post hoc * $p < 0.05$ and ** $p < 0.01$ as compared to the vehicle, # $p < 0.05$ and ## $p < 0.01$ as compared to TST vehicle, $n = 4$.

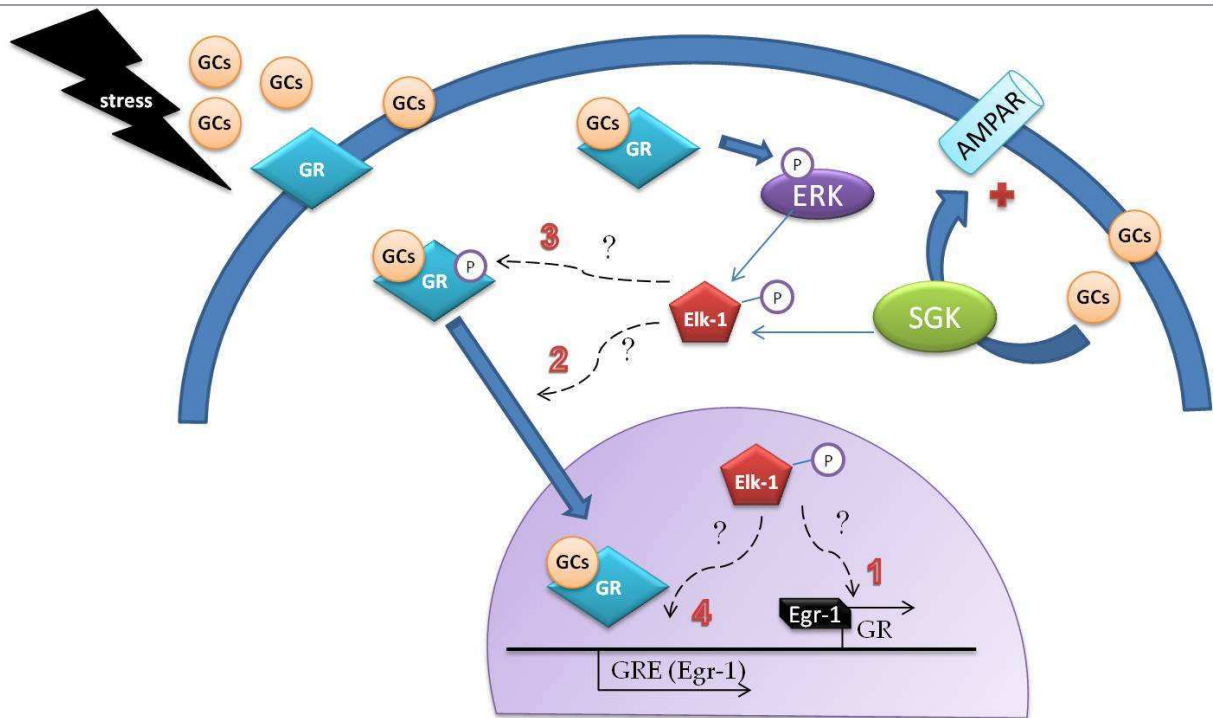
This indicates that activation of the ERK cascade after an acute stressor is GR-mediated and correlates with decreased immobility time in TST (Figure 2).



Consistent with a possible role of Elk1 at the interface between GR and glutamate signaling in the response to stress, findings in this thesis showed that acute selective inhibition of ERK/Elk1 signaling modifies response to stress. These modifications include inhibition of GR-mediated AMPA and NMDA trafficking, decreased expression of BDNF and GluR1 in hippocampus and increased expression of immediate early genes as a response to stress and correlate with behavioral output. From the data discussed so far, Elk1 seems to be implicated in the GR-mediated gene regulation as a response to stress.

This gives rise to the question: in which step of glucocorticoid signaling does Elk1 interfere?

