Dissertation zur Erlangung des Doktorgrades
der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

Rapid neuronal signaling cascades initiated by corticosterone

Yang, Silei

of Tieling, P. R. China

September, 2011

Erstgutachter:	Prof. Dr. Rainer Landgraf
Zweitgutachter:	Prof. Dr. Peter Geigenberger
Tag der mündlichen Prüfung:	25-09-2012

献给我挚爱的父母:杨波,刘建锐

To my beloved parents: Bo Yang, Jianrui Liu

克己而刚。

- 弗里德里希·威廉·尼采

That which does not kill us makes us stronger.

- Friedrich Wilhelm Nietzsche

TABLE OF CONTENTS

ABSTRACT

Besides inducing transcription by activating nuclear receptors, corticosterone (CORT) acts rapidly to alter cellular activity through multiple signaling cascades. Pharmacology-based experiments presented here show that nanomolar doses of CORT activate several pathways in primary hippocampal cultures within 20 minutes, a time-frame that excludes genomic mechanisms mediated by classical glucocorticoid (GR) and mineralocorticoid (MR) nuclear receptors. Moreover, none of these effects were subject to inhibition by either a series of structurally-unrelated antagonists of the classical GR and MR or by inhibitors of translation. Accordingly, a major aim of this work was to identify mechanisms proximal to the cell membrane that could potentially mediate these rapid actions of CORT.

Initial time-course studies showed that 10 nM CORT rapidly triggers protein tyrosine kinase (PTK) activation that can be blocked by the small molecule inhibitor, PP2. It was also found that Src kinase is rapidly activated by CORT through tyrosine phosphorylation at Y416 and dephosphorylation at Y527. These events were subject to regulation by another key PTK member, Pyk2 (proline-rich tyrosine kinase 2). A series of experiments designed to study the potential mechanisms that regulate Pyk2, revealed that CORT increases the phosphorylation status of at least three tyrosine sites within Pyk2, namely, Y402, Y579/580 and Y881. Further, pharmacological probing uncovered requisite roles for other signaling pathways, such as the classical PLC-PKC pathway and the “novel” PKA and PKB pathways.

Interestingly, further investigations revealed that the rapid CORT-induced activation of Pyk2/Src occurs in a G-protein dependent manner. This, together with the observation that membrane-impermeable BSA-conjugated CORT (CORT-BSA) produces similar responses profile to that obtained with CORT, led to pilot experiments to probe whether GPR30, a recently-described G protein-

coupled receptor that appears to transduce signals from other steroid ligands, might be the putative receptor for CORT. Support for this view was provided by the finding that the rapid actions of CORT and CORT-BSA on Pyk2/Src activation could be blocked by a novel inhibitor of GPR30. Nevertheless, further studies will be needed to establish the role of GPR30 more firmly.

Other studies sought to identify downstream targets of Pyk2/Src. Results show that Pyk2/Src regulates activation of c-Abl (another PTK) and RhoA, both of which are regulators of a number of cellular processes, including actin cytoskeleton remodeling. Furthermore, it was found that CORT triggers phosphorylation of the NR2B subunit of NMDAR, increases surface expression of NMDAR and activates downstream MAP kinases; all of these events depend on Src, one of whose direct substrates is the NR2B subunit.

In a summary, the evidence presented in this dissertation suggests that, by acting via a G protein-coupled receptor, rather than through classical nuclear receptor mechanisms, CORT rapidly activates a series of intracellular signaling cascades that lie proximal to the neuronal plasma membrane; Pyk2/Src are early kinases involved and beyond these divergent pathways come into play, ultimately influencing functions ranging from rearrangements of the actin cytoskeleton, spine structure, scaffold protein clustering and function, to synaptic plasticity and transcriptional regulation.

1. INTRODUCTION

1.1 Corticosteroids

Corticosteroids are steroid hormones synthesized and secreted by the adrenal cortex (**Figure 1),** and important mediators of homeostatic balance which they facilitate by altering glucose mobilization, immune function, and ingestive and sexual behaviors, as well as cell (including neuronal) proliferation, differentiation and apoptosis (Chrousos and Gold, 1992; Sousa and Almeida, 2002; Riedemann et al., 2010). Corticosteroids are small lipophilic molecules which can easily pass the blood-brain barrier and reach deep brain tissues such as the hypothalamus and hippocampus; both of these areas, but especially the hippocampus, are rich in corticosteroid receptors. In this way, corticosteroids can contribute to the regulation of emotional and cognitive behaviors, as well as physiological processes controlled by the brain. An example of the latter is the regulated secretion of corticosteroids through direct actions on neurons in the hippocampus and hypothalamus, as will be discussed again later. All neuronal layers of the hippocampus (granule and pyramidal) are sensitive to corticosteroids; in the hypothalamus, neurons that secrete the neuropeptides corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal system and thus the anterior pituitary, are particularly sensitive to corticosteroids.

Corticosteroids refer to two distinct classes of adrenal steroids: glucocorticoids (GC) and mineralocorticoids (MC). The term glucocorticoid comes from the role of these hormones in the regulation of glucose metabolism and the site of their synthesis (adrenal cortex). In most mammals, including humans, the predominant GC is cortisol. In other species (fish, reptiles, amphibians, birds and rodents), the main GC is corticosterone (CORT). GC are best known as “stress hormones” because their secretion is increased following the arrival of stressful (psychological or physical) stimuli. Mineralocorticoids get their name from the fact

that they are important for maintaining physiological levels of salt and water, and aldosterone is the most important endogenous MC in all species.

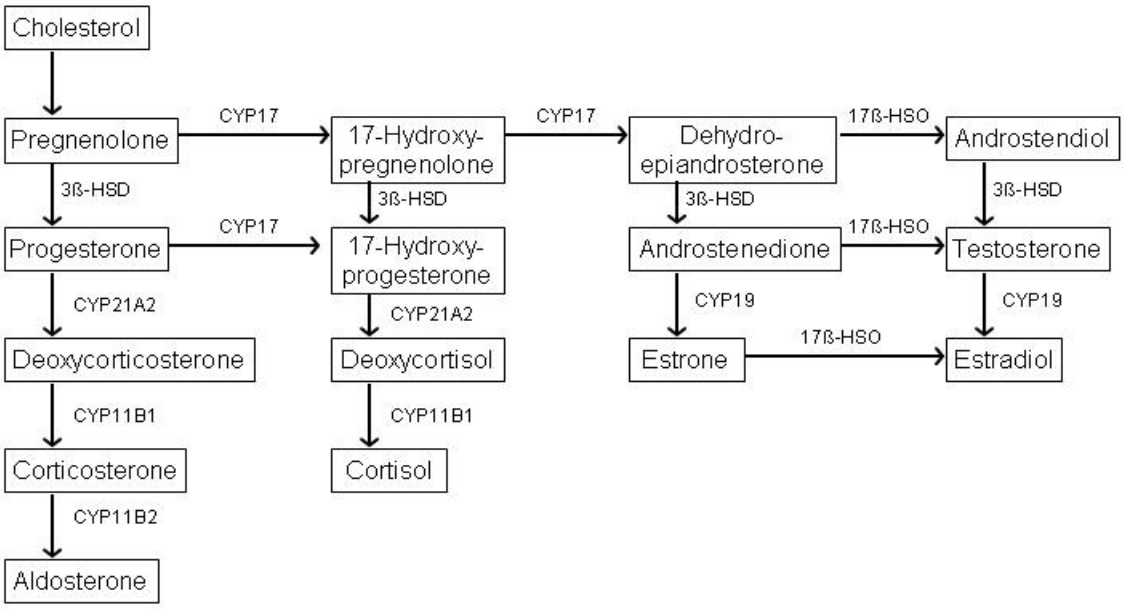


Fig. 1 Schematic representation of steroid biosynthetic pathways. Corticosteroids are synthesized from a common precursor hormone pregnenolone, which is produced from cholesterol by the cholesterol side chain cleavage. Corticosteroids include mineralocorticoids, glucocorticoids and androgens. 17β-HSO = 17β-hydroxysteroid oxidoreductase, 3β-HSD = 3β-hydroxysteroid dehydrogenase.

Like most other hormones, GC are secreted according to a robust circadian rhythm (in rodents: GC are high during the daily dark period of the light-dark cycle; in humans and other diurnally-active species, this pattern is reversed) under normal physiological conditions. (Reul et al. 1987; Young et al., 2004; Dickmeis et al., 2007). In the rodent, CORT is released from the adrenal cortex in a pulsatile manner, with each pulse lasting approximately 60 minutes; these regular pulses give rise to so-called ultradian rhythms upon which the circadian rhythm is superimposed (Jasper and Engeland 1991, 1994; Lightman et al., 2008). **Figure 2** illustrates this point but it should be noted that these rhythms may be altered according to physiological demands as well as physical or psychological stress stimulation. In addition, GC secretion under both basal and stressful conditions are modulated by other factors such as age and gender (van Cauter et al., 1996). Interestingly, recent studies have shown ultradian and

circadian rhythms of CORT in the rat brain, using microdialysis (Droste et al., 2008, 2009), suggesting that the activity of corticosteroid receptors in the brain changes in a rhythmic fashion.

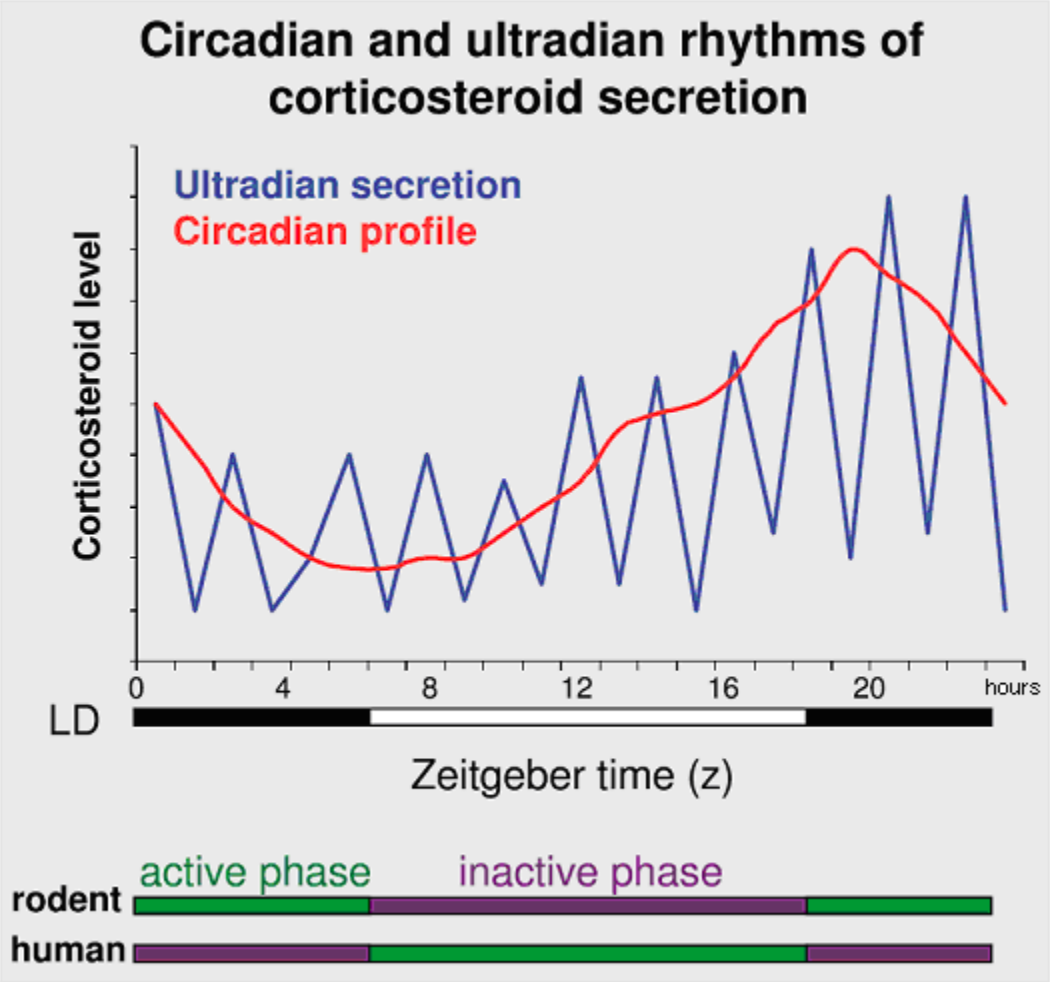


FIG.2 Corticosteroids are secreted rhythmically, displaying ultradian and circadian patterns. The circadian peak coincides with the onset of the daily activity cycle (dark phase in rodents, light phase in humans). While the physiological and behavioral significance of the ultradian rhythms of corticosteroid secretion is still unclear, it is plausible that they serve to dynamically fine-tune the regulation of the hypothalamo-pituitary-adrenal (HPA) axis and thus, to facilitate adaptive processes. LD: light-dark cycle. (from Riedemann et al. 2010)

The top-down control of GC secretion is illustrated in **Figure 3** where the hypothalamo-pituitary-adrenal (HPA) axis is central. Several brain regions are responsible for the positive regulation of adrenal function, but the hypothalamus, which communicates with the anterior pituitary (which contains adrenocorticotrophic hormone (ACTH)-secreting cells). The central drive on the

HPA axis is kept in check through negative feedback loop wherein GC act, in fast and slow modes, to inhibit the positive drive from the hypothalamic paraventricular nucleus (PVN, site of CRH and AVP neurons). Brain areas other than the hypothalamus that are important for this negative feedback include the prefrontal cortex and hippocampus. As already mentioned, maintenance of the negative feedback loop is important to regulate neuronal cell birth, differentiation and apoptosis, dendritic arborization and synaptic function, and thus, learning and memory as well as mood/emotional states (Sousa and Almeida, 2002); thus, the regulation of GC is considered highly relevant to a range of neuropsychiatric conditions, including anxiety and depression and mild and severe dementia (Sotiropoulos et al., 2008).

1.2. Nuclear receptors mediate slow and long-lasting effects of corticosteroids

Transcription-dependent actions of GC that persist (hours to years) have been observed at each of the GC feedback target sites of the HPA axis; for example, they regulate the expression of ACTH by the at pituitary (Birnberg et al., 1983), CRH and AVP in the neurons of the hypothalamus (Sawchenko, 1987), as well as GABAergic inhibitory synaptic inputs to CRH neurons of the PVN (Miklós and Kovács, 2002). Also, chronic increases of GC have been shown to induce long-term changes in the excitatory synaptic response properties of hippocampal CA1 neurons, suggesting their role in enhancing glutamate excitation (Karst and Joëls, 2005).

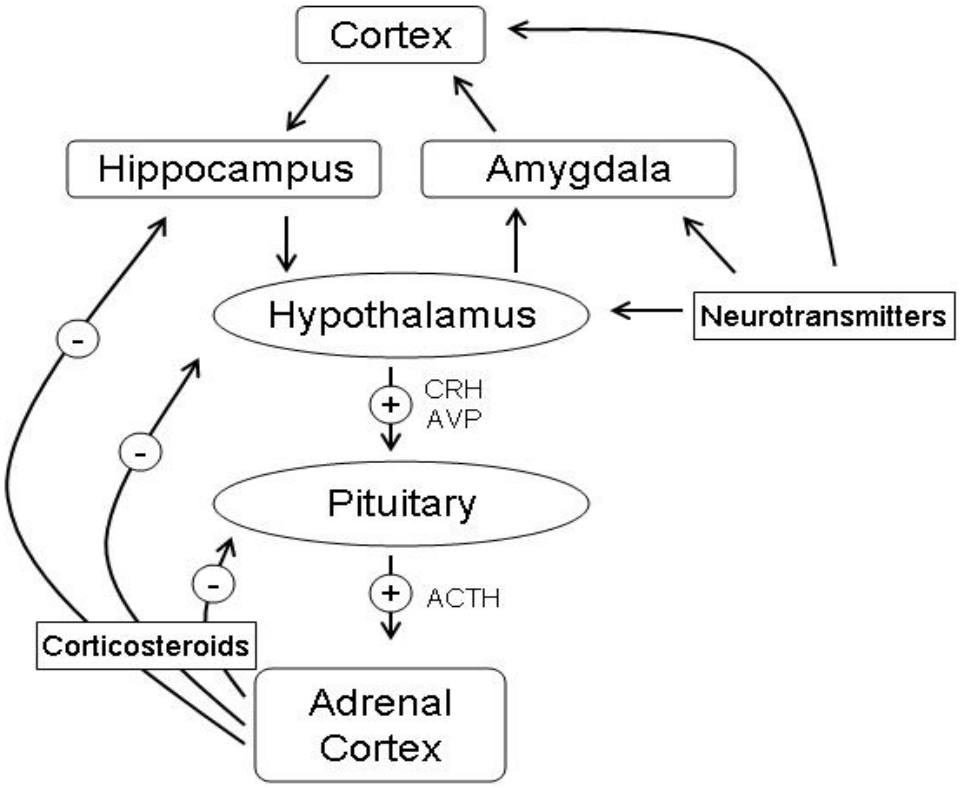


FIG.3 The hypothalamic-pituitary-adrenal (HPA) axis is a feedback loop that includes the hypothalamus, the pituitary and the adrenal glands. The main hormones that activate the HPA axis are corticotropin-releasing hormone (CRH), arginine vasopressin (AVP) and adrenocorticotropin hormone (ACTH). The loop is completed by the negative feedback of corticosteroids on the hypothalamus and pituitary. The simultaneous release of corticosteroids into the circulation has a number of effects, including elevation of blood glucose for increased metabolic demand. Corticosteroids also negatively affect the immune system and prevent the release of neurotransmitters. Interference from other brain regions (e.g. hippocampus and amygdala) can also modify the HPA axis, as can neuropeptides and neurotransmitters.

The best known biological effects of GC depend on gene transcription. When GC reach cells, they bind to corticosteroid receptors. These receptors belong to the superfamily of nuclear receptors. All nuclear receptors are ligand-activated transcription factors. Upon binding ligand (e.g. GC), they translocate from the cytoplasm to the nucleus and regulate gene transcription after binding to the promoter regions of their target genes. There are two types of corticosteroid receptors: glucocorticoid (GR) and mineralocorticoid (MR) receptors. Corticosteroids bind to GR with 10-fold lower affinity compared to MR (Reul and de Kloet, 1985). Therefore, GR only become activated when GC levels are high

(e.g. during stress) whereas MR are activated by low amounts of GC (e.g. under basal conditions). GR also become activated during the daily circadian peak in corticosterone secretion (Young et al., 2004). The biological effects mediated by GR and MR usually first become manifest after a few hours and all of them depend on alterations in protein synthesis. It should be noted that, depending on the HPA activity, CORT leads to opposite directions in the cells which express both GR and MR, since the activated respective receptors modulate transcription of different but overlapping CORT-targets genes via transactivation or transrepression (Datson et al., 2008).

Whereas the unliganded MR is mostly localized in the nucleus, unoccupied are found in the cytoplasm and translocate to the nucleus after ligand activation. This process depends on the dissociation of a host of chaperone and co-chaperone molecules such as heat shock protein 90 (HSP), as well as on the inclusion of a nuclear translocation signal in the receptor protein (Gronemeyer et al., 2004, Duma et al., 2006). Like other nuclear receptors, MR and GR are structured according to canonical modules, including a ligand-binding domain (LBD), a DNA-binding domain (DBD), and two activation functions (AF-1 and AF-2) at their N- and C-terminals, respectively. Interactions of the DBD with hormone-response elements (HRE) in the promoters of specific genes result in the induction or repression of gene transcription and subsequently, changes in the expression of proteins that influence cellular functions. Classically, the mechanisms of MR and GR in the neurons can be regulated in follow levels (**Figure 4**):

1. Corticosteroids are carried in the blood, bound to corticosteroid binding globulin (CBG); they penetrate into the brain. Endogenous corticosteroids are not excluded by the blood-brain-barrier by multiple drug resistance (MDR) p glycoprotein in the blood-brain barrier.
2. Upon entry into the cell and binding to GR, the GR dissociates from molecular chaperones (e.g. HSPs and FK506 binding proteins, FKBP), leading to conformation change of the receptor (Ratajczak et al. 2003), and eventually interactions with other transcription factors (TF) such as

nuclear factor-κB (NF- κB) and activator protein 1 (AP1) (De Bosscher et al., 2003), before entry into the nucleus.

3. Within the nucleus, MR and GR interact with co-regulators (co-repressors and co-activators) which then help induce, repress or transactivate gene expression (Privalsky, 2004; Rosenfeld et al., 2006).

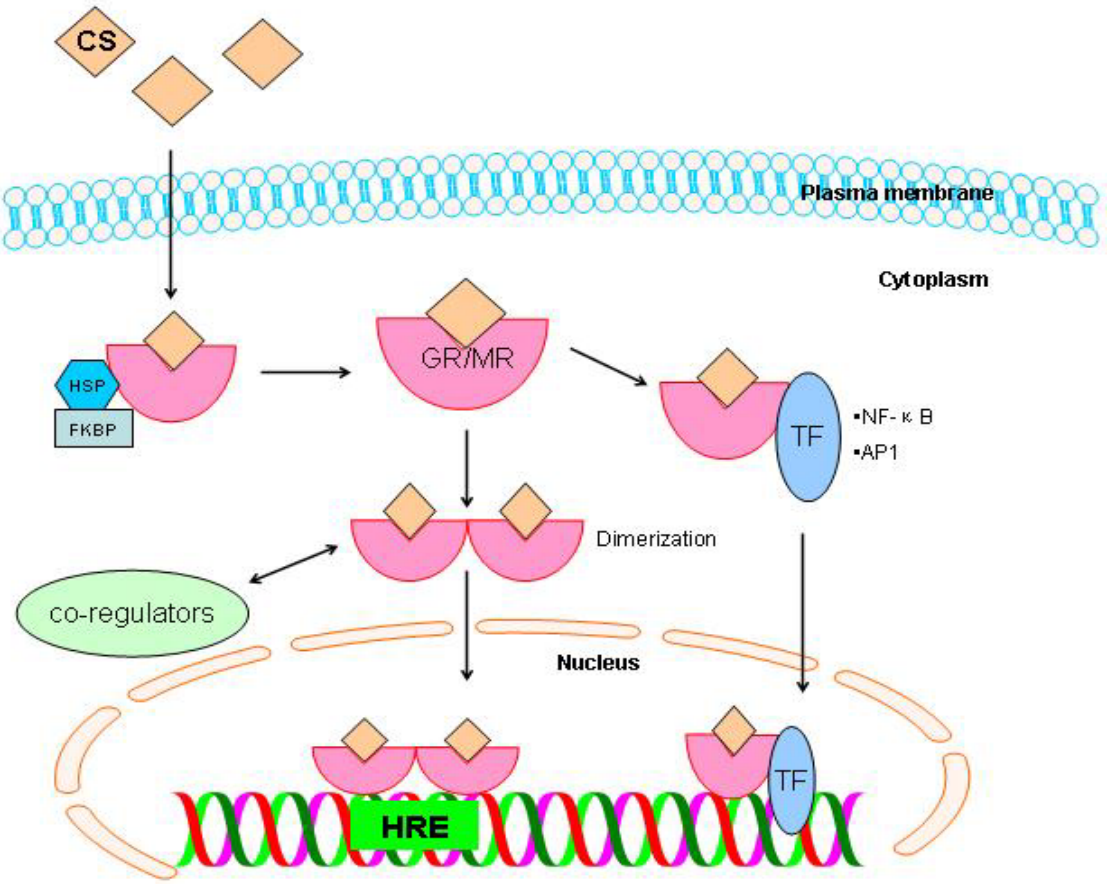


Fig. 4 Classical mechanisns of action of nuclear MR and GR. The MR or GR multimeric protein complex dissociates after binding corticosteroids. For the effects on gene expression, MR and GR functioning either as homodimers or heterodimers, interact at a hormone-response element (HRE) and recruit co-regulators, whereas GR monomers interact with stress-induced transcription factors to translocate into nucleus. CS: corticosteroids. HSP: heat shock protein. FKBP: FK506 binding protein. NF- κB: nuclear factor-κB. AP1: activator protein 1. HRE: hormone-response element. TF: transcription factors.

The MR and GR regulate the adaptive behavioral responses by altering the expression of related genes since both of them function as transcriptional regulators. Although the DBD of both MR and GR are highly conserved, the

genes to which they bind are not necessarily identical. Studies using serial analysis of gene expression (SAGE) in rat hippocampus, showed that CORT regulates the activation or repression of 203 responsive genes in a coordinated manner via MR and GR. 48% of these genes are responsive to MR, 35% are responsive to GR and only 16% are responsive to both receptors (Datson et al. 2001). These investigations suggest that both corticosteroids receptor mediate the expression of multiple genes whose function ranges from energy expenditure and cellular metabolism to protein synthesis and turnover, signal transduction and neuronal connectivity and synaptic transmission.

1.3. Corticosteroid actions are not solely mediated by nuclear receptors

Besides the genomic response, steroid hormones, including MC and GC have been recognized to exert rapid actions on cells from multiple organisms for long time. Such rapid actions have been well described for estrogens and, to some extent, progestins (Genazzani et al., 2000; Belcher and Zsarnovszky, 2001; Levin, 2008). The latter and their derivatives, for example, influence neuronal activity by interacting with an allosteric binding site on γ-aminobutyric acid type A ($GABA_A$) receptors located at the cell membrane (Frye and Walf, 2008). Estrogens have also been shown to act at mitochondrial and/or plasma membranes. These will be discussed in greater detail in the following section.

Interestingly, GC also exert acute effects and can act to rapidly to suppress HPA axis activation. For example, GC suppress CRH-induced ACTH secretion within minutes via a rapid and transcription-independent mechanism in the pituitary (Hinz and Hirschelmann, 2000). In the hypothalamus, GC have been reported to exert rapid inhibitory effects on spiking activity in PVN neurons (Chen et al., 1991), and to suppress stimulated AVP release, also via a transcription-independent mechanism (Liu et al., 1995). In the hippocampus, GC are reported to induce rapid, transcription-independent increases in glutamate release onto

CA1 pyramidal neurons, resulting in an increase of excitatory synaptic inputs, an effect that is thought to involve intracellular MC receptors (Karst et al., 2005).

GC have also been shown to rapidly enhance the functional expression of long-term potentiation (LTP) in the CA1 pyramidal cell layer; however, this can reportedly occur through mechanisms which are independent of the activation of both classical GC and MC receptors (Wiegert et al., 2006). Accompanying the emerging evidence described, the hippocampus, which contains the highest levels of receptors for corticosteroids, is becoming one of the best studied brain regions and a target for investigations of how fast adjustments in the activity of the HPA might occur. Since some neuropsychiatric diseases (e.g. anxiety, depression and Alzheimer's disease) are associated with impaired GC feedback actions (resulting in hypersecretion of GC, Sotiropoulos et al., 2008), more and more attention is being paid to the GC responses in the hippocampus. The importance of such studies in the hippocampus will be described in greater detail in **Section 1.5**.

1.4. Rapid actions of glucocorticoids

Steroids have been known for decades to trigger rapid responses (almost for as long as their nuclear receptor-mediated responses have been known). Corticosteroids, and especially GC, have been observed to exert a wide range of rapid functional effects on different cells and tissues (muscle, pancreas, heart, adipose tissue, the immune system and brain) as well as on behavioral responses in different vertebrate species. Some of the rapid, transcriptional-independent GC effects in tissues other than the central nervous system (CNS) are listed in **Table 1**; these studies indicated that the fast responses of GC in many organisms occur independently of, or simultaneous with, the transcriptional regulatory function of genomic GR. The use of membrane-impermeable GC preparations (e.g. bovine serum albumin conjugated to corticosterone, CORT-BSA) has played an important part in supporting the idea that GC can act at the cell membrane without penetration to access classical intracellular receptors.

Table 1. Examples of rapid glucocorticoids actions in non-neural tissues.

Tissue/System	Rapid effects	Reference
Trachea	Inhibit smooth muscle contraction via rapid actions that are not blocked by GR antagonist RU38486.	Sun et al., 2006
Pancreatic β-cells	Cause a rapid suppression of the stimulated release of insulin, which is opposite to the delayed increase in insulin levels caused by slow glucocorticoids actions.	Sutter-Dub, 2002
Heart	Induce endothelial nitric oxide release by stimulating nitric oxide synthase via PI3K and PKB pathway.	Hafezi-Moghadam et al., 2002
Adipose tissue	Stimulated fat cell production by facilitating the differentiation of preadipocyte precursors into adipocytes via a non-transcriptional suppression of a histone deacetylase complex.	Wiper-Bergeron et al., 2003
Immune system	The anti-inflammatory effects of glucocorticoids are partly independent of GR binding to DNA, indicating a non-transcriptional action.	Reichardt et al., 2001

In the CNS, GC have been found to exert effects on neuronal activity that can occur within seconds-to-minutes of exposure of cells to the steroids, whereas gene transcription and translation require at least 20-30 minutes or even longer for their effects to be apparent. **Table 2** shows some rapid GC-induced behavioral responses and physiological effects in different brain areas. These rapid effects of GC on brain function, often involving changes in structure, cannot be accounted for by transcriptional activation and are therefore referred to “non-genomic” effects (de Kloet et al. 2008; Riedemann et al. 2010).

Table 2. Rapid behavioral and physiological effects of glucocorticoids in the central nervous system. (Adapted from Riedemann et al. 2010)

Parameters	Rapid effects	Reference
Locomotion	Increased locomotion in novel environment which is independent on protein synthesis and mediated by nitric oxide.	Sandi et al. 1996a, 1996b
Aggressive behavior	Increased aggression in resident-intruder paradigm which is independent on protein synthesis	Mikics et al. 2004, 2007
Anxiety, risk-taking behavior	Increased risk assessment, but no anxiety-related behavior which is independent on protein synthesis	Mikics et al. 2005
Learning and memory	Enhanced taste conditioning	Kent et al. 2000
	Impaired retrieval contextual memory	Roozendaal et al. 2004
	Impaired memory in water maze task which is insensitive to transcriptional inhibition	Sajadi et al. 2006
	Impair memory retrieval via MR mediation which is independent on protein synthesis	Khaksari et al. 2007
	Impaired memory retrieval by acute stress which is mediated by putative membrane receptor	Chauveau et al. 2010
Neurochemical	Increased extracellular dopamine levels in nucleus accumbens via reward-motivational pathway	Piazza et al. 1996
	Increased NMDA-mediated firing of dopaminergic neurons in ventral tegmental area via reward-motivational pathway via GR	Cho and Little 1999
	Increased spike frequency in locus coeruleus neurons	Avanzino et al. 1987
	Inhibition of catecholamine uptake	Gründemann et al. 1998
	Increased p38 and JNK and ERK1/2 activation in PC12 cell line which is GR-independent	Qiu et al. 2001 Li et al. 2001
Neuroendocrine regulation	Triggers fast glucocorticoid negative feedback	Dallman et al. 1969
	Inhibits neuron firing rate in hypothalamic paraventricular nucleus	Kasai and Yamashita 1988
	Reduces excitatory glutamatergic input to PVN and increases endocannabinoid release via GPCR and retrograde endocannabinoid signaling	Di et al. 2003 Tasker et al. 2006
Electrophysiology	Suppress mEPSC in hypothalamus via GPCR	Di et al. 2003
	Increase mEPSC in hippocampus via membrane MR	Karst et al. 2005
	Suppress NMDA-dependent current via putative membrane receptors	Sato et al. 2004 Liu et al. 2007
	Increase NMDA-dependent neurotoxicity through the mediation of MAPK and membrane receptors	Xiao et al. 2010

Rapid GC actions have been shown to be mediated by multiple signal transduction pathways, such as guanosine triphosphate (GTP) -binding protein (G-protein) (Ffrench-Mullen, 1995; Qi et al., 2005; Hu et al., 2010), adenylyl cyclase (AC) (Kageyama et al., 2010), protein kinase C (PKC) and A (PKA) (Qiu et al., 2003a; Han et al., 2005; Qi et al., 2005), phosphatidylinositol-3-kinase (PI3K) (Hafezi-Moghadam et al., 2002), protein tyrosine kinase (PTK) and protein tyrosine phosphatases (PTP) (Nestler et al., 1989; Tian et al., 1998), nitric oxide synthase (NOS) (Sandi et al., 1996b; Hafezi-Moghadam et al. 2002), and mitogen-activated protein kinase (MAPK) (Qiu et al., 2001; Li et al., 2001; Qi et al., 2005; Xiao et al., 2005). These signaling pathways are located in the cytoplasm, some of them close to the plasma membrane; some are rapidly activated and eventually, many of their effects converge in the nucleus to regulate gene transcription and protein synthesis.

1.4.1. Rapid actions mediated by membrane receptors

G-protein-coupled receptors (GPCR) are the largest family of cell surface molecules involved in signal transduction; they mediate multiple sensory and chemical stimuli (such as odorants, light, neurotransmitters, hormones, ions), share a highly conserved backbone structure of a seven-transmembrane domain (Müller, 2000), and correspond to around 1-5% of protein content of the cell. Thus, GPCR are intensively investigated in pharmacology, especially in the CNS (Pierce et al., 2002). GPCR interact with G-proteins and activate downstream signaling cascades through well-studied mechanisms (Hur and Kim, 2002). Their binding with extracellular ligands causes conformational changes in their seven-transmembrane domains which, in turn, cause changes in the conformation of GPCR intracellular domains. These changes induce the specific association of the GPCR with distinct classes of heterotrimeric G-proteins composed of three subunits: the α-subunit that has a guanine-nucleotide binding site and GTPase activity, and the β- and γ-subunits that form a tightly-bound dimmer. Based on their amino acid similarities, G-protein α-subunits are further classified into four families: G_s, $G_{i/o}$, $G_{q/11}$, G_{12}. In general, stimulation of G_s activates AC and

voltage-sensitive calcium channels (VSCC), whereas stimulation of $G_{i/o}$ leads to inhibition. Stimulation of $G_{q/11}$ activates phospholipase C (PLC), and G_{12} is known to regulate small G-proteins and non-receptor tyrosine kinases.

Studies have shown that rapid GC effects are mediated, at least partly, by GPCR and their downstream intracellular signaling cascades in CNS. For example, GC were shown to trigger the effects of VSCC in hippocampal and dorsal root ganglion via a G-protein dependent mechanism (Ffrench-Mullen 1995; He et al., 2003). Further, GC inhibit ACTH release from AtT20 cells, a pituitary tumor-derived cell line, through a non-genomic mechanism that is blocked by pertussis toxin (PTX), a drug that specifically interferes with receptor coupling to most family members $G_{i/o}$ type G-proteins, indicating involvement of $G_{i/o}$ in mediation of the GC effects (Iwasaki et al. 1997). In addition, rapid GC effects have been shown to be dependent on activation of G_s in rat hypothalamic PVN neurons, suggesting that GC can positively regulate cyclic adenosine monophosphate (cAMP) production (Malcher-Lopes et al., 2006).

It is worth noting that the rapid and transcription-dependent effects of GC are very likely mediated not only by GPCR, but also by the classical intracellular corticosteroid receptors (GR and MR) or other putative receptors that are somehow linked with the plasma membrane. For example, one of the best studied steroid hormone receptors, the nuclear estrogen receptor (ER), or fragments thereof, appears to be present in the plasma membrane of multiple cell lines or tissues, including neuronal cells (Micevych and Dominguez, 2009). These immunoreactive ER are primarily associated with caveolae, invaginations of the plasma membrane (Razandi et al., 2002) composed of scaffolding proteins that associate with various signaling molecules, such as GPCR and Src, to facilitate rapid signal transduction (Cohen et al., 2004). Caveolin-1, a ubiquitously expressed caveolin, has been recently shown to regulate rapid signaling through binding of ER at the plasma membrane (Sud et al., 2010). Binding of caveolin-1 has also been suggested to be important for membrane translocation of other

steroid receptors (Lu et al., 2001; Pedram et al., 2007). In mouse hypothalamic neurons, nuclear ER have been reported to mediate rapid responses to estrogen in a G-protein dependent manner and via PKC/PKA signaling (Qiu et al., 2003b). On the other hand, some authors have suggested that the putative membrane ER-mediated rapid cell signaling may be a distinct GPCR that is intracellularly located on the endoplasmic reticulum membrane (Revankar et al., 2005). However, the question of whether the rapid estrogen effects are mediated by a separate GPCR or by classical intracellular (nuclear) receptors that directly associate with the plasma membrane remains open (Pedram et al., 2006).

The classical (nuclear) GR was reported, using electron and confocal microscopy, to be located on the membrane of rat neuronal cells where it was proposed to participate in signal transduction both at the cell membrane and nucleus (Liposits and Bohn, 1993; Johnson et al., 2005). However, questions still arise as to whether the rapid effects of GC could be mediated by classical intracellular receptors and about whether a nuclear receptor can indeed directly associate with elements of plasma membrane. At the same time, there are reports that the rapid GC-induced increase of glutamate miniature excitatory postsynaptic currents (mEPSCs) in hippocampal CA1 pyramidal neurons can be blocked by antagonist of intracellular MR, but not nuclear GR antagonists (e.g. RU38486 or mifepristone) (Karst et al., 2005). The same authors provided further evidence for mediation by the classical MR in that they failed to see GC modulation of mEPSCs in MR knock-out mice (Karst et al., 2005). Contrary to the suggested mediation of the rapid actions of GC by nuclear MR, other authors have proposed a role for classical GR. For example, while it was found that plasma membrane-impermeable CORT-BSA can enhance memory consolidation and impair working memory via a G-protein dependent mechanism, the actions of CORT-BSA were blocked by a GR, but not MR, antagonist (Barsegyan et al., 2010). Further support for GR involvement in the fast actions of GC comes from studies with the synthetic GR agonist dexamethasone (DEX): DEX was found to rapidly modulate the density and morphology of dendritic spines in CA1 pyramidal neurons in the

rat hippocampus, apparently mediated by postsynaptic GR in triton-insoluble synaptosomal fractions (Komatsuzaki et al., 2005).

Next, some of the possible membrane-proximal signaling molecules that could potentially mediate the fast actions of GC on neuronal function will be reviewed briefly as these will be considered in the Results section (**Chapter 4**) of this thesis.

1.4.2. Protein tyrosine kinase and phosphatase

Protein tyrosine kinases (PTK), including receptor tyrosine kinases (RTK) and non-receptor tyrosine kinases (NRTK), play an important role in the control of many fundamental cellular processes, such as cell cycle control, migration, metabolism and survival, as well as proliferation and differentiation, in multiple tissues and organs, including the CNS (Hubbard and Till, 2000).

RTK are a large family of transmembrane receptors that possess intrinsic tyrosine kinase activity. All of them contain an extracellular ligand-binding domain which is connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic domain contains a conserved PTK sequence and additional regulatory sequences that are themselves subject to auto-phosphorylation and that can be phosphorylated by other protein kinases (Hubbard et al., 1998). Almost all known RTK, except for the insulin receptor, exist as monomers in the cell membrane; examples of these are neurotrophic tyrosine receptor kinases (Trk), fibroblast growth factor (FGF) receptor, epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptor. Ligand binding induces dimerization of these receptors, resulting in autophosphorylation of their cytoplasmic domains which, in turn, activate various downstream signaling pathways (Jiang and Hunter, 1999).