Possible mechanisms of Elk1 implication in glucocorticoid signaling



Schematic representation of Elk1 implication in GR signaling. 1. Regulation of GR mRNA expression through Egr-1 (downstream target of Elk1); 2. Regulation of GR translocation; 3. Regulation of GR phosphorylation; 4. Co-regulator or co-repressor with GR in the transcription of GRE-containing genes. GCs:glucocorticoids; GR:glucocorticoid receptor; SGK: serum glucocorticoid kinase.

To answer this question, we conducted some preliminary studies examining the different phases of GR signaling and whether TAT-DEF-Elk1 peptide mediates these procedures under stress.

GRs lie in the cytoplasm in an inactivated form and upon activation (ligand binding) they translocate to the nucleus where they both upregulate or downregulate genes expression. We saw that the ERK cascade is activated by GRs during a stress.

We first assessed GR mRNA expression under acute stress in hippocampus (Figure 3). Acute stress did not significantly alter GR mRNA levels in hippocampus. The TAT-DEF-Elk1 peptide slightly decreased GR mRNA expression, but this effect is not statistically significant.

Subsequently we measured GR mRNA in hippocampus of chronically stressed mice (results part IIc). Chronic mild stress significantly decreased GR mRNA in vehicle-treated animals, however chronic administration of the TAT-DEF-Elk1 peptide did not inverse this effect, although it effectively reduced chronic-stress precipitated depression-like behaviors. Taken together, these data show that Elk1 is not mediating the regulation of the GR gene in response to stress.

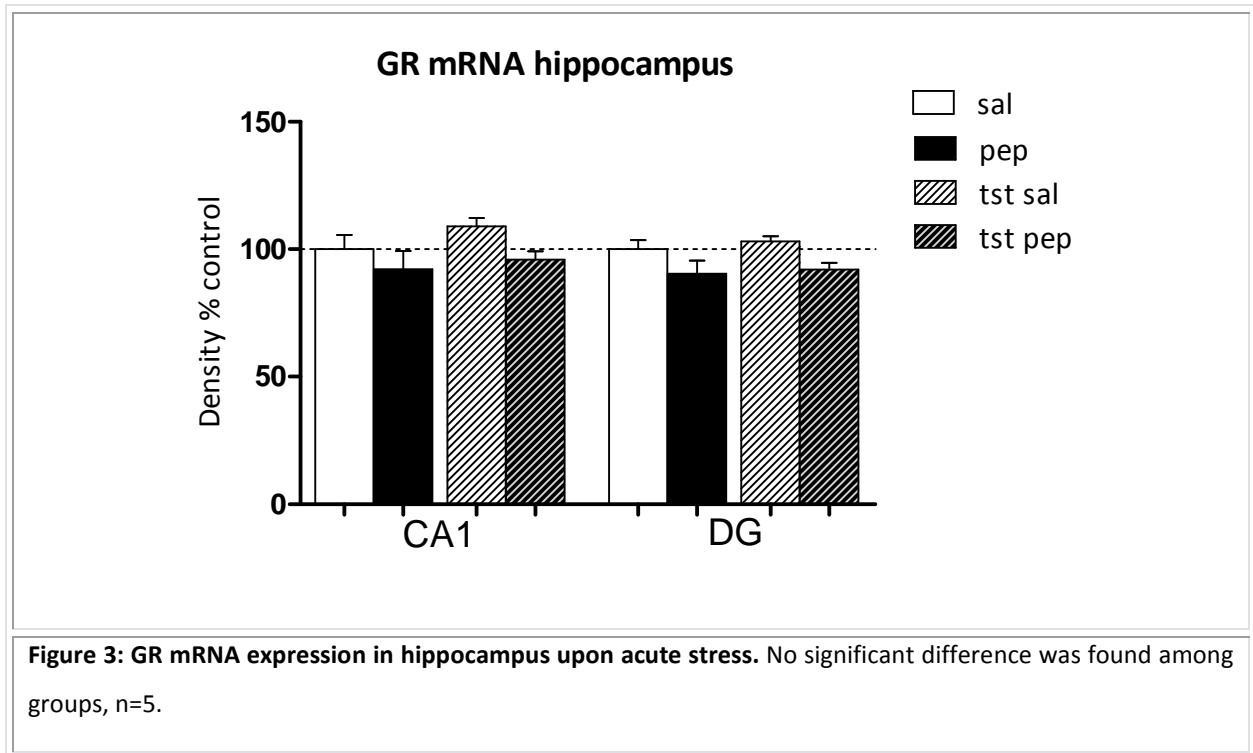
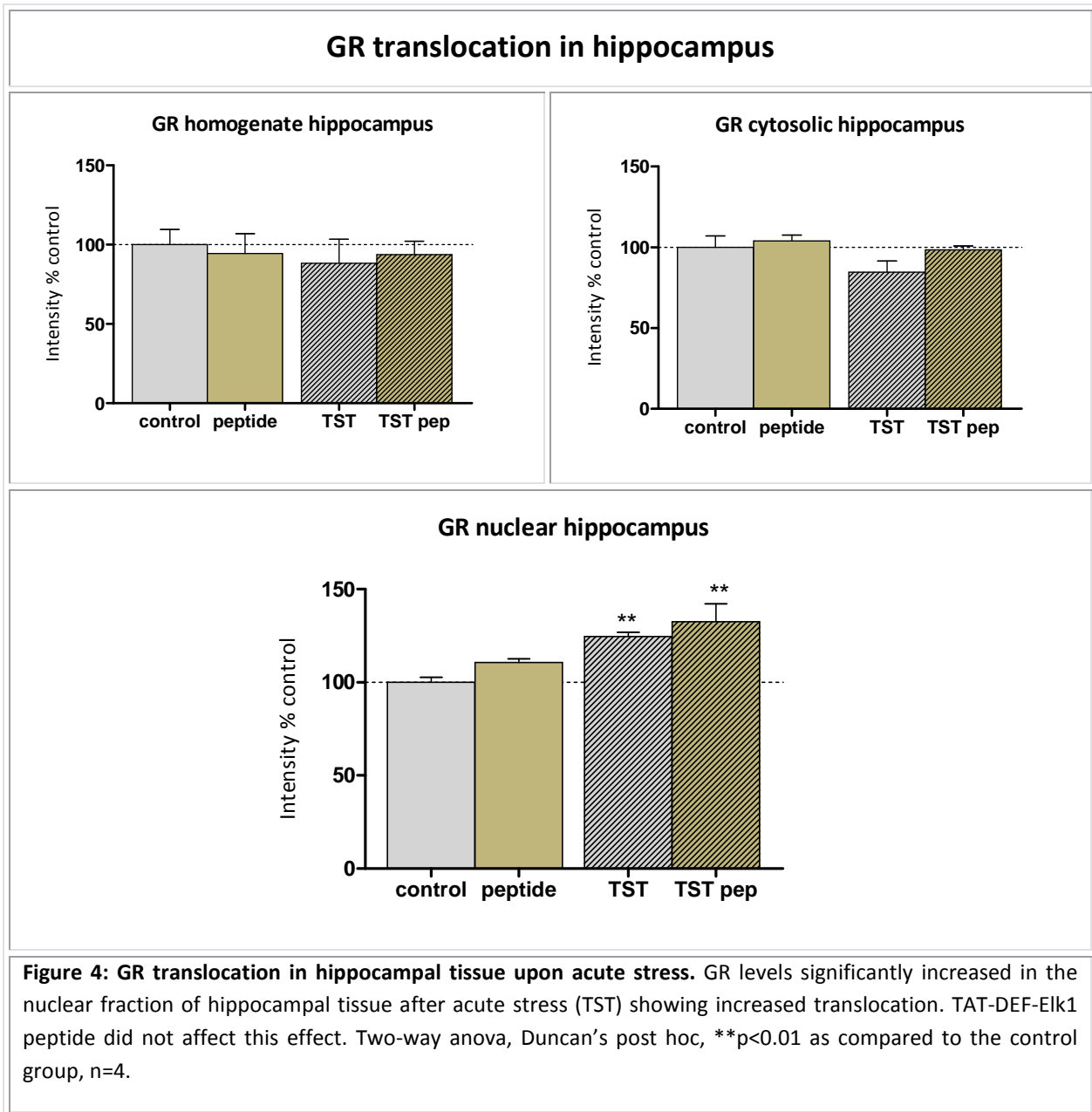


Figure 3: GR mRNA expression in hippocampus upon acute stress. No significant difference was found among groups, n=5.