NRTK are intracellular tyrosine-specific protein kinases which recruit activities to the plasma membrane but lack intrinsic kinase function. Most NRTK associate with subcellular structures permanently or transiently on the cytoplasmic side of the plasma membrane. These properties allow NRTK to play a role as intracellular effector molecules and to participate in fast signal transduction, either by receiving signals from activated membrane receptors or transmitting signals on to membrane-associated proteins, following phosphorylation or dephosphorylation of specific tyrosine residues. Thus, it is reasonable to assume that NRTK may play an important role in the mechanisms of rapid GC-induced actions. Three NRTK are particularly relevant to this thesis: sarcoma tyrosine kinase (Src), proline-rich tyrosine kinase 2 (Pyk2) and Abelson tyrosine kinase (Abl) (see later sub-sections).

Protein tyrosine phosphatase (PTP) is expressed in a wide variety of cell types and is an efficient regulator of cell adhesion, spreading and migration. PTP play a crucial role in modulating the activity of PTK and their subsequent signaling pathways. Like PTK, PTP are a structurally diverse family of enzymes including receptor-type and soluble-type forms which contain a highly-conserved catalytic domain and unique non-catalytic segments. This structural diversity reflects differences in the regulation and function of each enzyme. It has been reported that the activation of PTP controls cytoplasmic protein tyrosine phosphorylation to regulate PTK signaling pathways. Although the activity of RTK is down-regulated by the activation of PTP, PTP can exert a positive regulatory function in signal transduction through cleavage of the inhibitory phosphate residue of NRTK, such as the tyrosine phosphorylation site (Y) 527 of Src tyrosine kinase.

1.4.3. Src kinase

Src, a member of NRTK, has been studied as a proto-oncogene since its discovery in the chicken tumor-causing Rous sarcoma virus by the Nobel Prize winner Peyton Rous in 1911 (Roskoski, 2004). In the last decades, attention has been paid to Src, particularly investigations of its effects in the CNS. Src has

been shown to play an important role in neuronal differentiation and neurite out-growth, as well the regulation of ion channel activity, synaptic transmission, and N-methyl D-aspartate receptor (NMDAR)- dependent synaptic plasticity (Salter and Kalia, 2004). Much evidence indicates that Src may be involved in learning and memory, pain, epilepsy and neurodegeneration by serving as the signal transduction convergence point for pathways related to GPCR, RTK and cell adhesion receptors (Roskoski, 2004.).

Src is the prototype of the Src family of kinases (SFK) which is the largest family of mammal PTK. Five SFK members have been reported to be expressed throughout the mammalian CNS: Src, Fyn, Yes, Lck and Lyn (Salter and Kalia, 2004). All of these are proteins of 52-62 kDa in size that share a common domain structure (**Figure 5**), including a catalytic domain, the Src homology (SH) 2 domain and the SH3 domain. The catalytic domain exerts tyrosine kinase activity and contains the activation loop which embraces an auto-phosphorylation tyrosine residue that is important for the regulation of kinase activity. The SH2 domain has been found in many other PTK and contains around 90 amino acids which bind to specific peptide motifs such as phosphorylated tyrosine. The SH3 domain also mediates protein-protein interactions and contains only 60 amino acids which canonically bind to proline-rich sequences. Besides these highly-homologous domains, the SH4 domain shared by SFK members, albeit with lower homology, contains about 15 amino acids at the N-terminal that are important for anchoring the protein to cell membranes. A unique domain that exists between the SH3 and SH4 domains is a region with variable size and which shows poor conservation among SFK members.

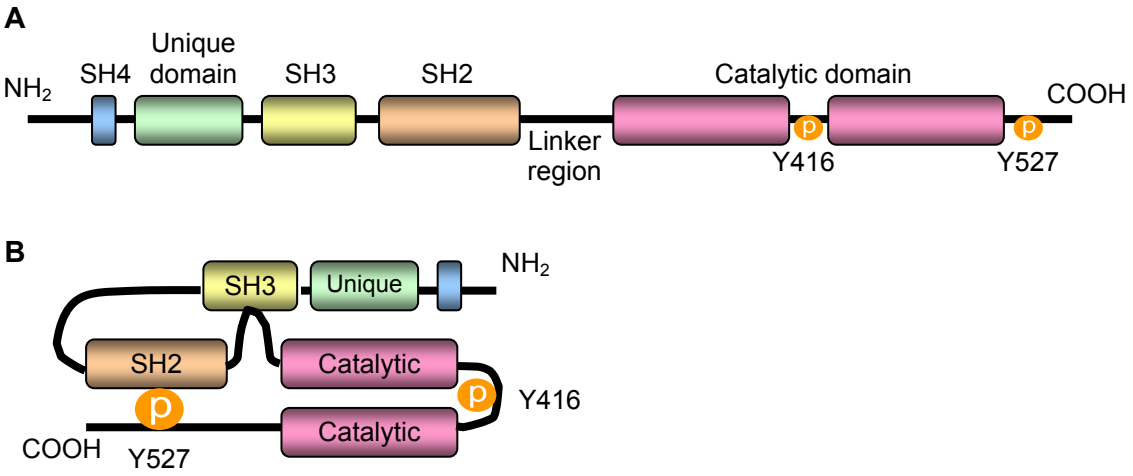


Figure 5. Structure and activation of Src family kinases (SFK). A: Members of SFK share a common domain structure which contains Src homolgy (SH) 4 domain, unique domain, SH3 domain, SH2 domain, linker region, and catalytic domain. The activation of Src is mediated by intramolecular interactions and tyrosine phosphorylation or dephosphorylation at tyrosine sites of kinase, such as the Y416 site in catalytic domain and Y527 in the C-terminal region. **B:** In the inactive conformation, the SH2 domain interacts with phosphorylated Y527 in the regulatory domain, the SH3 domain interacts with a ligand in the linker region, and autophosphorylation siteY416 in the activation loop is dephosphorylated. By convention, the amino-acid residue numbers shown are relative to chicken Src.

The catalytic activity of Src is dynamically mediated through the intramolecular interactions of the SH2 and SH3 domains and through the phosphorylation and dephosphorylation of the tyrosine kinase target sites, such as the tyrosine sites Y416 and Y527. As shown in **Figure 5**, under basal conditions, Src is inactivated and the Y527 of Src is phosphorylated by several PTK, such as the C-terminal Src kinase (Csk) and Csk homologous kinase (Chk). Src kinase activity is suppressed by intramolecular interactions, such as that of phosphorylated Y527 with the SH2 domain and SH3 domain within the linker region; this interaction provides the activation loop and embraces the autophosphorylation site Y416. Thus, at least two mechanisms are responsible for the activation of Src: the dephosphorylation of Y527 by kinases such as PTP, and the displacement of the intramolecular interaction by binding of SH2 and/or SH3 domain with other PTK. Both of these conformations lead to autophosphorylation of Y416, resulting in the full activation of Src kinase. Other modulators have also been shown to regulate Src kinase activity by interactions with the catalytic domain, e.g. the small

molecule inhibitor (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo [3,4-d]pyrimidine) PP2 (Hanke et al., 1996) and intracellular G_i-proteins (Ma et al., 2000).

In the CNS, an important function of SFK members is the regulation of ion channels activity, e.g. NMDA receptors (NMDAR) (Salter, 1998), VSCC (Cataldi et al., 1996), $GABA_A$ receptors (Moss et al., 1995), and nicotinic acetylcholine receptors (Wang et al., 2004). The NMDAR has been shown to participate in fast excitatory synaptic transmission via mechanisms mediated by SFK throughout the CNS. For example, Src has been shown to associate with and to upregulate the activity of the NMDAR in the hippocampus and spinal cord (Yu et al., 1997); moreover, Src-mediated upregulation of NMDAR induces LTP in the CA1 region of the rat hippocampus (Lu et al., 1998). More detailed information about NMDAR and its downstream pathways will be described later in this chapter.

1.4.4. Proline-rich tyrosine kinase 2

The proline-rich tyrosine kinase 2 (Pyk2), also known as related adhesion focal tyrosine kinase (RAFTK), cell adhesion kinase beta (CAK-β), calcium-dependent tyrosine kinase (CADTK) and focal adhesion kinase 2 (FAK2), has been identified as a member of focal adhesion kinase (FAK) family of NRTK (Avraham et al., 1995). Both members of the FAK family, FAK and Pyk2, have molecular masses that range between 110 and 125 kDa and exhibit approximately 48% amino acid identity over the length of the molecule. Although structural similarity exists, FAK and Pyk2 display significant different properties. For example, FAK is ubiquitously expressed whereas Pyk2 is mainly expressed in the CNS and in cells of the hematopoietic lineage (Avraham et al., 2000). While FAK is localized to focal adhesion sites in adherent cells, Pyk2 is mainly distributed throughout the cytoplasm and enriched in perinuclear regions (Schaller and Sasaki, 1997). These facts suggest that FAK and Pyk2 interact with different proteins and regulate different signaling pathways.

Although its detailed structure has not been fully characterized as yet, several functional domains of Pyk2 have been identified and characterized. As shown in **Figure 6**, these functional domains include an N-terminal band 4.1-ezrin-radixin-moesin (FREM) domain, a central tyrosine kinase domain, several proline-rich sequences and C-terminal focal adhesion targeting (FAT) domain. FREM domains are tight cloverleaf-shaped structures composed of three structural modules which mediate both protein-membrane targeting and protein-protein interactions to regulate Pyk2 function. It has been reported that Ca^{2+}/calmodulin induces Pyk2 activation directly or indirectly; this effect is abolished by mutations in the FREM domain, suggesting a role of the FREM domain in mediating Ca^{2+}/calmodulin binding (Kohno et al., 2008).

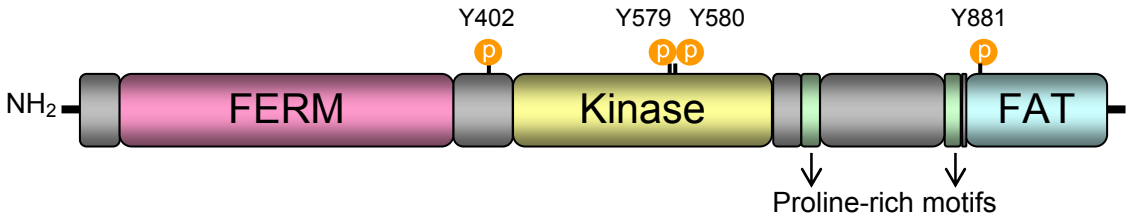


Figure 6. Schematic diagram of Pyk2 structure and its functional domains. Pyk2 contains an N-terminal band 4.1, ezrin, radixin, moesin (FERM) domain, a central tyrosine kinase domain, proline-rich motifs and a C-terminal focal adhesion targeting (FAT) domain. The phosphorylation of Y402 induces the binding with Src resulting in the phosphorylation of Y579/Y580 in the kinase domain, and the phosphorylation of Y881 in the FAT domain. Proline rich motifs (in green) mediate interaction with various proteins contained SH3 domain. The relative length of the domains and the positions of tyrosine sites are drawn to scale.

The central catalytic domain, which exerts tyrosine kinase activity, is connected to the FREM domain by a short linker segment which contains an auto-phosphorylation tyrosine site Y402. Phosphorylation at Pyk2 Y402 activates Src kinase by binding with the SH2 domain of Src to interfere with its intramolecular binding with phosphorylated Y527 of Src. The binding with Src results in the phosphorylation of Y579/Y580 in the Pyk2 catalytic domain to maximize Pyk2 activation through mechanisms that remain unclear (Park et al., 2004). The catalytic domain is followed by two proline-rich sequences which mediate the interaction of Pyk2 with proteins that contain SH3 domain. Notably, mutations of

proline-rich sequences in Pyk2 have been reported to have an exclusively nuclear localization, suggesting a potential role of Pyk2 in nucleus-located processes such as transcriptional regulation (Aoto et al., 2002). The C-terminal FAT domain of Pyk2 which is conserved with the corresponding FAT domain within FAK, which is necessary and sufficient for effective localization to focal adhesions, and has been reported to mediate the interactions with various proteins resulting in regulation of multiple cellular functions, such as actin cytoskeleton organization (Wang et al., 2003a) and scaffold protein function (Lulo et al., 2009). The phosphorylation site Y881 of Pyk2 locates to the FAT domain which binds to growth factor receptor-bound protein 2 (Grb2) after phosphorylation, and subsequently regulates downstream mitogen-activated protein kinase (MAPK) signaling pathways (Blaukat et al., 1999).

Over the last years, the role of Pyk2 in the CNS, especially in the hippocampus, has been intensively studied. It has been observed that the mechanisms of GPCR-activated MAPK signaling pathways are linked to activation of Pyk2 and Src via G_i- and G_q-coupled receptor dependent pathways in PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla and which exhibits neuronal properties when differentiated with (and maintained in) nerve growth factor (Dikic et al., 1996). Depolarization-induced activation of Pyk2, Src and ERK in a Ca^{2+}- and PKC-dependent manner have also been reported in rat hippocampal slices (Corvol et al., 2005). Activated Pyk2 has been reported to induce LTP which depends on downstream activation of Src to upregulate NMDAR in the CA1 region of the rat hippocampus (Huang et al., 2001); that observation suggests that the connection between Pyk2 with NMDAR might be linked by postsynaptic density protein 95 (PSD-95), one of the most prominent scaffolding proteins at postsynaptic sites, through the binding the SH3 domain of PSD-95 to the proline-rich region of Pyk2 (Seabold et al., 2003). A recent study in the CA1 region of the rat hippocampus indicated that NMDAR-mediated Ca^{2+} influx induces postsynaptic clustering and transactivation of Pyk2 by PSD-95

binding; further, this work suggested PKC involvement in this event, as well as pointing to a critical role of Pyk2 in learning and memory (Bartos et al., 2010).

1.4.5. Abelson tyrosine kinase

The Abelson tyrosine kinases (Abl) belong are NRTK and consist of c-Abl (also known as ABL1) and Arg (Abl-related gene, also known as ABL2), which are the only two known tyrosine kinases that directly interact with the cytoskeleton. Since the genes of Abl were first identified as proto-oncogenes from Abelson murine lymphosarcoma virus (Abelson and Rabstein, 1970), the signaling functions of c-Abl and its oncogenic fusion with the breakpoint-cluster region (BCR)-Abl have been extensively studied. c-Abl is ubiquitously expressed in vertebrates and has been found in several subcellular sites, including the nucleus, cytoplasm, mitochondria and endoplasmic reticulum where c-Abl interacts with multiple cellular proteins including PTK and PTP, cell-cycle regulators, transcription factors and cytoskeleton proteins, to mediate various cellular processes, e.g. regulation of cell growth and survival, oxidative stress and DNA-damage responses, actin dynamics and cell migration (Smith and Mayer 2002; Colicelli 2010).

As shown in **Figure 7**, like Src kinase, c-Abl contains a SH3, SH2 and catalytic domains; the latter includes an activation loop and exerts tyrosine kinase activities. Structurally, Abl and Src share 37% identity between their SH3 and SH2 domains and 52% identity in their catalytic domains (Nagar et al., 2003) and they both can auto-inhibited through intramolecular interactions (e.g. binding of SH3 domain with linker region and SH2 domain with C-terminal lobe of the kinase domain). The N-terminal of c-Abl contains a “Cap” region, equivalent to the unique domain of Src, which has different splice variants. The unique structure of c-Abl is its long C-terminal extension, also known as the last exon region, which contains various sites of interaction, including proline-rich motifs, a DNA-binding site, a globular (G)- and filamentous (F)-actin binding site, accounting for the diverse subcellular localization and specific functions of the

protein, such as the regulation of F-actin cytoskeleton in eukaryotic cells (Woodring et al., 2003). The last exon region has been reported to regulate tyrosine phosphorylation as well as activity of other cellular proteins (Hantschel and Superti-Furga, 2004).

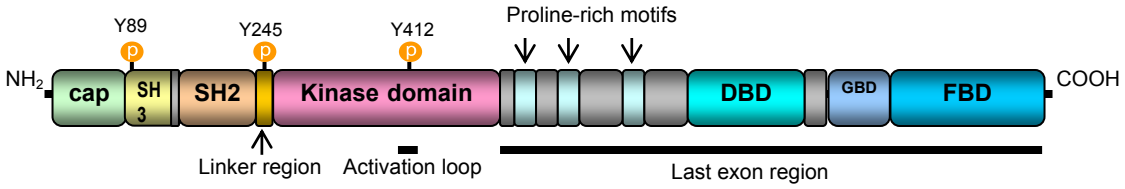


Figure 7. Schematic representation of the structure of c-Abl and its functional domains. c-Abl contains a "cap" region in N-terminal, SH3 domain, SH2 domain and a central tyrosine kinase domain. The last exon region follows the kinase domain in C-terminal extension, including proline-rich motifs, DNA binding domain (DBD), G-actin binding domain (GBD) and F-actin binding domain (FBD). Only tyrosine sites of Y89 in SH3 domain, Y245 in linker region and Y412 in the activation loop of kinase domain are shown.

Nine tyrosine phosphorylation sites have been identified in Abl by mass spectrometry (Steen et al., 2003). Two of these have received considerable attention since they have been correlated with increased c-Abl kinase activity: Y245, which resides in the linker region between the SH2 and kinase domains, and Y412, which resides in the activation loop of the kinase domain. The c-Abl kinase is activated by the auto-phosphorylation of kinase domain tyrosine residues *in trans* or by other protein kinases such as Src kinase. Phosphorylation of Y245 and Y412 are required for full kinase activation (Brasher and Etten, 2000; Tanis et al., 2003) of Abl. Furthermore, SFK-induced phosphorylation of Y89, which lies in the SH3 domain of c-Abl, has been reported to prevent the intramolecular binding of the c-Abl SH3 domain with the linker region, resulting in enhancement of c-Abl kinase activity and subsequent cellular signaling (Chen et al., 2008).

The pharmacological compound STI-571 (imatinib mesylate), also known as Gleevec®, is a 2-phenyl-amino-pyrimidine derivative which shows very high affinity and specificity for Abl tyrosine kinase, originally designed to inhibit the Abl

protein tyrosine kinase for the treatment of BCR-Abl positive leukaemias (Druker et al., 1996). STI-571 binds to the kinase domain of Abl and forces it into an inactive and non-phosphorylable conformation. Although the sequences of the kinase domain are highly conserved between c-Abl and Src, STI-571 was found to inhibit the catalytic activity of c-Abl, but not that of Src (Nagar et al., 2003), making this drug an effective tool to study the cellular functions of Abl tyrosine kinase.

There are emerging evidences that suggest that Abl family kinases are important in diverse contexts in CNS development and function. For example, genetic studies showed that Abl is required for the proper morphogenesis of most neurons in the developing nervous system of fruit flies (Gertler et al., 1989). In vertebrates, c-Abl has been implicated in different neuronal process, such as neurite outgrowth and synaptic function (Moresco and Koleske, 2003). It was shown that the activation of c-Abl induces the phosphorylation of cyclin-dependent kinase 5 (Cdk5), which is a small serine/threonine kinase that plays a crucial role during development of the CNS, e.g. in promoting neurite outgrowth (Zukerberg et al., 2000). Abl-induced neurite outgrowth has also been observed to occur through a Rho-dependent pathway in cultured hippocampal neurons (Jones et al., 2004). Other work has reported that c-Abl localizes to the presynaptic terminals and dendritic spines of neurons in the CA1 region of the mouse hippocampus where it regulates neurotransmitter release at Schaffer collateral-CA1 synapses (Moresco et al., 2003). c-Abl is reportedly activated by the PDGF receptor, a RTK that regulates various signal transduction pathways in hippocampal neurons in a Src-dependent manner, serving to regulate changes in the neuronal cytoskeleton which eventually influence NMDA receptor signaling (Beazely et al., 2008). One recent study has shown that c-Abl regulates synapse formation by mediating tyrosine phosphorylation and clustering of the scaffold protein PSD-95 in the post-synaptic density of rat hippocampal neurons (de Arce et al., 2010). Together, these results suggest an important role for Abl kinases in

linking signal transduction pathways that induce cytoskeletal rearrangement with the regulation of neuronal morphogenesis and synaptic function.

1.4.6. Other mechanisms related to this study

PTP-PEST

Protein tyrosine phosphatase (PTP) plays just a crucial role as PTK in the regulation of cellular tyrosine phosphorylations. The proline-, glutamic acid-, serine- and threonine-rich (PEST) family of PTP is a soluble-type PTP and contains three intracellular phosphatases: PTP-PEST, proline-enriched phosphatase (PEP)/lymphoid tyrosine phosphatase and PTP-hematopoietic stem cell fraction. All family members reside in the cytoplasm and share a common domain structure, including N-terminal catalytic domain, several proline-rich motifs and a conserved C-terminal tail. Unlike other members of the family whose expression is restricted in immune and hematopoietic cells, PTP-PEST is ubiquitously expressed in various cell types, including neurons (Veillette et al., 2009). Two serine residues, (S) 39 and S435, are the major phosphorylation sites of PTP-PEST. The phosphorylation of PTP-PEST at S39 was shown to down-regulate PTP-PEST activity by decreasing its affinity for substrate through the meditation of PKC and PKA; however, phosphorylation at PTP-PEST (S435) does not have any influence over its activity (Garton and Tonks 1994; Nakamura et al., 2010). The substrates of PTP-PEST are predominantly cytoskeletal and focal adhesion proteins and include Src (Chellaiah et al., 2009), Pyk2 (Lyons et al., 2001; Davidson et al., 2010) and c-Abl (Cong et al., 2000). Notably, it was shown that Y402, the major autophosphorylation site of Pyk2, as well as the activation loop tyrosine residues, Y579 and Y580, are regulated by PTP-PEST (Lyons et al., 2001), thus implicating PTP-PEST as important player in the mediation of Pyk2 actions.

N-methyl D-aspartate receptor signaling

The N-methyl D-aspartate receptor (NMDAR), a specific type of ionotropic glutamate receptor, has been intensively studied. It is widely expressed in the

CNS, shows specialized characteristics such as voltage-dependent block by magnesium, calcium permeability and slow deactivation kinetics, and plays a role in controlling synaptic plasticity and memory function (Cull-Candy et al., 2001). The NMDAR, comprised of NR1, NR2 and NR3 subunits which form the central conductance pathway, are tightly associated with various proteins that mediate NMDA signaling; together with its interacting partners (calmodulin kinase II (CaMKII), PKA and PKC, Src and Pyk2, scaffolding proteins and trafficking proteins, it forms the so-called NMDAR complex (Stephenson, 2006). Several tyrosine phosphorylation sites are located in NR2 (including NR2A and NR2B), but not in the NR1 subunit. Among these sites, Y1472 in the NR2B C-terminal cytoplasmic domain has been shown to be the primary substrate in NR2B that is targeted by Src kinase (Cheung and Gurd, 2001).

Electrophysiological studies showed that NMDAR currents in neurons are regulated by the phosphorylation and dephosphorylation of tyrosines of various proteins; thus inhibiting endogenous PTP activity or introducing exogenous PTK into cells enhances NMDAR currents, and *vice versa* (Wang and Salter, 1994). Several SFK, especially Src, have been found to be endogenous up-regulators of NMDAR activity in a tyrosine phosphorylation-manner. For example, synaptic NMDAR-mediated currents have been reported to be up-regulated by Src kinase in cultured neurons and slices of hippocampus (Yu et al., 1997; Lu et al., 1998). In addition, there is evidence to suggest that the calcium-dependent activation of Src and Pyk2 may serve as a convergence point of multiple signaling pathways (e.g. GPCR, PTK and Ras pathways) that regulate divergent downstream mechanisms including NMDAR signaling which ultimately leads to LTP (Salter and Kalia, 2004).

It has been well demonstrated that elevated GC suppress hippocampal synaptic potentiation (Smriga et al., 1996; Xu et al., 1998; Alfarez et al., 2002) by modulating NMDAR signaling (Kim et al., 1996; Wiegert et al., 2005); several of these studies have suggested mediation of these GC effects by classical nuclear

GR. Notably, the ability of CORT to modulate NMDAR function appear to vary according to neuronal type in the mouse hippocampus; thus, while the steroid rapidly influences NMDA-induced Ca^{2+} elevation in the CA1, but not in CA3 region and dentate gyrus (Sato et al., 2004). Other studies have reported that CORT rapidly modulates NMDA currents through the mediation of the PKA signaling pathway (Liu et al., 2007). Lastly, CORT has been recently shown to rapidly enhance NMDA neurotoxicity by increasing intracellular Ca^{2+} levels and attenuating NR2A-ERK-mediated neuroprotective signaling in rat hippocampus (Xiao et al., 2010).

Mitogen-activated protein kinase

In mammals, the mitogen-activated protein kinases (MAPK), a family of serine/threonine protein kinases which have classically been studied as regulators of cell proliferation and differentiation (Seger and Kreb, 1995), include three members: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAP kinase. The two ERK isoforms, p44 MAPK (ERK1) and p42 MAPK (ERK2), have been shown to ubiquitously express in the CNS and may mediate effects of growth factors or neurotransmitters on the dendritic cytoskeleton (Fiore et al., 1993). ERK 1/2, as the prototype of MAPK family, has been best studied for its intracellular signaling pathways which mediate the transmission of signals induced by various extracellular stimuli, such as cytokines, growth factors, neurotransmitters and hormones; thus, they transducer signals from the cell surface to cytoplasmic and nuclear effectors. Classically, the ERK signaling pathway contains a three-step protein kinase activation cascade, including the MAPK kinase kinases, RAS and RAF which sequentially activate the MAPK kinase MEK, which in turn activates ERK. Activated ERK translocates into the nucleus where it directly or indirectly activates downstream transcription factors such as early growth response-1 (Egr-1), which was shown to be target of activated GR, and cAMP response element-binding (CREB) protein; the latter is strongly implicated in synaptic plasticity and learning among a large spectrum of

other functions necessary for survival (Rubinfeld and Seger 2005; Hardingham et al., 2001; Revest et al., 2005; Davis and Laroche, 2006).

The important role of the MAPK family in rapid GC effects has been appreciated for years. GC have been reported to trigger rapid and non-genomic activation of the MAPK family members p38, JNK, and ERK in PC12 cells (Li et al., 2001; Qiu et al., 2001) and cultured hippocampal neurons (Qi et al., 2005; Xiao et al., 2005) via a putative membrane receptor through a PKC-dependent signaling mechanism. GC were also shown to rapidly increase the frequency of mEPSC in mouse CA1 pyramidal neurons via membrane-located non-genomic MR involving MEK/ERK signaling pathways (Olijslagers et al., 2008). Interestingly, ERK is rapidly activated via a NMDAR-dependent pathway to induce synapse plasticity in rat hippocampus (Kaphzan et al., 2006).

Rho family

The Rho family GTPases are low-molecular weight guanine nucleotide-binding proteins, which generally function as binary molecular switches that cycle between an active GTP-bound and inactive guanosine diphosphate (GDP) - bound state. Their activity can be positively influenced by guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDI) (Jaffe and Hall, 2005). Rho GTPases are regulated by direct phosphorylation or ubiquitination (Lang et al., 1996; Wang et al., 2003b), but the detailed mechanisms which may mediate these post-translational modifications are not fully characterized. Currently, 22 Rho family members have been identified in the mammalian genome, the most studied members being Cdc42, Rac1 and RhoA (Aspenström et al., 2004). These proteins regulate signaling pathways related to actin-dependent cellular processes, such as the assembly and organization of the actin cytoskeleton (Hall, 1998; Etienne-Manneville and Hall, 2002) and regulate cellular functions that

include gene transcription, the cell cycle, cell morphogenesis and cell migration (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

Drebrin

The filamentous actin (F-actin) is one of the major structural elements of dendritic spines and critical for the regulation of spine plasticity (Matus, 2000). Drebrin, a typical neuron-specific F-actin-binding protein, is abundantly expressed in dendritic spines, the postsynaptic elements that receive the majority of excitatory glutamatergic inputs in the CNS, and plays an important role in spine morphogenesis and morphology (Sekino et al., 2007). In developing hippocampal neurons, drebrin was shown to be involved not only in clustering of PSD-95 (Takahashi et al., 2003), but also in activity-dependent synaptic targeting of NMDAR (Takahashi et al., 2006). Interestingly, drebrin levels were shown to be markedly decreased in the brains of patients with Alzheimer's disease (Kojima and Shirao, 2007), in keeping with the view that Alzheimer's disease reflects synaptic dysfunction (Roselli et al., 2011).

1.5. The hippocampus as a model to study the rapid actions of corticosteroids

The adaptive response to stress needs not only the regulation of the HPA axis, but also the coordination of limbic system structures, including the amygdala, prefrontal cortex and hippocampus. The adaptations in these structures involve rapid mechanisms of synaptic plasticity. The hippocampus has proven an ideal structure in which to investigate molecular, signaling cascades and electrophysiological aspects of how information is encoded, transmitted and stored in neuronal circuits to regulate behavior. In particular, the simple laminar structure of the hippocampus has facilitated the use of extracellular recording techniques to record synaptic events *in vivo* (Tsien et al., 1996); notably, the hippocampus has been extensively studied in the context of long-term potentiation (LTP) and long-term depression (LTD), which have been strongly implicated to correlates with learning and memory (Bliss and Collingridge 1993). While most electrophysiological experiments are carried out on hippocampal slices, methods are well established for preparing primary cultures of hippocampal neurons from both embryonic and postnatal rodents.

As shown in **Figure 8**, the hippocampus proper comprises pyramidal neurons of the *Cornus Ammon,* subdivided into 3 layers (CA3, CA2 and CA1) pyramidal cell regions), granule neurons of the dentate gyrus, and basket and interneurons of the hilus. The hippocampus proper[1] is innervated by the entorhinal cortex via the perforant pathway that mainly terminates on granule cells in the dentate gyrus but also distal dendrites of CA3 pyramidal cells which further project to the CA1 region via Schaffer collaterals.

[1] Although most researchers use the term hippocampus to refer to the hippocampus proper, the neuroanatomical literature strictly reserves this term to refer to the inter-connected entorhinal cortex, the hippocampus proper and the s subiculum (main output of the hippocampus); alternatively, this collection of structures may be referred to as the hippocampal formation. However, in this work, the common usage of the term hippocampus (instead of hippocampus proper) will be applied for convenience.

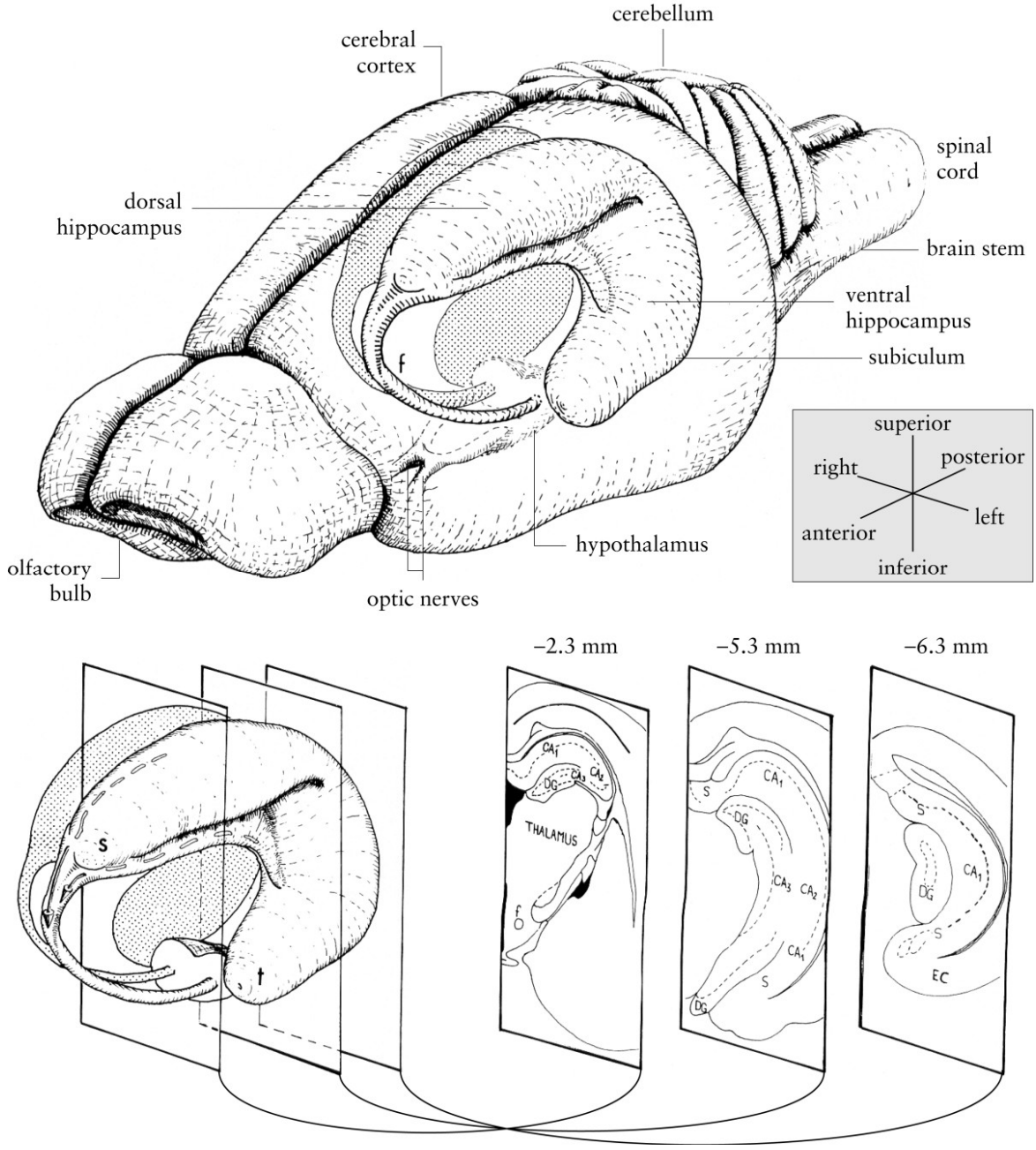


Figure 8. Diagram of the anatomy of the rat hippocampus. This figure is showing the location and organization of the hippocampus. Three coronal sections through the left hippocampus are shown at the bottom right of the figure, with their approximate anteroposterior coordinate relative to bregma. CA1, CA2, CA3: cornu ammonis fields 1–3; DG: dentate gyrus; EC: entorhinal cortex; f: fornix; s: septal pole of the hippocampus; S: subiculum; t: temporal pole of the hippocampus. (Adapted from Cheung and Cardinal, 2005)

The hippocampus is a major target of corticosteroids. Of all brain regions, it is endowed with the greatest density of classical (nuclear) MR and GR, and corticosteroids cause both reversible and irreversible changes in hippocampal structure and hippocampus-related memory (Sousa and Almeida, 2002; Joëls 2008). There is a large literature that describes the actions of GC on synaptic plasticity in the hippocampus, although the mechanisms remain largely unknown. For example, low levels of corticosterone alter synaptic plasticity in the CA1 subfield and dentate gyrus (Alfarez et al., 2003); such effects have also been seen after chronic stress and can be reversed with brief treatment with the GR antagonist, RU38486 (mifepristone) (Krugers et al., 2006). In another study, acute stress was found to increase voltage-dependent calcium currents in rat hippocampus through the mediation of GR (Karst and Joëls., 2007). In contrast, the rapid regulation of NMDA-induced Ca^{2+} influx in hippocampal slices by CORT was ascribed to mediation by putative membrane receptors, the nature of which was not specified (Sato et al., 2004). CORT has also been reported to enhance the electrophysiological activity of hippocampal CA1 neurons and to reduce paired-pulse facilitation via a membrane MR-dependent and transcription-independent manner, suggesting a hormone-dependent enhancement of glutamate-release probability (Karst et al., 2005). On the other hand, acute GC stimulation in hippocampal slices was shown to rapidly enhance LTP of Schaffer collateral inputs to CA1 pyramidal neurons in an MR- and GR-independent fashion (Wiegert et al., 2006).

Although the transcriptional actions of GC have been proved to regulate long-term hippocampal neurons responses of afferent inputs, the rapid action of GC in the hippocampus, which may be mediated though putative membrane receptors, provides possible mechanisms for the acute effects of GC in various cellular functions.

Generally, much evidence indicates that corticosteroids, especially GC, are tightly associated with hippocampal cognitive impairment, cellular changes and

neuronal survival. Hippocampus, as the main target of stress-induced hormones, has been found to relate to many diseases and disorders, such as depression, amnesia, epilepsy, schizophrenia and Alzheimer's disease (de Kloet et al., 2005; Sotiropoulos et al., 2008). To investigate the mechanisms, either genomic or non-genomic, of GC-induced modulation on hippocampal functions provides approaches to understand how stress affects the brain and cognition.

2. AIMS OF THIS STUDY

Much emerging evidence indicates that corticosterone (CORT) exerts rapid non-genomic effects in multiple tissues and cell types, including the hippocampus. The role of classical (nuclear) corticosteroid receptors and putative membrane receptors in the mediation of rapid CORT signaling are controversial. Although some authors suggest the involvement of G-proteins, PTK, NMDAR and MAPK in the signaling pathways of the non-genomic effects of CORT, the detailed mechanisms that potentially regulate these cascades in neuronal cells remain largely unknown at the present time. Understanding the rapid signaling pathways induced by CORT are important because this hormone produces fast changes in physiology or behavior. In the present study, experiments were undertaken to investigate the following specific questions:

- Is rapid CORT-induced signaling initiated at a membrane receptor in hippocampal neuronal cells? What are the roles of classical corticosteroids receptors (MR and GR) in these signaling cascades?

- How is the secondary signal transduction of CORT regulated in the cytoplasm? What might be the interactions between various intracellular mediators (G-protein, PTK and MAPK) in the final responses to rapid CORT singling?

- How does rapid CORT-induced signaling influence cellular functions such as gene transcription, cell morphogenesis and synapse function?

3. MATERIALS AND METHODS

3.1. Primary hippocampus neuronal culture

Primary neurons from the rat hippocampus were cultured according to a method described by (Roselli et al., 2005), with slight modifications. Cultures were prepared from Wistar rat pups aged 4-5 days; pups were born to mothers obtained from Charles River (Sulzfeld Germany) on gestation day 18-19, and held under standard laboratory conditions. All animal experiments were conducted in accordance with German laws on the use of animals in research and were approved by the local ethics committees.

On the day of culture, pups were sacrificed by rapid decapitation and the brains quickly taken out and placed in a 5 cm Petri dish containing ice-cold dissection solution (Solution I)[2].