Our second question was if ERK cascade, in particular Elk1, is implicated in the regulation of GR translocation from the cytoplasm to the nucleus. We studied GR translocation 30min after an acute stress (10min TST) and the effects of a prior administration of the TAT-DEF-Elk1 peptide (2mg/kg, i.p. 90min before stress). GR amounts were quantified in cytoplasmic and nuclear fractions of hippocampal tissue homogenate by Western blotting. Stress significantly increased GR translocation to the nucleus but this was not affected by the TAT-DEF-Elk1 peptide (Figure 4).



These preliminary data showed that inhibition of Elk1 phosphorylation during an acute stress does not affect GR translocation.

Although some questions answered, there are still important points left to elucidate. Is Elk1 important for GR phosphorylation? Once translocated to the nucleus, GRs act as transcription factors that regulate transcription of genes containing a glucocorticoid response element (GRE) in their promoter. Is Elk1 implicated, as co-activator or co-repressor, in GRE-mediated gene expression? And finally, is Elk1 necessary for GR effects on AMPA/NMDA synaptic movement during stress?

CONCLUSIONS

In this thesis animal models of depression and antidepressant action have been thoroughly studied. Plasticity based targets showed antidepressant action in acute and chronic models, early onset of antidepressant action and concomitant BDNF increase in hippocampus. Glutamatergic system, in particular through AMPA receptors, plays a major role in the pathophysiology of depression and regulates motivational aspects. AMPA receptors may constitute important pharmacological compounds for some subtypes of depression and/or adjunct for conventional antidepressant treatment for acceleration of onset of therapeutic action. Beyond synapse and membrane signals, attention is drawn to intracellular signal transduction pathways that directly regulate gene expression as a response to extracellular messages. ERK/MAPK cascade has been implicated mainly in BDNF signaling and in BDNF gene regulation via CREB. BDNF has a prominent role in pathophysiology of depression as well as in therapeutic activity.

Antidepressant treatments initially targeted membrane and/or extracellular components resulting in a synaptic increase of monoamines. Since the discovery of implication of intracellular signal transduction pathways in the antidepressant action (rolipram, DARPP-32) and modulation of receptors activity via cytoplasmic modules, antidepressant activity was directly linked to gene regulation and plasticity changes. Today, we can go one step further, targeting directly transcription factors and thus gene expression into the nucleus.

Regarding our understanding of the fundamental mechanisms of stress and mood regulation this work reveals a prominent role of the ternary complex factor Elk1. Many questions arise and further studies are necessary to completely elucidate Elk1 network in the pathophysiology of depression.

From a therapeutic strategy perspective, this work provides unequivocal experimental evidence on the antidepressant actions of the TAT-DEF-Elk1 peptide. Using this peptide technology to analyze and validate the Elk-1 pathway as a drugable target will potentially lead to novel therapeutic opportunities.