Hippocampi from both hemispheres were dissected out, and transferred to a second Petri dish containing fresh, ice-cold Solution I. After dissection of the last hippocampus, hippocampal explants were sliced (250 µm) using a McIlwain tissue chopper (Camden Instruments, Leics, UK). Slices (with minimal Solution I) were placed in trypsin digestion solution (Solution II)[3] in a 50 mL tube and incubated in a shaking water bath (37 °C, 200 rpm) for 10 minutes. Thereafter,

[2] Solution I, **Dissection solution**

B27 (GIBCO), 50X	1 mL
NeuroBasal A (GIBCO)	49 mL

[3] Solution II, **Trypsin digestion solution**

		Final concentration
Trypsin (GIBCO), 2.5%	0.1 mL	0.05 %
BSA (Sigma), 7.5%	0.2 mL	3 mg/mL
DNase (Worthington), 2000U/ml	0.125 mL	50 U/mL
EBSS	4.575 mL	

trypsin inhibitor solution (Solution III)[4], was added and slices were gently triturated using a 10 mL round-edged, plastic pipette (Sarstedt, Ulm, DE). Following brief centrifugation (70 *g*, 30 seconds), the pellet was re-suspended in Solution III and filtered through a 30 µm nylon mesh (VWR, München, DE). Dissociated cells (in filtrate) were then centrifuged (200 *g*, 6 minutes) and the pellet was resuspended in 3 mL Solution I to which 0.125mL DNase (2000U/mL; GIBCO) and 0.3 mL BSA (7.5%; Sigma) was added. Dead cells and cell debris were subsequently excluded using a density gradient (Solution IV)[5]. After centrifugation (70 g, 6 minutes), the resulting pellet was resuspended gently in pre-warmed Growing Medium[6]. An aliquot of the cell suspension was used to estimate the number of cells using a Neubauer hemocytometer (Strober, 2001). This estimate was used to dilute the cell suspension in Growing Medium to give a cell density of, respectively 300 cells/mm^2 or 450 cells/mm^2 when plated onto pre-coated glass coverslips (0.13-0.17 mm,12X12 mm Marienfeld, DE) or 6-well plates (NUNC).

Glass coverslips were pre-cleaned by washing in chromosulfuric acid (Merck, Darmstadt, DE) for 2 hours, sequential washing in tap and distilled water to remove all traces of acid, boiling in absolute ethanol for 1 h, and overnight drying

[4] Solution III, **Trypsin inhibitor solution**

		Final concentration
Soy bean trypsin inhibitor (Sigma), 4mg/ml	1.5 mL	0.4 mg/mL
BSA (Sigma), 7.5%	0.6 mL	3 mg/mL
FCS (GIBICO)	0.3 mL	
B27 (GIBCO), 50X	1 mL	1 X
NeuroBasal A (GIBCO)	12.3 mL	

[5] Solution IV, **Density gradient solution**

BSA (Sigma) 7.5%	3 mL
Solution I	2 mL

[6] **Growing Medium**

		Final concentration
B27 (GIBCO) **,** 50X	1 mL	1 X
GlutamaxI (GIBCO), 200 mM	0.25 mL	1 mM
bFGF (GIBCO), 4 µg/ml	0.125 mL	10 ng/mL
Kanamycin (GIBCO), 100X	0.5 mL	1 X
NeuroBasal A (GIBCO)	48.25 mL	

at 200 °C; prior to plating, coverslips were placed individually in the wells of 6-well plates (NUNC). Coverslips and 6-well plates were coated with gelatine[7] (2 hours, room temperature) before coating (at least 2 hours, room temperature) with poly-D-lysine (PDL; MW: 70,000 – 150,000; Sigma)[8]. Subsequently, the PDL was aspirated, and coverslips and plates were washed thoroughly (3 times) with sterile dH_2O. Coverslips and plates were then stored in a incubator (37 °C; 95% relative humidity) until used.

Cells were plated in 1 mL Growing Medium and allowed to attach for 30 minutes after which a further 1 mL of Growing Medium was added to each well. After plating, cells were grown in 2 mL Growing Medium; every 3 days, 1 mL of medium was aspirated off gently and replaced by 1 mL of fresh (37 °C). Cells were maintained for 10-13 days before treatments were applied.

Characterization of the cultures by immunocytochemistry revealed the presence of cells with the following phenotypes: mature, MAP-2- (microtubule-associated protein, a dendritic marker of mature neurons) positive cells and NeuN- (neuronal nucleus, a vertebrate neuron-specific nuclear protein) positive cells; these cells accounted for around 45% and 55% of the cultures, respectively (based on counting of all cells, labeled with Hoechst 33342, a marker of DNA) at 12 days *in vitro* (**Fig 3.1**).

[7] **Gelatin solution**, 0.08%

Gelatin (Sigma)	0.04 g
Borate Buffer (0.024M, pH 8.2-8.4)	50 mL
Sterilize using 0.2 μm filter	

[8] **Poly-D-Lysine (PDL) solution**, 0.031 mg/mL

PDL (Sigma), 1mg/mL	1 mL
Borate Buffer (0.024M), sterilized	32.2 mL

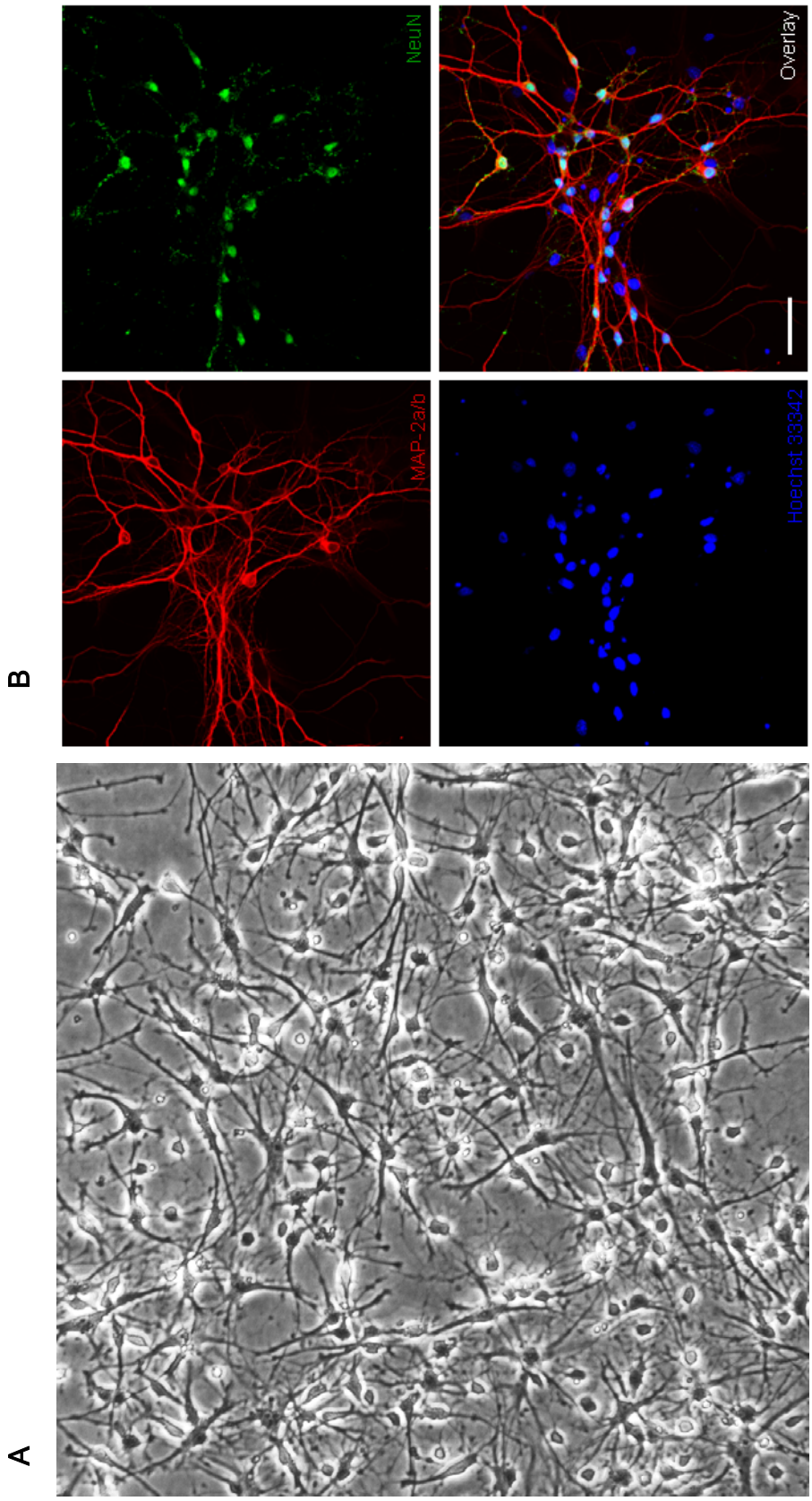


Fig. 3.1: Morphology and immunophenotypes of rat postnatal hippocampal neuronal cell cultures. A: Phase contrast images (20×) of cultured hippocampal cells were taken at 10 days *in vitro* (DIV), using an Olympus IX50 inverted microscope (Olympus, Tokyo, Japan). **B:** Confocal images (40×; Olympus FluoView 1000) of cultured hippocampal neuronal cells were taken at 12 DIV. Parafomaldehyde-fixed cells were stained with the antibodies for the dendritic marker (MAP-2a/b, red), the neuronal nucleus-specific marker (NeuN, green) and for cell nuclei (Hoechst 33342, blue). Compared to Hoechst 33342 positive cells, about 45% of all cells were MAP-2a/b-positive cells and approximately 55% of cells were NeuN-positive cells (from three independent cultures). Scale bar represents 50 µm.

3.2. Pharmacological treatments

Before any drug application, cells were "starved" to remove the potentially interfering effects of growth and other factors in the Growing Medium; this was achieved by placing cells in unsupplemented NeuroBasal A (GIBCO) for 3 hours. Drugs were applied to cells for 30 minutes unless specifically stated otherwise. Appropriate vehicles (and controls) were used for each drug (see **Table 3.1**); these were deimethylsulfoxide (DMSO), 40% cyclodextrin or, in certain cases, 100% ethanol; vehicle concentrations were kept to a maximum of 0.1% (final concentration) in order to avoid confounding effects.

3.3. Immunocytochemistry

Immunocytochemistry was used to phenotypically characterize the cultures, but also to detect the localization, distribution and expression level of specific proteins in cultured cells. After treatments, cells on coverslips were briefly washed with pre-warmed (37 °C) phospho-buffered saline (PBS)[9] and fixed with ice-cold 4% paraformaldehyde[10] for 10 minutes at room temperature. Following 3 temperature to reduce non-specific binding of primary antibodies; the BSA was

[9] **Phosphate buffered saline** (PBS)

		Final concentration
NaCl (Roth)	8.0 g	137 mM
KCl (Merck)	0.2 g	2.7 mM
$Na_2HPO_4 \cdot 7H_2O$ (Roth)	2.16 g	8.0 mM
KH_2PO_4 (Merck)	0.2 g	1.5 mM
dH_2O	Up to 1 L	

Adjust pH to 7.4, sterilize through 0.2 μm filter

[10] **4% Paraformaldehyde** (PFA)

		Final concentration
$Na_2HPO_4 \cdot 7H_2O$ (Roth)	2.16 g	0.08 M
NaH_2PO_4 (Roth)	0.24 g	0.02 M
Paraformaldehyde (SIGMA)	4 g	4%
dH_2O	Up to 100 mL	

Adjust pH to 7.4, Heat at 56°C to dissolve. Prepare freshly before use.

washes (5 minutes each), cells incubated in 3% BSA for 1 hour at room usually diluted in PBS or in PBST[11] when cell surface antigens were to be visualized; in the latter case, this permeabilization step preceded the incubation with BSA (see above). Cells were subsequently incubated with specific primary antibodies (see **Table 3.2**.) diluted in blocking buffer (3% BSA in PBS) at 4 °C overnight. After extensive washing with PBS at room temperature (3 times, 30 minutes each), cells were incubated with appropriate fluorescent-tagged secondary antibodies (see **Table 3.3**) diluted in PBST (again, those for cell surface protein staining were diluted in PBS) for1 hour at room temperature. Specimens were then washed (PBS, 3 times, 30 minutes each) and incubated with the DNA-intercalating dye Hoechst 33342 (Invitrogen; diluted in PBST for 10 min at room temperature, to visualize cell nuclei. After extensive washing with PBS at room temperature, cells were mounted onto glass slides with anti-fade mounting medium[12], dried overnight and then examined by microscopy.

[11] **Phosphate buffered saline- Triton** (PBST)

		Final concentration
Triton-X100 (Roth)	300 µL	0.3 %
PBS	Up to 100 mL	

The concentration of Triton-X100 was further optimized when using certain antibodies.

[12] **Mounting Medium**

Glycerol (Sigma)	6 g
Mowiol 4-88 (Calbiochem)	2.4 g
dH_2O	6 mL
Tris buffer (0.2 M), pH 8.5	12 mL

Store at -20°C.

Table 3.1 Drugs used in this study

Drugs	Description	Dose	Vehicle	Source
2-Aminoethyl diphenylborinate, 2-APB	Modulator of intracellular IP_3-induced calcium release	100 µM	EtOH	Sigma, Deisenhofen, DE
AG1478	Selective inhibitor of EGFR	10 µM	DMSO	Sigma
API-2	Selective inhibitor of Akt/PKB	30 µM	DMSO	Tocris, Bristol, UK
BAPTA-AM	Selective chelator of intracellular Ca^{2+}	13 µM	H_2O	Sigma
CaMKII inhibitor (CI)	Inhibitor of Ca^{2+}/calmodulin (CaMKII)	10 µM	H_2O	Calbiochem, La Jolla, CA
Corticosterone-BSA, (CORT-BSA)	Cell-impermeable conjugate of BSA and CORT	100 nM	H_2O	ABiox, Newberg, OR
2-hydroxypropyl-β-cyclodextrin corticosterone	Water-soluble CORT (CORT:HBC)	10 nM	H_2O	Sigma
CORT 182097	Selective antagonist for GR	100 nM	EtOH	Corcept Therapeutics, CA
Cycloheximide, CHX	Protein synthesis inhibitor	10 µM	DMSO	Sigma
Eplerenone	Selective MR antagonist (<AR, PR and ER)	100 nM	DMSO	Tocris
Forskolin	Cell-permeable activator of adenylate cyclase	10 µM	DMSO	Tocris
G-15	Selective GPR30 receptor antagonist	1 µM	DMSO	Tocris
GDP-β-S	Non-hydrolysable GDP analog	100 µM	H_2O	Sigma
Gö 6983	PKC inhibitor	5 µM	DMSO	Sigma
TDZD-8	Selective inhibitor of GSK-3β	10 µM	DMSO	Merck, Darmstadt, DE
GW 5074	Selective c-Raf1 kinase inhibitor	1 µM	DMSO	Tocris
H-89	Selective inhibitor of PKA	1 µM	DMSO	Calbiochem
J2700	GR antagonist	100 nM	DMSO	Gift, Jenpahram, Jena/DE
JAK inhibitor	Potent inhibitor of Janus tyrosine kinases.	1 µM	DMSO	Calbiochem
K252a	Specific and potent inhibitor of Trk	1 µM	DMSO	Sigma
MK801	Selective NMDAR antagonist.	10 µM	H_2O	Tocris
NMDA	Prototypic NMDAR agonist	100 nM	H_2O	Tocris
PD 98059	Specific inhibitor of MAPK	25 µM	DMSO	Tocris
Pertussis toxin (PTX)	Gi- and Go- protein inhibitor	500 mg/L	H_2O	List Biologicals, CA
PF431396	Potent pyrimidine-based Pyk2 inhibitor	3 µM	DMSO	Symansis, Timaru, NZ
PP2	Selective inhibitor of the Src family	1 µM	DMSO	Calbiochem
PP3	Negative control for the PP2	1 µM	DMSO	Calbiochem
RU 28318	Selective antagonist for MR	100 nM	H_2O	Tocris

Drugs	Description	Dose	Vehicle	Source
RU 38486	PR and GR antagonist	100 nM	CyD	Sigma
Spironolactone, SPIRO	Competitive MR and AR antagonist	100 nM	DMSO	Tocris
STI-571	Selective inhibitor of c-Abl and PDGFR	1 µM	DMSO	Cayman, Ann Arbor, MI
SU6656	Potent Src family kinase inhibitor	1 µM	DMSO	Calbiochem
Syk inhibitor	Potent Spleen tyrosine kinase inhibitor	1 µM	DMSO	Calbiochem
Thapsigargin	Inhibitor of endoplasmic reticulum Ca^{2+}-ATPases	1 µM	DMSO	Tocris
U 73122	PLC inhibitor	5 µM	DMSO	Tocris
U0126	Selective inhibitor of MAPK kinase.	10 µM	DMSO	Tocris
Verapamil	Inhibitor of L-type Ca^{2+} channels	50 µM	EtOH	Sigma
W7	Calmodulin antagonist	50 µM	DMSO	Tocris
Wortmannin	Selective inhibitor of PI3K	4 µM	DMSO	Tocris

DMSO: Dimethyl sulfoxide (SIGMA); CyD: 40% Cyclodextrin (SIGMA); EtOH: 100% Ethanol

Table 3.2. Primary Antibodies used for Immunocytochemistry and/or Immunoblotting

Antibody	Host	WB	ICC	Source
Actin	Mouse	1:5000		Millipore, Billerica, MA
c-Abl Phospho-Tyr 412	Rabbit	1:1000		Novus, Littleton, CO
CaMKII, Phospho-Thr286	Rabbit	1:1000		Cell Signalling, Danvers, MA
CREB Phospho-Ser133	Rabbit	1:1000		Cell Signalling
Drebrin	Rabbit		1:5000	IBL, Hamburg, DE
EGR 1	Rabbit	1:1000		Cell Signalling
JNK Phospho	Rabbit	1:1000		Cell Signalling
Map2	Rabbit		1:200	Millipore
Map2a/b	Mouse		1:500	Sigma, Deisenhofen, DE
Neuronal Nuclei (NeuN),	Mouse		1:200	Chemicon
NMDA-NR2B ($pTyr^{1472}$)	Rabbit	1:1000		Cell Signalling
NMDA-R1	Mouse		1:100	BD Biosciences, San Jose, CA
NR2B glutatmate rec	Rabbit		1:200	Sigma
p38 Phospho-spec	Rabbit	1:1000		Cell Signalling
p44/42, Erk 1/2	Rabbit	1:2000		Cell Signalling
p44/42, Erk 1/2 Phospho	Rabbit	1:1000		Cell Signalling
PSD-95	Mouse		1:400	Acris, Herford, DE
PTP-PEST Phospho-Ser39	Rabbit	1:1000		Dr. K. Mashima, Rikkyo University, JP
P-Tyr-100, Phospho-Tyrosine	Mouse	1:1000		Cell Signalling
Pyk2	Rabbit	1:2000		Cell Signalling
Pyk2 Phospho-Tyr402	Rabbit		1:200	Biosource
Pyk2 Phospho-Tyr402	Rabbit	1:1000		Invitrogen, Darmstadt, DE
Pyk2 Phospho-Tyr579	Rabbit	1:1000		Invitrogen
Pyk2 Phospho-Tyr580	Rabbit	1:1000		Invitrogen
Pyk2 Phospho-Tyr881	Rabbit	1:1000		Invitrogen
Src antibody	Rabbit	1:2000		Cell Signaling
Src Phospho-Tyr416	Rabbit	1:1000		Cell Signalling
Src Phospho-Tyr527	Rabbit	1:1000		Cell Signalling
Synapsin 1	Rabbit		1:750	Sigma

Table 3.3. Secondary antibodies used in ummunocytochemistry

Antibody (primary) target	Dilution	Source
HRP-conjugated anti mouse	1:2000	Jackson Labs, Bar Harbor, ME
HRP-conjugated anti rabbit	1:2000	Jackson Labs
Alexa Fluor 488 anti mouse	1:400	Molecular Probes/Invitrogen
Alexa Fluor 594 anti mouse	1:400	Molecular Probes
Alexa Fluor 488 anti rabbit	1:400	Molecular Probes
Alexa Fluor 594 anti rabbit	1:400	Molecular Probes

3.4 Confocal imaging and deconvolution analysis

Fluorescent imaging of immunohistochemical staining of cultured cells was performed on an Olympus FV-1000 laser scanning microscope with Fluoview software (Olympus, Tokyo, Japan). Images were obtained from 5-7 optical fields chosen across individual coverslips and slices using a Kalman filter and sequential scanning mode; standard (saved) settings for laser power; photomultiplier gain and offset were used. In the case of double or triple labeling, each channel was recorded consecutively to avoid cross-excitation and emission. Z-stack images were obtained from each optical field and each z-stack image was ultimately collapsed into a single plane to create a 2 dimensional image. Usually, 7-9 z-stack images were taken for a single cell at a resolution of 1024 x 1024 pixels with a 40× water-immersion objective (NA 1.15, Olympus) or a 60 x water-immersion objective (NA 1.20, Olympus).

NIH ImageJ (Rasband, 1997–2011) software was used to process and analyze the captured images. Images were imported into ImageJ software, converted to 8-bit grayscale; unified thresholds were used for all images. When identifying colocalized puncta, the ‘Co-localization Highlighter’ plugin was used and analysis was done with the ‘Analyze Particle’ module of the ImageJ program.

3.5 Protein sample preparation

Cells from 6-well plates were briefly washed with ice-cold PBS, followed by 150 µL ice-cold Lysis Buffer[13]. After gently shaking, the cells were left on ice with Lysis Buffer for 5 minutes before being harvested with a commercial plastic cell scraper. Scraped cells in buffer were then quickly transferred into pre-chilled 1.5 mL Eppendorf tubes and briefly sonicated (Branson). Finally, lysates were centrifuged at 4 °C, (13,200 rpm) for 10 minutes and supernatants were stored at -80 °C for further analysis.

Aliquots of the cell lysates were used to determine protein concentrations in the lysates using the Lowry method (Lowry et al., 1951). The reagents for the assay are provided in the footnote[14]. For the assay, samples were diluted (e.g. 1:50) in 150 µL deionized (DI) H_2O. A 1 mg/mL BSA (Sigma) solution was used to provide

[13] **Lysis Buffer,** 1 mL

		Final concentration
Tris-Cl (ROTH), 1 M, pH 7.5	50 µL	50 mM
NaCl (ROTH), 5 M	30 µL	150 mM
EDTA (SIGMA), 0.5 M	2 µL	1 mM
$MgCl_2$ (SIGMA), 1M	5 µL	5 mM
Nonidet P40 (Fluka), 10%	100 µL	1%
Sodium deoxycholate, (SIGMA), 5%	100 µL	0.5%
Protease Inhibitor (Roche), 50x	20 µL	1x
Phosphatase Inhibitor 1, (SIGMA)	10 µL	
Phosphatase Inhibitor 2, (SIGMA)	10 µL	
Deionized (DI) H_2O	673 µL	

Prepare fresh on ice before use.

[14] **Lowry solution A**

Na_2CO_3 (SIGMA)	2 g
0.1 M NaOH (SIGMA)	Up to 100 mL
Lowry solution B	
$KNaC_4H_4O_6$ (SIGMA)	2 g
DI H_2O	Up to 100 mL
Lowry solution C	
$CuSO_4$ (SIGMA)	1 g
DI H_2O	Up to 100 mL

linear concentrations (also in 150 µL DI H_2O) for use as standards. The assay was conducted by adding 750 µL of Lowry reagent to standards and samples, mixing and incubation for 15 minutes at room temperature. Thereafter, 75 µL of Folin-Ciocalteus Phenol Reagent (Merck; 1:2 dilution in DI H_2O) and the mixture was allowed to stand for 1 hour at room temperature. Finally, the absorbance of standards and samples were measured at 750 nm with a Synergy-HT (Bio-Tek) plate reader.

3.6 Immunoblotting

3.6.1. SDS-PAGE

The first and critical step in immunoblotting is separating out proteins according to size using SDS-polyacrylaminde gel electrophoresis (SDS-PAGE). To this end, appropriate volumes of each sample were diluted 1:1 in 1 x Laemmli Buffer[15] to a volume of 30 µL. Samples were then boiled at 95 ºC for 10 min to denature and linearise the proteins. After boiling, samples were briefly mixed and centrifuged at 13,200 rpm for 30 seconds, after which 50 µg protein was carefully loaded onto 8% or 10 % (according to target protein size) Tris-Cl polyacrylamide gels (PAGE

[15] **Laemmli Buffer**,6x

Stacking Gel Buffer, Stock	3.5 mL
Glycerol (SIGMA)	1.5 mL
Dithiothreitol (DTT, SIGMA)	0.465 g
SDS (SIGMA)	0.5 g
Bromphenol blue	0.6 mg
DI H_2O	Up to 5 mL

Store at –20°C

gels, see **Table 3.4**), along with a pre-stained protein molecular weight marker (Fermentas, St. Leon-Rot, DE).

Table 3.4 The preparation of SDS-PAGE Gel.

Separating Gel Mix,.1.5 mm thickness gel	**8 % gel**	**10 % gel**
Rotiphorese Gel 30, (Roth), 30%	2,1 mL	2,7 mL
[16]Separating Gel Buffer Stock	2 mL	2 mL
DI H_2O	3,9 mL	3,3 mL
[17]10% Ammonium Persulfate (APS)	40 µL	40 µL
Tetramethylethylenediamine (TEMED, SIGMA)	6 µL	6 µL
[18]**Stacking Gel Mix,**.1.5 mm thickness gel		
stacking gel buffer		3 mL
10% APS		20 µL
TEMED		3 µL

The gels were run in a Bio-Rad electrophoresis system (Bio-Rad, Hercules, USA) filled with Electrophoresis Chamber Buffer[19] (both inner and outer chambers). A constant voltage of 75 V was applied to the gel until the tracking dye migrated towards the bottom of the gel. Next, the gel was briefly washed in ice-cold

[16]**Separating Gel Buffer, Stock**	
Tris-Cl (ROTH)	91 g
SDS (SIGMA)	2 g
DI H_2O	Up to 500 mL

[17] **Ammonium Persulfate** (APS), 10%	
Ammonium Persulfate (SIGMA)	10 g
DI H_2O	Up to 100 mL

[18] **Stacking Gel Buffer, Mix**	
Rotiphorese Gel 30, (Roth), 30%	13.3 mL
Stacking Gel Buffer, stock (see below)	25 mL
DI H_2O	Up to 100 mL

Stacking Gel Buffer, Stock	
Tris-Cl (ROTH)	6.05 g
SDS (SIGMA)	0.4 g
DI H_2O	Up to 100 mL

[19] **Electrophoresis Chamber Buffer**	
Tris-Cl (ROTH)	3 g
Glycine (ROTH)	14.4 g
SDS (SIGMA)	1 g
DI H_2O	Up to 1 L

Transfer Buffer before transfer of electrophoresed proteins to nitrocellulose membranes (Bio-Rad, Hercules, USA) in a Bio-Rad blotting apparatus (18 V for 45 minutes, 60 minutes or 90 minutes, depending on target protein size), using Transfer Buffer[20]. Successful transfer was confirmed by Ponceau staining of the membrane. The membrane was then quickly rinsed with DI H_2O twice and TBST[21] once (5 minutes each) and incubated, with shaking, in Blocking Buffer[22] for 60 minutes at room temperature. After briefly washing 3 times with DI H_2O, the membrane was incubated with primary antibody in 1% to 5% BSA in TBST or Blocking Buffer (optimized for each primary antibody) for 2 to 4 hours at room temperature or overnight at 4 ºC. Antibodies were affinity-purified and used at 1000 to 5000-fold dilutions, as specified in Table 3.2. Unbound primary antibodies were removed by 3 X 10 minute washes in TBST. The membranes were then incubated with respective secondary antibodies conjugated to horseradish peroxidase (HRP, GE Healthcare, Freiburg) at room temperature for 1 hour. The dilution of secondary antibodies was 1:2000 in Blocking Buffer in

[20] **Transfer Buffer**

Tris-Cl (ROTH)	3 g
Glycine (ROTH)	14.4 g
20% SDS (SIGMA)	2 mL
Methanol	100 mL
DI H_2O	Up to 1 L

Store at 4°C

[21] **Tris-buffered saline-Tween** (TBS-T)

Tris-Cl (ROTH)	12.1 g
NaCl (ROTH)	17.6 g
Tween-20 (ROTH)	20 mL
DI H_2O	Up to 2 L

Adjust pH to 8.0.

[22] **Blocking Buffer,** 5%

No-fat Milk Powder (Roth)	5 g
TBST	Up to 100 mL

most cases, but this was sometimes adjusted for individual primary antibodies. After further washing (3 times, 7 minutes) in TBST to remove unbound secondary antibodies, proteins were visualized using electrochemiluminescence (ECL; Roche, Mannheim, DE), according to the protocol from manufacturer. Membranes were then exposed to blue-light sensitive X-ray film (λmax = 428 nm; GE Healthcare) which were subsequently processed using Kodak developer and fixer (both from Sigma) according to the manufacturer's protocol.

3.6.2 Semi-quantitative analysis of Immunoblots

Subsequently, protein bands revealed on film (from at least 3 identical but independent experiments) were scanned with an EPSON scanner (fixed brightness/contrast settings) and analyzed using TINA software (Raytest, Straubenhardt, Germany). A fixed rectangular region of interest (ROI) was used to measure all bands and the optical density within each ROI was measured after background density was subtracted. All data were entered into Microsoft Excel files and subjected to further analysis.

3.7 G-protein linked immunosorbent assay (GLISA) test

3.7.1 Assay Principle

G-LISA is a technique based on Enzyme-linked immunosorbent assay (ELISA) that allows efficient measurement of the activation of small GTPase, such as members of the Rho family, in cell or tissue samples. The basic steps of assay are shown in **Fig 3.2**.

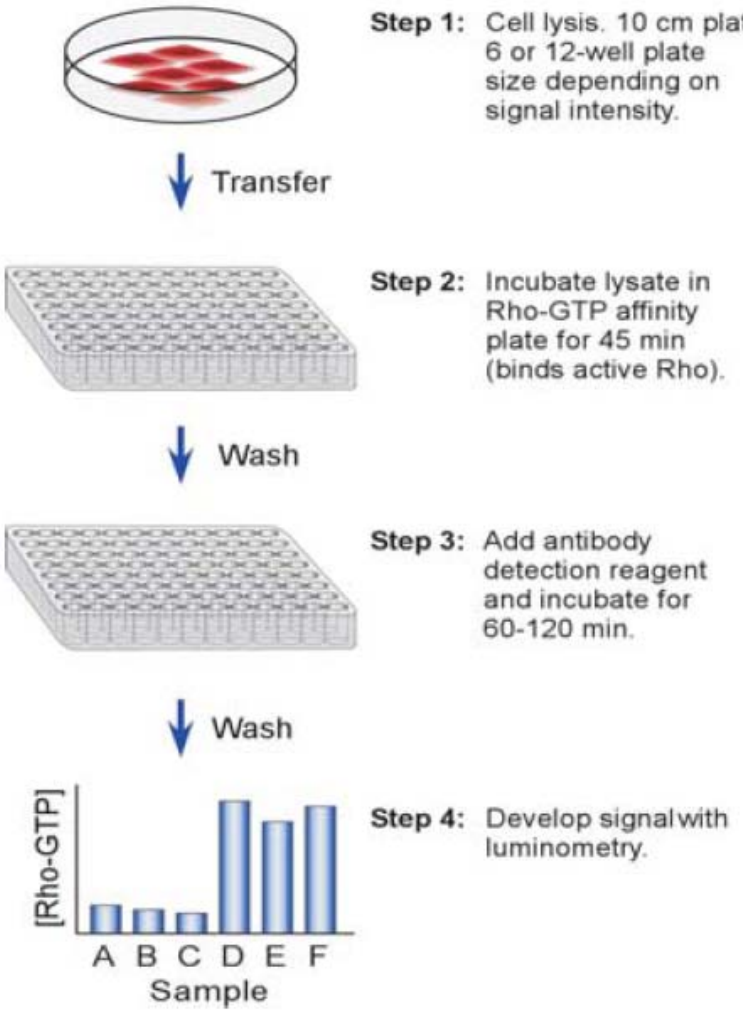


Fig 3.2 A basic schematic of the steps involved in the G-LISA (Cytoskeleton Inc., Denver, CO, USA).

Briefly, a 96-well plate covalently coated with a Rho-GTP-binding protein is used. Active GTP-bound Rho in cell lysates binds to the coated wells and inactive GDP-bound Rho is removed by washing steps. The bound active RhoA is then detected with a RhoA specific antibody and chemiluminescence. The degree of RhoA activation can be estimated by comparing readings from activated versus non-activated cell lysates (in these studies, using the culture 'starvation' protocol described in **Section 3.1.**).

3.7.2 Assay Protocol

G-LISA assay kits for assessment of RhoA activation were purchased from (Cytoskeleton Inc., Denver, CO) and used according, with slight optimization, to the manufacturer's protocol with optimization. Cells were washed once with ice cold PBS and harvested (see **Section 3.5**) with lysis buffer provided by the manufacturer. Lysates (drug-treated and control) were applied to the assay plates at a final concentration of 0.5 mg/mL, on ice. The plate was then placed on a cold orbital microplate shaker at 4 °C, 400 rpm for 30 minutes. After washing twice with Washing Buffer (provided in kit), each well was incubated with Antigen Presenting Buffer (provided in kit) at room temperature for 2 minutes after which the solution was removed and the plate washed 3 times with Washing Buffer. Next, anti-RhoA primary antibody (provided in the assay kit; 1:500) was added to each well, and the plate was incubated on a microplate shaker at room temperature (400 rpm, 45 minutes). After three washes, secondary HRP-labeled antibody (provided in kit; 1:500) was added to each well, and the plate was again incubated (microplate shaker, room temperature, 400 rpm, 45 minutes). Following removal of the secondary antibody and 3 washes, HRP detection reagent (provided in kit) was added and luminescence signal was detected within 5 minutes, using a Bio-Tek Synergy HT microplate reader (gain setting fixed at 100, integration time at 0.1 second; filters optimized to 590/30 to obtain reasonable relative luminescence units (RLU) that, in positive controls were 6-9 fold higher than background). Data were transferred to Microsoft Excel worksheets and subjected to further analysis.

3.8 Statistics

All immunoblotting data are depicted as mean ± standard deviation (SD) from 3-5 independent experiments. Immunofluorescence data derive from evaluation of a minimum of 600 synapses (N) in each of 8-10 neurons (n). Data were analyzed for statistical significance using ANOVA and appropriate post hoc tests (Student-Keuls or Kruskal-Wallis multiple comparison procedures, as appropriate) where $p < 0.05$ was set as the minimum level of significance. The SigmaStat software package (Systat Software GmbH, Erkrath, DE) was used for the statistical analysis.

4. RESULTS

4.1 Primary hippocampal cultures as a valid model for studying the fast actions of CORT

Previous studies describing the the rapid and non-genomic actions of CORT were done in a rat phaeochromocytoma cell line (PC12). They showed that CORT rapidly induces the activation of MAPK family signaling cascades (Li et al., 2001; Qiu et al., 2001). In a later study, Qi et al. (2005) showed that CORT also triggered MAPK activation in neuronal cultures derived from the embryonic rat hippocampus. It is now known that the ERK/MAPK signaling cascade is regulated through numerous signal transduction regulators, including such as GPCR, PTK and ion channels and that the consequences of this activation include alterations in cellular excitability and the activation of transcription factors (and subsequently changes in gene expression) which, together, serve to induce synaptic plasticity, a phenomenon important for memory formation (Sweatt, 2004) as well as the expression of other behaviors. In the present work, a first step was to reproduce the earlier reports on CORT-induced activation of MAPK in primary hippocampal neurons obtained from postnatal rats.

Given that the Growing Medium used for culturing primary hippocampal neurons contains various hormones and growth factors (see **3.2.1** for recipe) which could potentially activate the MAPK cascade, protocols had to be optimized to test whether “starvation” would overcome this potential confound. Experiments were thus designed in which cells were transiently deprived of Growing Medium and were instead maintained in NeuroBasal A medium (free from supplements as well as phenol red – an activator of estrogen receptors which are also known to potently activate the MAPK pathway - and glutamine). Results in **Fig 4.1A** show a time-course study from which a starvation period of 3 hours was determined to be sufficient so as to suppress endogenous expression of one MAPK, extracellular

signaling kinase 1/2 (ERK1/2), under basal (drug-free) conditions. Moreover, this paradigm did not cause any significant alteration in the morphology (signs of apoptosis) of the cells, determined by Hoechst staining and microscopic examination (data not shown). This condition was used in all further experiments described in this thesis. To ensure that the vehicles used to apply the various drugs used in this work (see **Table 3.1**) also did not interfere with ERK1/2 activity, a screening of the effects of DMSO, 40% Cyclodextrin, and 100% ethanol was also carried out. The results, shown in **Fig 4.1B** reveal that the levels of total and activated ERK1/2 were not changed by any of the vehicle treatments.

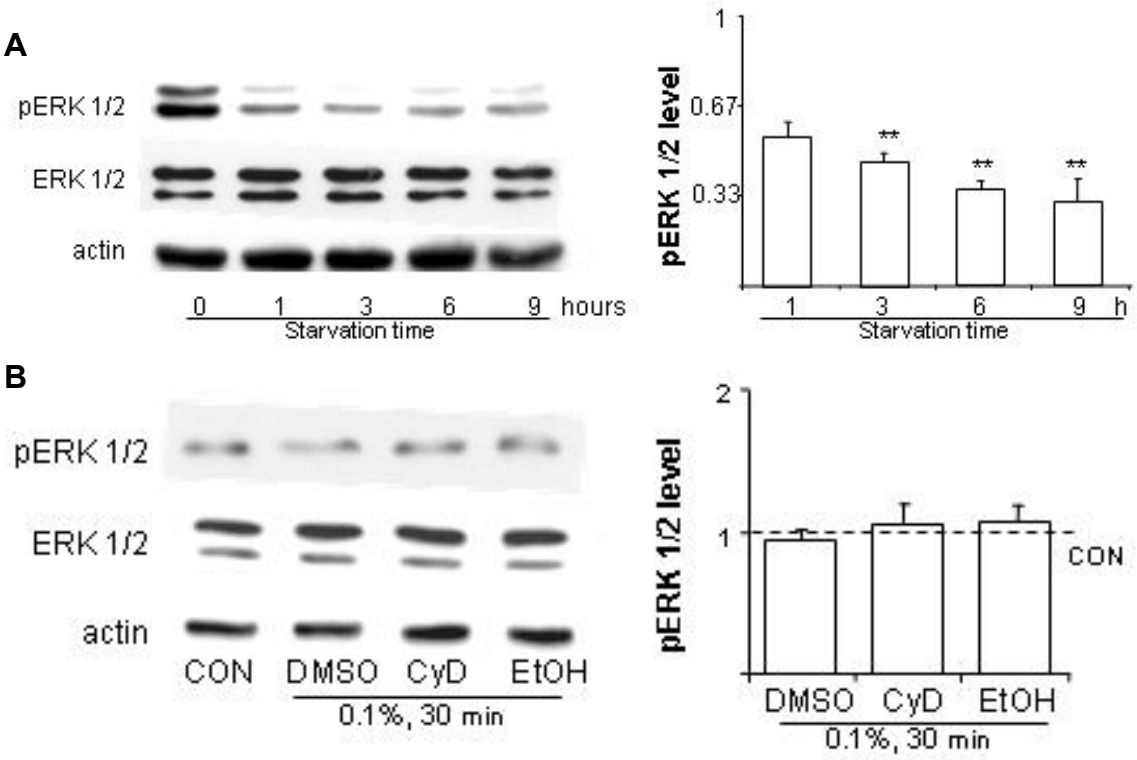


Fig. 4.1: A. Deprivation of primary hippocampal neuronal cultures cells of Growing Medium ("starvation") and replacement with supplement-free NeuroBasal A medium for 3 h suppresses endogenous activation of ERK 1/2. **B**. Treatment of cultures with DMSO, 40% cyclodextrin (CyD) and 100% ethanol (EtOH) (all at final concentrations of 0.1%) did not interfere with ERK 1/2 activity. Compared to untreated cells (CON), no significant changes of phosphorylated ERK 1/2 were observed. Levels of total ERK 1/2 and activated (phosphorylated) ERK 1/2 were determined by immuno- blotting. Total ERK 1/2 levels were not altered under any of the conditions tested.
The immunoblot images shown are representative of at least 3 independent experiments. ** indicates $p < 0.001$ vs. untreated (CON) cells.

Next, experiments were performed to test the dose-dependency and temporal effects of CORT on ERK1/2 activation in hippocampal cultures. For this, cells were treated with different concentrations of CORT (range from 1 nM to 1 µM) for between 0 and 120 minutes. As shown in **Fig. 4.2**, significant phosphorylation (activation) of ERK1/2 was first seen at a CORT dose of 10 nM ($p \leq 0.001$) without any accompanying changes in the total levels of the (inactive) kinase.

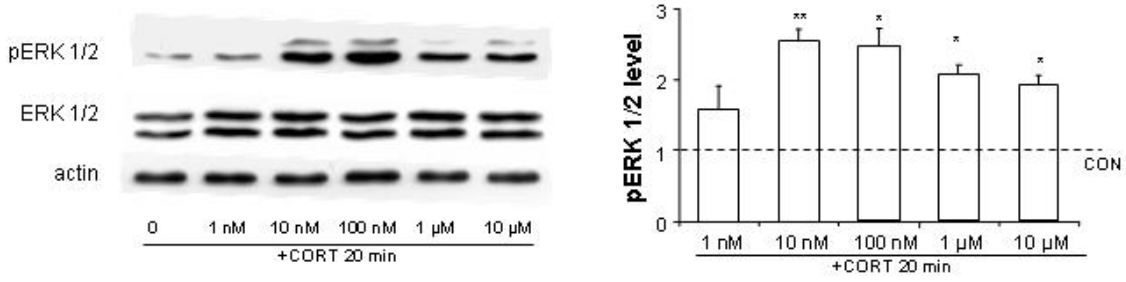


Fig. 4.2. CORT dose-dependently alters the levels of activated (pERK) in primary hippocampal cultures.
Cell lysates were immunoblotted were analyzed 20 minutes after treatment of cells with various doses of CORT. Total ERK 1/2 levels were unchanged.
The immunoblot image depicted is representative of at least 3 experiments that yielded similar results. * indicates $p < 0.05$, **, indicates $p < 0.001$, both compared to untreated (CON) cells.

Using this first effective dose of CORT (10 nM), subsequent experiments on the time-course of effects showed that the ERK1/2-activating actions of CORT can be seen as early as 10 minutes after application of the steroid **(Fig. 4.3)**. Maximum increases in phospho-ERK1/2 (pERK) were observed at 20 minutes ($p \leq 0.001$); these levels returned to baseline after 120 minutes. Analysis of two other MAPK family members, p38 and JNK, revealed their significant phosphorylation by CORT within 10 minutes, the effects remaining elevated for at least 120 minutes **(Fig. 4.3)**.

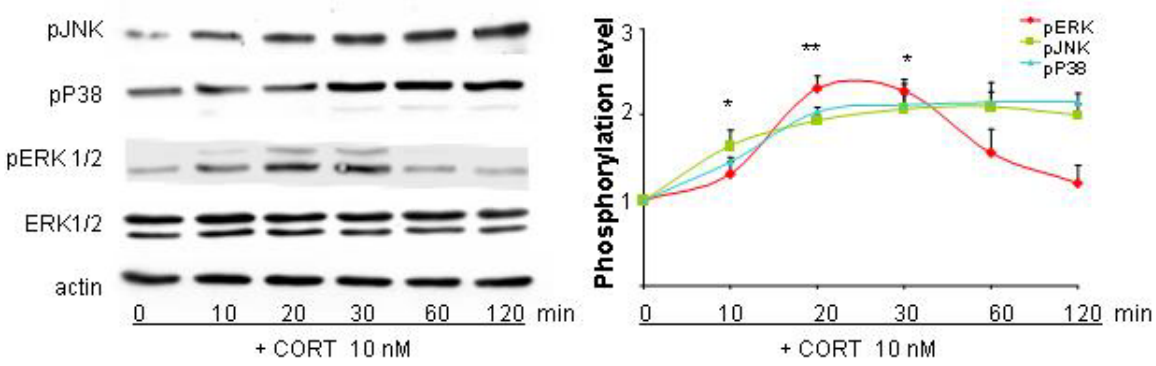


Fig. 4.3. Time course of CORT-induced activation of key MAPK family members.
Primary hippocampal cells were treated with CORT (10 nM) for between 0 and 120 minutes. CORT induced significant increases in the phosphorylation state of ERK 1/2, JNK and p38, with maximum activation being seen after 20 minutes of exposure to the steroid. Total ERK 1/2 level were unchanged.
The immunoblot image is representative of at least 3 independent experiments. *: $P<0.05$, **: $P<0.001$ vs untreated cells. For reasons of clarity the significance of increases in the phosphorylation levels of JNK and P38 are not shown.

The above-mentioned earlier studies suggested that the rapid actions of CORT could not involve gene transcription (i.e. that CORT has non-genomic actions) and/or *de novo* protein synthesis (Qiu et al., 2001; Qi et al., 2005; Xiao et al., 2005). Also, those earlier works hypothesized that CORT might act through a putative membrane-bound receptor (Qi et al., 2005), similar to hypotheses about the actions of other steroid hormones (e.g. estrogen, progesterone; Ke and Ramirez, 1990; Revankar et al., 2005) that are well characterized as ligands of classical nuclear receptors. Since CORT is small (MW 346.5 g/mol) and lipophilic, it rapidly crosses cell membranes; however, it can be made membrane-impermeable by conjugation to a large protein such as BSA (Xiao et al., 2005; 2010). Results shown in **Fig. 4.4A** demonstrate that CORT-BSA is similarly effective to unconjugated CORT in stimulating ERK1/2 activation in terms of magnitude and temporal pattern of effects. But the peak level of phosphorylated ERK1/2 was induced by 100 nM CORT-BSA. No detectable change induced by BSA alone was observed (**Fig. 4.4B**).

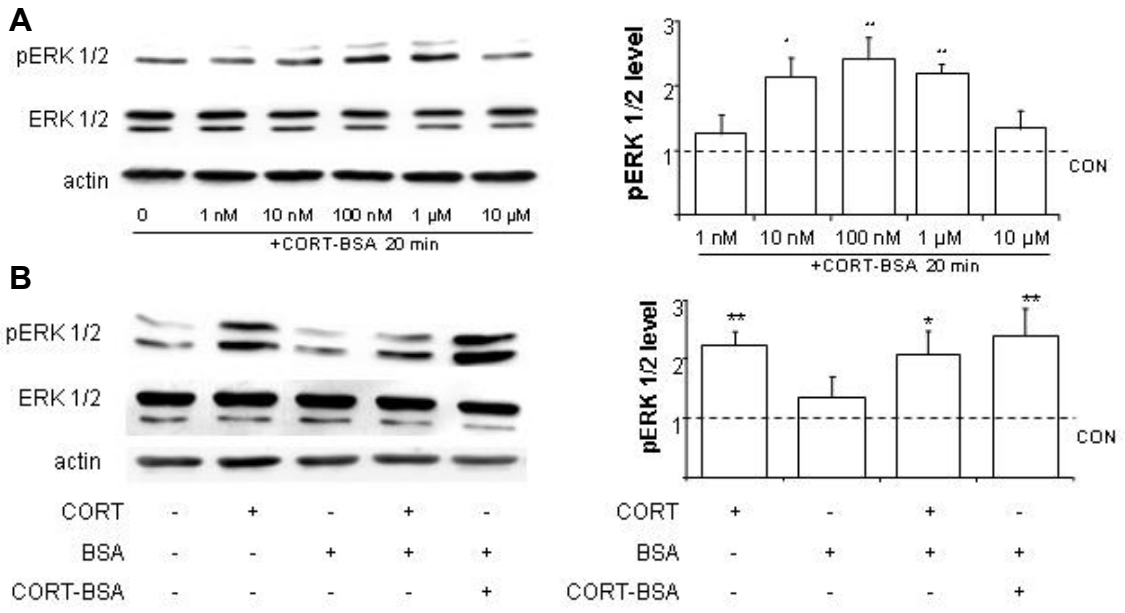


Fig. 4.4. Membrane-impermeable CORT-BSA induces ERK 1/2 phosphorylation.
A. Similarly to unconjugated CORT which can readily permeate the plasma membrane, a CORT-BSA conjugate (membrane-impermeable, mainly because of its size (> 65,000 Da) and large protein moiety) induced phosphorylation of ERK 1/2 within 20 minutes of application to hippocampal neurons in culture. Both CORT and CORT-BSA were applied at a dose of 10 nM (with correction for BSA component). **B**. BSA itself (500 nM, 20 minutes) with/out CORT or CORT-BSA did not influence the levels of phosphorylated ERK 1/2. Total ERK 1/2 level were not changed in any of the experiments.
The immunoblots images are representative of at least 3 independent experiments. * indicates $p < 0.05$, ** indicates $p < 0.001$ vs untreated (CON) cells.

To address dependency of the above effects on *de novo* protein synthesis, experiments shown in **Fig. 4.5** were carried out in which hippocampal cells were exposed to 10 nM CORT in the presence of cycloheximide (CHX; 10 μM), a inhibitor of protein translation. The results show that CORT induces pERK1/2 even in the presence of CHX.

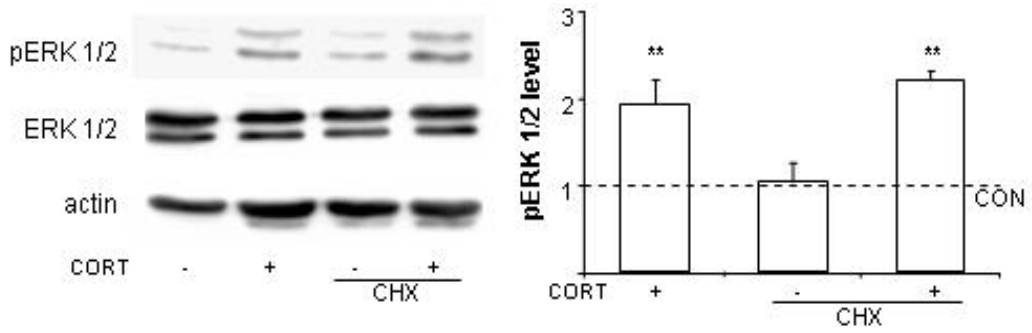


Fig. 4.5. CORT-induced activation of ERK 1/2 does not require *de novo* protein synthesis. The protein synthesis inhibitor, cycloheximide (CHX; 10 μM, 30 minutes) did not affect the phosphorylation of ERK 1/2 induced by CORT (10 nM, 20 minutes). Total ERK 1/2 levels were unchanged.
The immunoblot images shown represents results obtained in independent replications (n = 3). ** denotes $p < 0.001$ vs untreated (CON) cells.

In summary, the above results show that primary hippocampal neurons from postnatal rats respond to CORT with significant increases in activated ERK1/2 with 20 minutes. These effects are dose-dependent and do not require the synthesis of new proteins. Further, they can also be induced by membrane-impermeable CORT, supporting the view that a membrane-bound mechanism transduces the effects of CORT to cytoplasmic signaling pathways such as ERK1/2. Having obtained these data, and given that MAPK signaling pathways are likely to lie considerably downstream from the plasma membrane, it was of interest to examine the potential involvement of more proximal mechanisms. In the next set of experiments therefore, the Src family kinase signaling pathway was analyzed.

4.2 CORT activates Src family kinase signaling pathway

As described in **1.4.2**, protein tyrosine kinase (PTK) plays an important role in the cellular signal transduction (Hubbard and Till, 2000). In order to screen for the activation of PTK in response to CORT application, the global level of tyrosine phosphorylation was assessed in hippocampal neuronal cells. Immunoblots of whole-cell lysates, probed with an anti-phosphotyrosine antibody, detected a

large number of bands in the range of 20-200 kDa that were significantly upregulated within 5 minutes of CORT application at a dose of 10 nM ($p \leq 0.05$); tyrosine phosphorylation levels peaked at 20 minutes ($p \leq 0.001$) and thereafter decreased slowly although they were still significantly higher ($p \leq 0.05$) than under basal conditions after 60 minutes (**Fig 4.6A**). Notably, the effects of CORT on tyrosine phosphorylation were not prevented by the presence of the protein synthesis inhibitor, cycloheximide (CHX), implying a signaling mechanism that does not require classical transcriptional mechanisms (**Fig 4.6B**).

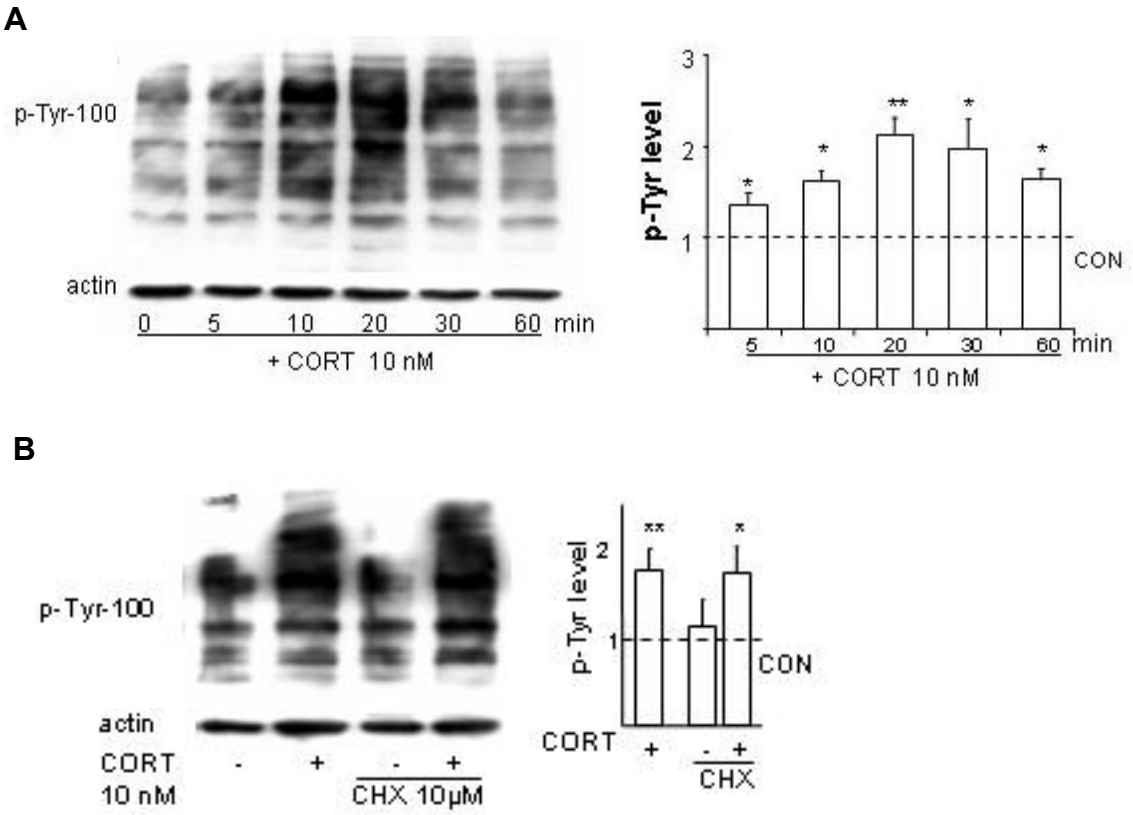


Fig. 4.6. Rapid induction of phosphorylation of protein tyrosine kinases (PTK) by CORT.
A: The levels of phosphorylated PTK (p-Tyr-100) were significantly increased after 5 minutes of CORT treatment (10 nM), and were sustained above control levels for at least 60 minutes. **B.** The phosphorylation of PTK induced by CORT (10 nM, 20 min) was not affected by cycloheximide (CHX; 10 µM, 30 minutes).
The blots shown are representative of at least 3 experiments, all of which gave similar results. *: P<0.05, **: P<0.001 vs untreated (CON) cells.

Next, small molecule inhibitors of non-receptor protein kinases (NRTK) were screened in an attempt to identify the candidate PTK that could be potentially responsible for the observed CORT-induced increases in phosphotyrosine levels. Specifically, we tested a Src family kinase inhibitor (PP2), an inhibitor of Abl (imatinib, STI-571), as well as inhibitors of the JAK-STAT and Syk pathways (**Fig 4.7A**); in addition, two receptor tyrosine kinase (RTK) inhibitors were tested: a TrkB inhibitor (K252a) and an epidermal growth factor (EGF) inhibitor (AG1478) (**Fig 4.7B**). Apart from PP2 which completely abolished the CORT-triggered upregulation of phosphotyrosine, none of the other inhibitors tested exerted a significant effect on the ability of CORT to influence this parameter. Importantly, PP3, an inactive analog of PP2 did not significantly interfere with the actions of CORT **Fig 4.7C**). These findings point to a central role of the Src family kinases in mediating the actions of CORT that originates at the plasma membrane. Indeed, this view is supported by additional data obtained when a structurally unrelated (compared to PP2) Src-family inhibitor (SU6656) was used **Fig 4.7C**.

The importance of Src family activation was next further explored. Immunoblotting assays confirmed that CORT efficiently upregulates of Src phosphorylation and revealed that this occurs at Y416, a tyrosine residue which determines the activation of the kinase (**Fig 4.8A**). This activation was detectable within 10 minutes of CORT application at a dose of 10 nM ($p \leq 0.05$), with peak activation being seen after 30 minutes (**Fig 4.8A**). Interestingly, phosphorylation of Y527, an epitope responsible for triggering an the auto-inhibitory loop (Roskoski, 2005), was not immediately affected (10 minute time-point) although phosphorylation of this epitope was significantly reduced after 30 and 60 minutes of exposure to CORT (**Fig 4.8B**). It should be noted that the levels of total Src were not influenced by any of the above treatments (not shown).

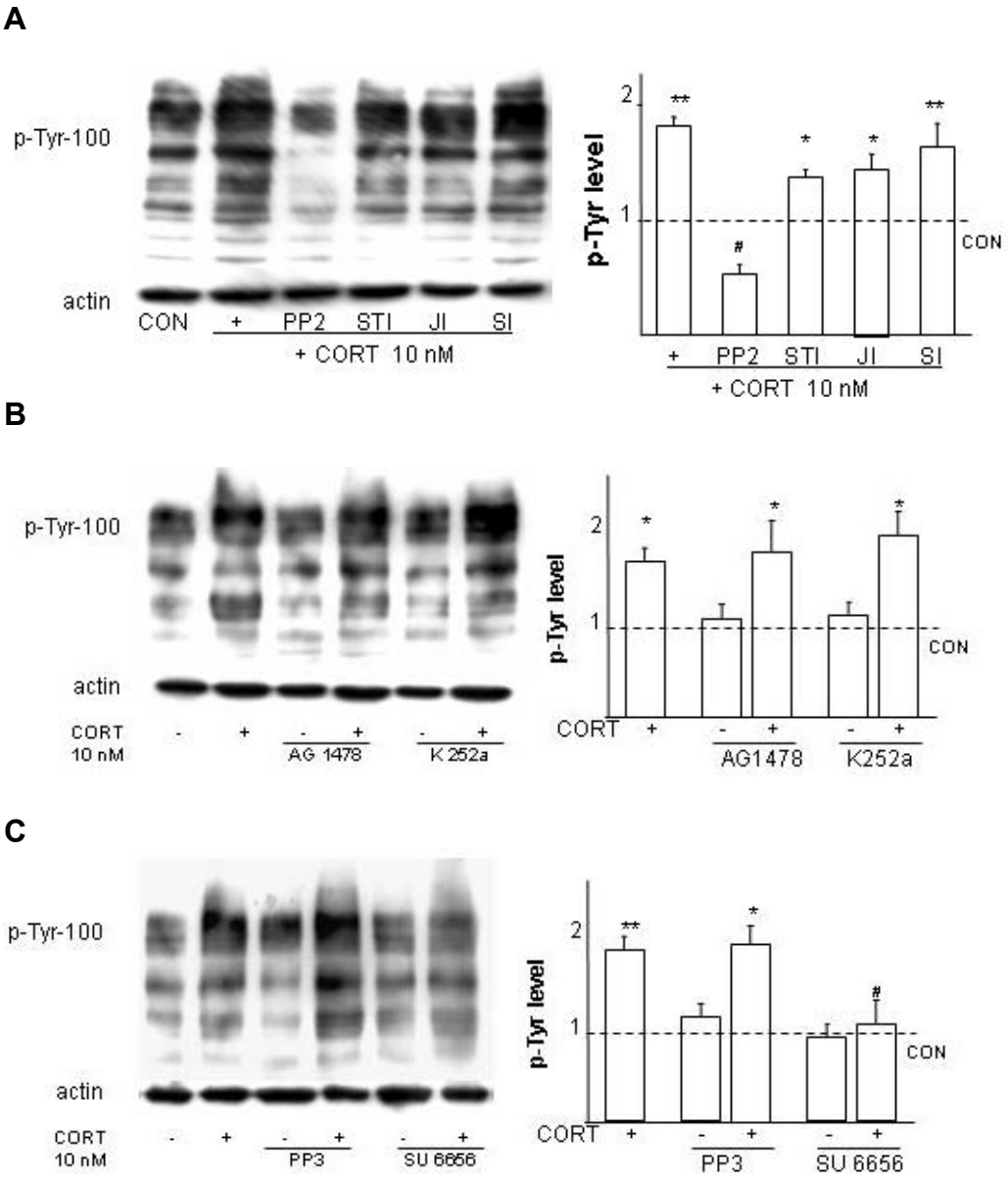


Fig. 4.7. Small molecule inhibitors of PTK screening. A. Hippocampal neuronal cultures were pretreated with various inhibitors of non-receptor protein kinase for 30 minutes before CORT application (10 nM, 20 minutes). Only the Src inhibitor, PP2 (1 μM) significantly abrogated the ability of CORT to induce PTK phosphorylation. The Abl inhibitor (STI, 1 μM), JAK-STAT inhibitor (JI, 1 μM) and Syk inhibitor (SI, 1 μM) were non-effective at preventing PTK phosphorylation by CORT. **B**. Two receptor protein kinase inhibitors, the EGF inhibitor AG1478 (10 μM, 30 minutes) and TrkB inhibitor K252a (1 μM, 30 minutes) also did not counteract the actions of CORT. **C**. The inactive analogue of PP2, PP3 (1 μM, 30 minutes), failed to inhibit CORT-induced phosphorylation of PTK, but another Src family inhibitor, SU 6656 (1 μM, 30 minutes) yielded results that were similar to those found with PP2.
The immunoblots shown are representative of results obtained in at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05 vs CORT-treated cells.

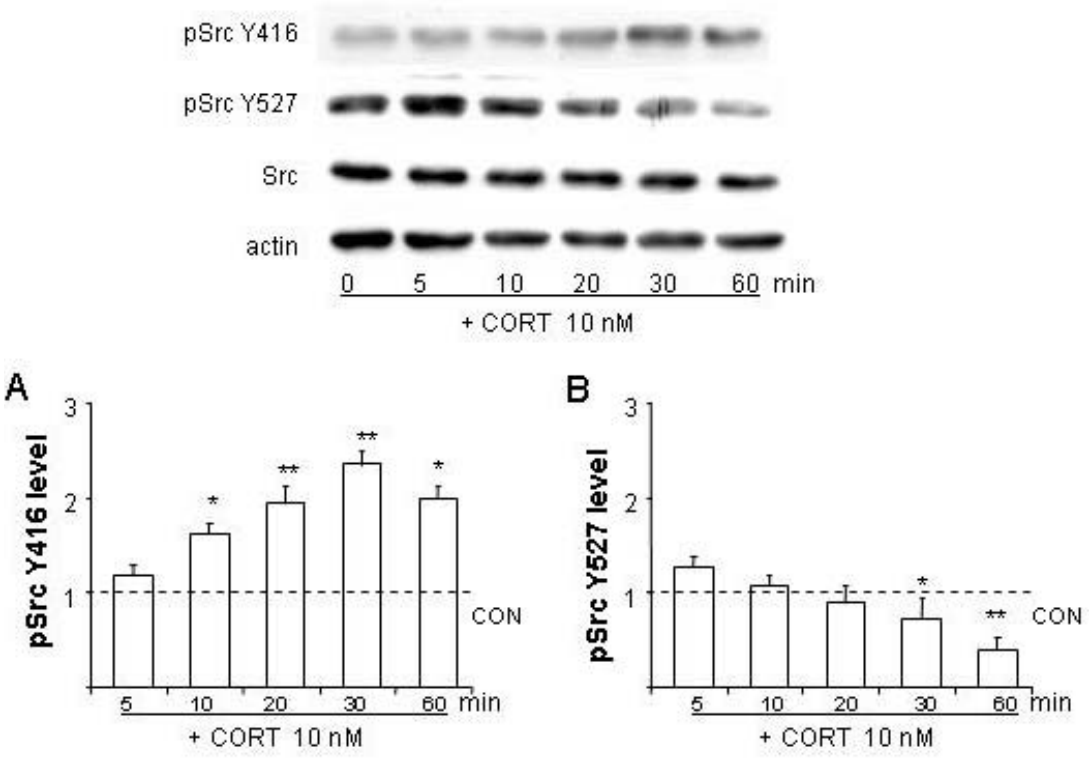


Fig. 4.8. CORT rapidly activates Src. A: After CORT treatment (10 nM), the phosphorylation level of Src tyrosine site 416 (Y416) was significantly increase from as early as 10 minutes and peaked at 30 minutes. **B**: Within 30 minutes of treatment, CORT significantly induced the dephosphorylation of Src at tyrosine site 527 (Y527). The total levels of Src level were not affected by CORT (not shown).
The image of Western blot represents results obtained in at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells.

Together, the above sets of results established activation of the Src kinase family as an essential step in the rapid signaling effects of CORT and provided the impetus to follow this line of investigation further.

4.3 Pyk2 phosphorylation mediates CORT-induced Src activation

In neurons, the Src family kinases are activated by a large number of stimuli and mechanisms (Kalia et al. 2004), with the PTK-scaffold protein Pyk2 being identified as a leading direct activator of Src (Huang et al., 2001). In light of this,

Pyk2 was next investigated as a player in the mechanisms leading to the manifestation of rapid actions of CORT.

As shown in Fig **4.9A**, CORT was found to cause robust activation of Pyk2 for between 10 and 30 minutes after application; this was evidenced by increased phosphorylation of the Y402 residue in Pyk2. Further, CORT-induced activation of Src was prevented in the presence of PF431396, a selective inhibitor of the Pyk2 kinase (**Fig 4.9B**), confirming a major role of Pyk2 in CORT-induced Src family activation.

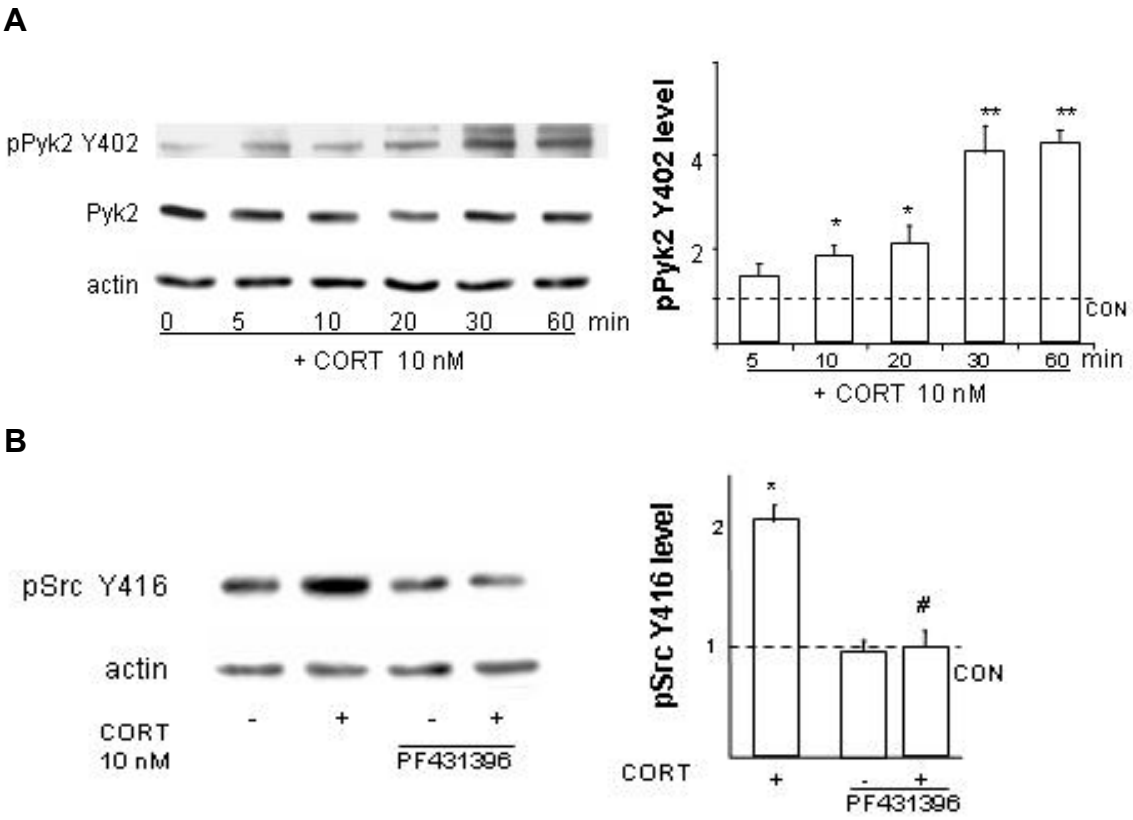
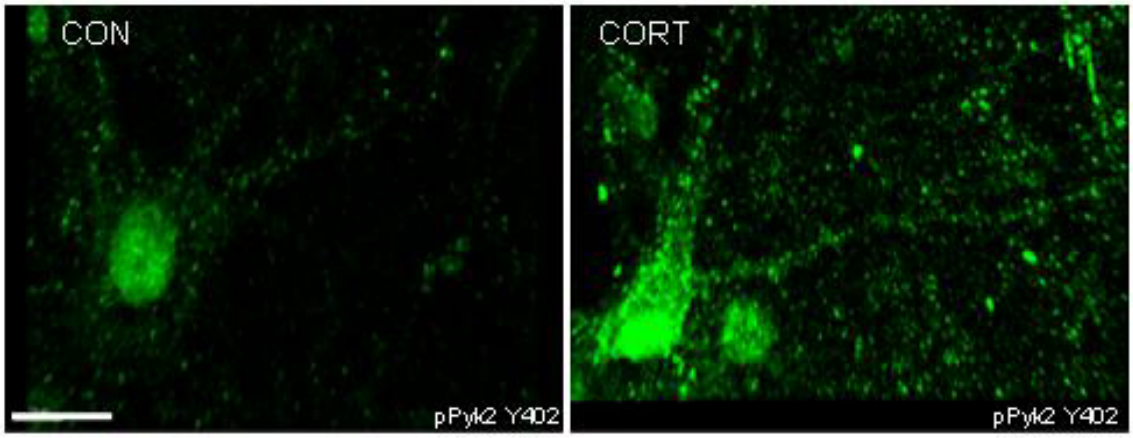


Fig. 4.9. Phosphorylation of Pyk2 occurs rapidly CORT application to hippocampal neurons. A: CORT (10 nM) induced the phosphorylation of Pyk2 at the tyrosine site 402 (Y402) epitope within 10 minutes of application(peaking after 30 minutes, while total levels of Pyk2 were unchanged. **B**: The Pyk2 inhibitor, PF431396 (3 μM, 30 minutes), abolished the CORT-induced phosphorylation of Src Y416, indicating regulation of Src activation by Pyk2. Immunoblots are representative of at least 3 different experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05 vs CORT treated cells.

Confirmation that CORT induces phosphorylation of Pyk2 was obtained by immunocytochemical analysis. For this, hippocampal neurons were subject to immunostain with a specific anti-Pyk2 antibody. Whereas only weak immuno-reactivity was detectable in the soma and nucleus in cells under baseline conditions, CORT (10 nM) treatment caused a marked increase in the intensity of immunostaining (**Fig 4.10A)**; in the latter case, the Pyk2 staining was punctuate and was seen to occur in distinct clusters along dendrites (**Fig 4.10B**).

A



B

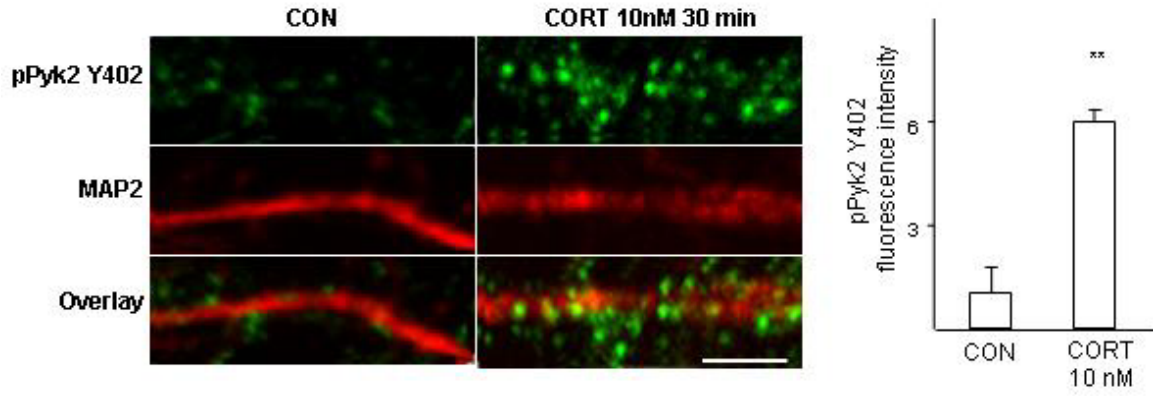


Fig. 4.10. Immunostaining of hippocampal cultures for pPyk2 (Y402). A: Treatment with 10 nM CORT (30 minutes) caused an increase in the intensity of immunostaining of the Pyk2 that was phosphorylated at tyrosine residue 402 (Y402). Immunoreactivity was observed in both, cell soma and nuclei. Scale bar represents 20 µm **B**: CORT application (10 nM, 30 minutes) caused a significant increase in the punctuate staining of pPyk2 (Y402), suggesting that Pyk2 activation was taking place in discrete subdomains along dendrites. Scale bar represents 2 µm
**: P<0.001 vs untreated (CON) cells.

The activation of Pyk2 was further characterized by analyzing the phosphorylation of four phospho-epitopes: the autophosphorylation site Y402, the kinase domain sites Y579 and Y580 (whose phosphorylation is required for full kinase activity) and Y881 (located in the C-terminal FAT domain). CORT was found to significantly trigger the phosphorylation of all four tyrosine residues (**Fig 4.11**). Phosphorylation on Y402 (site of autophosphorylation; Park et al., 2004) proved sensitive to blockade with PF431396 (Pyk2 inhibitor), but not Src inhibitors, PP2. On the other hand, phosphorylation of the Y579 residue was found to depend on activated Src since it was prevented when cells were co-treated with PP2. Importantly, CORT-induced phosphorylation on Y580 and Y881 was only partially affected by Src blockade (**Fig 4.11**), indicating the existence of additional regulatory mechanisms for the phosphorylation of these tyrosine residues.

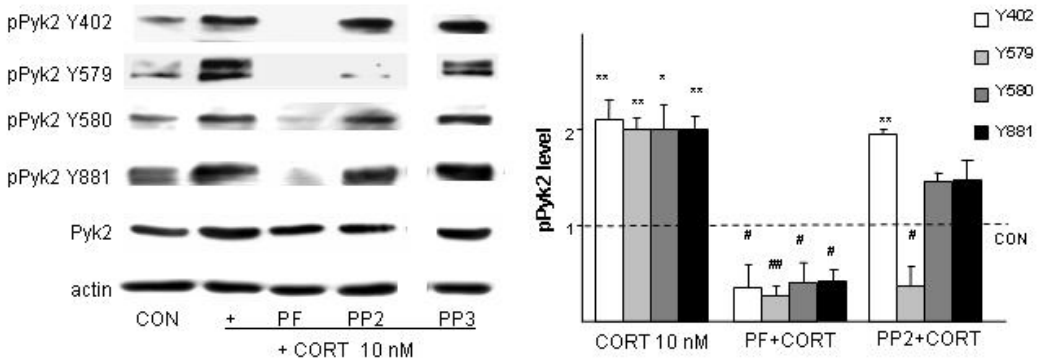


Fig. 4.11. Analysis of phosphorylated Pyk2 epitopes after CORT treatment of hippocampal cultures. The phosphorylation levels of all four Pyk2 phosphoepitopes were significantly increased after CORT treatment (10 nM, 20 minutes). These events were abolished by pretreatment with the Pyk2 inhibitor PF431396 (PF, 3 μM, 30 minutes). The Src inhibitor PP2 (1 μM, 30 minutes) did not inhibit phosphorylation of Y402, but fully blocked phosphorylation of Y579 and partly blocked phosphorylation of the Y580 and Y881 residues. The inactive analogue of PP2, PP3, did not exert any effects on any of the potentially phosphorylable Pyk2 epitopes. None of the treatments influenced the levels of total Pyk2.
The Western blot image shown is representative of at least 3 different experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

The above sets of data strongly suggest that activation of Pyk2, a downstream target of Src kinase, is likely to be a key mediator of the fast, non-genomic signalling cascade triggered by CORT at the plasma membrane.

4.4 PLC-PKC, PKA and PKB-dependent pathways mediate CORT-induced activation of Pyk2 in a convergent manner

Previous work has shown that Pyk2 is a signaling hub that is activated by multiple pathways and second messengers, most of which are Ca^{2+}- and PKC-dependent (Avraham et al., 2000). Here, CORT-induced autophosphorylation of Pyk2 on Y402 was shown to be mediated by the PLC-PKC signaling cascade; specifically, and as depicted in **Fig. 4.12A**, Pyk2 phosphorylation on Y402 was prevented when hippocampal neurons were treated with inhibitors of either PLC inhibitor (U 73122), PKC (Gö 6983) or Calmodulin (W7). Moreover, these events were shown to depend on the mobilization of intracellular Ca^{2+} stores: blockade of Ca^{2+} release by either a Ca^{2+} chelator (BAPTA-AM) or IP-3 receptor antagonist (2-APB) significantly abrogated CORT-induced activation of Pyk2 and Src (**Fig 4.12B**). Reciprocally, short-term application of the Ca^{2+} pump blocker thapsigargin (whose acute application results in increased Ca^{2+} release) mimicked the effects of CORT, as seen by the increased phosphorylation of Pyk2 and Src following application of the steroid (**Fig 4.12B**).