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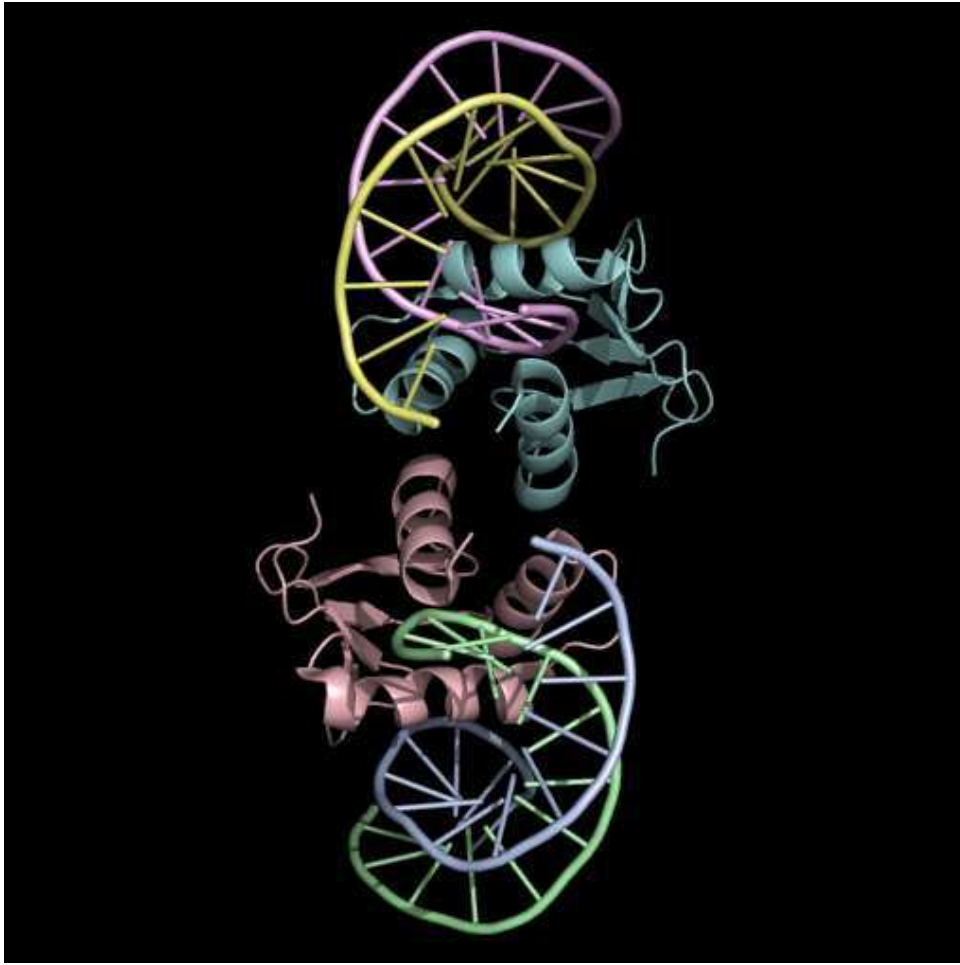
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Elk 1



Plasticity based targets for the treatment of depression

Abstract

Major depression is a devastating disease that affects up to 20% of world population and is classified today as a leading cause of disability-adjusted life years. Since late 50s with the serendipitous discovery of the first antidepressant agents, pathophysiology and therapeutics of depression are governed by the monoaminergic hypothesis. Monoaminergic-based treatment, although still in use today, was proven inefficient to treat a significant proportion of cases and presents a delayed onset of action.

Recent research has unveiled an array of new mechanisms through which antidepressant medication helps restore neuronal plasticity and neurotransmission that is disrupted in mood disorders and in animal models of depression. Glutamatergic transmission, in particular AMPA receptor, and signal transduction cascades have been implicated both in antidepressant action and the pathophysiology of depression, as here and now regulators that mediate persistent changes.

In this study, AMPA receptors positive modulators demonstrated antidepressant-like effects in a chronic model of depression and preliminary data suggest a faster onset of action than conventional antidepressants. The ERK/MAPK signaling pathway was also studied as a major integrator of synaptic plasticity modifications that links extracellular signals to gene expression regulation via its downstream molecular partners. We used a new class of inhibitors of the ERK pathway, whose design was based on the particular property of ERK to bind to its downstream targets via specific docking domains. A considerable amount of data provided evidence for an antidepressant action of selective inhibition of the ERK/Elk1 signaling complex in multiple animal models of depression.

Overall, the findings of this work reveal novel, promising targets for the treatment of depression.

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