Further analysis in which the non-genomic CORT-induced autophosphorylation of Pyk2 was investigated by using inhibitors of either PKA (PI and H89), PI-3K (wortmannin) or PKB (API-2). The results indicated that PKC is the sole regulator of CORT-induced Pyk2 autophosphorylation since none of the above inhibitors were able to effectively abrogate the actions of CORT (**Fig 4.13A**). Given that autophosphorylation on Y402 is the earliest event in Pyk2 phosphorylation (Park et al., 2004), it was not surprising to find that the PKC inhibitor Gö 6983 also blocked he phosphorylation on Y579, Y580 and Y881 (**Fig 4.13B-D**). Interestingly, however, a different picture emerged upon inspecting the effects of PKA and PKB on the Y579 and Y580 phosphoepitopes on Pyk2. Both, PKA and PI-3K/PKB inhibitors prevented CORT-induced phosphorylation of these residues which are located in the kinase activation loop and whose phosphorylation is required for full kinase activity (**Fig 4.13B and 4.13C**). Thus, it would appear that CORT recruits other regulatory pathways that impinge on these sites.

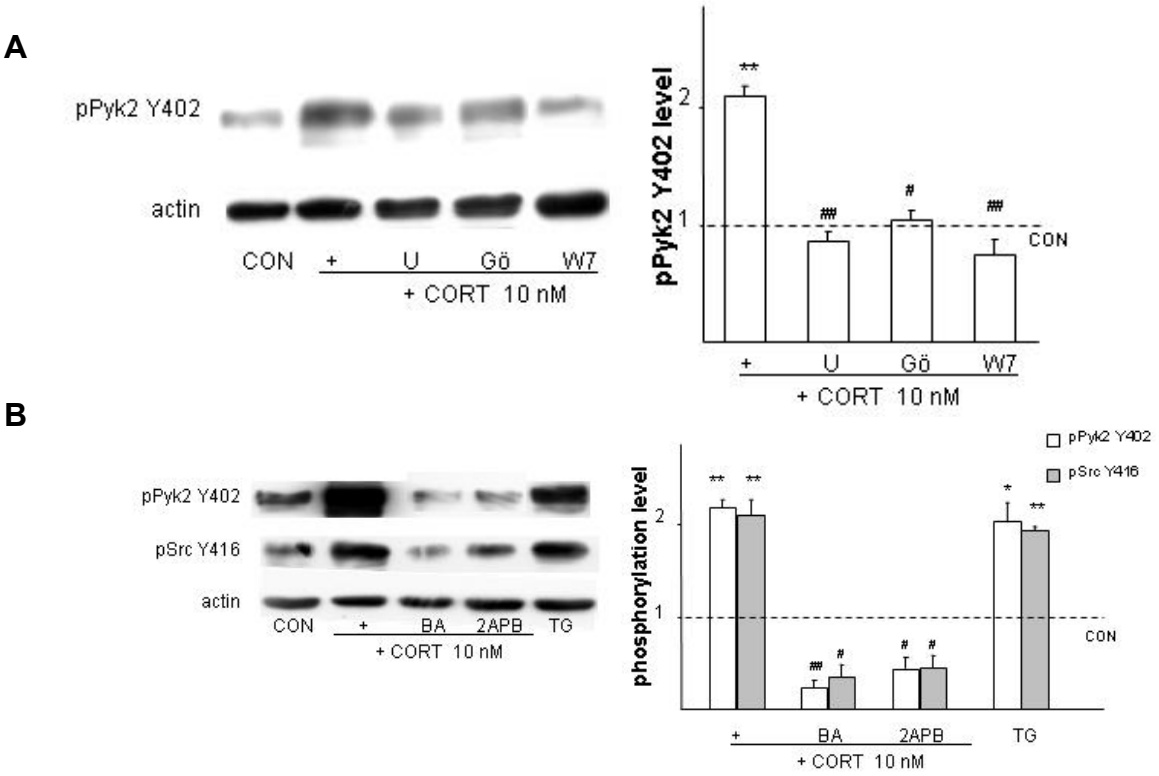


Fig. 4.12. The role of PLC-PKC and calcium ions in the actions of CORT. A: The phosphorylation of Pyk2 Y402 by CORT (10 nM, 20 minutes) was significantly inhibited by the pharmacological inhibitors of PLC (U 73122; U, 5 μM), PKC (Gö 6983; Gö. 5 μM) and calmodulin (W7, 50 μM), all present for 30 minutes. **B**: The Ca^{2+} chelator BAPTA-AM (BA. 13 μM) and IP-3 receptor antagonist 2-APB (100 μM) both prevented CORT-induced phosphorylation of Pyk2 Y402 and Src Y416 (both drugs available for 30 minutes). However, the Ca^{2+} pump blocker thapsigargin (TG, 1 μM, 30 minutes) acted like CORT in inducing phosphorylation of Pyk2 Y402.
The immunoblots shown represent results from at least 3 different experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells (CORT).

Another important finding from this set of experiments was that, although phosphorylation of the Y881 site occurs independently of PKA but depends on activation of the PI-3K/PKB pathway; specifically, while H89 did not prevent CORT-induced phosphorylation at Y881, both wortmannin and API-2 abrogated the actions of CORT at this phospho-epitope (**Fig 4.13D**). The demonstrated role of PKA and PKB in the regulation of phosphorylation of Pyk2 tyrosine residues implies involvement of an additional mediator. Accordingly, in a subsequent step, we tested the importance of PTP-PEST (see Chapter 1) since this molecule was previously shown to regulate Pyk2 (Lyons et al., 2001; Davidson et al., 2010).

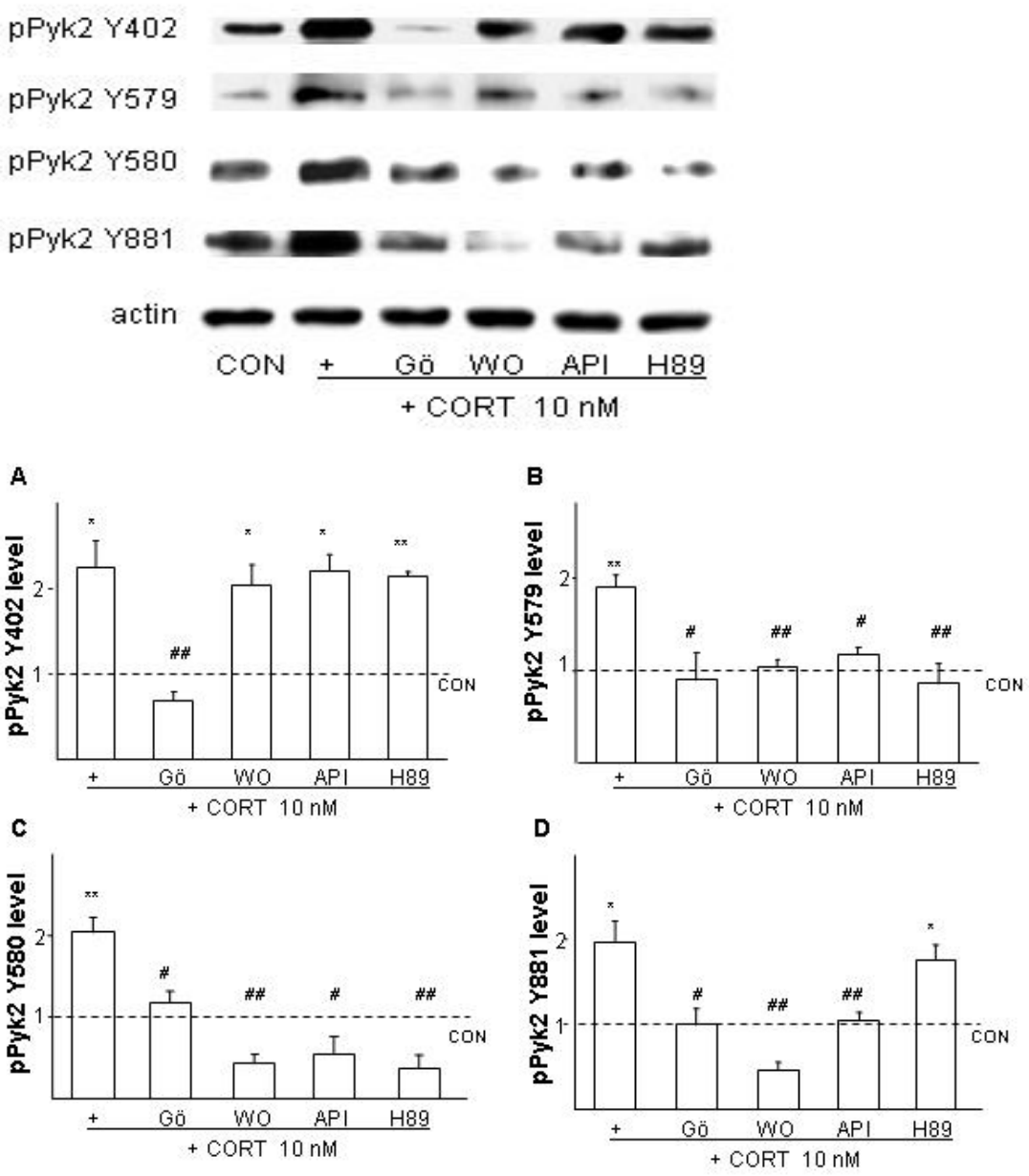


Fig. 4.13. The role of PKC, PKB and PKA in regulating Pyk2 epitope phosphorylation. A. The CORT-triggered (10 nM, 20 minutes) phosphorylation of Pyk2 at Y402 was significantly inhibited by a PKC inhibitor (Gö 6983, Gö, 5 μM, 30 minutes), but not by inhibitors of PI3K (wortmannin, WO 4 μM, 30 minutes), PKB (API-2, API. 30 μM, 30 minutes) or PKA (H89, 1 μM, 30 minutes). **B**. All of inhibitors abolished the ability of CORT to induce phosphorylation of Pyk2 at Y579 and **C**, all inhibitors exerted similar effects on Pyk2 Y580. **D**: The Pyk2 Y881 phosphorylation induced by CORT was inhibited by inhibitors of PKC, PI3K and PKB, but not by inhibitors of PKA.
Th immunoblot shown is representative of at least 3 separate experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

Results shown in **Fig. 4.14A** demonstrate that CORT (10 nM) induces phosphorylation of PTP-PEST at serine 39 (S39) within 5 minutes of application (peak of effect at 20-30 minutes). Importantly, phosphorylation of this specific site has previously been reported to result in an inhibition of PTP-PEST activity (Garton and Tonks, 1994; Nakamura et al., 2010). Notably, inhibitors of both PKA and PKB prevented CORT-induced PTP-PEST phosphorylation in hippocampal neurons (**Fig 4.14B**).

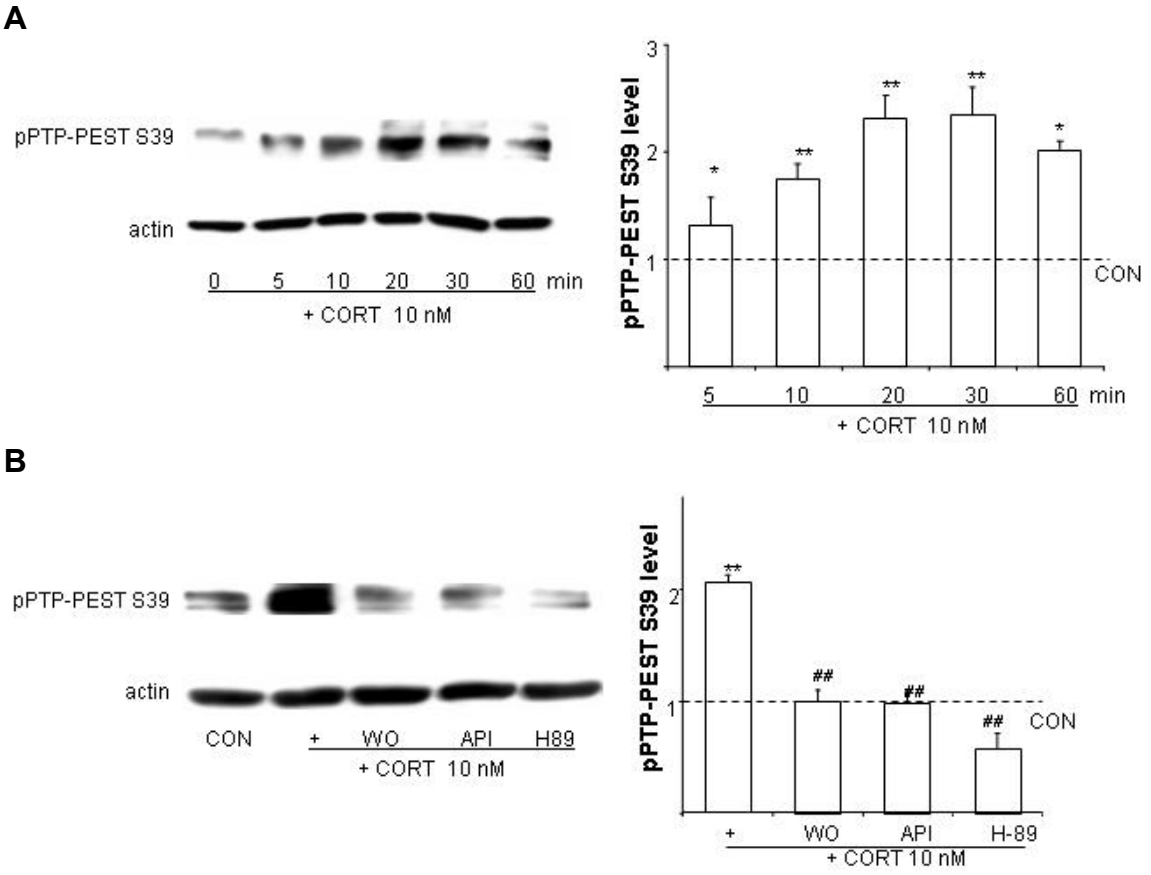


Fig. 4.14. Role of the cytoplasmic protein tyrosine phosphatase, PTP-PEST. A. Treatment of hippocampal cultures with CORT (10 nM) induced phosphorylation of PTP-PEST at serine 39 (S39) within 5 minutes and the effect peaked at 20-30 minutes. **B**: The inhibitors of PI3K (wortmannin, WO, 4 µM, 30 minutes), PKB (API, 30 µM, 30 minutes) and PKA (H89, 1 µM, 30 minutes) abolished the above response to CORT.
The immunoblots are representative of at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

To sum up, the above series of experiments suggest that Pyk2 is activated within a short time of exposure of hippocampal neurons to CORT. This activation of Pyk2 occurs through the participation of several signalling pathways: one, the PLC-PKC pathway induces autophosphorylation of Pyk2; the other pathways involved are PKA and PKB pathways which appear to regulate Pyk2 activity by inhibiting the activity of PTP-PEST.

4.5 Involvement of a membrane-bound G-protein coupled receptor in the rapid signaling effects of CORT

The above-described involvement of the PLC-PKC and PKA pathways in the fast actions of CORT raised the intriguing possibility that a G-protein-mediated mechanism may initiate the non-genomic rapid signaling actions of CORT. To address this, hippocampal neuronal cells were treated with GDP-β-S, a non-hydrolysable GDP analogue that traps G-proteins in an inactive conformation. This treatment blocked the ability of CORT to induce phosphorylation of Pyk2 and Src (**Fig 4.15A**), a result that strongly pointed to involvement of G-proteins. This view was further confirmed in experiments in which hippocampal neurons were exposed to CORT in the presence or absence of pertussis toxin (PTX), a potent inhibitor of Gi/o proteins, as depicted in (**Fig 4.15B**).

To strengthen the hypothesis that a G-protein coupled receptor (GPCR) might mediate the fast, non-genomic actions of CORT, studies were next performed using cell-impermeable CORT-BSA. The latter triggered both Pyk2 and Src phosphorylation to the same extent as unconjugated CORT, albeit in a PTX-sensitive manner; this action of CORT-BSA was also subject to inhibition when a PKC inhibitor (Gö 6983) was co-applied (**Fig 4.16**).

In contrast, CORT-BSA-activated signaling persisted even in the presence of antagonists of classical (nuclear) GR (RU38486 and J2700) and MR (RU28318, Eplerenone and Spironolactone), as shown in **Fig 4.17A**. In addition, a novel GR

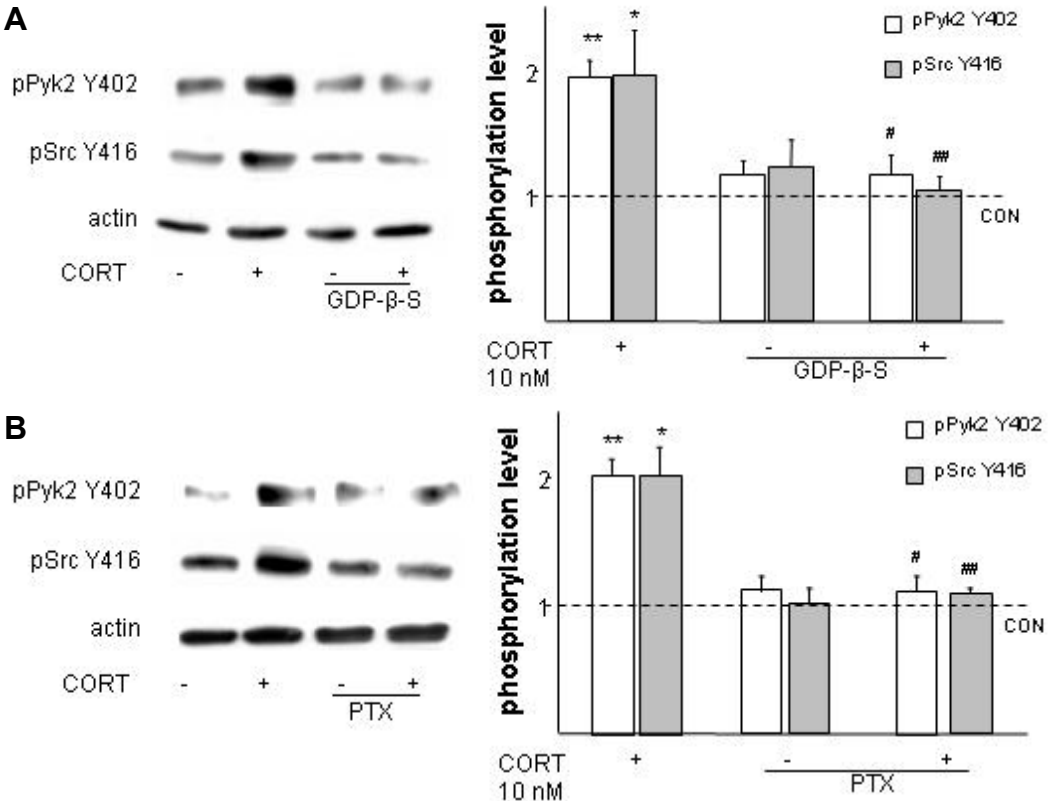


Fig. 4.15. Involvement of a G-protein-mediated mechanism. A. Treatment of hippocampal cells with the non-hydrolysable GDP analogue, GDP-β-S (100 µM, 30 minutes), abolished CORT-induced phosphorylation of both Pyk2 Y402 and Src Y416. **B**. Similar results were obtained after treatment with Gi/o protein inhibitor, pertussis toxin (PTX, 500 ng/mL, 120 minutes).
The images shown are representative of results obtained in at least in at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells (CORT).

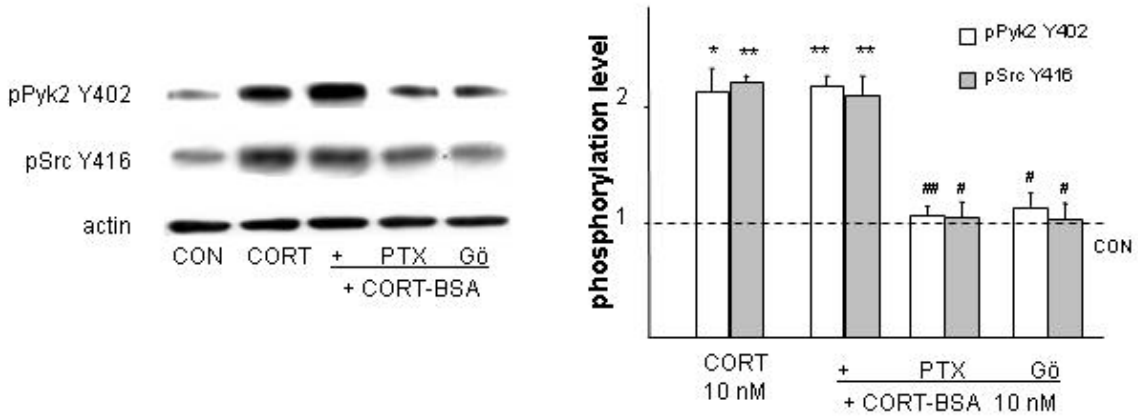


Fig. 4.16. A cell impermeable CORT-BSA conjugate (10 nM, 20 minutes) rapidly induced the activation of both Pyk2 and Src, effects that were blocked by pertussis toxin (PTX, 500 ng/mL, 120 minutes) an inhibitor of Gi/o proteins and Gö 6983, Gö (5 µM, 30 minutes) a PKC inhibitor.
The immunoblot shown is representative of at least 3 individual experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT-BSA treated cells.

antagonist, CORT 108297, failed to influence the effects of both CORT and CORT-BSA in terms of activation of Pyk2 and Src (**Fig 4.17B**).

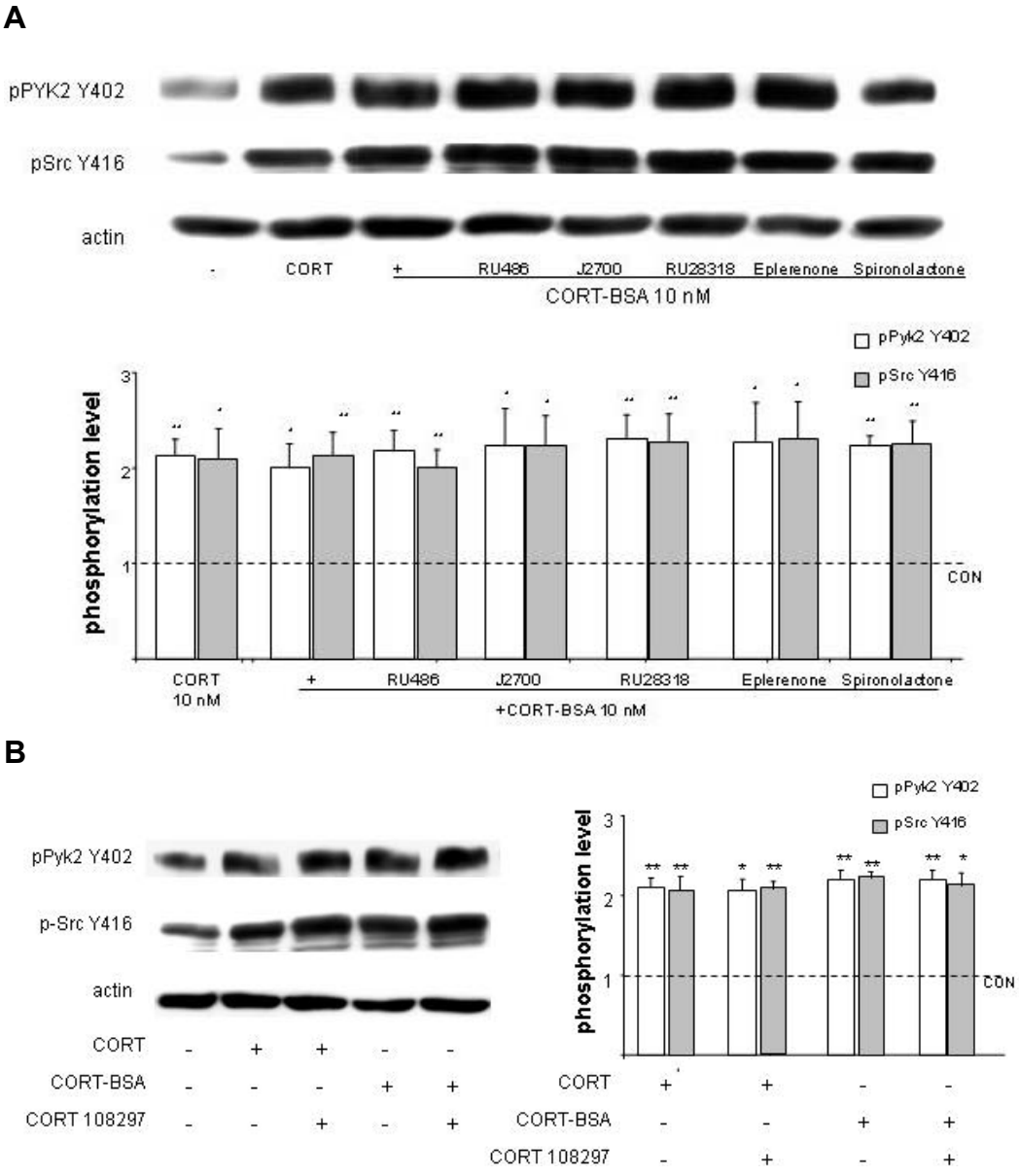


Fig. 4.17. Lack of effects of antagonists of nuclear GR and MR. A. Co-incubation of cells with CORT-BSA and either nuclear GR (RU 486 and J2700, 100 nM, 30 minutes) or nuclear MR (RU28318, Eplerenone and Spironolactone,100 nM, 30 minutes) antagonists suggested that nuclear GR and MR are not required for manifestation of the rapid phosphorylation of Pyk2 Y402 and Src Y416 by CORT-BSA (10 nM, 20 minutes); these drugs also did not exert any effect on the actions of CORT (not shown). **B.** Similarly, a novel GR antagonist (CORT 108297, 100 nM, 30 minutes) did not affect the rapid effects of either CORT or CORT-BSA.
The immunoblot images are representative of results obtained in at least 3 experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells.

However, both CORT- and CORT-BSA-induced phosphorylation of Pyk2 and Src was prevented when cultured hippocampal neurons were treated in the presence of G15, a novel antagonist of the putative GPCR (termed GPR30) that is increasingly considered to mediate estrogen signaling at the cell surface (**Fig 4.18**).

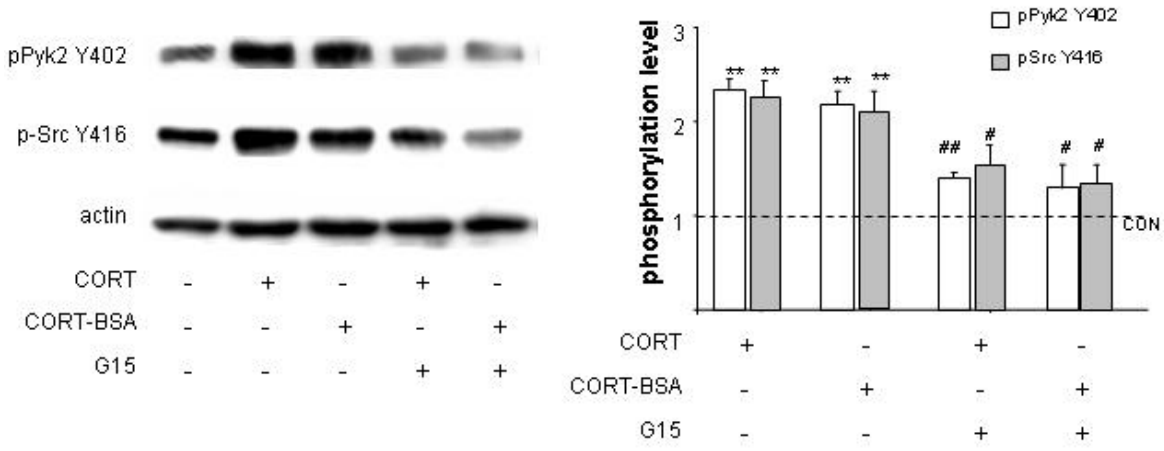


Fig. 4.18. Attenuation of the induction of Pyk2 Y402 and Src Y416 by CORT and CORT-BSA by G15. The antagonist of the G-protien-coupled receptor GPR30, G15 (1 µM, 30 minutes) caused a significant reduction of the effects of CORT and CORT-BSA on the induction of Pyk2 Y402 and Src Y416, suggesting mediation of the steroid effects by GPR30- A representative immunoblot from at least 3 independent experiments is shown. *: P<0.05, **: P < 0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT or CORT-BSA treated cells.

Together, the evidence described here goes against the idea that classical (nuclear) receptors (GR or MR) mediate the rapid actions of CORT on intracellular signalling pathways. Rather, the results presented in this section imply that the fast actions of CORT are mediated by a Gi/o protein. Moreover, the data suggest that the GPCR that mediates CORT actions at the plasma membrane maybe be identical, or very similar, to GPR30.

4.6 Pyk2 mediates CORT induces activation of c-Abl and RhoA

Lying downstream of many GPCR, the cellular oncogene c-Abl is a non-receptor tyrosine kinase. It is localized in dynamic actin structures where, by phosphorylating various target proteins, it serves to regulate cytoskeleton remodeling in many contexts, including cell differentiation, cell division and cell adhesion. Very recently, c-Abl has been implicated in Alzheimer's disease pathology since it is a potent Tau kinase (Schlatterer et al., 2011). The latter authors noted that c-Abl is only found in neurons (not in glial cells) and suggested its activation might depend on neuronal activity. Lastly, in the context of the present work, it is worth mentioning that c-Abl is recruited to the phosphorylated Y881 site of Pyk2 (Zrihan-Licht et al., 2004), making it a relevant candidate for further investigation.

Here, hippocampal neurons were treated with CORT (10 nM) up to 60 minutes and the levels of active c-Abl (i.e. phosphorylated on Y412) were probed using a phospho-specific antibody. Application of CORT triggered c-Abl phosphorylation within 20 minutes and peak (> 3-fold) increases were observed after 60 minutes. Temporally, the increase in activated c-Abl was delayed in comparison to that of phospho-Pyk2 and phospho-Src whose upregulation was detectable 5-10 minutes after treatment of neurons with CORT (**Fig 4.19A**). In fact, as shown in **Fig 4.19B,** activation of c-Abl required Pyk2 and Src activity: the appearance of activated c-Abl was blocked in the presence of inhibitors of Pyk2 (PF431396) and Src (PP2).

Interestingly, the specific antagonist of NMDAR, MK801, failed to inhibit CORT-induced activation of c-Abl (**Fig 4.19C**). This observation indicates that the actions of CORT do not depend on activation of the NMDA receptor (NMDAR) and that the non-genomic signaling cascade triggered by CORT involves activation of c-Abl through Pyk2 and Src kinases.

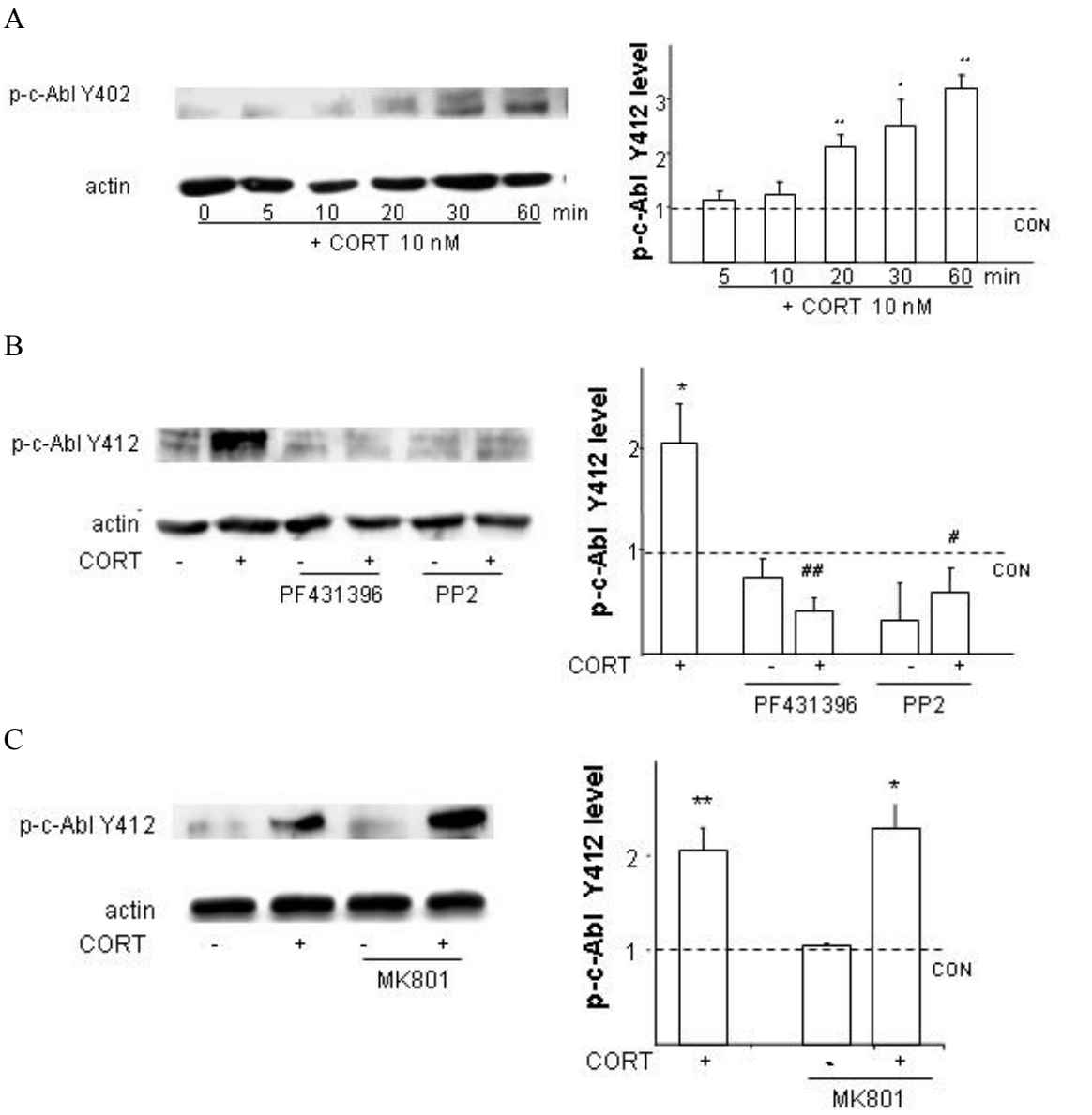


Fig. 4.19. c-Abl is a further downstream target of CORT actions. A. Exposure of hippocampal cultures to CORT (10 nM) was followed by a rapid increase (within 20 minutes) in the levels of phosphorylated c-Abl Y412; the increase in phosphorylated c-Abl Y412 reached a peak after 60 minutes. **B**. Both, a Pyk2 inhibitor (PF431396, 3 μM, 30 minutes) and an inhibitor of Src (PP2, 1 μM, 30 minutes) abolished this CORT-induced effect on CORT-induced c-Abl. **C**. The NMDAR antagonist, MK801 (10 μM, 30 minutes) did not inhibit the activation of c-Abl by CORT.
The immunoblot images shown are representative of at least 3 experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

Since Abl is a major regulator of the actin cytoskeleton (Colicelli, 2010) and of the postsynaptic density (PSD; de Arce et al., 2010), the ability of CORT to influence integrity of the PSD through the mediation of activated c-Abl. To this end, hippocampal neurons were treated with CORT (10 nM, 30 minutes) for detecting the expression of drebrin, an F-actin binding protein was monitored by confocal microscopy (Takahashi et al., 2006). At baseline, immunostained drebrin was largely punctuated and strongly apposed to synapsin-positive puncta, as predicted by the localization of drebrin in dendritic spines (**Fig 4.20A**). Drebrin immunoreactivity in the dendritic spines was strongly enhanced in the presence of CORT (**Fig 4.20B**), with a marked increase in the spine-to-shaft ratio. Importantly, inhibitors of Pyk2 and Src kinases prevented these effects of CORT (**Fig 4.20C,D**), as did the c-Abl inhibitor (STI-571) (**Fig 4.20E**); notably, STI-571 also reversed the spine-to-shaft gradient of drebrin localization. In parallel, CORT triggered an increase in PSD-95 cluster size (**Fig 4.21F,G**), an effect that was prevented by pharmacological inhibition of Pyk2, Src and c-Abl (**Fig 4.21H-J**).

RhoA, a member of the small GTPase Rho family, has been extensively studied for its role in cellular processes and functions, including remodeling of the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Using an advanced enzyme-linked immunosorbent assay for small GTPase, RhoA activation was shown to be detectable in cultured hippocampal neuronal cells. As shown in **Fig 4.21,** RhoA activation was significantly increased in cells pretreated with CORT (10 nM, 30 minutes) compared to normal cells. This effect of CORT was abolished by the inhibitors of G-proteins (PTX), Pyk2 (PF431396) and Src (PP2), but not by the c-Abl inhibitor, STI-571, suggesting that the RhoA activation pathway induced by CORT is regulated by G-protein and Py2/Src kinases, but not by c-Abl kinase.

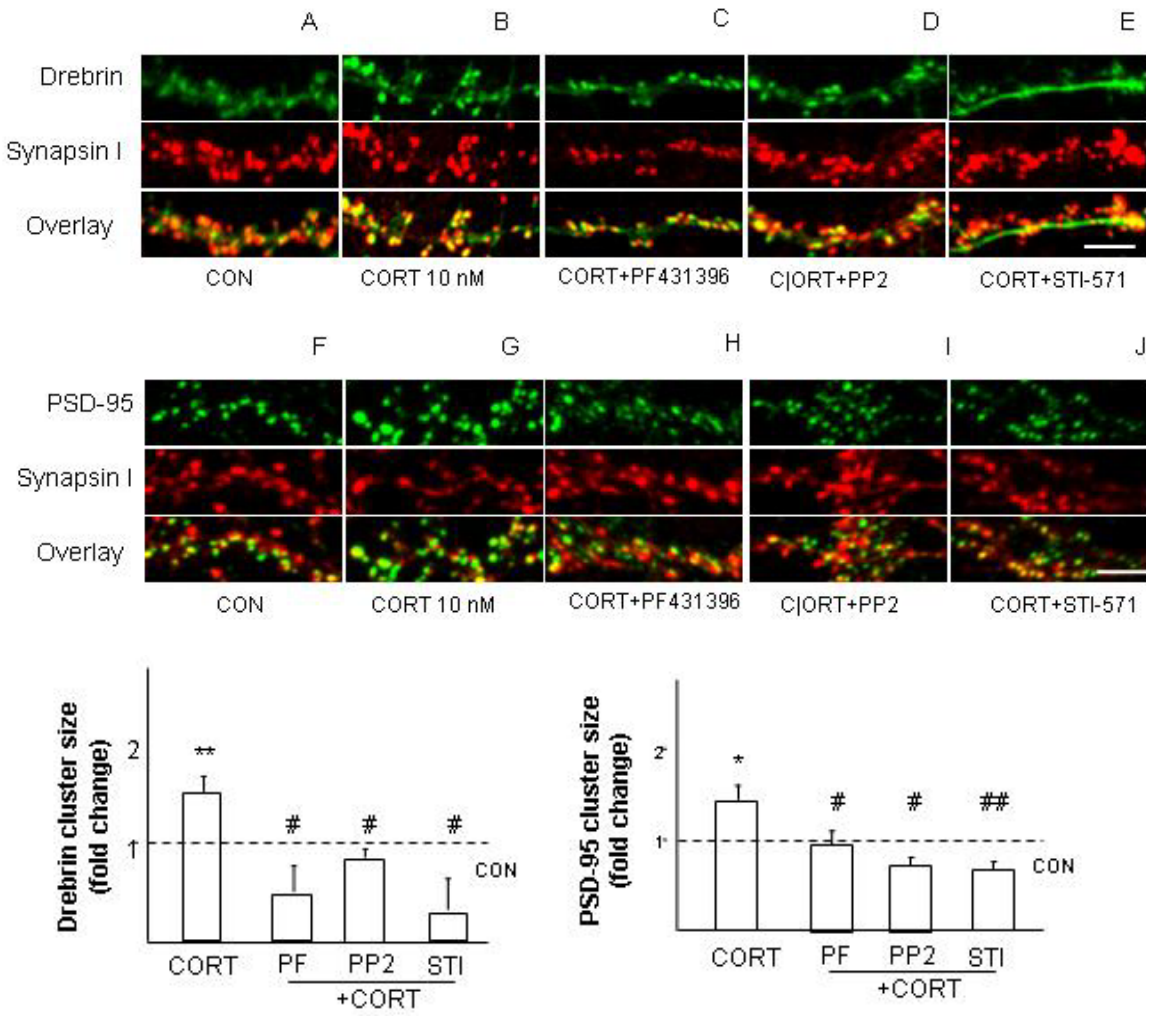


Fig. 4.20. Confocal microscopical analysis of drebrin and PSD-95 immunostaining in hippocampal cultures. A: Under baseline conditions, drebrin co-localized with synapsin-positive puncta in dendritic spines. **B**: Exposure of cultures to CORT (10 nM, 30 minutes) resulted in enhanced the drebrin immunoreactivity and increased size of drebrin clusters. **C:** The effects of CORT were abolished by the Pyk2 inhibitor PF431396 (PF, 3 μM, 30 minutes) and, as shown in **D,** by the Src inhibitor (PP2, 1 μM, 30 minutes). **E**: The c-Abl inhibitor, STI-571 (STI, 1 μM, 30 minutes) also abrogated the effects of CORT. Scale bar represents 2 μm. Similar effects were also observed in immunostaining of PSD-95: **F.** PSD-95 cluster size in dendritic spine of normal hippocampal cells. **G**: CORT (10 nM, 20 minutes) significantly size of PSD-95 clusters. **H**: The effects of CORT were attenuated by the Pyk2 inhibitor PF431396 (PF, 3 μM, 30 minutes), **I,** by the Src inhibitor (PP2, 1 μM, 30 minutes), and **J,** by he c-Abl inhibitor, STI-571 (STI, 1 μM, 30 minutes). Scale bar represents 2 μm.
*: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001vs CORT treated cells (CORT).

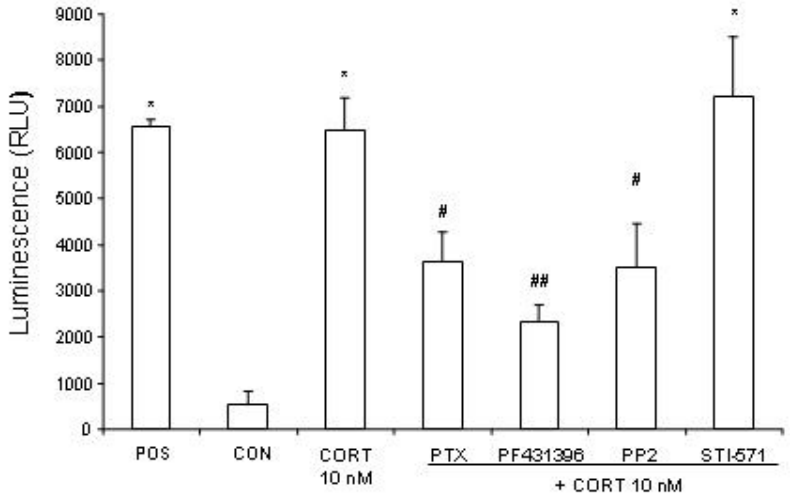


Fig. 4.21. Measurement of RhoA activation using GLISA kit. RhoA activation was significantly increased after CORT treatment (10 nM, 30 minutes). The inhibitors of G-protein (PTX, PTX, 500 ng/mL, 120 minutes), Pyk2 (PF431396, 3 μM, 30 minutes) and Src (PP2, 1 μM, 30 minutes) abolished this effect, but c-Abl inhibitor, STI-571 (1 μM, 30 minutes), did not affect RhoA activation. The experiments were repeated three times in independent cultures. The results are presented in terms of relative luminescence units (RLU). POS: positive control, provided by kit manufacturer.
*: $P<0.05$ vs untreated (CON) cells. #: $P<0.05$, ##: $P<0.001$vs CORT treated cells (CORT).

The data presented in this section show that CORT sequentially activates Pyk2, Src and c-Abl. These events do not require activation of NMDAR. The activation of c-Abl has an impact on both drebrin and PSD structures, most likely through the actin cytoskeleton-remodelling actions of c-Abl. Additionally, the activation of RhoA is caused by CORT, through a Pyk2/Src-regulated but c-Abl-independent pathway.

4.7 Functional consequence of non-genomic CORT signaling in hippocampal neurons

Having identified several key events in the non-genomic signaling cascades initiated by CORT, potential functional endpoints of the cascade was investigated. Given the above-mentioned changes in PSD structure, the study focused on the NMDAR. Previous work suggests that NMDAR subunits are the most abundant phosphotyrosine proteins present in synapses (Trinidad et al., 2008) and, at the same time, are substrates of several Src family kinases. It is also known that tyrosine phosphorylation of the NR2 subunit regulates NMDAR trafficking and membrane insertion (Salter and Kalia, 2004). The question asked here was whether CORT affect NMDAR trafficking and clustering through tyrosine kinase activation.

The above question was addressed by treating cultured hippocampal neurons were treated with CORT (10 nM) before immunostaining surface NR1 subunits under non-permeabilizing conditions using a monoclonal antibody directed against the extracellular epitope of NR1. It was found that 20 minutes treatment with 10 nM CORT markedly upregulates surface NR1 levels (**Fig 4.22A,C**) as well as synaptic NR2B (**Fig 4.22B,D**). These increases are subject to abrogation by inhibitors of Pyk2 and Src, but not by the inhibitor of c-Abl (ST-571). In support of these results, it was also found that CORT increases the phosphorylation of Y1472 on the cytoplasmic tail of the NR2B subunit, an event known to block endocytosis and increases surface trafficking of the receptor (Lavezzari et al., 2004; Goebel et al., 2005).

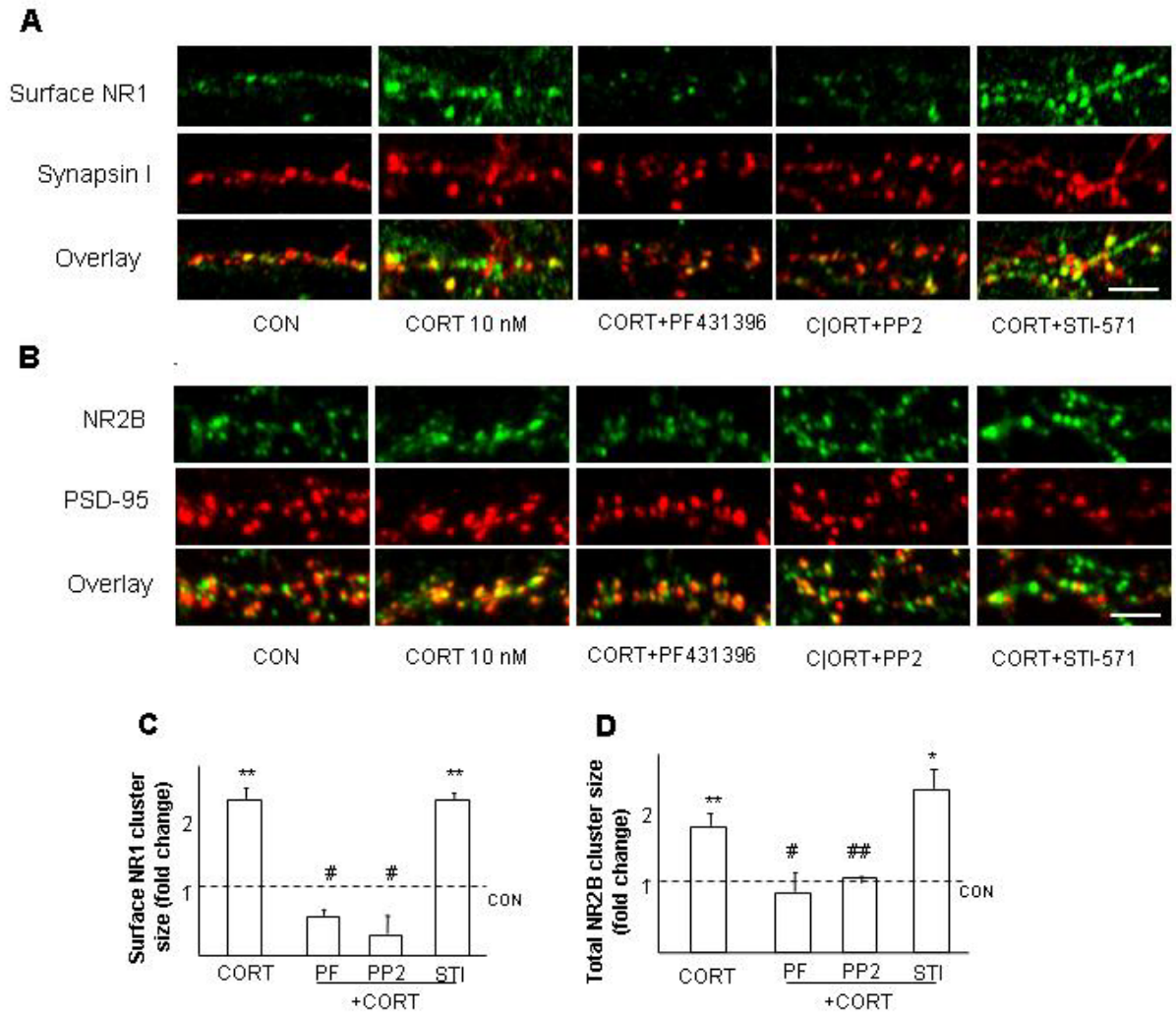


Fig. 4.22. Immunostaining of surface NR1 and synaptic NR2B in hippocampal neuronal cultures treated with CORT. A, C Surface expression of NR1 was significantly increased after CORT treatment (10 nM, 20 minutes) in manner that was sensitive to inhibition by inhibitors of Pyk2 (PF431396; PF, 3 μM, 30 minutes) Src (PP2; 1 μM, 30 minutes). However, the c-Abl inhibitor, STI-571 (STI, 1 μM, 30 minutes) did not interfere with the actions of CORT. Scale bar represents 2 μm. **B, D** Similar effects were observed upon inspection of synaptic expression of NR2B; specifically, CORT induced synaptic levels of NR2B that were subject to inhibition by inhibitors of Pyk2 and Src, but not of c-Abl. Scale bar represents 2 μm.
*:P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT-treated cells.

The latter effect is sensitive to inhibition by inhibitors of both Pyk2 and Src, but not of c-Abl (**Fig 4.23A**). Additionally, CORT-induced phosphorylation of NR2B Y1472 was abolished by another Src inhibitor SU6656, but not by PP3, an inactive form of the Src inhibitor PP2, further confirming the regulatory role of Src kinase in NR2B Y1472 phosphorylation (**Fig 4.23B**).

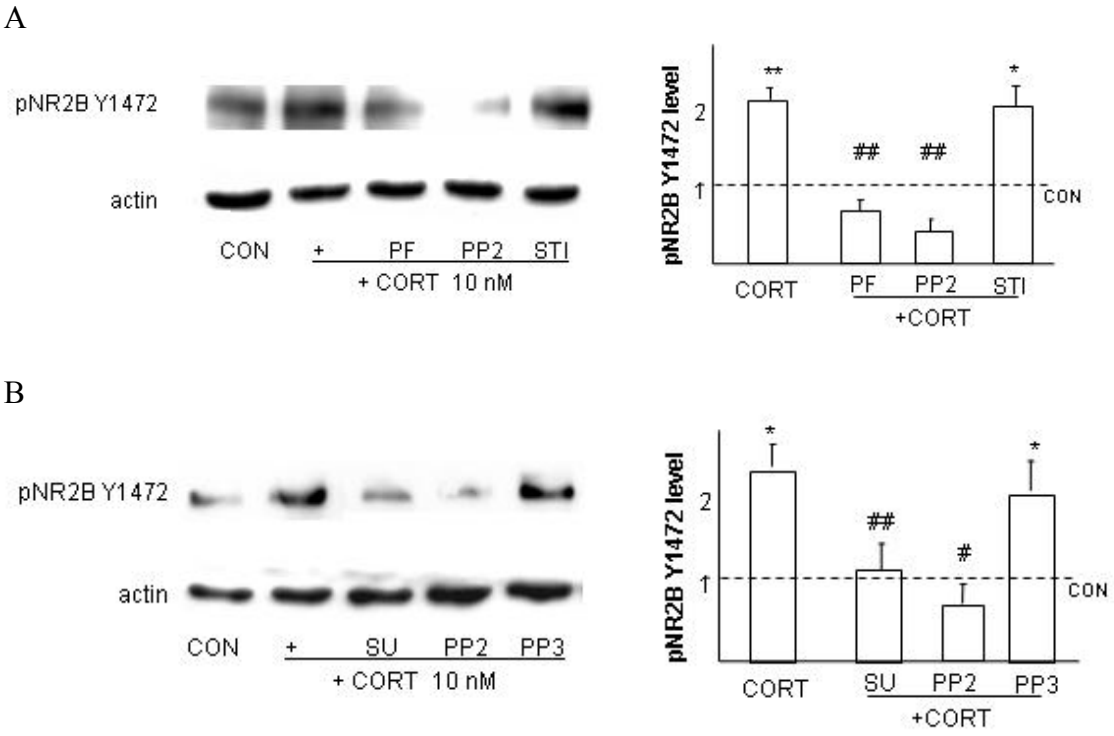


Fig. 4.23. Phosphorylation of NR2B Y1472 induced by CORT. A. Treatment of hippocampal cultures with CORT (10 nM, 20 minutes) led to an increase in the levels of phosphorylated NR2B at tyrosine site 1472 (Y1472), an effect abolished by both a Pyk2 inhibitor (PF431396, 3 μM, 30 minutes) and Src inhibitor (PP2, 1 μM, 30 minutes), but not the inhibitor of c-Abl inhibitor, STI-571 (STI, 1 μM, 30 minutes). **B**. Both Src inhibitors, SU6656 (SU, 1 μM, 30 minutes) and PP2 (1 μM, 30 minutes), inhibited CORT-induced NR2B phosphorylation; PP3 (1 μM, 30 minutes), the inactive analogue of PP2, had no effect.
The Western blot images are representative of at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

As a consequence of the CORT-upregulated surface expression, there was a significant increase in the levels of activated ERK1/2 (**Fig 4.24A**), which was mentioned in **Section 4.1**. This increase was completely blocked when the NMDAR antagonist MK801 was present (**Fig 4.24B**).

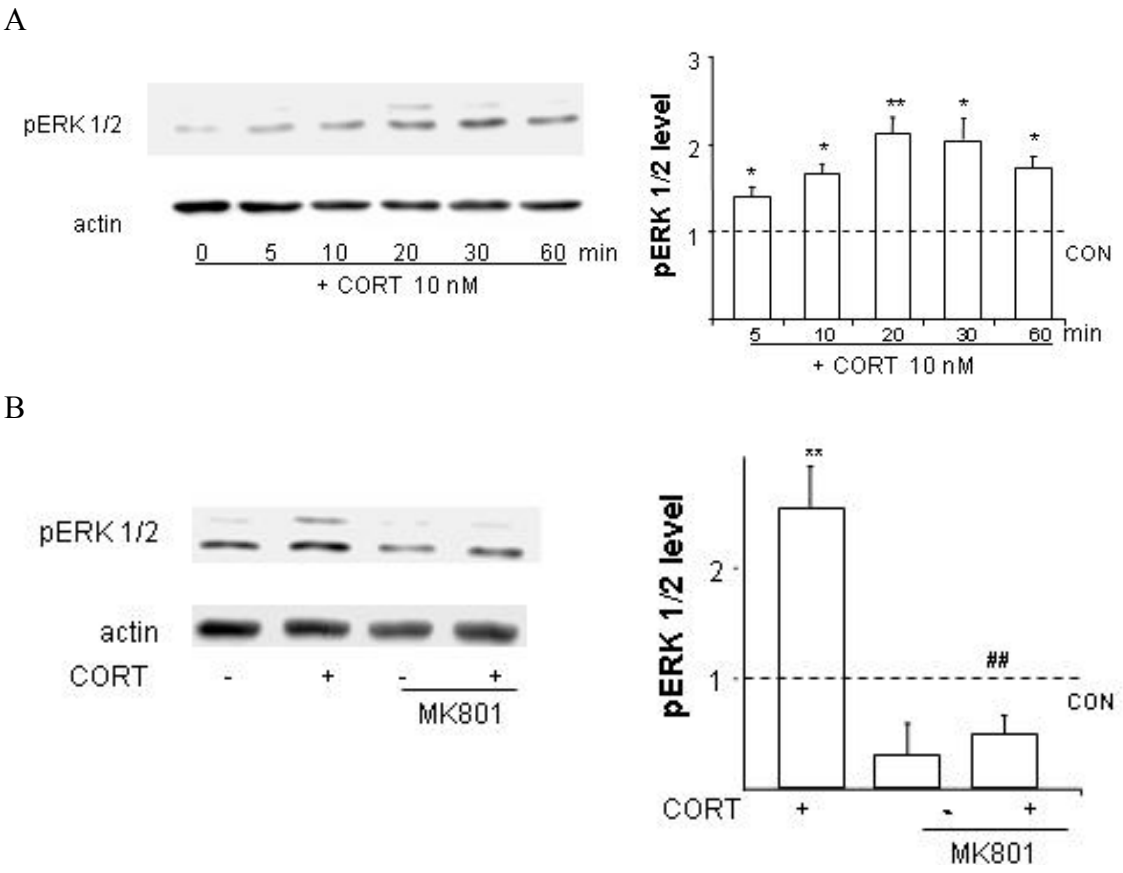


Fig. 4.24. CORT induction of ERK phosphorylation. A. CORT (10 nM) stimulated ERK 1/2 phosphorylation within 5 minutes of application, the maximum effects occurring at 20 minutes. **B**. The activation of ERK 1/2 was completely abrogated in the presence of the NMDAR antagonist, MK801 (10 μM, 30 minutes).
The immunoblot images are representative of at least 3 experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

Interestingly, when hippocampal neurons that had been pretreated with CORT (10 nM, 20 minutes) were challenged with NMDA (100 nM, 5 minutes), resulted in only a minor change (no significant change was observed) in the levels of phosphorylated ERK 1/2 phosphorylation (**Fig 4.25**); this observation suggests that the initial exposure to CORT occluded further agonist-induced NMDAR activation.

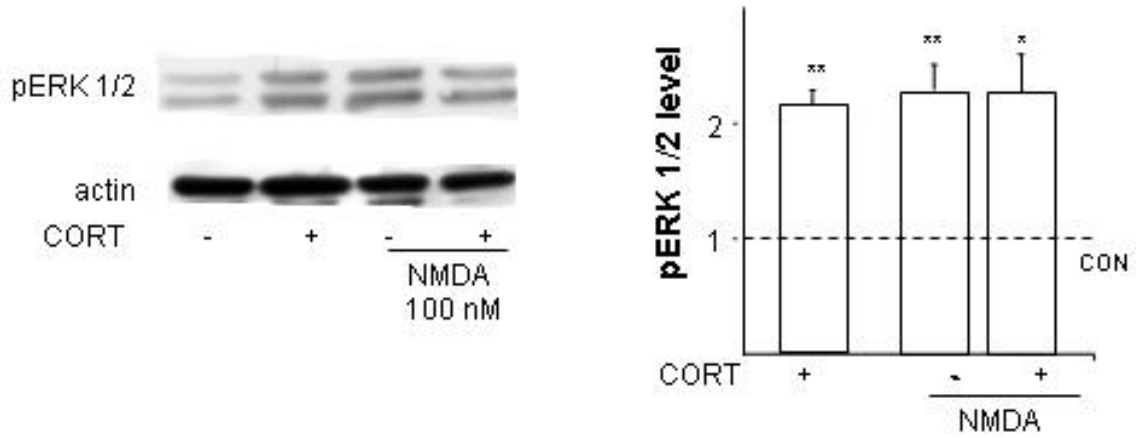


Fig. 4.25. NMDAR activation produces effects on ERK 1/2 phosphorylation that are similar to those observed with CORT. Hippocampal cultures exposed to NMDA (100 nM, 5 minutes) showed an induction of ERK 1/2 phosphorylation. However, application of NMDA (100 nM, 5 minutes) after initial treatment with CORT (10 nM, 20 minutes) did not elicit a greater response than either drug alone.
The immunoblot image is representative of at least 3 independent experiments. *: $P<0.05$, **: $P<0.001$ vs untreated (CON) cells.

Together, this last set of data show that CORT actions at the neuronal plasma membrane can lead to tyrosine phosphorylation of NR2B; this results in an upregulation of the amount of NMDAR on the cell surface and amplification of the NMDAR-mediated signalling cascade.

4.8 The activation of MAPK cascade and transcriptional events.

As mentioned in **Section 4.1**, CORT rapidly induces phosphorylation of all three MAPK family members. One of them, ERK 1/2 is known to induce synaptic plasticity in the hippocampus in an NMDAR-dependent manner (Kaphzan et al. 2006). Since CORT-induced ERK 1/2 phosphorylation was abolished in the presence of the NMDAR antagonist, MK801, and not increased after further challenging with NMDA after CORT treatment, it appears that the activation of ERK 1/2 induced by CORT is mediated by a cascade of events downstream of the NMDAR. As CaMKII, an NMDAR-associated protein (Yan et al. 2011) was previously shown to regulate activation of ERK/MAPK signaling in PC 12 cells (Liu et al. 2009), the involvement of this kinase was investigated next.

Treatment of hippocampal neuronal cultures with CORT (10 nM) rapidly induced phosphorylation of CaMKII at the autophosphorylation threonine 286 site (T286) that allows full kinase activity (Yang and Schulman, 1999), with a peak increase appearing as early as 5 minutes (**Fig 4.26A**) after application of CORT. Importantly, the CORT-induced effect was attenuated by MK801, the NMDAR antagonist (**Fig 4.26B**), indicating NMDAR mediation of the effect.

The cAMP response element-binding (CREB), an important transcription factor in neurons, has been reported to be regulated by synaptic NMDAR and to play an important role in transcription-dependent memory and cognitive functions (Hardingham et al. 2001). To investigate the impact of CORT on CREB, inhibitors of CaMKII (CI), RAF (GW 5074) and MAPK (PD98059 and U0126) were applied in combination with CORT (10 nM, 20 min). As shown in **Fig 4.27A,** CORT-induced phosphorylation of CaMKII was inhibited by the CaMKII inhibitor, but not by the inhibitors of RAF and MAPK; Further, the CORT-induced activation of ERK 1/2 was attenuated in the presence of inhibitors of CaMKII and RAF and abolished in the presence of both MAPK inhibitors (**Fig 4.27B**). Lastly, CORT induced significant phosphorylation of CREB, an effect that was abrogated by all of four inhibitors (**Fig 4.27C**).

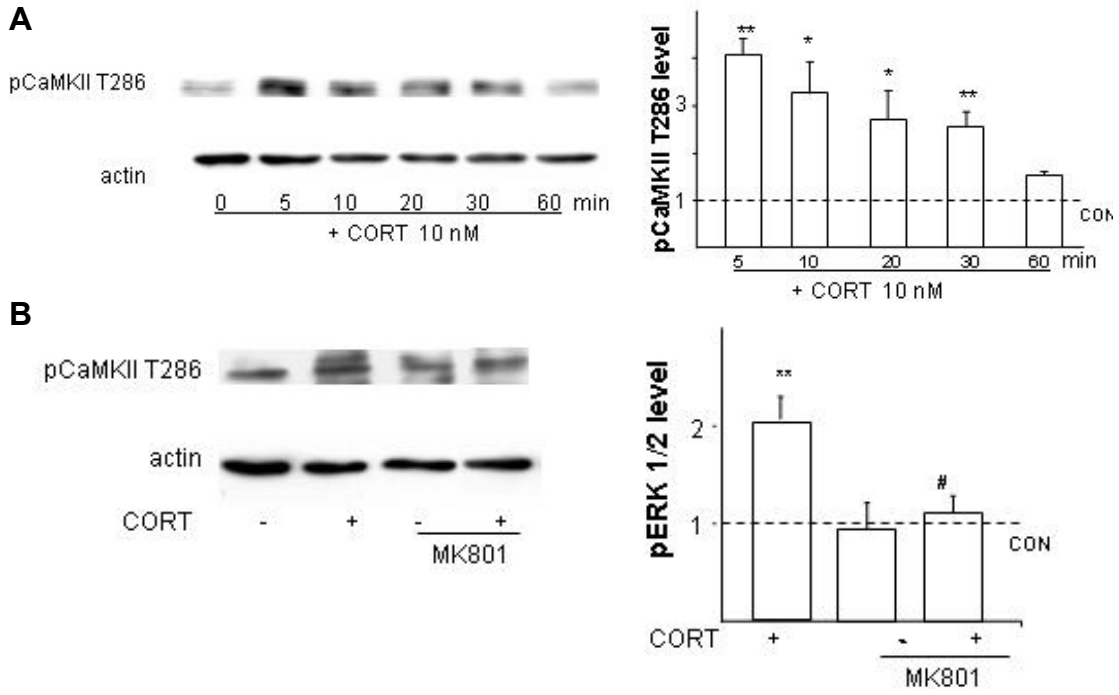


Fig. 4.26. CORT induces phosphorylation of CaMKII. A: CORT (10 nM) induced a significant increase in the phosphorylation of CaMKII at its threonine 286 site (T286) within 5 minutes of application. **B.** The CORT-induced phosphorylation of CaMKII was inhibited by the NMDAR antagonist, MK801 (10 μM, 30 minutes).
The immunoblot images are representative of results obtained in at least 3 different experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT treated cells.

Another ERK- related transcription factor, early growth response-1 (Egr-1), has been described as of possible target of activated GR (Revest et al. 2005). Here, 10 nM CORT induced an increase in the expression levels of Egr-1 in hippocampal neuronal cultures. As shown in **Fig 4.28**, the first significant increase appeared at 30 minutes, peaked at 60 minutes, and slightly decreased at 120 minutes.

These results show that CORT-induced phosphorylation of the transcription factor CREB is regulated via a CaMKII-MAPK mechanism that lies downstream of NMDAR. CORT also stimulated Egr-1 expression, which was suggested to be a GR-targeted transcription factor. Thus, it appears that membrane and nuclear signalling mechanisms can converge.

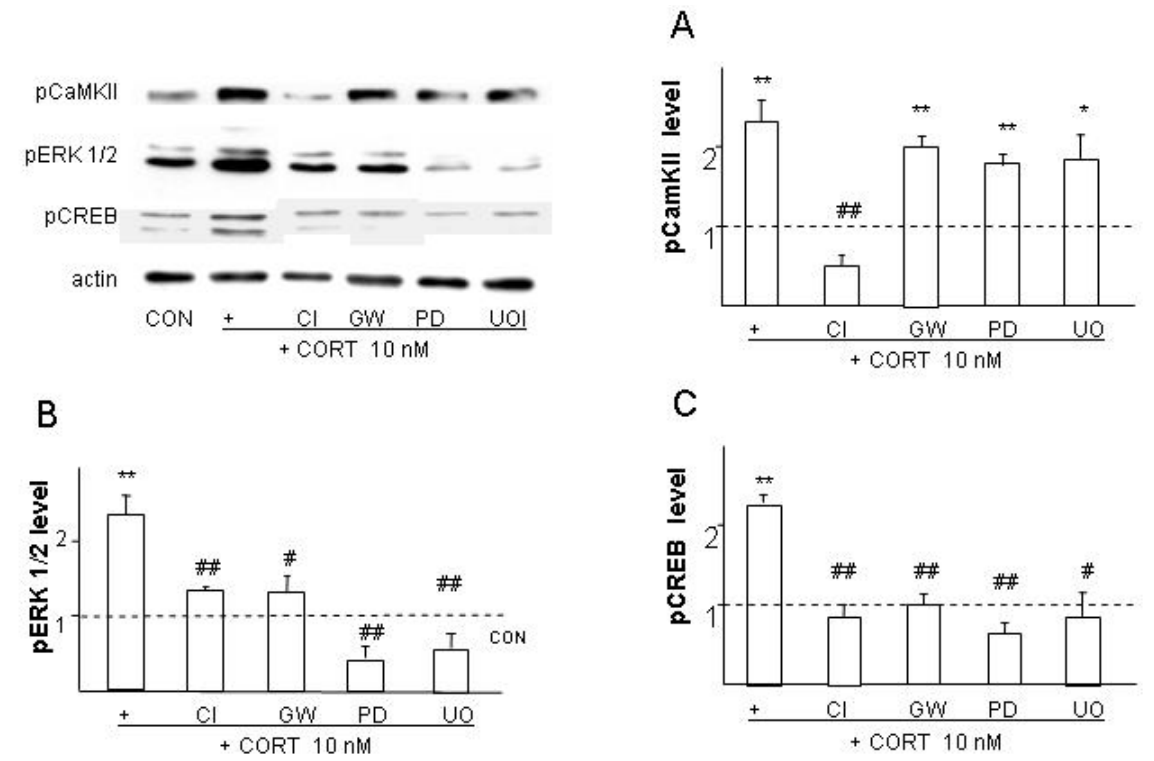


Fig. 4.27. CaMKII and MAPK mediate the effects of CORT on CREB phosphorylation. A. CORT (10 nM, 20 minutes) induced a significant increase in the phosphorylation of CaMKII T286, an effect abolished in the presence of an inhibitor of CaMKII (CI, 10 μM, 30 minutes) but the inhibitor RAF inhibitor GW 5074 (GW, 1 μM, 30 minutes) or two MAPK inhibitors PD 98059 (PD; 25 μM) and UO126, (UO, 10 μM), both drugs added for 30 minutes. **B**. The CORT-induced phosphorylation of ERK 1/2 was also reduced by inhibitors of CaMKII and RAF and abolished by both MAPK inhibitors. **C.** The phosphorylation of CREB induced by CORT was abrogated by the inhibitors of CaMKII, RAF and ERK 1/2.
The Western blot images shown are representative of results from at least 3 independent experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells. #: P<0.05, ##: P<0.001 vs CORT-treated cells.

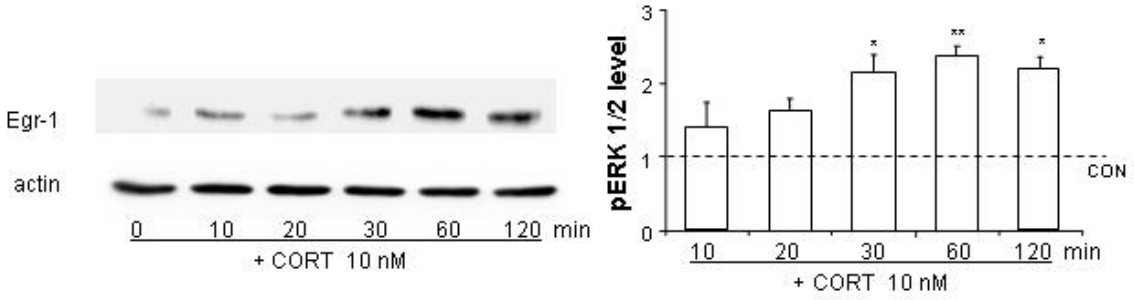


Fig. 4.28. CORT at a dose of 10 nM was sufficient to enhance the expression of Egr-1, an ERK 1/2 target gene. The first significant increase appeared at 30 minutes, peaked at 60 minutes, and was maintained at levels significantly higher than under baseline conditions for at least 120 minutes.
The immunoblot shown is representative of results obtained in at least 3 experiments. *: P<0.05, **: P<0.001 vs untreated (CON) cells.

5. DISCUSSION

Glucocorticoids (GC) are the main hormonal mediators of stress. In addition to assuring physiological adaptations (e.g. glucose mobilization and immuno-suppression), GC influence behavior through actions exerted directly on neurons. For example, GC can affect neuronal birth, differentiation and survival as well as alter dendritic arborization and synaptic activity in a number of brain regions, including the hippocampus, amygdala and prefrontal cortex. Further, GC actions in both the brain (prefrontal cortex, hippocampus and hypothalamus) and pituitary are important regulators of the endocrine response to stress, including its curtailment once the stressor is no longer present. In most cases, GC serve an adaptive function, but in some circumstances (e.g. when their levels remain high for long periods), they can cause damage in peripheral and neural tissues. For this reason, they are of particular interest in the filed of psychiatry because mood and anxiety disorders are closely associated with elevated levels of glucocorticoids in patients.

The long-term actions of GC are mediated by nuclear receptors, the glucocorticoid (GR) and mineralocorticoid (MR) receptors. These receptors are in fact ligand-activated transcriptional factors. Most studies on GC actions relate to their transcriptional effects that eventually lead to the *de novo* synthesis of proteins from about one hour after arrival of the GC signal. On the other hand, research in the last two decades has indicated that GC, like some other steroid hormones whose actions are also normally mediated through nuclear receptors (the best example is the estrogen, estradiol), induce rapid changes in neural activity as well as in behavior. Most of the studies have involved electrophysiological recordings but several have also shown that plasma membrane-proximal signaling cascades can be activated within a few minutes of GC application. Such signaling mechanisms that do not immediately involve gene transcription and protein synthesis are referred to as the rapid or non-genomic actions of GC. The purpose of the work described in this thesis was to

obtain more detailed information concerning the mechanisms that rapidly transduce GC signals from the cell membrane.

Primary neuronal cultures from the rat were chosen as the model to investigate the fast signaling cascades triggered by GC. The hippocampus is widely used for such studies because it is a known target of GC actions in the brain, in particular the central control of activity of the hypothalamo-pituitary-adrenal (HPA) axis and behavioral functions such as learning and memory. On the other hand, the hippocampus strongly expresses the GC-activated nuclear receptor (GR), a fact that, in some ways, compromised the use of this cellular model to understand GC actions that might not involve these receptors; this limitation was overcome however by the use of potent antagonists of the nuclear GR (e.g. RU 38486, J2700 and the novel CORT 108297 from Corcept Therapeutics). In fact, all of the investigations reported in this thesis were based on pharmacological approaches. Although the use of certain genetic manipulations were considered, these were not feasible primarily because of the difficulties associated with introducing genes by transfection into post-mitotic neurons. At the analytical level, besides the use of confocal microscopy for verification purposes, the studies were mainly carried out by immunoblotting. Such methods are standard for the analysis of kinase signaling pathways, in particular the phosphorylation of substrate proteins since cells can be rapidly lysed in phosphatase inhibitor-containing buffers to prevent spontaneous dephosphorylation. Another approach sometimes used to detect phosphorylated proteins is enzyme-linked immuno-adsorbent assay (ELISA). While ELISA provides relatively high throughput quantitative measurements, its cost is a major limiting factor. Fluorescence resonance energy transfer assays (FRET) and time-resolved fluorescence assays may also be used to study cell signaling, but such methods lack sufficient throughput. Phospho-proteomics, based on mass spectrometry (MS), probably represents the best state-of-the-art approach; however, sample preparation is tedious and the assays have low throughput and require significant bioinformatics support.

The present study provides a comprehensive description of the signaling cascade set in motion by non-classical glucocorticoid signaling and report the functional consequences at hippocampal neuronal synapses. In this work, a cascade of events was identified, namely, a membrane phase, a divergent early signaling phase, a convergence phase resulting in activation of a “hub” of tyrosine kinases, and a late-divergent phase during which tyrosine kinases influence multiple downstream effectors and targets to finally alter NMDA receptor function and the actin cytoskeleton.

Although the rapid effects of steroids have been shown to share some common secondary signaling pathways (Hammes and Levin, 2007), detailed studies of intracellular pathways involved in the rapid actions of steroids, and corticosteroids in particular, in neurons are still poorly understood. For example, several groups have observed that the phosphorylation of ERK 1/2 can be rapidly (within minutes) induced by the stimulation of aldosterone, corticosterone, estradiol, androgens and vitamin D in (mostly) non-neuronal cells or in an undifferentiated neural (PC12) cell line (Qiu et al., 2001; Pedram et al., 2007; Grossmann and Gekle, 2009). It has been shown that all three members of MAPK family (ERK 1/2, JNK and P38) can be activated by CORT or membrane impermeable CORT conjugated to BSA (CORT-BSA) in cultured rat embryonic hippocampal neuronal cells; these effects are seen at concentrations ranging from 10^{-10} to10^{-7} M, occur within 15 minutes and cannot be blocked by antagonists of the classical (nuclear) GR (RU38486) or MR (spironolactone). Also, they occur even in the presence of protein synthesis inhibitor (cycloheximide, CHX), strongly implicating the involvement of MAPK activation and demonstrating that these effects do not directly involve activation and/or repression of genes. Rather, it has been hypothesized that these effects are mediated by novel membrane-bound receptors, rather than classical GR or MR (Xiao et al., 2005). Moreover, researchers from latter group also suggested that G-protein and PKC, but not PKA, are part of the intracellular signal transduction

pathways that are induced by CORT or CORT-BSA, at least in the case of MAPK activation (Qi et al., 2005).

This work was started by carrying out a series of dose-response and time course experiments to examine how CORT acts in rapid mode to influence intracellular signaling cascades in postnatal rat hippocampal neuronal cells (dissociated cultures). By these experiments, it was intended to reproduce some of the earlier work in non-hippocampal cells and to test the suitability and applicability of our cellular model. In that work, the focus was on a key member of the MAPK family, ERK1/2. Cultures were treated with CORT and ERK phosphorylation was measured as the end-point. It was found that within 20 minutes of treatment, CORT at a dose of 10 nM induced maximum activation of ERK 1/2. A similar pharmacological profile was obtained with membrane-impermeable CORT-BSA. Importantly, the presence of CHX did not alter the activation of ERK 1/2 induced by CORT. Additionally, another researcher from our laboratory found that CaMKII, an upstream regulator of MAPK (**Fig 4.27**), can also be activated by CORT within 20 minutes in rat hippocampal slices, and that these effects are blocked by neither CHX nor the transcriptional inhibitor anisomycin (T. Riedemann, unpublished data). Thus, the non-genomic MAPK activation induced by CORT can be reproduced in hippocampal neurons, allowing further investigations of the potential mechanisms or pathways through which they may be mediated.

A comment regarding the use of steroid-BSA conjugates in implications of membrane receptor-mediated of a steroid is required at this point. As noted by Chambliss at al., (2010), such preparations may contain traces of free (unconjugated) steroid and may be biodegradable over time. Further, other authors (Stevis et al., 1999; Temple and Wray, 2005) have noted that the high molecular weight conjugates that result from conjugation, together with the fact that the linked BSA may mask the steroid's ability to bind to ligand-binding sites on the cognate nuclear receptor. It should be noted that, despite these criticisms, steroid-BSA conjugates (e.g. CORT-BSA) remain a standard tool until

dendrimers of CORT and other steroids, such as those being developed for estrogen (Chambliss et al., 2010) become available. While the limitation of free steroid contaminants should not be disregarded, the fact that conjugated steroids may not be able to bind to their corresponding nuclear receptor(s) would, actually be an advantage in the type of investigations performed in this thesis since they would exclude the participation of nuclear receptors. The problem of biodegradability is an important one (especially with respect to pre-pared conjugates, but this issue is not easily resolved given the cost and difficulties involved in consistently preparing conjugates with a pre-defined steroid-BSA ratio. On the other hand, since the present experiments were carried out with the aim of following signaling processes initiated within 20-60 minutes, the degradation of the complex after addition to cultured cells (lacking all the normal degradation and detoxification mechanisms found in the live animal) would be expected to be minimal. Lastly, it should be mentioned that being haptens, steroids need to be covalently linked to BSA, using linkers such as thyroglobulin, O-carboxy-methyl-oxime (CMO), or hemi-succinate; in our case, CORT was conjugated to BSA via CMO.

Together with the results obtained with CORT-BSA and CHX, our data point to the possibility that the fast actions of CORT are non-genomic and that CORT most likely initiate signaling through non-classical (non-nuclear) membrane-bound receptor(s). On the other hand, electron and confocal microscopy approaches led to the description of postsynaptic membrane-bound classical GR (Johnson et al. 2005) and MR (Prager et al. 2010) in the neurons in the rat amygdala. These findings complement with other reports that CORT induces neurotransmitter release and modulates synaptic plasticity through receptors which are sensitive to antagonists of classical MR and/or GR (Komatsuzaki et al., 2005; Wang and Wang, 2009). Also worth noting is the work by Joëls and colleagues which suggests involvement of classical GR and MR in the electrophysiological actions of CORT; in those studies, the authors used mouse mutants in which either the nuclear GR is non-functional due to deletion of the

dimerization domain of the receptor (Karst et al., 2000) or in which the MR gene was knocked out (Karst et al., 2005; Olijslagers et al., 2008). The subject of whether classical (nuclear) receptors might mediate steroid signaling initiated at the membrane remains highly controversial at present and the subject of intensive research, especially with respect to membrane mediators of the estrogen actions (Maggiolini and Picard, 2010; Chambliss et al., 2010). Questions remain about whether the membrane receptor shares identity with nuclear receptors and, if so, how receptors that are normally localized in the nucleus (or are translocated there after ligand activation, as in the case of the GR) are trafficked and inserted into the membrane – the latter seems unlikely from a biophysical perspective.

At least in the present work (and similar to some other studies, e.g. Qi et al., 2005; Xiao et al., 2005; Hu et al., 2010; Xiao et al., 2010), the rapid actions of CORT were not subject to blockade with antagonists of the classical nuclear receptors (MR and GR). It should be noted that in each case, three structurally distinct antagonists were examined (GR: RU38486, J2700, CORT 108297; MR: RU28318, spironolactone, eplerenone). Additionally, classical GR-unrelated effects, that could not be antagonized by GR antagonists, have been reported at the cellular (Di et al., 2003; 2009) and behavioral (Sandi et al., 1996a; Mikics et al., 2005) levels. Those effects could be potentially mediated by a G protein-coupled receptor (GPCR) since G-proteins have been shown to take part in other rapid effects of CORT (Ffrench-Mullen, 1995; Qi et al., 2005; Hu et al., 2010). In an elegant set of studies, using fluorescence correlation spectroscopy, Maier et al., (2005) showed that binding of fluorescein-labeled dexamethasone (a synthetic potent glucocorticoid that normally shows high selectivity for nuclear GR) on cell membranes of the mouse pituitary cell line (AtT20) sensitive to pertussis toxin (PTX), a G-protein inhibitor, but not the GR antagonist RU38486. These results provide strong evidence of high affinity binding (Kd of 1.8 nM) of glucocorticoids on cell membranes and that this binding requires G-proteins. However, this study did not examine whether G-proteins are required for the

triggering of intracellular signaling cascades downstream of the putative membrane receptor.

More recently, GPR30, a GPCR originally suggested to mediate estrogen effects and localized in the membranous endoplasmic reticulum (Thomas et al., 2005; Revankar et al., 2005), was identified as a possible mediator of the rapid signaling induced by aldosterone (a steroid closely-related to CORT and the natural ligand of the classical MR) in endothelial cells. Briefly, Gros et al., (2011) showed that the activation of ERK induced by aldosterone could be inhibited by an antagonist of GPR30 and suggested GPR30 might be an alternative aldosterone receptor (Gros et al., 2011). The previous work on GPR30 as a mediator of estrogen actions has raised much debate, with some authors claiming that it does not display any properties expected of an estrogen receptor (Otto et al., 2008). However, an emerging view is that GPR30 acts as a collaborator of estrogen receptor in signaling cascades and may also function downstream of cellular events induced by steroid compounds which are certainly not mediated by classical receptors (Levin 2009; Langer et al., 2010). Although most studies related to GPR30 were performed in carcinoma cell lines, the expression of GPR30 has been reported to be abundant in rodent brain, especially in hippocampus (Matsuda et al., 2008; Hazell et al., 2009).

In this work, the GPR30 antagonist (G-15) significantly prevented the rapid effects induced by CORT and CORT-BSA, adding support for the view that this GPR30 has a mediatory role in the activation of membrane-proximal signaling cascades by CORT. Attempts here to localize GPR30 in hippocampal neurons however failed; this could be because of need to further optimize immunostaining and cell fixation conditions. On the other hand, since it was reported that other types of cultured cells tend to express less GPR30 with increasing time in culture (Chambliss et al., 2010), caution will still be needed in interpreting results that indicate GPR30 mediation of effects observed. Nevertheless, this discussion will proceed on the assumption that technical difficulties contributed to the failure to

detect the protein in our hippocampal neuronal cultures, especially in light of evidence shown in this study that PTX blocks the rapid actions of CORT, a result that implies a role of G (Gi/o) proteins, as shown in **Fig 4.15 and Fig 4.16**.

Here, protein tyrosine kinase (PTK) was considered a good candidate to investigate as a starting point for the potential secondary signaling cascades which potentially mediate non-genomic signal transduction by CORT. Previously, the mediatory role of PTK, such as Pyk2 and Src, were shown be involved in the signaling pathways triggered in GPCR-dependent MAPK activation in PC12 cells (Dikic et al., 1996) and hippocampal slices (Corvol et al., 2005). The results of the present research (**Fig 4.6A**) showed that CORT rapidly induces a global increase of tyrosine phosphorylation levels in hippocampal neuronal cells. These effects were not affected by pretreatment with CHX (**Fig 4.6B**), suggesting a crucial role of tyrosine kinase in execution of the non-genomic effects of CORT. This finding prompted us to next analyze the specific tyrosine kinases involved.

Earlier studies have reported that activation of divergent second messengers (e.g. Ca^{2+}) and classical pathways (e.g. PKC, PKA and PI-3K/PKB) can converge to produce full activation of the scaffold tyrosine kinase Pyk2. Notably, Ca^{2+}- and PKC-dependent mechanisms were described to be fundamental for the activation of Pyk2 in multiple contexts (Della Rocca et al., 1997; Avraham et al., 2000; Huang et al., 2001), Pyk2 being a key player in neuronal signaling mediated by various GPCR (MacDonald et al., 2005; Sayas et al., 2006; Gaddini et al., 2009; Nicodemo et al., 2010; Yang et al., 2010). In the present study, CORT was shown to regulate the activation of Pyk2 at several “checkpoints”. Specifically, the steroid induced PKC-dependent autophosphorylation of Pyk2, regulated the inhibitory control of PTP-PEST by PKB and PKA (**Fig 4.13**), and influenced the phosphorylation of the C-terminal interaction domain (focal adhesion targeting domain) of Pyk2 via PKA through additional, but as yet unknown mechanisms. In this context, it is interesting that the simultaneous activation of multiple, converging pathways was shown by other authors to contribute to the strong phosphorylation of Pyk2 in synapses (Huang et al., 2001; Hsin et al., 2010);

based on those findings it appears that CORT may cause a sudden low-to-high switch in Pyk2 activity, thus allowing it to serve as a regulator of metaplasticity. The convergence of multiple signaling pathways on Pyk2 confers to this non-receptor tyrosine kinase a major role in integrating multiple signaling inputs, in particular allowing a strong interaction with other pathways (e.g. those from neurotransmitter receptors), leading to PKA or PKB activation. Taken together, these results represent new levels of integration of rapid CORT signaling with other cascades; previously, such convergence of CORT signaling has been restricted to transcriptional regulation (Schmidt et al., 2001).

On the other hand, activated Pyk2 serves as a major divergence point in the signaling cascades: Pyk2 not only functions as kinase that phosphorylates a number of substrates, adaptors and other kinases but it also acts as a scaffold protein to trigger the formation of transduction complexes (Avraham et al., 2001). In the present study, both Src kinase and c-Abl kinase were phosphorylated upon treating hippocampal neurons with CORT; these effects were dependent on Pyk2 activation (**Fig 4.9B and Fig 4.19B**). Interestingly, Src kinase was found to involved in an amplification loop: after phosphorylation of Src by Pyk2 auto-phosphorylation, phosphorylated Src in turn, led to further kinase activity on other residues in Pyk2 kinase and scaffold domains (**Fig 4.11**), as was described before for other system (Avraham et al., 2001; Park et al., 2004).

Since synapses are enriched with Pyk2, it seems likely that the Pyk2/Src module may be an important modulator of synaptic physiology. In line with this view, Cheung and Gurd (2001) reported that the cytoplasmic tail of the NR2 subunit of NMDAR to be a major target of tyrosine phosphorylation. Among several tyrosine phosphorylation sites, the tyrosine 1472 (Y1472) site of the NR2B appears to be a crucial regulator of NMDAR endocytosis since it provides the binding site for the endocytic adapter AP1 (Roche et al., 2001; Prybylowski et al., 2005). Accordingly, plasticity-inducing stimuli trigger Pyk2 activation, following Src activation, leading to major changes in the amount of surface NMDAR (Huang et al., 2001; Heidinger et al., 2002; Hsin et al., 2010). As shown in **Fig 4.22**, CORT

induces a marked increase in synaptic surface-localized NMDAR, an effect that involves increased phosphorylation of NR2B Y1472; both CORT-induced events were required the Pyk2/Src cascade (**Fig 4.23**). These findings provide molecular evidence to explain the previously-reported fast actions of CORT on electrophysiological activity of hippocampal neurons (Takahashi et al., 2002).

An interesting result obtained in this work was that CORT caused a significant increase in levels of phosphorylated CaMKII at its threonine 286 site within just a few minutes of application; phosphorylation of this site has been described to result in persistent activation of CaMKII (Yang and Schulman, 1999). A similar effect was observed in other work on hippocampal acute slices in our laboratory (T. Riedemann, unpublished). Since CaMKII serves to regulate ERK/MAPK (Liu et al. 2009), it is interesting that in this present study, CORT-induced phosphorylation of ERK 1/2 could be blocked by an inhibitor of CaMKII (**Fig 4.27B**). Furthermore, inhibitors of both CaMKII and MAPK attenuated the activation of cAMP response element-binding protein (CREB) induced by CORT (**Fig 4.27C**), indicating that CORT-triggered transcriptional events occur through a CaMKII-MAPK-CREB pathway; a similar pathway was identified in PC12 cells (Banno et al., 2008). Lastly, CORT led to increased expression of early growth response-1 (Egr-1) expression in hippocampal neuronal cells (**Fig 4.28**), a MAPK-regulated transcription factor that is also considered to be a target of classical GR (Revest et al. 2005; Benz et al., 2010)..

NMDAR play a critical role in the regulation of synapse plasticity, effects that are mediated by CaMKII (Barria and Malinow, 2005) and ERK1/2 (Kaphzan et al., 2006). In the work describe in this dissertation, NMDAR antagonists abolished CORT-induced activation of both CaMKII and ERK1/2, implying that CORT can also impact on the NMDAR- regulated CaMKII-MAPK-CREB pathway. These observations indicate that CORT signaling can cause transactivation of the NMDAR, setting the stage for the convergence and integration of intracellular signals that ultimately regulate gene transcription.

Further studies undertaken as part of this research aimed at gaining an insight into the functional meaning of the above results. The C-terminal interaction domain of Pyk2 contains binding sites for several adaptor proteins, including RhoGEF (Ying et al., 2009), the vav/sos complex (Gao and Blystone, 2009) and c-Abl (Zrihan-Licht et al., 2004). It was decided to first focus on RhoA, a member of the Rho family, implicated in the regulation of various cellular processes and, in particular, the actin cytoskeleton (Hall, 1998; Etienne-Manneville and Hall, 2002). The actin cytoskeleton is the major structural component of both pre- and postsynaptic synapses (spines). Dynamic changes in the actin cytoskeleton are believed to be mainly responsible for the morphological changes that accompany synaptic plasticity, usually measured in terms of presynaptic neurotransmitter release and altered postsynaptic electrophysiological activity. In addition the actin cytoskeleton plays an important role in the exo- and endocytic trafficking and anchorage of receptors such as NMDAR at the synaptic surface (Dillon and Goda, 2005; Cingolani and Goda, 2008; Roselli et al., 2011). In the present investigation (**Fig 4.21**), CORT induced activation of RhoA, an effect that was blocked by inhibitors of G-protein, Pyk2 and Src. However, inhibition of c-Abl, another actin-regulating pathway, did not interfere with the effects of CORT on RhoA activity. Similar findings were reported in another study on rat hippocampal neuronal cells in which a glutathione S-transferase pull-down approach was used for analysis (Jones et al., 2004). These observations therefore suggest that the Rho pathway either acts independently or in cooperation with c-Abl kinases.

As a powerful regulator, c-Abl has been shown to integrate multiple signals to regulate the actin cytoskeleton through the phosphorylation of Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) complexes (Woodring et al, 2003; Colicelli, 2010). The clinical relevance of c-Abl is reflected in the fact that it was identified as a potent Tau kinase; Tau hyperphosphorylation represents a neuropathological hallmark of Alzheimer's disease pathology (Schlatterer et al., 2011), and was shown in our laboratory to mediate the effects of adverse effects of stress on neuronal structure and

function thus also implicating it causally in depression (Catania et al., 2009; Sotiropoulos et al., 2011). Additionally, recent evidence suggests that c-Abl can modulate the clustering of postsynaptic density (PSD) proteins (de Arce et al., 2010). In this study, it was seen that c-Abl is activated by CORT via a cascade originating at Pyk2, but independently of the NMDAR-triggered cascade (**Fig 4.19**). Compared to the activation of Pyk2 and Src by CORT, phosphorylation of c-Abl appears with a slight delay. Use of STI571, a highly specific inhibitor pf c-Abl, revealed at least two sets of changes that may be attributed to signaling through c-Abl: as shown in **Fig 4.20**, the remodeling of the actin cytoskeleton (as revealed by altered clustering and localization of drebrin) and increased clustering of PSD-95, a key synaptic protein that serves to maintain PSD structure and couple receptors with signaling modules (Roselli et al., 2005). The results obtained this suggest that the reported fast actions of CORT on spine morphology (Komatsuzaki et al., 2005) may be due to its ability elicit intracellular signaling pathways that underlie actin remodeling and PSD-95 clustering. It should be noted that PSD-95 reportedly interacts with several non-receptor tyrosine kinases, including Pyk2 and Src (Seabold et al., 2003) and may thus serve as an additional point of integration of PTK signaling.

A schematic, representing the main steps through which CORT can elicit rapid responses from hippocampal neurons is summarized in **Figure 5.1.** The model proposes that CORT interacts with a putative membrane GPCR (GPR30), which, through Gi/o protein-dependent mechanism transduces the signal into cytoplasm. Besides increasing of intracellular Ca^{2+} levels, autophosphorylation of Pyk2 at its Y402 residue occurs via the PLC/PKC pathway. This is followed by autophosphorylation of the Src kinase at its Y416 site which, in turn, facilitates full activation of Pyk2 through phosphorylation of its Y579/580 and Y881 residues under the control of PTP-PEST; PI-3K/PKB and PKA pathways participate in the latter. Ultimately, activated Pyk2/Src kinases trigger multiple downstream signaling mechanisms including RhoA and c-Abl; among other functions, the latter serves to regulate dynamics of the actin cytoskeleton and PSD-95

clustering, as well as NMDAR cascades. Acting through the CaMKII pathway, NMDAR-mediated signaling activates MAPK, eventually impacting on transcriptional factors such as Egr-1 and CREB.

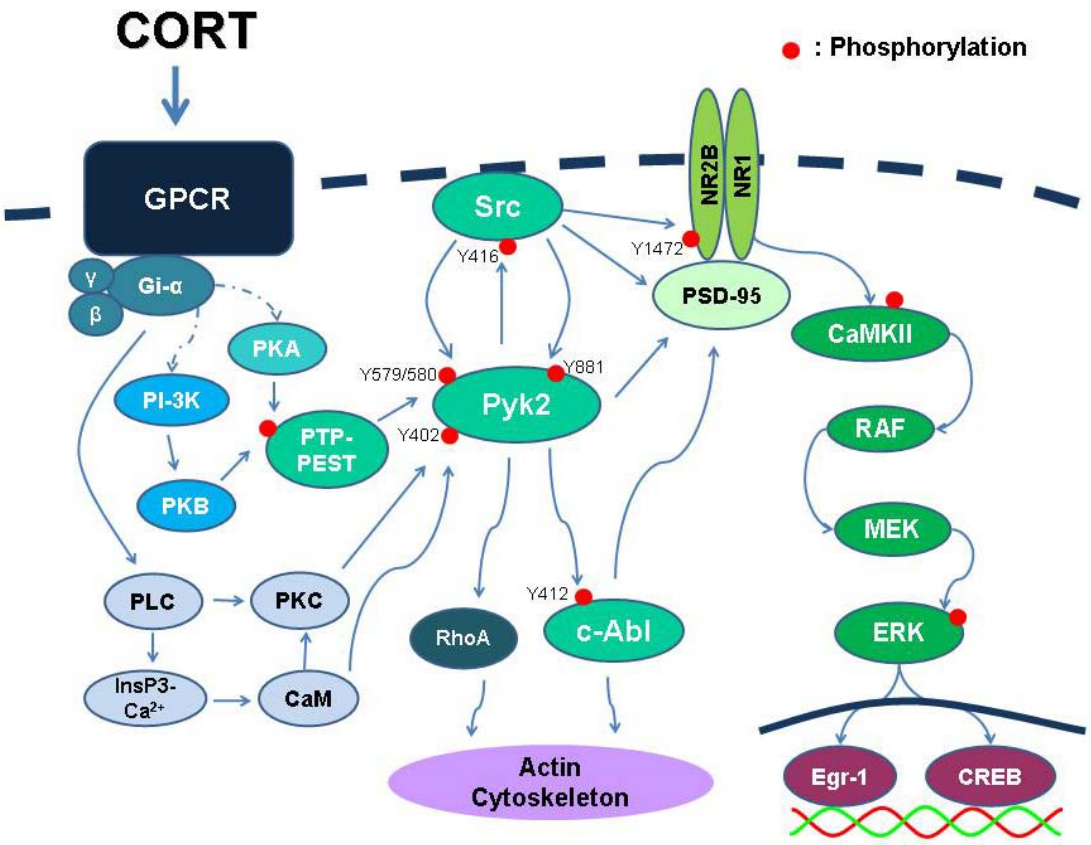


FIG.5.1 A working model to represent how CORT may induce rapid signaling events in hippocampal neurons, based on results obtained in the present study.

The present dissection of membrane-initiated signaling by CORT signaling in neurons has added new information to the field. It has led to interconnections between a putative GPCR for CORT to a major signaling hub; the latter, comprised of non-receptor tyrosine kinases links to downstream functional targets such as NMDAR and the actin cytoskeleton, through to transcriptional events. Together, these findings demonstrate how CORT-initiated non-nuclear receptor-mediated pathways and events can influence synaptic function that may extend to complex remodeling of brain networks (Ferries and Stolberg, 2010).

Notably, c-Abl can exert distinct cytoplasmic and nuclear functions (Liberatore et al., 2009; Colicelli, 2010) and may provide another possibility for transcriptional integration of classical GR and non-genomic pathways. In a very recent study, c-Abl has been implicated in Alzheimer's disease pathology since it is a potent Tau kinase (Schlatterer et al., 2011)

The identification and characterization of receptors responsible for non-genomic effects of corticosteroids would be an important filed to focus for the further studies, the better understating of secondary mechanisms of these fast corticosteroids effects may also deserve more attention because it may not only be useful to identify the pharmacological targets of clinical treatments for disorders in the nervous system, but also may bring hints for studies of mechanisms regulated other steroid hormones rapid effects. Advances in this direction are essential to be able to provide irrefutable arguments against claims that the rapid actions of CORT at the plasma surface are mediated by classical nuclear MR and/or GR (see review by Groeneweg et al., 2011). Recently, as reviewed in an instructive article by Krug et al., (2011), it has emerged that fragments of the classical MR may associated with the plasma membrane (perhaps explaining reports of MR/GR immunoreactivity in neuronal cell membrane, e.g. Johnson et al., 2005; Prager et al., 2010). One idea, shown to apply to the estrogen receptor, is that the liganded (holo)-nuclear receptor, or fragments thereof, may become palmitoylated and eventually embedded in calveolae containing a variety of molecules, including steroid hormone receptors, growth factor receptors, and molecules involved in rapid steroid signalling (Krug et al., 2011).

Lastly, it deserves mentioning that in this dissertation and in the literatures, the terms genomic and non-genomic signaling are often used; the term "genomic actions" refers to transcriptional regulation mediated by classical nuclear receptors, whereas those events that are triggered by membrane signaling (e.g. the fast or rapid actions of CORT) are considered "non-genomic actions". Strictly,

this terminology is inappropriate since signals interacting with signaling cascades originating at the cell membrane, cytoplasm and nucleus will all ultimately converge to regulate gene expression. This is illustrated by the observation that CORT can lead to the expression of transcription factors such as CREB and Egr-1 by activating MAPK/ERK; CREB itself can modulate GR activity through CREB binding protein (CPB) and p300 (Kino et al., 1999) and both CREB (Chen et al., 2010) and Egr-1 (Lu et al., 2011) can activate cyclin-dependent kinase 5 (CDK5) an important kinase in neurons. Interestingly, in preliminary data obtained at a late stage of this study found that CORT rapidly leads to the phosphorylation of nuclear GR within 5 minutes (Yang and Almeida, unpublished data). Previous studies by Kino demonstrated that CDK5 induces phosphorylation of nucleus-acting GR (Kino et al., 2007) and MR (Kino et al., 2010) and regulate receptor-related transcriptional activity through physical interaction. Finally, it is relevant to note that the phosphorylation of CDK5 has also found to be induced by the activation of c-Abl (Zukerberg et al., 2000). On the other hand, data from a preliminary study showed that CORT rapidly (with 20 minutes) induces phosphorylation of GR at serine 232 (a site known to increase the transcriptional activity of GR) can be prevented by treatment of hippocampal neurons with antagonists of the classical nuclear GR (Yang and Almeida, unpublished data). Although these findings need to be confirmed they hint at how membrane-mediated signals may serve to modulate the activity of nuclear receptors.

In conclusion, the studies presented in this thesis indicate that extra-nuclear signaling initiated by CORT, serving as a “starter” of multiple kinase cascades, can converge on gene regulatory pathways that are activated by CORT binding to nuclear MR and GR. Among the many questions that remain to be tackled in future research, the identity of the putative membrane receptor for CORT remains the most important and also most challenging.

6. REFERENCES

1 Abelson HT, Rabstein LS. 1970. Lymphosarcoma: virus-induced thymic-independent disease in mice. Cancer Res. 30(8):2213-22.

2 Alfarez DN, Joëls M, Krugers HJ. 2003. Chronic unpredictable stress impairs long-term potentiation in rat hippocampal CA1 area and dentate gyrus in vitro. Eur J Neurosci. 17(9):1928-34.

3 Alfarez DN, Wiegert O, Joëls M, Krugers HJ. 2002. Corticosterone and stress reduce synaptic potentiation in mouse hippocampal slices with mild stimulation. Neuroscience. 115(4):1119-26.

4 Aoto H, Sasaki H, Ishino M, Sasaki T. 2002. Nuclear translocation of cell adhesion kinase beta/proline-rich tyrosine kinase 2. Cell Struct Funct. 27(1):47-61.

5 Aspenström P, Fransson A, Saras J. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J. 377(Pt 2):327-37.

6 Avanzino GL, Ermirio R, Cogo CE, Ruggeri P, Molinari C. 1987. Effects of corticosterone on neurones of the locus coeruleus, in the rat. Neurosci Lett, 80:85-88.

7 Avraham H, Park SY, Schinkmann K, Avraham S. 2000. RAFTK/Pyk2-mediated cellular signalling. Cell Signal. 12(3):123-33.

8 Avraham S, London R, Fu Y, Ota S, Hiregowdara D, Li J, Jiang S, Pasztor LM, White RA, Groopman JE, Avraham H. 1995. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. J Biol Chem. 270(46):27742-51.

9 Banno Y, Nemoto S, Murakami M, Kimura M, Ueno Y, Ohguchi K, Hara A, Okano Y, Kitade Y, Onozuka M, Murate T, Nozawa Y. 2008. Depolarization-induced differentiation of PC12 cells is mediated by phospholipase D2 through the transcription factor CREB pathway. J Neurochem. 104(5):1372-86.

10 Barria A, Malinow R. 2005. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron. 48(2):289-301.

11 Barsegyan A, Mackenzie SM, Kurose BD, McGaugh JL, Roozendaal B.2010. Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. Proc Natl Acad Sci U S A. 107:16655-60.

12 Bartos JA, Ulrich JD, Li H, Beazely MA, Chen Y, Macdonald JF, Hell JW. 2010. Postsynaptic clustering and activation of Pyk2 by PSD-95. J Neurosci. 30(2):449-63.

13 Beazely MA, Weerapura M, MacDonald JF. 2008. Abelson tyrosine kinase links PDGFbeta receptor activation to cytoskeletal regulation of NMDA receptors in CA1 hippocampal neurons. Mol Brain. 1:20.

14 Belcher SM, Zsarnovszky A. 2001. Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms. J Pharmacol Exp Ther. 299(2):408-14.

15 Benz AH, Shajari M, Peruzki N, Dehghani F, Maronde E. 2010. Early growth response-1 induction by fibroblast growth factor-1 via increase of mitogen-activated protein kinase and inhibition of protein kinase B in hippocampal neurons. Br J Pharmacol. 160(7):1621-30.

16 Birnberg NC, Lissitzky JC, Hinman M, Herbert E. 1983. Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. Proc Natl Acad Sci U S A. 80(22):6982-6.

17 Blaukat A, Ivankovic-Dikic I, Grönroos E, Dolfi F, Tokiwa G, Vuori K, Dikic I. 1999. Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. J Biol Chem. 274(21):14893-901.

18 Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature , 361:31-39.

19 Brasher BB, Van Etten RA. 2000. c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. J Biol Chem. 275(45):35631-7.

20 Cataldi M, Taglialatela M, Guerriero S, Amoroso S, Lombardi G, di Renzo G, Annunziato L. 1996. Protein-tyrosine kinases activate while protein-tyrosine phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. J Biol Chem. 271(16):9441-6.

21 Catania C, Sotiropoulos I, Silva R, Onofri C, Breen KC, Sousa N, Almeida OF. 2009. The amyloidogenic potential and behavioral correlates of stress. Mol Psychiatry. 14(1):95-105.

22 Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS, Thomas GD, Mineo C, Yuhanna IS, Kim SH, Madak-Erdogan Z, Maggi A, Dineen SP, Roland CL, Hui DY, Brekken RA, Katzenellenbogen JA, Katzenellenbogen BS, Shaul PW. 2010. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. J Clin Invest. 120(7):2319-30. doi: 10.1172/JCI38291.

23 Chauveau F, Tronche C, Piérard C, Liscia P, Drouet I, Coutan M, Béracochéa D. 2010. Rapid stress-induced corticosterone rise in the hippocampus reverses serial memory retrieval pattern. Hippocampus. 20(1):196-207.

24 Chellaiah MA, Schaller MD. 2009. Activation of Src kinase by protein-tyrosine phosphatase-PEST in osteoclasts: comparative analysis of the effects of bisphosphonate and protein-tyrosine phosphatase inhibitor on Src activation in vitro. J Cell Physiol. 220(2):382-93.

25 Chen MC, Lin H, Hsu FN, Huang PH, Lee GS, Wang PS. 2010. Involvement of cAMP in nerve growth factor-triggered p35/Cdk5 activation and differentiation in PC12 cells. Am J Physiol Cell Physiol. 299(2):C516-27.

26 Chen S, O'Reilly LP, Smithgall TE, Engen JR. 2008. Tyrosine phosphorylation in the SH3 domain disrupts negative regulatory interactions within the c-Abl kinase core. J Mol Biol. 383(2):414-23.

27 Chen YZ, Hua SY, Wang CA, Wu LG, Gu Q, Xing BR. 1991. An electrophysiological study on the membrane receptor-mediated action of glucocorticoids in mammalian neurons. Neuroendocrinology. 53 Suppl 1:25-30.

28 Cheung HH, Gurd JW. 2001. Tyrosine phosphorylation of the N-methyl-D-aspartate receptor by exogenous and postsynaptic density-associated Src-family kinases. J Neurochem. 78(3):524-34.

29 Cheung TH, Cardinal RN. 2005. Hippocampal lesions facilitate instrumental learning with delayed reinforcement but induce impulsive choice in rats. BMC Neurosci. 6:36.

30 Cho K, Little HJ. 1999. Effects of corticosterone on excitatory amino acid responses in dopamine-sensitive neurons in the ventral tegmental area. Neuroscience, 88:837-845.

31 Chrousos GP, Gold PW. 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA. 267(9):1244-1252.

32 Cingolani LA, Goda Y. 2008. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nat Rev Neurosci. 9(5):344-56.

33 Cohen AW, Hnasko R, Schubert W, Lisanti MP. 2004. Role of caveolae and caveolins in health and disease. Physiol Rev. 84(4):1341-79.

34 Colicelli J. 2010. ABL tyrosine kinases: evolution of function, regulation, and specificity. Sci Signal. 3(139):re6.

35 Cong F, Spencer S, Côté JF, Wu Y, Tremblay ML, Lasky LA, Goff SP. 2000. Cytoskeletal protein PSTPIP1 directs the PEST-type protein tyrosine phosphatase to the c-Abl kinase to mediate Abl dephosphorylation. Mol Cell. 6(6):1413-23.

36 Corvol JC, Valjent E, Toutant M, Enslen H, Irinopoulou T, Lev S, Hervé D, Girault JA. 2005. Depolarization activates ERK and proline-rich tyrosine kinase 2 (PYK2) independently in different cellular compartments in hippocampal slices. J Biol Chem. 80(1):660-8. Epub 2004 Nov 10.

37 Cull-Candy S, Brickley S, Farrant M. 2001. NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol. 11(3):327-35.

38 Dallman MF, Yates FE. 1969. Dynamic asymmetries in the corticosteroid feedback path and distribution-metabolism-binding elements of the adrenocortical system. Ann NY Acad Sci, 156:696-721.

39 Datson NA, Morsink MC, Meijer OC, de Kloet ER. 2008. Central corticosteroid actions: Search for gene targets. Eur J Pharmacol. 583(2-3):272-89.

40 Datson NA, van der Perk J, de Kloet ER, Vreugdenhil E. 2001. Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. Eur J Neurosci. 14(4):675-89.

41 Davidson D, Shi X, Zhong MC, Rhee I, Veillette A. 2010. The phosphatase PTP-PEST promotes secondary T cell responses by dephosphorylating the protein tyrosine kinase Pyk2. Immunity.33(2):167-80.

42 Davis, S., and Laroche, S. 2006. Mitogen-activated protein kinase/extracellular regulated kinase signalling and memory stabilization: a review. Genes Brain Behav. 5 (Suppl 2), 61–72.

43 de Arce KP, Varela-Nallar L, Farias O, Cifuentes A, Bull P, Couch BA, Koleske AJ, Inestrosa NC, Alvarez AR. 2010. Synaptic clustering of PSD-95 is regulated by c-Abl through tyrosine phosphorylation. J Neurosci. 30(10):3728-38.

44 de Bosscher K, Vanden Berghe W, Haegeman G. 2003. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev. 24(4):488-522.

45 de Kloet ER, Joëls M, Holsboer F. 2005. Stress and the brain: from adaptation to disease. Nat Rev Neurosci. 6(6):463-75.

46 de Kloet ER, Karst H, Joëls M. 2008. Glucocorticoids hormones in the central stress response: quick-and-slow. Front Neuroendocrinol. 29(2):268-72.

47 Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. 1997. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. J Biol Chem. 272(31):19125-32.

48 Di S, Malcher-Lopes R, Halmos KC, Tasker JG. 2003. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J Neurosci, 23:4850-4857.

49 Di S, Maxson MM, Franco A, Tasker JG. 2009. Glucocorticoids regulate glutamate and GABA synapse-specific retrograde transmission via divergent nongenomic signaling pathways. J Neurosci. 29(2):393-401.

50 Dickmeis T, Lahiri K, Nica G, Vallone D, Santoriello C, Neumann CJ, Hammerschmidt M, Foulkes NS. 2007. Glucocorticoids play a key role in circadian cell cycle rhythms. PLoS Biol. 5(4):e78.

51 Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. 1996. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature. 383(6600):547-50.

52 Dillon C, Goda Y. 2005. The actin cytoskeleton: integrating form and function at the synapse. Annu Rev Neurosci. 28:25-55.

53 Droste SK, de Groote L, Atkinson HC, Lightman SL, Reul JM, Linthorst AC. 2008. Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. Endocrinology. 149(7):3244-53.

54 Droste SK, de Groote L, Lightman SL, Reul JM, Linthorst AC. 2009. The ultradian and circadian rhythms of free corticosterone in the brain are not affected by gender: an in vivo microdialysis study in Wistar rats. J Neuroendocrinol. 21(2):132-40.

55 Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. 1996. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 2(5):561-6.

56 Duma D, Jewell CM, Cidlowski JA. 2006. Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J Steroid Biochem Mol Biol. 102(1-5):11-21.

57 Etienne-Manneville S, Hall A. 2002. Rho GTPases in cell biology. Nature. 420(6916):629-35.

58 Ferris CF, Stolberg T. 2010. Imaging the immediate non-genomic effects of stress hormone on brain activity. Psychoneuroendocrinology. 35(1):5-14.

59 Ffrench-Mullen JM. 1995. Cortisol inhibition of calcium currents in guinea pig hippocampal CA1 neurons via G-protein-coupled activation of protein kinase C. J Neurosci. 15(1 Pt 2):903-11.

60 Fiore RS, Bayer VE, Pelech SL, Posada J, Cooper JA, Baraban JM. 1993. p42 mitogen-activated protein kinase in brain: prominent localization in neuronal cell bodies and dendrites. Neuroscience. 55(2):463-72.

61 Frye CA, Walf AA. 2008. Membrane actions of progestins at dopamine type 1-like and GABAA receptors involve downstream signal transduction pathways. Steroids. 73(9-10):906-13.

62 Gaddini L, Villa M, Matteucci A, Mallozzi C, Petrucci TC, Di Stasi AM, Leo L, Malchiodi-Albedi F, Pricci F. 2009. Early effects of high glucose in retinal tissue cultures Renin-Angiotensin system-dependent and -independent signaling. Neurobiol Dis. 35(2):278-85.

63 Gao C, Blystone SD. 2009. A Pyk2-Vav1 complex is recruited to beta3-adhesion sites to initiate Rho activation. Biochem J. 420(1):49-56.

64 Garton AJ, Tonks NK. 1994. PTP-PEST: a protein tyrosine phosphatase regulated by serine phosphorylation. EMBO J. 13(16):3763-71.

65 Genazzani AR, Stomati M, Morittu A, Bernardi F, Monteleone P, Casarosa E, Gallo R, Salvestroni C, Luisi M. 2000. Progesterone, progestagens and the central nervous system. Hum Reprod. 15 Suppl 1:14-27.

66 Gertler FB, Bennett RL, Clark MJ, Hoffmann FM. 1989. Drosophila abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with disabled. Cell. 58(1):103-13.

67 Goebel SM, Alvestad RM, Coultrap SJ, Browning MD. 2005. Tyrosine phosphorylation of the N-methyl-D-aspartate receptor is enhanced in synaptic membrane fractions of the adult rat hippocampus. Brain Res Mol Brain Res. 142(1):65-79.

68 Groeneweg FL, Karst H, de Kloet ER, Joëls M. 2011. Mineralocorticoid and glucocorticoid receptors at the neuronal membrane, regulators of nongenomic corticosteroid signalling. Mol Cell Endocrinol. 2011 Jun 28.

69 Gronemeyer H, Gustafsson JA, Laudet V. 2004. Principles for modulation of the nuclear receptor superfamily. Nat Rev Drug Discov. 3(11):950-64.

70 Gros R, Ding Q, Sklar LA, Prossnitz EE, Arterburn JB, Chorazyczewski J, Feldman RD. 2011. GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. Hypertension. 2011 Mar;57(3):442-51.

71 Grossmann C, Gekle M. 2009. New aspects of rapid aldosterone signaling. Mol Cell Endocrinol. 308(1-2):53-62.

72 Gründemann D, Schechinger B, Rappold GA, Schömig E. 1998. Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. Nat Neurosci, 1:349-351.

73 Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK. 2002. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat Med 8:473–479

74 Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science. 279(5350):509-14.

75 Hammes SR, Levin ER. 2007. Extranuclear steroid receptors: nature and actions.Endocr Rev. 28(7):726-41.

76 Han JZ, Lin W, Chen YZ. 2005. Inhibition of ATP-induced calcium influx in HT4 cells by glucocorticoids: involvement of protein kinase A. Acta Pharmacol Sin. 26(2):199-204.

77 Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem. 271(2):695-701.

78 Hantschel O, Superti-Furga G. 2004. Regulation of the c-Abl and Bcr-Abl tyrosine kinases. Nat Rev Mol Cell Biol. 5(1):33-44.

79 Hardingham GE, Arnold FJ, Bading H. 2001. Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. Nat Neurosci. 4(3):261-7.

80 Hazell GG, Yao ST, Roper JA, Prossnitz ER, O'Carroll AM, Lolait SJ. 2009. Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. J Endocrinol. 202(2):223-36.

81 He LM, Zhang CG, Zhou Z, Xu T. 2003. Rapid inhibitory effects of corticosterone on calcium influx in rat dorsal root ganglion neurons. Neuroscience. 116(2):325-33.

82 Heidinger V, Manzerra P, Wang XQ, Strasser U, Yu SP, Choi DW, Behrens MM. 2002. Metabotropic glutamate receptor 1-induced upregulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. J Neurosci. 22(13):5452-61.

83 Hinz B, Hirschelmann R. 2000. Rapid non-genomic feedback effects of glucocorticoids on CRF-induced ACTH secretion in rats. Pharm Res. 17(10):1273-7.

84 Hsin H, Kim MJ, Wang CF, Sheng M. 2010. Proline-rich tyrosine kinase 2 regulates hippocampal long-term depression. J Neurosci. 30(36):11983-93.

85 Hu W, Zhang M, Czéh B, Flügge G, Zhang W. 2010. Stress impairs GABAergic network function in the hippocampus by activating nongenomic glucocorticoid receptors and affecting the integrity of the parvalbumin-expressing neuronal network. Neuropsychopharmacology. 35(8):1693-707.

86 Huang Y, Lu W, Ali DW, Pelkey KA, Pitcher GM, Lu YM, Aoto H, Roder JC, Sasaki T, Salter MW, MacDonald JF. 2001. CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. Neuron. 29(2):485-96.

87 Hubbard SR, Mohammadi M, Schlessinger J. 1998. Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem. 273(20):11987-90.

88 Hubbard SR, Till JH. 2000. Protein tyrosine kinase structure and function. Annu Rev Biochem. 69:373-98.

89 Hur EM, Kim KT. 2002. G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. Cell Signal. 14(5):397-405.

90 Iwasaki Y, Aoki Y, Katahira M, Oiso Y, Saito H. 1997. Non-genomic mechanisms of glucocorticoid inhibition of adrenocorticotropin secretion: possible involvement of GTP-binding protein. Biochem Biophys Res Commun. 235(2):295-9.

91 Jaffe AB, Hall A. 2005. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 21:247-69.

92 Jasper MS, Engeland WC. 1991. Synchronous ultradian rhythms in adrenocortical secretion detected by microdialysis in awake rats. Am J Physiol. 261(5 Pt 2):R1257-68.

93 Jasper MS, Engeland WC. 1994. Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. Neuroendocrinology. 59(2):97-109.

94 Jiang G, Hunter T. 1999. Receptor signaling: when dimerization is not enough. Curr Biol. 9(15):R568-71.

95 Joëls M. 2008. Functional actions of corticosteroids in the hippocampus. Eur J Pharmacol. 583(2-3):312-21.

96 Johnson LR, Farb C, Morrison JH, McEwen BS, LeDoux JE. 2005. Localization of glucocorticoid receptors at postsynaptic membranes in the lateral amygdala. Neuroscience. 136(1):289-99.

97 Jones SB, Lu HY, Lu Q. 2004. Abl tyrosine kinase promotes dendrogenesis by inducing actin cytoskeletal rearrangements in cooperation with Rho family small GTPases in hippocampal neurons. J Neurosci. 24(39):8510-21.

98 Kageyama K, Akimoto K, Suda T. 2010. Corticotrophin-releasing factor gene transcription is directly activated after deprivation of glucocorticoids in hypothalamic cells. J Neuroendocrinol. 22(9):971-8.

99 Kalia LV, Gingrich JR, Salter MW. 2004. Src in synaptic transmission and plasticity. Oncogene. 23(48):8007-16.

100 Kaphzan H, O'Riordan KJ, Mangan KP, Levenson JM, Rosenblum K. 2006. NMDA and dopamine converge on the NMDA-receptor to induce ERK activation and synaptic depression in mature hippocampus. PLoS One. 1:e138.

101 Karst H, Berger S, Turiault M, Tronche F, Schütz G, Joëls M. 2005. Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. Proc Natl Acad Sci U S A. 102(52):19204-7.

102 Karst H, Joëls M. 2005. Corticosterone slowly enhances miniature excitatory postsynaptic current amplitude in mice CA1 hippocampal cells. J Neurophysiol. 94(5):3479-86.

103 Karst H, Joëls M. 2007. Brief RU 38486 treatment normalizes the effects of chronic stress on calcium currents in rat hippocampal CA1 neurons. Neuropsychopharmacology. 32(8):1830-9.

104 Karst H, Karten YJ, Reichardt HM, de Kloet ER, Schütz G, Joëls M. 2000. Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. Nat Neurosci. 3(10):977-8.

105 Kasai M, Yamashita H. 1988. Inhibition by cortisol of neurons in the paraventricular nucleus of the hypothalamus in adrenalectomized rats; an in vitro study. Neurosci Lett, 91:59-64.

106 Ke FC, Ramirez VD. 1990. Binding of progesterone to nerve cell membranes of rat brain using progesterone conjugated to 125I-bovine serum albumin as a ligand. J Neurochem , 54:467-472.

107 Kent WD, Cross-Mellor SK, Kavaliers M, Ossenkopp KP. 2000. Acute effects of corticosterone on LiCl-induced rapid gustatory conditioning in rats: a taste reactivity analysis. Neuroreport , 11:3903-3908.

108 Khaksari M, Rashidy-Pour A, Vafaei AA. 2007. Central mineralocorticoid receptors are indispensable for corticosterone-induced impairment of memory retrieval in rats. Neuroscience. 149(4):729-38.

109 Kim JJ, Foy MR, Thompson RF. 1996. Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc Natl Acad Sci U S A. 93(10):4750-3.

110 Kino T, Ichijo T, Amin ND, Kesavapany S, Wang Y, Kim N, Rao S, Player A, Zheng YL, Garabedian MJ, Kawasaki E, Pant HC, Chrousos GP. 2007. Cyclin-dependent kinase 5 differentially regulates the transcriptional activity of the glucocorticoid receptor through phosphorylation: clinical implications for the nervous system response to glucocorticoids and stress. Mol Endocrinol 21: 1552–1568

111 Kino T, Jaffe H, Amin ND, Chakrabarti M, Zheng YL, Chrousos GP, Pant HC. 2010. Cyclin-dependent kinase 5 modulates the transcriptional activity of the mineralocorticoid receptor and regulates expression of brain-derived neurotrophic factor. Mol Endocrinol. 24(5):941-52.

112 Kino T, Nordeen SK, Chrousos GP. 1999. Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300. J Steroid Biochem Mol Biol. 70(1-3):15-25.

113 Kohno T, Matsuda E, Sasaki H, Sasaki T. 2008. Protein-tyrosine kinase CAKbeta/PYK2 is activated by binding Ca2+/calmodulin to FERM F2 alpha2 helix and thus forming its dimer. Biochem J. 410(3):513-23.

114 Kojima N, Shirao T. 2007. Synaptic dysfunction and disruption of postsynaptic drebrin-actin complex: a study of neurological disorders accompanied by cognitive deficits. Neurosci Res. 58(1):1-5.

115 Komatsuzaki Y, Murakami G, Tsurugizawa T, Mukai H, Tanabe N, Mitsuhashi K, Kawata M, Kimoto T, Ooishi Y, Kawato S. 2005. Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus. Biochem Biophys Res Commun. 335:1002-7.

116 Krug AW, Pojoga LH, Williams GH, Adler GK. 2011. Cell membrane-associated mineralocorticoid receptors? New evidence. Hypertension. 57(6):1019-25.

117 Krugers HJ, Goltstein PM, van der Linden S, Joëls M. 2006. Blockade of glucocorticoid receptors rapidly restores hippocampal CA1 synaptic plasticity after exposure to chronic stress. Eur J Neurosci. 23(11):3051-5.

118 Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J. 15(3):510-9.

119 Langer G, Bader B, Meoli L, Isensee J, Delbeck M, Noppinger PR, Otto C. 2010. A critical review of fundamental controversies in the field of GPR30 research. Steroids. 75(8-9):603-10.

120 Lavezzari G, McCallum J, Dewey CM, Roche KW. 2004. Subunit-specific regulation of NMDA receptor endocytosis. J Neurosci. 24(28):6383-91.

121 Levin ER. 2008. Rapid signaling by steroid receptors. Am J Physiol Regul Integr Comp Physiol. 295(5):R1425-30.

122 Levin ER. 2009. G protein-coupled receptor 30: estrogen receptor or collaborator? Endocrinology. 150(4):1563–5.

123 Li X, Qiu J, Wang J, Zhong Y, Zhu J, Chen Y. 2001. Corticosterone-induced rapid phosphorylation of p38 and JNK mitogen-activated protein kinases in PC12 cells. FEBS Lett, 492:210-214.

124 Liberatore RA, Goff SP, Nunes I. 2009. NF-kappaB activity is constitutively elevated in c-Abl null fibroblasts. Proc Natl Acad Sci U S A. 106(42):17823-8.

125 Lightman SL, Wiles CC, Atkinson HC, Henley DE, Russell GM, Leendertz JA, McKenna MA, Spiga F, Wood SA, Conway-Campbell BL. 2008. The significance of glucocorticoid pulsatility. Eur J Pharmacol. 583(2-3):255-62.

126 Liposits Z, Bohn MC. 1993. Association of glucocorticoid receptor immunoreactivity with cell membrane and transport vesicles in hippocampal and hypothalamic neurons of the rat. J Neurosci Res. 35(1):14-9.

127 Liu J, Zhou R, He Q, Li WI, Zhang T, Niu B, Zheng X, Xie J. 2009. Calmodulin kinase II activation of mitogen-activated protein kinase in PC12 cell following all-trans retinoic acid treatment. Neurotoxicology. 30(4):599-604.

128 Liu L, Wang C, Ni X, Sun J. 2007. A rapid inhibition of NMDA receptor current by corticosterone in cultured hippocampal neurons. Neurosci Lett. 420(3):245-50.

129 Liu X, Wang CA, Chen YZ. 1995. Nongenomic effect of glucocorticoid on the release of arginine vasopressin from hypothalamic slices in rats. Neuroendocrinology. 62(6):628-33.

130 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.1951, Protein measurement with the Folin phenol reagent. J Biol Chem., 193:265-75.

131 Lu ML, Schneider MC, Zheng Y, Zhang X, Richie JP. 2001. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J Biol Chem. 276(16):13442-51.

132 Lu Y, Li T, Qureshi HY, Han D, Paudel HK. 2011. Early growth response 1 (Egr-1) regulates phosphorylation of microtubule-associated protein tau in mammalian brain. J Biol Chem. 286(23):20569-81.

133 Lu YM, Roder JC, Davidow J, Salter MW. 1998. Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science. 279(5355):1363-7.

134 Lulo J, Yuzawa S, Schlessinger J. 2009. Crystal structures of free and ligand-bound focal adhesion targeting domain of Pyk2. Biochem Biophys Res Commun. 383(3):347-52.

135 Lyons PD, Dunty JM, Schaefer EM, Schaller MD. 2001. Inhibition of the catalytic activity of cell adhesion kinase beta by protein-tyrosine phosphatase-PEST-mediated dephosphorylation. J Biol Chem. 276(26):24422-31

136 Ma YC, Huang J, Ali S, Lowry W, Huang XY. 2000. Src tyrosine kinase is a novel direct effector of G proteins. Cell. 102(5):635-46.

137 Macdonald DS, Weerapura M, Beazely MA, Martin L, Czerwinski W, Roder JC, Orser BA, MacDonald JF. 2005. Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires G alpha q, protein kinase C, and activation of Src. J Neurosci. 25(49):11374-84.

138 Maggiolini M, Picard D. 2010. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol. 204(2):105-14.

139 Maier C, Rünzler D, Schindelar J, Grabner G, Waldhäusl W, Köhler G, Luger A. 2005. G-protein-coupled glucocorticoid receptors on the pituitary cell membrane. J Cell Sci. 118(Pt 15):3353-61.

140 Malcher-Lopes R, Di S, Marcheselli VS, Weng FJ, Stuart CT, Bazan NG, Tasker JG. 2006. Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release. J Neurosci. 26(24):6643-50.

141 Matsuda K, Sakamoto H, Mori H, Hosokawa K, Kawamura A, Itose M, Nishi M, Prossnitz ER, Kawata M. 2008. Expression and intracellular distribution of the G protein-coupled receptor 30 in rat hippocampal formation. Neurosci Lett. 441(1):94-9. Epub 2008 Jun 5.

142 Matus A. 2000. Actin-based plasticity in dendritic spines. Science. 290(5492):754-8.

143 Micevych P, Dominguez R. 2009. Membrane estradiol signaling in the brain. Front Neuroendocrinol. 30(3):315-27.

144 Mikics E, Barsy B, Barsvári B, Haller J. 2005. Behavioral specificity of non-genomic glucocorticoid effects in rats: effects on risk assessment in the elevated plus-maze and the open-field. Horm Behav , 48:152-162.

145 Mikics E, Barsy B, Haller J. 2007. The effect glucocorticoids on aggressiveness in established colonies of rats. Psychoneuroendocrinology, 32:160-170.

146 Mikics E, Kruk MR, Haller J. 2004. Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats. Psychoneuroendocrinology, 29:618-635.

147 Miklós IH, Kovács KJ. 2002. GABAergic innervation of corticotropin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopy. Neuroscience. 113(3):581-92.

148 Moresco EM, Koleske AJ. 2003. Regulation of neuronal morphogenesis and synaptic function by Abl family kinases. Curr Opin Neurobiol. 13(5):535-44.

149 Moresco EM, Scheetz AJ, Bornmann WG, Koleske AJ, Fitzsimonds RM. 2003. Abl family nonreceptor tyrosine kinases modulate short-term synaptic plasticity. J Neurophysiol. 89(3):1678-87.

150 Moss SJ, Gorrie GH, Amato A, Smart TG. 1995. Modulation of GABAA receptors by tyrosine phosphorylation. Nature. 377(6547):344-8.

151 Müller G. 2000. Towards 3D structures of G protein-coupled receptors: a multidisciplinary approach. Curr Med Chem. 7(9):861-88.

152 Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bornmann W, Clarkson B, Superti-Furga G, Kuriyan J. 2003. Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell. 112(6):859-71.

153 Nakamura K, Palmer HE, Ozawa T, Mashima K. 2010. Protein phosphatase 1alpha associates with protein tyrosine phosphatase-PEST inducing dephosphorylation of phospho-serine 39. J Biochem. 147(4):493-500.

154 Nestler EJ, Terwilliger RZ, Halm E. 1989. Corticosterone increases protein tyrosine kinase activity in the locus coeruleus and other monoaminergic nuclei of rat brain. Mol Pharmacol. 35(3):265-70.

155 Nicodemo AA, Pampillo M, Ferreira LT, Dale LB, Cregan T, Ribeiro FM, Ferguson SS. 2010. Pyk2 uncouples metabotropic glutamate receptor G protein signaling but facilitates ERK1/2 activation. Mol Brain. 3:4.

156 Olijslagers JE, de Kloet ER, Elgersma Y, van Woerden GM, Joëls M, Karst H. 2008. Rapid changes in hippocampal CA1 pyramidal cell function via pre- as well as postsynaptic membrane mineralocorticoid receptors. Eur J Neurosci. 27(10):2542-50.

157 Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, Brittain D, Langer G, Bader B, Prelle K, Nubbemeyer R, Fritzemeier KH. 2008. G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. Endocrinology. 149(10):4846-56.

158 Park SY, Avraham HK, Avraham S. 2004. RAFTK/Pyk2 activation is mediated by trans-acting autophosphorylation in a Src-independent manner. J Biol Chem. 279(32):33315-22

159 Pedram A, Razandi M, Levin ER. 2006. Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol. 20(9):1996-2009.

160 Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC, Levin ER. 2007. A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem. 282(31):22278-88.

161 Piazza PV, Rougé-Pont F, Deroche V, Maccari S, Simon H, Le Moal M. 1996. Glucocorticoids have state-dependent stimulant effects on the mesenceph-alic dopaminergic transmission. Proc Natl Acad Sci USA, 93:8716-8720.

162 Pierce KL, Premont RT, Lefkowitz RJ. 2002. Seven-transmembrane receptors. Nat Rev Mol Cell Biol. 3(9):639-50.

163 Prager EM, Brielmaier J, Bergstrom HC, McGuire J, Johnson LR. 2010. Localization of mineralocorticoid receptors at mammalian synapses. PLoS One. 5(12):e14344.

164 Privalsky ML. 2004. The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu Rev Physiol 66:315–360

165 Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ. 2005. The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 47(6):845-57

166 Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ. 2005. The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 47(6):845-57.

167 Qi AQ, Qiu J, Xiao L, Chen YZ. 2005. Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. J Neurosci Res. 80(4):510-7.

168 Qiu J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK, Kelly MJ. 2003b. Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. J Neurosci. 23(29):9529-40.

169 Qiu J, Wang CG, Huang XY, Chen YZ. 2003a. Nongenomic mechanism of glucocorticoid inhibition of bradykinin-induced calcium influx in PC12 cells: possible involvement of protein kinase C. Life Sci. 72(22):2533-42.

170 Qiu J, Wang P, Jing Q, Zhang W, Li X, Zhong Y, Sun G, Pei G, Chen Y. 2001. Rapid activation of ERK1/2 mitogen-activated protein kinase by cortico-sterone in PC12 cells. Biochem Biophys Res Commun, 287:1017-1024.

171 Rasband, W.S.1997-2011. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/.

172 Ratajczak T, Ward BK, Minchin RF. 2003. Immunophilin chaperones in steroid receptor signalling. Curr Top Med Chem. 3(12):1348-57.

173 Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. 2002. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol Endocrinol. 16(1):100-15.

174 Reichardt HM, Tuckermann JP, Gottlicher M, Vujic M, Weih F, Angel P, Herrlich P, Schutz G. 2001. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J 20:7168–7173

175 Reul JM, de Kloet ER.1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology. 117:2505-11.

176 Reul JM, van den Bosch FR, de Kloet ER. 1987. Differential response of type I and type II corticosteroid receptors to changes in plasma steroid level and circadian rhythmicity. Neuroendocrinology. 45(5):407-12.

177 Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science.307(5715):1625-30.

178 Revest JM, Di Blasi F, Kitchener P, Rougé-Pont F, Desmedt A, Turiault M, Tronche F, Piazza PV. 2005. The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. Nat Neurosci. 8(5):664-72.

179 Riedemann T, Patchev AV, Cho K, Almeida OF. 2010. Mol Brain. Corticosteroids: way upstream. 3:2.

180 Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ. 2001. Molecular determinants of NMDA receptor internalization. Nat Neurosci. 4(8):794-802.

181 Roozendaal B, de Quervain DJ, Schelling G, McGaugh JL. 2004. A systemically administered beta-adrenoceptor antagonist blocks corticosterone induced impairment of contextual memory retrieval in rats. Neurobiol Learn Mem, 81:150-154.

182 Roselli F, Livrea P, Almeida OF. 2011. CDK5 Is Essential for Soluble Amyloid β-Induced Degradation of GKAP and Remodeling of the Synaptic Actin Cytoskeleton. PLoS One. 6(7):e23097.

183 Roselli F, Tirard M, Lu J, Hutzler P, Lamberti P, Livrea P, Morabito M, Almeida OF. 2005. Soluble beta-amyloid1-40 induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. J Neurosci. 25(48):11061-70.

184 Rosenfeld MG, Lunyak VV, Glass CK 2006 Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptionaf response. Genes Dev 20:1405–1428

185 Roskoski R Jr. 2004. Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun. 324(4):1155-64.

186 Roskoski R Jr. 2005. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun. 331(1):1-14.

187 Rubinfeld H, Seger R. 2005. The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol. 31(2):151-74.

188 Sajadi AA, Samaei SA, Rashidy-Pour A. 2006. Intra-hippocampal micro-injections of anisomycin did not block glucocorticoid-induced impairment of memory retrieval in rats: an evidence for non-genomic effects of glucocorticoids. Behav Brain Res, 173:158-162.

189 Salter MW, Kalia LV. 2004. Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci. 5(4):317-28.

190 Salter MW. 1998. Src, N-methyl-D-aspartate (NMDA) receptors, and synaptic plasticity.Biochem Pharmacol. 56(7):789-98.

191 Sandi C, Venero C, Guaza C. 1996a, Novelty-related rapid locomotor effects of corticosterone in rats. Eur J Neurosci, 8:794-800.

192 Sandi C, Venero C, Guaza C. 1996b. Nitric oxide synthesis inhibitors prevent rapid behavioral effects of corticosterone in rats. Neuroendocrinology,63:446-453.

193 Sato S, Osanai H, Monma T, Harada T, Hirano A, Saito M, Kawato S. 2004. Acute effect of corticosterone on N-methyl-D-aspartate receptor-mediated Ca2+ elevation in mouse hippocampal slices. Biochem Biophys Res Commun. 321(2):510-3.

194 Sawchenko PE. 1987. Evidence for differential regulation of corticotropin-releasing factor and vasopressin immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. Brain Res. 437(2):253-63.

195 Sayas CL, Ariaens A, Ponsioen B, Moolenaar WH. 2006. GSK-3 is activated by the tyrosine kinase Pyk2 during LPA1-mediated neurite retraction. Mol Biol Cell. 17(4):1834-44.

196 Schaller MD, Sasaki T. 1997. Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. J Biol Chem. 272(40):25319-25.

197 Schlatterer SD, Acker CM, Davies P. 2011. c-Abl in Neurodegenerative Disease. J Mol Neurosci. Epub ahead of print

198 Schmidt P, Holsboer F, Spengler D. 2001. Beta(2)-adrenergic receptors potentiate glucocorticoid receptor transactivation via G protein beta gamma-subunits and the phosphoinositide 3-kinase pathway. Mol Endocrinol. 15(4):553-64.

199 Seabold GK, Burette A, Lim IA, Weinberg RJ, Hell JW. 2003. Interaction of the tyrosine kinase Pyk2 with the N-methyl-D-aspartate receptor complex via the Src homology 3 domains of PSD-95 and SAP102. J Biol Chem. 278(17):15040-8.

200 Seger R, Krebs EG. 1995. The MAPK signaling cascade. FASEB J. 9(9):726-35.

201 Sekino Y, Kojima N, Shirao T. 2007. Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochem Int. 51(2-4):92-104.

202 Smith JM, Mayer BJ. 2002. Abl: mechanisms of regulation and activation. Front Biosci. 7:d31-42.

203 Smriga M, Saito H, Nishiyama N. 1996. Hippocampal long- and short-term potentiation is modulated by adrenalectomy and corticosterone. Neuroendocrinology. 64(1):35-41.

204 Sotiropoulos I, Catania C, Pinto LG, Silva R, Pollerberg GE, Takashima A, Sousa N, Almeida OF. 2011. Stress acts cumulatively to precipitate Alzheimer's disease-like tau pathology and cognitive deficits. J Neurosci. 31(21):7840-7.

205 Sotiropoulos I, Cerqueira JJ, Catania C, Takashima A, Sousa N, Almeida OF. 2008. Stress and glucocorticoid footprints in the brain-the path from depression to Alzheimer's disease. Neurosci Biobehav Rev. 32(6):1161-73.

206 Sousa N, Almeida OF. 2002. Corticosteroids: sculptors of the hippocampal formation. Rev Neurosci. 13(1):59-84.

207 Steen H, Fernandez M, Ghaffari S, Pandey A, Mann M. 2003. Phosphotyrosine mapping in Bcr/Abl oncoprotein using phosphotyrosine-specific immonium ion scanning. Mol Cell Proteomics. 2(3):138-45.

208 Stephenson FA. 2006. Structure and trafficking of NMDA and GABAA receptors. Biochem Soc Trans. 34(Pt 5):877-81.

209 Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE. 1999. Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology. 140(11):5455-8.

210 Strober W. 2001. Monitoring cell growth. Curr Protoc Immunol. Appendix 3:Appendix 3A.

211 Sud N, Wiseman DA, Black SM. 2010. Caveolin 1 is required for the activation of endothelial nitric oxide synthase in response to 17beta-estradiol. Mol Endocrinol. 24(8):1637-49.

212 Sun HW, Miao CY, Liu L, Zhou J, Su DF, Wang YX, Jiang CL. 2006. Rapid inhibitory effect of glucocorticoids on airway smooth muscle contractions in guinea pigs. Steroids. 71(2):154-9.

213 Sutter-Dub MT. 2002. Rapid non-genomic and genomic responses to proges progestogens, estrogens, and glucocorticoids in the endocrine pancreatic B cell, the adipocyte and other cell types. Steroids 67:77–93

214 Sweatt JD. 2004. Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol.14(3):311-7.

215 Takahashi H, Mizui T, Shirao T. 2006. Down-regulation of drebrin A expression suppresses synaptic targeting of NMDA receptors in developing hippocampal neurones. J Neurochem. 97 Suppl 1:110-5.

216 Takahashi H, Sekino Y, Tanaka S, Mizui T, Kishi S, Shirao T. 2003. Drebrin-dependent actin clustering in dendritic filopodia governs synaptic targeting of postsynaptic density-95 and dendritic spine morphogenesis. J Neurosci. 23(16):6586-95.

217 Takahashi T, Kimoto T, Tanabe N, Hattori TA, Yasumatsu N, Kawato S. 2002. Corticosterone acutely prolonged N-methyl-d-aspartate receptor-mediated Ca2+ elevation in cultured rat hippocampal neurons. J Neurochem. 83(6):1441-51.

218 Tanis KQ, Veach D, Duewel HS, Bornmann WG, Koleske AJ. 2003. Two distinct phosphorylation pathways have additive effects on Abl family kinase activation. Mol Cell Biol. 23(11):3884-96.

219 Tasker JG, Di S, Malcher-Lopes R. 2006. Minireview: rapid glucocorticoid signaling via membrane-associated receptors. Endocrinology, 147:5549-5556.

220 Temple JL, Wray S. 2005. Bovine serum albumin-estrogen compounds differentially alter gonadotropin-releasing hormone-1 neuronal activity. Endocrinology. 146(2):558-63.

221 Thomas P, Pang Y, Filardo EJ, Dong J. 2005. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 146(2):624-32.

222 Tian L, Knaus HG, Shipston MJ. 1998. Glucocorticoid regulation of calcium-activated potassium channels mediated by serine/threonine protein phosphatase. J Biol Chem. 273(22):13531-6.

223 Trinidad JC, Thalhammer A, Specht CG, Lynn AJ, Baker PR, Schoepfer R, Burlingame AL. 2008. Quantitative analysis of synaptic phosphorylation and protein expression. Mol Cell Proteomics. 7(4):684-96.

224 Tsien JZ, Huerta PT, Tonegawa S. 1996. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell.;87(7):1327-38.

225 van Cauter E, Leproult R, Kupfer DJ. 1996. Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. J Clin Endocrinol Metab. 81(7):2468-73.

226 Veillette A, Rhee I, Souza CM, Davidson D. 2009. PEST family phosphatases in immunity, autoimmunity, and autoinflammatory disorders. Immunol Rev. 228(1):312-24.

227 Wang CC, Wang SJ. 2009. Modulation of presynaptic glucocorticoid receptors on glutamate release from rat hippocampal nerve terminals. Synapse. 63(9):745-51.

228 Wang HR, Zhang Y, Ozdamar B, Ogunjimi AA, Alexandrova E, Thomsen GH, Wrana JL. 2003b. Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science. 302(5651):1775-9.

229 Wang K, Hackett JT, Cox ME, Van Hoek M, Lindstrom JM, Parsons SJ. 2004. Regulation of the neuronal nicotinic acetylcholine receptor by SRC family tyrosine kinases. J Biol Chem. 279(10):8779-86

230 Wang Q, Xie Y, Du QS, Wu XJ, Feng X, Mei L, McDonald JM, Xiong WC. 2003a. Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin. J Cell Biol. 160(4):565-75.

231 Wang YT, Salter MW. 1994. Regulation of NMDA receptors by tyrosine kinases and phosphatases. Nature. 369(6477):233-5.

232 Wiegert O, Joëls M, Krugers H. 2006. Timing is essential for rapid effects of corticosterone on synaptic potentiation in the mouse hippocampus. Learn Mem. 13(2):110-3.

233 Wiegert O, Pu Z, Shor S, Joëls M, Krugers H. 2005. Glucocorticoid receptor activation selectively hampers N-methyl-D-aspartate receptor dependent hippocampal synaptic plasticity in vitro. Neuroscience. 135(2):403-11.

234 Wiper-Bergeron N, Wu D, Pope L, Schild-Poulter C, Hache RJ. 2003. Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex. EMBO J 2:2135–2145

235 Woodring PJ, Hunter T, Wang JY. 2003. Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. J Cell Sci. 116(Pt 13):2613-26.

236 Xiao L, Feng C, Chen Y. 2010. Glucocorticoid rapidly enhances NMDA-evoked neurotoxicity by attenuating the NR2A-containing NMDA receptor-mediated ERK1/2 activation. Mol Endocrinol. 24(3):497-510.

237 Xiao L, Qi A, Chen Y. 2005. Cultured embryonic hippocampal neurons deficient in glucocorticoid (GC) receptor: a novel model for studying nongenomic effects of GC in the neural system. Endocrinology. 146(9):4036-41.

238 Xu L, Holscher C, Anwyl R, Rowan MJ. 1998. Glucocorticoid receptor and protein/RNA synthesis-dependent mechanisms underlie the control of synaptic plasticity by stress. Proc Natl Acad Sci U S A. 95(6):3204-8.

239 Yan JZ, Xu Z, Ren SQ, Hu B, Yao W, Wang SH, Liu SY, Lu W. 2011. Protein Kinase C Promotes N-Methyl-D-aspartate (NMDA) Receptor Trafficking by Indirectly Triggering Calcium/Calmodulin-dependent Protein Kinase II (CaMKII) Autophosphorylation. J Biol Chem. 286(28):25187-200.

240 Yang E, Schulman H. 1999. Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. J Biol Chem. 274(37):26199-208.

241 Yang K, Lei G, Jackson MF, Macdonald JF. 2010 . The involvement of PACAP/VIP system in the synaptic transmission in the hippocampus. J Mol Neurosci. 42(3):319-26.

242 Ying Z, Giachini FR, Tostes RC, Webb RC. 2009. PYK2/PDZ-RhoGEF links Ca2+ signaling to RhoA. Arterioscler Thromb Vasc Biol. 29(10):1657-63.

243 Young EA, Abelson J, Lightman SL. 2004. Cortisol pulsatility and its role in stress regulation and health. Front Neuroendocrinol.25(2):69-76.

244 Yu XM, Askalan R, Keil GJ 2nd, Salter MW. 1997. NMDA channel regulation by channel-associated protein tyrosine kinase Src. Science. 275(5300):674-8.

245 Zrihan-Licht S, Avraham S, Jiang S, Fu Y, Avraham HK. 2004. Coupling of RAFTK/Pyk2 kinase with c-Abl and their role in the migration of breast cancer cells. Int J Oncol. 24(1):153-9.

246 Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA, Tsai LH. 2000. Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron. 26(3):633-46.

7. ABBREVIATIONS

2-APB	2-Aminoethyl diphenylborinate
Abl	Abelson tyrosine kinase
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
AF	activation functions
APS	Ammonium Persulfate
Arg	Abl-related gene
AVP	arginine vasopressin
BCR	breakpoint-cluster region
BSA	bovine serum albumin
CADTK	calcium-dependent tyrosine kinase
CAKβ	cell adhesion kinase beta
CaMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid binding globulin
Cdk5	Cyclin-dependent kinase 5
Chk	Csk homologous kinase
CHX	cycloheximide
CNS	central nervous system
CNS	central nervous system
CORT	corticosterone
CORT-BSA	bovine serum albumin conjugated to corticosterone
CREB	cAMP response element-binding
CRH	corticotrophin releasing hormone
CS	corticosteroids
Csk	C-terminal Src kinase
CyD	40% cyclodextrin
DBD	DNA binding domain
DEX	dexamethasone
dH_2O	Distilled water

DI H_2O	deionized water
DIV	days *in vitro*
DMSO	Dimethyl sulfoxide
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr-1	early growth response-1
ELISA	Enzyme-linked immunosorbent assay
ER	estrogen receptors
ERK 1/2	extracellular signal-regulated kinase p44/p42
EtOH	Ethanol
F-actin	filamentous-actin
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FBD	F-actin binding domain
FGF	fibroblast growth factor
FKBP	FK506 binding proteins
FREM	band four point one, ezrin, radixin, moesin
GABA A	gamma-aminobutyric acid type A
G-actin	globular-actin
GAP	GTPase activating proteins
GBD	G-actin binding domain
GC	glucocorticoids
GDI	guanine nucleotide dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GLISA	G-protein linked immunosorbent assay
GPCR	G-protein coupled receptors
G-protein	GTP-binding protein
GR	glucocorticoids receptor

Grb2	growth factor receptor-bound protein 2
GSK-3	Glycogen synthase kinase 3
GTP	guanosine triphosphate
HPA	hypothalamo-pituitary-adrenal
HRE	hormone response elements
HRP	horse radish peroxidase
HSP	heat shock protein
ICC	Immunocytochemistry
JAK	Janus protein tyrosine kinases
JNK	c-Jun N-terminal kinase
kDa	kilo daltons
L	liter
LBD	ligand binding domain
LTD	long-term depression
LTP	long-term potentiation
M	molar
m	meter
MAPK	mitogen-activated protein kinase
MC	mineralocorticoids
MDR	multiple drug resistance
mEPSCs	miniature excitatory postsynaptic currents
MR	mineralocorticoids receptor
NF- κB	nuclear factor-κB
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
NOS	nitric oxide synthase
NRTK	non-receptor tyrosine kinases
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline- Triton-X100
PDGF	platelet-derived growth factor
PDL	Poly-D-Lysine
PEP	proline-enriched phosphatase

PEST	proline-, glutamic acid-, serine- and threonine-rich family
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PP2	(4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine)
PSD-95	postsynaptic density protein 95
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatases
PTX	pertussis toxin
PVN	paraventricular nucleus
Pyk2	proline-rich tyrosine kinase 2
RAFTK	related adhesion focal tyrosine kinase
RLU	relative luminescence units
rpm	Revolutions per minute
RTK	receptor tyrosine kinases
S	serine
SAGE	serial analysis of gene expression
SD	standard division
SDS	Sodium dodecyl sulfate
SFK	Src tyrosine kinase
SH	Src homology
Src	sarcoma tyrosine kinase
Syk	Spleen tyrosine kinase
T	threonine
TBST	Tris-buffered saline-Tween-20
TEMED	Tetramethylethylenediamine
TF	transcription factors
Trk	neurotrophic tyrosine receptor kinases
VSCC	voltage-sensitive calcium channel
WASP	Wiskott–Aldrich syndrome protein

WAVE	WASP-family verprolin-homologous protein
Y	tyrosine

8. ACKNOWLEDGEMENTS

My supervisor, Dr. Osborne Almeida (Neuroadaptations Group, Max-Planck Institute of Psychiatry, Munich), deserves special appreciation. Osborne's His generous encouragement, wide knowledge, constant support and understanding make the past years become some of the best time in my life. Not only did he teach me the ways of scientific thinking, but also the proper attitude to face the unpredictable future. Osborne's mentorship will undoubtedly guide me in my future career and entire life.

I cannot thank Dr. Francesco Roselli enough. Francesco provided excellent and patient supervision and introduced me to a number of biochemical and microscopic techniques that form the backbone of this thesis. This work gained much from his wide and insightful knowledge that always served to "rescue" even the most frustrating experiments! Francesco's rapid and intelligent suggestions – even after he had moved to Basel – merit admiration and special thanks.

I would also like to thank Prof. Dr. Rainer Landgraf for accepting to support me through my studies as a PhD student at the Ludwig-Maximilians-Universität München; without his kind advice and support the final stages of my PhD studies would not be possible.

Others in the Neuroadaptations Group also contributed to my studies and deserve special thanks. Dr. Shuang Yu was always patient and encouraging and enthusiastically introduced me to neurobiological experiments. Her guidance and encouragement helped me bridge difficult periods. I would also like to express sincere gratitude to Dr. Alexandre Patchev and Therese Riedemann for their many suggestions and criticism that helped improve the design and interpretation of my experiments. I am very thankful to Dieter Fischer and Rainer Stoffel for their always friendly help and advice and for contributing to the smooth lab environment.

I am deeply thankful to Frau Carola Hetzel for her kindness, optimism and ever-ready-to-help attitude. Carola, you really helped to make my lonely life abroad so much better!

I would like to thank Prof. Dr. Dr. Dr. h. c. Florian Holsboer for enabling me to complete my studies by providing generous resources and the opportunity to work in this excellent Institute.

Finally, I would like to thank my most beloved parents, Dr. Bo Yang and Jianrui Liu, for their consistent love and belief in me, as well as my uncle, Prof. Ze Yang, for his encouragement and guidance. I would not be here without them. And I deeply grateful to my girlfriend, Huan Chen; she was there at all the right moments, giving me enormous support and comfort. Although she could not join me in Munich, I always felt her love and encouraging spirit.

CURRICULUM VITAE

Given Name: Silei Family Name: Yang
Date of Birth: December 20, 1978
Place of Birth: Tieling, Province of Liaoning, P. R. China
E-mail: sileiyang@ yahoo.com

Education

2007.09 - Present Ph.D. student,
Max Planck Institute of Psychiatry, Munich
supervised by Prof. Dr. Rainer Landgraf and Dr. Osborne Almeida;
registered for PhD degree from **Ludwig-Maximilians-Universität München**

2002.09 – 2003.07 Master degree,
Majored in Molecular Medicine and Genetic Epidemiology
University of Pavia, Italy

1997.09 – 2001.07 Bachelor degree,
Majored in Biochemistry Engineering,
Minzu University of China, China

Experience

2006.10 - Present Ph.D. student
Neuroadaptations Group (Head: Osborne Almeida)
Max Planck Institute of Psychiatry, Munich, Germany

2005.03-2006.08 Research Assistant
International Joint Cancer Institute
Second Military Medical University, Shanghai, China

2003.08 – 2006.07 Research Assistant
Department of Medical Genetics,
National Institute of Geriatrics, Beijing, China

2002.10 - 2003.07 Research Assistant
Laboratory of Genetic Epidemiology
Department of Scienze Sanitarie Applicate e Psicocomportamentali,
University of Pavia, Pavia, Italy

2002.02 - 2002.07 Research Assistant
Department of Medical Genetics,
National Institute of Geriatrics, Beijing, China

Publications

Zhao J, Zhang X, Shi M, Xu H, Jin J, Ni H, **Yang S,** Dai J, Wu M, Guo Y.TIP30 inhibits growth of HCC cell lines and inhibits HCC xenografts in mice in combination with 5-FU. *Hepatology.* 2006 Jul;44(1):205-15.

Yang S, Patchev AV, Roselli F, Almeida OFX *(in preparation)*. Signaling pathways downstream of the rapid actions of glucocorticoids mediated by putative membrane receptors. *(In preparation)*

Yu S, **Yang S**, Holsboer F, Sousa N, Almeida OFX (2011) Glucocorticoid regulation of astrocytic fate and function. PLoS One. 6:e22419.